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A molecular technique for detecting the liberation of intracellular zinc in cultured neurons

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Abstract

We have previously reported that oxidative stimuli liberate Zn^{2+} from metalloproteins, a phenomenon that can trigger neuronal cell death. Excessive intracellular Zn^{2+} in many cell types triggers the expression of genes that encode metal binding proteins, such as metallothionein, via the activation and nuclear translocation of metal response element (MRE)-binding transcription factor-1 (MTF-1). Cd^{2+} strongly induces nuclear translocation of MTF-1 in non-neuronal cells, but it does so by displacing Zn^{2+} from its metal binding sites within the cell and increasing the intracellular concentration of this ion. Here, we describe the use of MRE-driven expression of a luciferase reporter gene as a sensitive molecular assay for detecting increases in intracellular zinc concentrations. MRE transactivation was induced in primary cortical neurons upon brief exposure to Zn^{2+} or Cd^{2+}. Enhanced MRE transactivation was observed upon co-exposure of neurons to Cd^{2+} together with NMDA, as this metal can permeate through the receptor channel. Luciferase expression was observed regardless of whether or not neurons had been co-transfected with an MTF-1-containing plasmid, suggesting the presence of an endogenous MTF-1-like protein. Indeed, RT–PCR revealed that MTF-1 mRNA is present in neurons. In contrast, MTF-1 deficient dko7 cells were only observed to have MRE transactivation when co-transfected with MTF-1. Our results indicate that Cd^{2+} can effectively induce transactivation of MRE in neurons by liberating Zn^{2+} from its intracellular binding sites.

Keywords: Cadmium; Zinc; Cortical neurons; Metal response element; Metal response element-binding transcription factor; Metallothionein; Luciferase; Transfection

1. Introduction

Metal response element (MRE)-binding transcription factor-1 (MTF-1) is a six-zinc finger protein that is responsible for inducing metallothionein-I and -II gene expression following zinc exposure (Palmiter, 1994; Westin and Schaffner, 1988). Zinc, but not other heavy metals, increases the binding activity of MTF-1 to MRE, which is present in multiple copies in the promoter region of metallothionein genes (Bittel et al., 1998; Koizumi et al., 1992, 1999; Zhang et al., 2003). As such, MTF-1 serves as a selective intracellular Zn^{2+} sensor to activate MT gene expression (Giedroc et al., 2001; Smirnova et al., 2000; Zhang et al., 2003). It has been demonstrated that in the absence of zinc, approximately 80% of cellular MTF-1 is found in the cytosol, and is unable to bind DNA (Smirnova et al., 2000). Upon Zn^{2+} exposure, nearly all of the cellular content of MTF-1 translocates to the nucleus. Using the transient expression of an MTF-1-FLAG construct in a double MTF-1 knock out cell line (dko7), Smirnova et al. (2000) showed that Zn^{2+}could preferentially induce the rapid translocation of the transcription factor from the cytoplasm into the nucleus. Although the dynamics of MRE transactivation have been well documented in many cell types, virtually nothing is known about the activation of this process in neurons.

Here, we have evaluated whether nuclear translocation of MTF-1, as measured by the transactivation of an MRE-luciferase construct (Chen et al., 2004), could be detected in primary neurons in tissue culture following the displacement of Zn^{2+} from its intracellular binding sites (Fig. 1). If so, this method could serve as a sensitive

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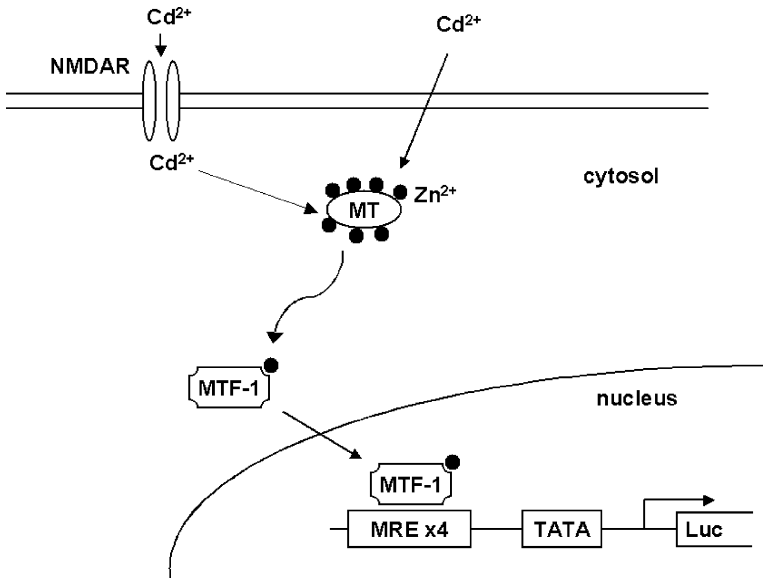

Fig. 1. Diagram of the transactivation assay used to detect the intracellular liberation of Zn^{2+} in neurons. Cd^{2+} enters cells via a non-specified pathway or through activated *N*-methyl-D-aspartate (NMDA) receptors. Once inside, Cd^{2+} can displace bound Zn^{2+} from metallothionein (MT). The liberated Zn^{2+}, in turn, binds to metal response element (MRE)-binding transcription factor-1 (MTF-1). MTF-1 then translocates into the nucleus and transactivates an MRE-driven luciferase reporter gene.

molecular assay for detecting increases in the intracellular concentrations of Zn^{2+} with very high degree of specificity (Chen et al., 2004; Zhang et al., 2003). We have utilized Cd^{2+} as a trigger to liberate Zn^{2+}, as the former metal has a higher affinity than the latter for metal binding proteins such as metallothionein. A main objective of this work was to establish the presence Zn^{2+} displacement in neurons with molecular techniques since the liberation of this metal from its binding sites can lead to the activation of cell death-inducing pathways in neurons (Aizenman et al., 2000; Du et al., 2002; McLaughlin et al., 2001; Pal et al., 2003). We report here that Cd^{2+} can effectively induce MRE transactivation in neurons via a process that does not require expression of exogenous MTF-1. As such, we demonstrate the existence of endogenous MTF-1 message in our neuronal preparation.

2. Materials and methods

2.1. Reagents

Reagents were obtained from Sigma (St. Louis, MO) unless otherwise noted. Tissue culture supplies and SuperScript First-Strand Synthesis System for RT–PCR were purchased from Invitrogen (Carlsbad, CA), except for heat-inactivated and iron-supplemented bovine calf serum (Hyclone, Logan, UT). *Renilla* Luciferase reporter gene (pRL-TK) and Dual-Glo luciferase assay system were purchased from Promega (Madison, WI). Rneasy Mini kit was purchased from Qiagen (Valencia, CA). MTF-1 deficient dko7 cells were a gift from W. Schaffner (ESBATech, Zurich, Switzerland); G. Andrews (University of Kansas Medical Center, Kansas City, KS) kindly provided the MTF-1 expression vector (pCMV-MTF-1-FLAG); the MRE-luciferase construct (pLuc-MCS/4MREa) was the gift of D. Giedroc (Texas A&M University, College Station, TX; Chen et al., 2004).

2.2. Cell culture procedures

Cortical cultures from embryonic day 16 Sprague–Dawley rats were prepared as described previously (Hartnett et al., 1997). Cells were plated onto poly-L-lysine-coated glass coverslips (12 mM) at a cell density of 3–5 $\times$ 10^5 cells/ml in growth medium containing DMEM, 10% F12 nutrients and 10% bovine calf serum (heat-inactivated and iron-supplemented) and maintained at 37 °C in 5% CO_2. Glial cell proliferation was inhibited at 2 weeks with 1–2 μM cytosine arabinoside, at which time the serum in the culture medium was reduced to 2%, while F12-nutrients were altogether removed. Neuron-enriched cultures were prepared as outlined earlier (Aizenman et al., 2000; McLaughlin et al., 2001): cells were plated as described above, but glial cell proliferation was inhibited after 48 h in culture with 1–2 μM cytosine arabinoside. After 3 days in vitro (DIV), the serum-containing medium was replaced with a serum-free solution comprised of Neurobasal medium, B27 supplement, and antimycotic/antibiotic mixture. Dko7 cells, derived from the homozygous knockout of the MTF-1 gene (MTF-$1^{-/-}$), were grown in DMEM supplemented with 10% FBS, 24 U/ml penicillin, 24 U/ml streptomycin and 2 mM L-glutamine.

2.3. Luciferase reporter assay

Cells were transfected with MTF-1 expression plasmid (pCMV-MTF-1-FLAG), MRE-firefly luciferase reporter (pLuc-MCS/4MREa) and *Renilla* luciferase reporter (pRL-TK). *Renilla* luciferase was used as internal, non-inducible reporter standard to monitor transfection efficiency. Neurons were transfected at 25–29 days in vitro with the plasmids (1 μg pLuc-MCS/4MREa, 0.4 μg pRL-TK and/or 0.1 μg pCMV-MTF-1-FLAG) using Lipofectamine 2000 (Pal et al., 2003). Twenty-four hours later, the cultures were exposed to Zn^{2+} and Cd^{2+} and/or NMDA and glycine for 10 min in MEM (plus 0.01% BSA and 25 mM HEPES). For transfection of the plasmids into dko7 cells, these cells were seeded into 24-well culture plate at a cell density 3.5×10^4 cells/well. The next day dko7 cells were transfected with the plasmids (300 ng pLuc-MCS/4MREa, 30 ng pRL-TK and/or 5 ng pCMV-MTF-1-FLAG) using Lipofectamine. Twenty-four hours later, the medium was replaced with DMEM supplemented with 1% FBS, and the cells were maintained for an additional 24 h. Cells were exposed to Zn^{2+} or Cd^{2+} for 30 min in MEM (plus 0.01% BSA and 25 mM HEPES). After exposure, cortical neurons and dko7 cells were incubated for 5–6 h. Luciferase activity was measured using Dual-Glo luciferase assay system. Results were expressed as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

2.4. RNA preparation and RT–PCR

Total RNA was extracted from neuron-enriched cultures, mixed cultures, rat cerebral cortex and rat liver using Rneasy Mini kit. First strand cDNA was synthesized from 2 μg total RNA using SuperScript First-Strand Synthesis System for RT–PCR. Aliquots of cDNA (1 μl) were amplified with primers specific for MTF-1 (forward primer, 5′-TGCGAGTGCACACAAAGGAGAA-3′; reverse primer, 5′-TGAGACTGTACTGAGTGCTAAA-3′ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer, 5′-GTGGACATTGTTGCCATCAACGAC-3′; reverse primer 5′-TTTCTCGTGGTTCACACCCATCAC-3′). The PCR reaction mixture (25 μl) contained 1 × PCR buffer, 1 mM $MgSO_4$, 0.3 μM each of forward and reverse primers, 0.3 mM dNTP and 1 U Platinum *Pfx* DNA polymerase. PCR was carried out as follows: 2 min at 94 °C, one cycle; 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, 30 cycles. Aliquots of the PCR mixtures were separated on 2% agarose gel (agarose-1000, Invitrogen), stained with ethidium bromide and photographed. The identity of the band was confirmed by sequencing.

2.5. Statistical analysis

All data were analyzed using one-way ANOVA, followed by ether Bonferroni or Dunnett multiple comparisons test. A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Zn^{2+} and Cd^{2+} activate MRE in cortical neurons

To determine whether Zn^{2+} and Cd^{2+} increase MRE activation in neurons, we exposed cortical neurons that had been previously transfected with MTF-1 and the MRE reporter plasmid to 100 μM Zn^{2+} or 20 μM Cd^{2+} for 10 min. Five hours after the exposure to either metal, MRE transactivation was determined by the measurement of luciferase activity (Chen et al., 2004). As shown in Fig. 2, both metals effectively activated MRE-driven luciferase expression. This result demonstrated that both metals could induce the transactivation of MRE in cortical neurons. Moreover, we found that both metals increased MRE activation in neurons that had not been transfected with the MTF-1 expression plasmid. This suggests that an endogenous MTF-1-like transcription factor is likely to be expressed in the cultured neurons.

Before we confirmed the presence of MTF-1 message in neurons, we established that MTF-1 was indeed required for MRE-driven luciferase expression in our hands. We thus examined whether Zn^{2+} could drive MRE-luciferase expression in cells lacking endogenous MTF-1. We utilized dko7 cells, which are derived from embryos with homozygous knockout of the MTF-1 gene (MTF-$1^{-/-}$; Radtke et al., 1995). Dko7 cells that had been previously transfected

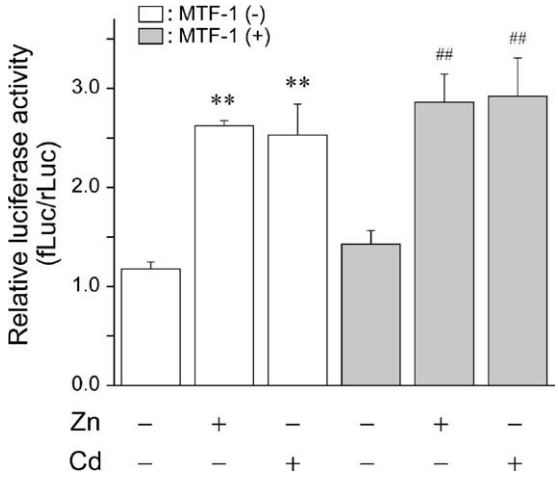

Fig. 2. Zn^{2+} and Cd^{2+} activate MRE in cortical neurons. Neurons (21–25 DIV) were transfected with pLuc-MCS/4MREa (1 μg), pRL-TK (0.4 μg) with or without pCMV-MTF-1-FLAG (0.1 μg). Twenty-four hours later, neurons were exposed to 100 μM Zn^{2+} or 20 μM Cd^{2+} for 10 min. The measurement of firefly and *Renilla* luciferase activities was carried out 5 h after the exposure to either metal. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Values represent mean ± S.E.M. ($n = 3$); $^{**}P < 0.01$ compared to control cells untransfected with MTF-1 and $^{\#\#}P < 0.01$ compared to control cells transfected with MTF-1.

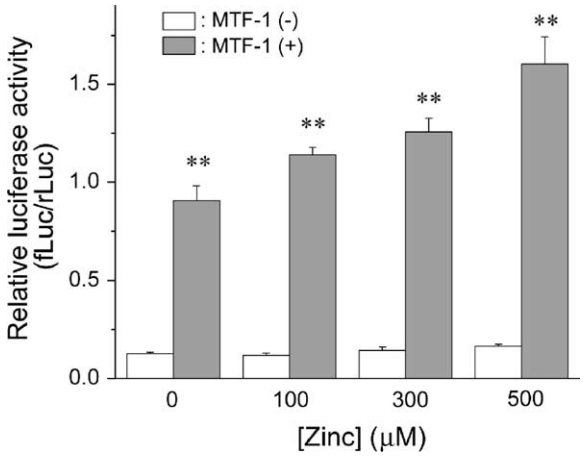

Fig. 3. Zn^{2+} induces the transactivation of MRE via MTF-1. Dko7 cells were transfected with pLuc-MCS/4MREa (300 ng), pRL-TK (30 ng) with or without pCMV-MTF-1-FLAG (5 ng). The next day the medium was replaced to DMEM supplemented with 1% FBS; cells were maintained for an additional 24 h. Cells were exposed to 100, 300 and 500 μM Zn^{2+} for 30 min. The measurement of firefly and *Renilla* luciferase activities was carried out 5 h after the exposure to Zn^{2+}. Firefly luciferase activity was normalized to the *Renilla* luciferase activity. Values represent mean ± S.E.M. ($n = 3$); $^{**}P < 0.01$ compared to control cells untransfected with MTF-1.

with either empty vector or with MTF-1 expression plasmid were exposed to various concentrations of Zn^{2+} for 30 min. As shown in Fig. 3, Zn^{2+} induced a concentration-dependent activation of MRE, but only in cells that had been co-transfected with MTF-1. In addition, the mere inclusion of MTF-1 was sufficient to induce substantial MRE activation in the absence of any exogenously-added zinc, suggesting that background levels of this metal were sufficiently elevated to trigger the reporter system. These results confirmed the fact that Zn^{2+} could activate MRE, but only when MTF-1 is present, essentially as described by prior studies (Chen et al., 2004; Heuchel et al., 1994). To rule out the possibility that cadmium activates MRE independently of MTF-1, we treated dko7 cells (transfected with only the MRE-expression vector) with various concentrations of Cd^{2+} (25–100 μM). Cd^{2+} did not induce activation of MRE in MTF-1-deficient dko7 cells (data not shown).

3.2. Expression of MTF-1 in cortical neurons

We utilized RT–PCR to determine whether MTF-1 message was present in cortical neurons in vitro. Total RNA was extracted from mixed cortical cultures (20% neurons) and also neuron-enriched cultures (>95% neurons; McLaughlin et al., 2001; Rosenberg and Aizenman, 1989). Amplified PCR products for MTF-1 message were found in both types of cultures (Fig. 4), in addition to intact rat brain and liver tissue. We confirmed by sequencing that the bands were indeed derived from MTF-1. These results indicated that endogenous MTF-1 expressed in cultured cortical neurons. Our

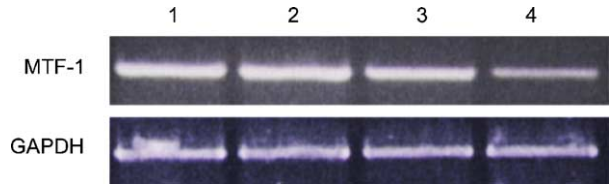

Fig. 4. RT–PCR analysis of MTF-1 mRNA expression in cultured neurons. Total RNAs were extracted from neuron-enriched (lane 1) and mixed (lane 2) cortical cultures, rat cerebral cortex (lane 3) and rat liver (lane 4) and RT–PCR was carried out according to the procedure in Section 2.

observations, however, do not rule out the possibility that MTF-1 is also expressed in cultured glia.

3.3. NMDA enhances cadmium-influx and Zn^{2+}-displacement in cortical neurons

Cd^{2+} readily permeates through the NMDA subtype of glutamate receptors (Usai et al., 1999). We thus hypothesized that MRE-driven luciferase expression would be enhanced upon NMDA exposure in the presence of Cd^{2+}, as a higher concentration of this metal could be achieved in the cytosol under these conditions. Cortical cultures transfected with MRE reporter and MTF-1 expression plasmids were exposed to 20 μM Cd^{2+} for 10 min in the absence and presence of 50 μM NMDA and 10 μM glycine. As shown in Fig. 5, NMDA enhanced Cd^{2+}-induced MRE activation, whereas NMDA alone was without a measurable effect. The enhancement of Cd^{2+}-induced MRE activation by NMDA

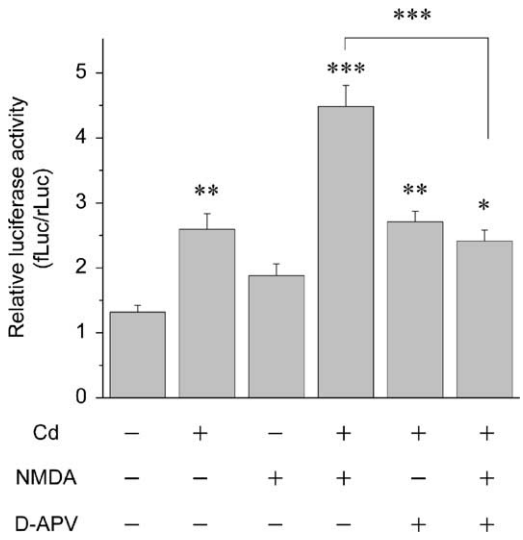

Fig. 5. NMDA enhances influx of Cd^{2+} and MRE transactivation in cortical neurons. Neurons (21–25 DIV) were transfected with pLuc-MCS/4MREa (1 μg), pRL-TK (0.4 μg) and pCMV-MTF-1-FLAG (0.1 μg). Twenty-four hours later, the neurons were pretreated with or without 50 μM D-APV for 10 min, and then exposed to 20 μM Cd^{2+} in the presence or absence of 50 μM NMDA and 10 μM glycine for 10 min ± 50 μM D-APV. The measurement of firefly and *Renilla* luciferase activities was carried out 5 h after drug exposures. Firefly luciferase activity was normalized to the *Renilla* luciferase activity. Values represent mean ± S.E.M. ($n = 3$–6); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

was completely inhibited by the presence of the competitive NMDA antagonist D-APV (50 μM). These results suggest that MRE-transactivation levels may closely reflect the degree of available displaced Zn^{2+} in the cytosol, upon its liberation from intracellular binding sites.

4. Discussion

Cd^{2+} and thiol oxidants such as peroxide have been shown to effectively activate MRE in an MTF-1-dependent fashion in non-neuronal cells or in cell-free assays (Dalton et al., 1996; Saydam et al., 2002; Zhang et al., 2003). It is generally believed, however, that MRE activation as a result of exposure to these agents is due to displacement of Zn^{2+} from intracellular binding sites (Palmiter, 1994; Zhang et al., 2003). Zn^{2+}, in turn, can directly bind to MTF-1 and promote its nuclear translocation (Chen et al., 2004; Smirnova et al., 2000), although it has also been suggested that Zn^{2+} can bind to, and inactivate a yet unidentified inhibitor of the translocation process itself (Palmiter, 1994). Nonetheless, MTF-1 translocation to the nucleus and transactivation of MRE can serve as selective molecular indicators of elevations of intracellular Zn^{2+} (Giedroc et al., 2001; Smirnova et al., 2000). An important objective of this work was to demonstrate the feasibility of using a molecular reporter for the liberation of Zn^{2+} from intracellular binding sites in primary neuronal cultures. We were interested in showing this process in neurons as we have previously observed that changes in intracellular Zn^{2+} following its liberation from intracellular sites (Aizenman et al., 2000) can result in the activation of MAPK-dependent cell death pathways (Du et al., 2002; McLaughlin et al., 2001; Pal et al., 2003). These molecular pathways may therefore be also activated, albeit indirectly, by Cd^{2+}, a highly neurotoxic metal (López et al., 2003). However, since there are no available chelators that can effectively distinguish between Cd^{2+} and Zn^{2+}, we are unable to determine at this point the relative contribution of Zn^{2+} liberation to Cd^{2+} neurotoxicity. Nonetheless, the fact that MRE transactivation can be induced by Cd^{2+} exposure indicates that Cd^{2+} can induce Zn^{2+} displacement in neurons. In addition, we show that MRE-driven luciferase expression (Chen et al., 2004) provides a sensitive assay to detect the liberation of Zn^{2+} from intracellular binding sites in primary neurons in vitro. This relatively qualitative, albeit highly sensitive assay will complement the vast array of novel fluorescent Zn^{2+} indicators that have recently become available for quantifying the intracellular concentrations of this ion (Frederickson, 2003). It must be mentioned, however, that some technical issues exist that may limit the usefulness of these fluorescent indicators as precise quantitative tools (Dineley et al., 2002). For instance, although the specificity of these dyes for heavy metals over Ca^{2+} or Mg^{2+} is fairly good, we know of no dye that can effectively distinguish between Zn^{2+} and other metals such as Cu^{2+}. Also, the relatively high intracellular concentration of indicator needed to achieve a measurable signal may effectively limit their sensitivity to small changes in intracellular Zn^{2+}. Furthermore, the high concentration of the dyes may also significantly perturb the normal handling of Zn^{2+} by the cell. Since we show that MTF-1 is normally expressed by neurons, the assay described here does not require the inclusion of an additional, potential Zn^{2+} buffer. Nonetheless, fluorescent indicators have the distinct advantage of offering spatial resolution of Zn^{2+} transients within the neuron.

Our results are also of general interest as they represent the first demonstration of MRE transactivation in a primary neuron. This result suggests that metal homeostatic elements that have been characterized in non-neuronal cells are also in play in CNS neurons. In addition, the fact that Cd^{2+} can effectively set in motion MRE activation strongly indicates that there is a high degree of bound Zn^{2+} in metal-containing proteins within neurons and that, importantly, there is little, if any, metallothionein normally present in its thionein or metal-free form in these cells. This suggests that a very delicate balance exists in intracellular Zn^{2+} homeostatic mechanisms and that a slight disruption of this balance can quickly set in motion Zn^{2+}-dependent activation of cell death pathways.

It has been reported that MTF-1 plays an important role in the regulation of gene expression associated with zinc homeostasis, including metallothionein (Giedroc et al., 2001) and the zinc transporter Znt-1 (Langmade et al., 2000). In neurons, regulation of the expression of these proteins may have important consequences on neuronal survival. For example, metallothionein overexpression has been shown to have protective effects in several models of brain injury (Giralt et al., 2002; van Lookeren Campagne et al., 1999). Tsuda et al. (1997) demonstrated accumulation of Zn^{2+} in hippocampal neurons following transient ischemia, resulting in the induction of Znt-1. Increased expression of this protein has also been shown to have neuroprotective actions against Zn^{2+} toxicity (Tsuda et al., 1997; Kim et al., 2000). Hence, as MTF-1 is essential for both basal and metal-induced expression these genes, it is important to understand the fundamental properties of MTF-1 regulation in neurons. We report here that MTF-1 is indeed expressed in neurons and that its activity can be regulated upon metal exposure.

Using fluorescent indicators, Usai et al. (1999) demonstrated that the NMDA subtype of glutamate receptors could mediate Cd^{2+} influx in mammalian neurons. In previous work, Ascher and Nowak (1988) had shown that Cd^{2+}, unlike Co^{2+} and Mn^{2+}, did not mimic the effects of Mg^{2+} in blocking the receptor-associated channel. Our observation that NMDA application can substantially enhance the effects of Cd^{2+} on MRE-driven luciferase expression further support the notion that the NMDA receptor can provide an important route of entry for this potential neurotoxic metal. This observation may have important consequences by linking excitotoxic processes to metal exposure, and may provide an important therapeutic avenue to minimize or prevent metal-induced neurodegeneration.

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