Overexpression of human metallothionein-III prevents hydrogen peroxide-induced oxidative stress in human fibroblasts

Ho Jin You[a,d,*], Kyung Jin Lee[b], Hye Gwang Jeong[c,d,*]

[a] *Department of Pharmacology, School of Medicine, Chosun University, Kwangju, South Korea*
[b] *Department of Biology, Chonnam National University, Kwangju, South Korea*
[c] *Department of Pharmacy, College of Pharmacy, Chosun University, 375 Seosuk-dong, Kwangju 501-759, South Korea*
[d] *Research Center for Proteineous Materials, Chosun University, 375 Seosuk-dong, Kwangju 501-759, South Korea*

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Abstract Metallothioneins (MT) are ubiquitous low molecular weight metal binding proteins that may act as antioxidants. In the present study, the cloned human MT-III coding region was permanently transfected into GM00637 cells in order to investigate the antioxidative effects of this brain-specific MT isoform. GM00637/MT-III cells overexpressed MT-III mRNA versus pcDNA3 plasmid-transfected control cells (GM00637). When challenged with H_2O_2, the GM00637/MT-III cells displayed significantly more resistance than the GM00637 cells, as determined by cell cytotoxicity, lactate dehydrogenase leakage, and lipid peroxidation. In addition, the GM00637/MT-III cells were highly protected from the H_2O_2-induced production of reactive oxygen species and DNA damage. These results directly support the antioxidative role of MT-III, and demonstrate MT-III can scavenge free oxygen radicals and protect cells from oxidative stress. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Metallothionein-III; Hydrogen peroxide; Antioxidant

1. Introduction

Oxygen-containing free radicals are considered to be involved in many important biological reactions. Several harmful reactions such as DNA degradation, membrane peroxidation and the destruction of endothelial cells have been attributed to oxygen-derived free radicals [1]. DNA damage by oxygen free radical species can initiate carcinogenesis or feature in the pathogenesis of neurodegenerative diseases [2]. Moreover, specific cellular enzymatic defense mechanisms, mediated by superoxide dismutase, glutathione peroxidase and catalase, have been suggested to protect from oxygen free radical species [3]. It has also been suggested that sulfhydryl-containing agents help to protect against radical-induced damage [4].

Metallothioneins (MTs) are low molecular weight (≤ 7 kDa), cysteine-rich metal binding proteins [5]. In mammalians, four members of the MT family have been identified [5,6]. MT-I/II are expressed in virtually all tissues, whereas MT-III and MT-IV are localized mainly in the central nervous system and the stratified squamous epithelia, respectively. MTs may be involved in the detoxification of potentially toxic heavy metal ions and in the homeostasis of essential trace metals [5,6]. However, growing interest has been focused on a role of MTs as antioxidants because of their high thiol content, which is exclusively involved in the formation of diamagnetic metal–thiolate clusters, and of removing metal ions such as copper and iron, which may prevent Fenton reaction [5,6]. Several studies have suggested that MT-I/II could have significant antioxidant capacity, and it has been suggested that MT-I/II provide cellular protection against various oxidative stress-induced cytotoxic injuries [5–9]. Furthermore, reports on transgenic mice that overexpress MT-I/II and MT-I/II knockout mice support such roles [10,11].

Recently, more evidence points towards the neuroprotective role of MT-III, the brain-specific member of the MT family. MT-III was found at lower levels in Alzheimer's disease brains [12,13], and it has been proposed that MTs in the brain can protect neuronal cells from the cytotoxicity of reactive oxygen species (ROS) [14,15]. In addition, it has recently been shown that mice deficient in MT-III are more susceptible to seizures induced by kainic acid and that they exhibit greater neuronal injury than normal mice [16]. These results suggest that MT-III could have a neuroprotective role; however, the mechanisms underlying such a protective role are unknown. Moreover, MT's proposed protective role as a free radical scavenger may be of relevance in brain neurological diseases. Therefore, MT-III, which is a unique protein, warrants further study to further define its antioxidant nature and its involvement in neuroprotection. The present study was undertaken to determine whether brain-specific MT-III, as a neuroprotective agent, can protect from oxidative stress. For this purpose, we developed a stable human MT-III-overexpressing cell line, GM00637/MT-III, which shows reduced cytotoxic and nuclear DNA oxidative damage when induced by H_2O_2.

2. Materials and methods

2.1. Materials

Chemicals and cell culture materials were obtained from the following sources: H_2O_2, Earle's minimum essential medium (EMEM), trypsin, G418, fetal bovine serum, and penicillin–streptomycin solution from Life Technologies; and dichlorodihydrofluorescein diacetate from Molecular Probes. Other chemicals were of the highest commercial grade available.

*Corresponding authors. Fax: (82)-62-230 6639.
E-mail address: hgjeong@mail.chosun.ac.kr (H.J. You).

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2.2. Cell culture

The human fibroblast GM00637 cell line was purchased from the Coriell Institute for Medical Research and maintained in EMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator.

2.3. Overexpression of MT-III

The human MT-III coding region was amplified from human cerebral cortical cDNA (Clontech, USA) by PCR. A sense primer including the *Eco*RI restriction site (5′-AGCAGAATTCATGGACCCTGAGACCTGCCCCTGC-3′) and a reverse primer with the *Not*I restriction site (5′-GCC GCGGCCGCTCACTGGCAGCAGCTGCACTTCTC-3′) were designed to amplify MT-III cDNA [17]. A single PCR product was inserted in pcDNA3 plasmid (Invitrogen) and sequenced by the dideoxynucleotide chain termination procedure. Cells transfected with this plasmid are referred to as GM00637/MT-III. Cells were transfected with the pcDNA3/MT-III plasmid or pcDNA3 plasmid using a gene pulser transfection apparatus (Bio-Rad). Forty-eight hours after transfection, the cells were incubated with complete medium containing 400 μg/ml of G418 for 5 weeks. Cell clones resistant to G418 were isolated and analyzed.

2.4. MT-III mRNA measurement by RT-PCR

RNA was extracted using a total RNA separator kit, treated with DNase I and reverse transcribed into cDNA before PCR was performed, as previously described [18]. The sense and reverse primers described above without restriction sites were used to amplify human MT-III cDNA. RT-PCR of human MT-III mRNA was performed as previously described [18].

2.5. Cell cytotoxicity

Cell cytotoxicity was assessed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay kit and a lactate dehydrogenase (LDH) assay kit (Roche), according to the manufacturer's instructions.

2.6. Lipid peroxidation

Lipid peroxidation in cells was determined using the thiobarbituric acid-reactive substances (TBARS) reaction with malondialdehyde and related compounds as previously described [19]. Lipid peroxidation was expressed as TBARS nmol/mg protein.

2.7. Intracellular production of ROS

The fluorescent probe dichlorodihydrofluorescein diacetate was used to monitor the intracellular generation of ROS by H_2O_2 [20].

2.8. Comet assay

Oxidative DNA damage was evaluated using the Comet assay. Culture medium was aspirated from the cell monolayer and cells were then exposed to different concentrations of H_2O_2 in phosphate-buffered saline (PBS) for 5 min on ice. Following oxidant exposure, cells were washed twice with ice-cold PBS. The cells were then detached from the culture dishes and processed for the Comet assay as previously described [21]. To quantitate the Comet assay, ethidium bromide-stained nucleoids were examined under a Nikon fluorescence microscope using Image-Pro 4.5 software (Media Cybernetics). One hundred comets per slide were visually scored according to the amount of DNA present in the tail. Under these conditions the tail moment Tm = [(fluorescence intensity of the tail)/(fluorescence intensity of the head) × tail length] reflected DNA damage.

2.9. Statistical analyses

All experiments were repeated at least three times. Student's *t*-test was used to assess the statistical significance of differences. A confidence level of < 0.05 was considered significant.

3. Results

3.1. Metallothionein overexpression

Clones of GM00637 cells permanently expressing the human MT-III coding region were obtained, and confirmed by RT-PCR. Fig. 1 shows the results of a representative clone. PCR amplification of the pcDNA3 plasmid containing the MT-III coding region and RT-PCR amplification of GM00637/MT-III cells yielded a single band of approximately 207 bp (lanes 3 and 4). No amplification was observed in samples of GM00637/pcDNA3 (GM00637) cells (lanes 1 and 2). GM00637 and GM00637/MT-III cell growths were similar (data not shown).

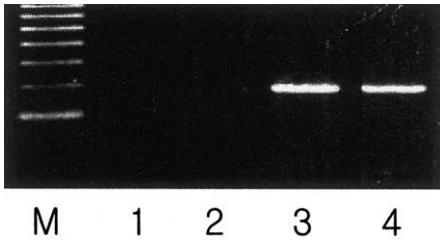

Fig. 1. Expression of human MT-III mRNA in stably transfected GM00637/MT-III cells. The cells were lysed and total RNA was prepared for RT-PCR analysis of gene expression. The PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. One of three representative experiments is shown. Lane M, 100 bp DNA ladder; lanes 1 and 2, GM00637 cells; lanes 3 and 4, GM00637/MT-III cells.

3.2. Cell cytotoxicity

Sensitivity of GM00637 and GM00637/MT-III cells to H_2O_2-mediated cytotoxicity was quantified by the MTT assay. The overexpression of MT-III was associated with resistance to H_2O_2 (Fig. 2A). The LC_{50} of GM00637/MT-III cell to H_2O_2 was two- to three-fold greater than that of GM00637 cells. To more precisely quantify the different resistances of the transfected cells to H_2O_2, we performed LDH assays, and it was found that consistent with the MTT results GM00637/MT-III cells withstood higher H_2O_2 doses than GM00637 cells (Fig. 2B). These results show that GM00637/MT-III cells were less sensitive to the cytotoxic effects of H_2O_2 than GM00637 cells.

3.3. Levels of lipid peroxidation

TBARS were used to determine the antioxidant properties of MT-III against H_2O_2-induced lipid peroxidation. No significant differences in the basal lipid peroxidation levels were found in these cells. In the GM00637 cells, lipid peroxidation levels were found to gradually increase with time, whereas lipid peroxidation levels in GM00637/MT-III cells increased 3 h after exposure to H_2O_2 (Fig. 2C). Moreover, lipid peroxidation levels in the GM00637 cells were significantly higher than those of the GM00637/MT-III cells at 2, 3 and 6 h. Accordingly, MT-III accumulation in GM00637/MT-III cells might increase resistance to oxidative stress.

3.4. Intracellular ROS production

Believing that differences in intracellular MT-III content affect defense against oxidative stress, we further evaluated the antioxidative properties of MT-III against H_2O_2. Intracellular ROS production was assessed by monitoring dichlorodihydrofluorescein (DCF) fluorescence. Prior to H_2O_2 addition, no difference was seen between the DCF signals of GM00637 and GM00637/MT-III. Rapid increases in intracellular oxidant levels were noted in both cell types after H_2O_2 treatment, as assessed by increased DCF fluorescence, but the oxidant burden after H_2O_2 exposure was greater in the GM00637 cells (Fig. 2D). These results demonstrate that the

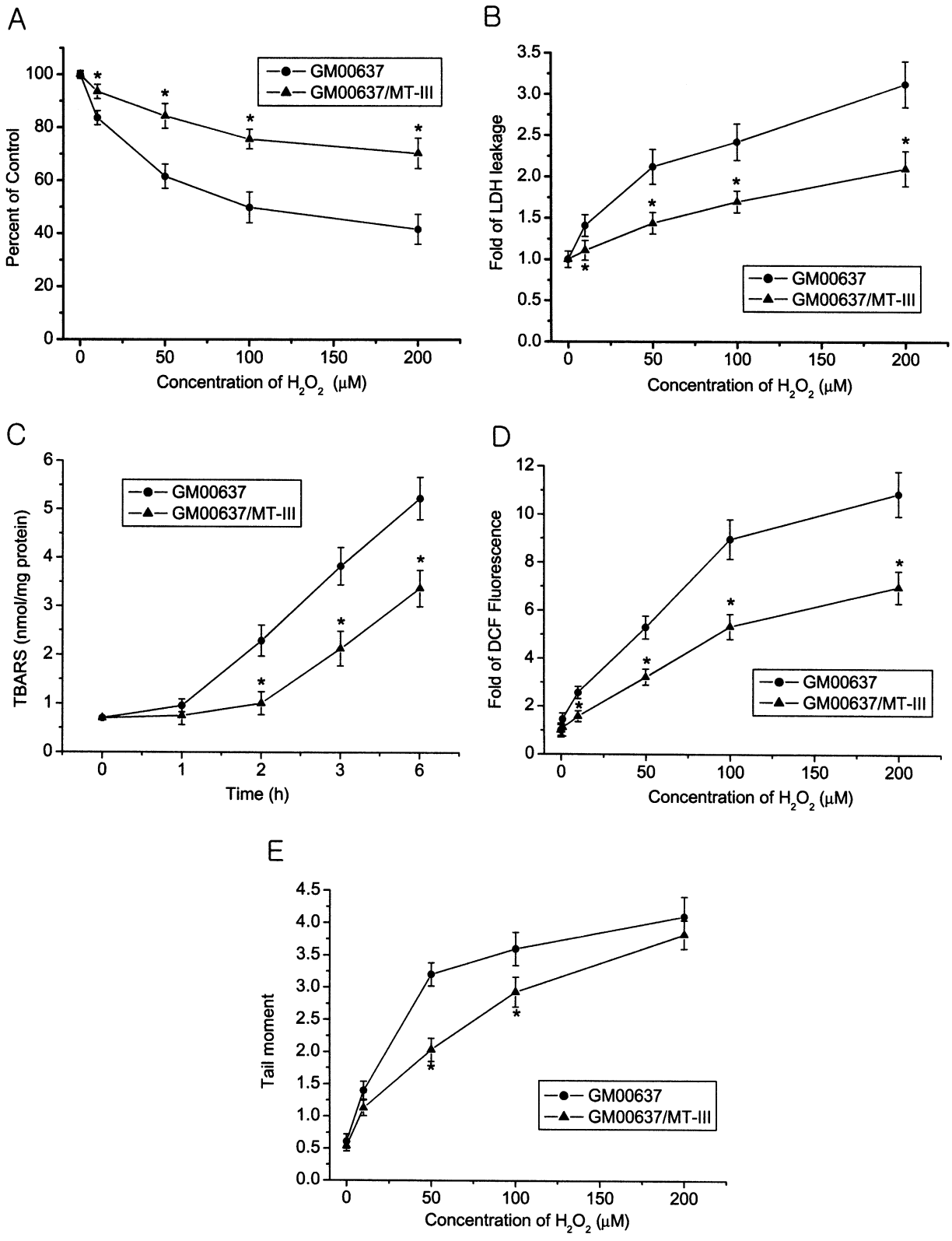

Fig. 2. The cells were treated with different concentrations of H_2O_2 for 3 h. Medium containing H_2O_2 was replaced with fresh medium without H_2O_2, and cellular cytotoxicity was measured using a MTT-based colorimetric assay kit (A) and LDH activity measured using a LDH kit (B). The cells were treated with 100 μM of H_2O_2 for 6 h and the extent of lipid peroxidation in the cells was determined by the TBARS reaction (C). The cells were treated with 10 μM of dichlorodihydrofluorescein diacetate for 10 min and the medium was replaced by fresh medium containing different concentrations of H_2O_2. After 15 min of treatment, intracellular ROS were measured by monitoring fluorescence increases (D). The cells were treated with different concentrations of H_2O_2 for 5 min and oxidative DNA damage was evaluated using the Comet assay (E). Each value represents the mean ± S.D. of three independent experiments, performed in triplicate. $^*P < 0.05$, significantly different from the GM00637 cells.

GM00637/MT-III cells had significantly higher antioxidant activity than the GM00637 cells.

3.5. Oxidative DNA damage

The Comet assay was used to assess the effects of MT-III overexpression on oxidative DNA damage. The Comet assay detects single- and double-stranded DNA breaks in naked supercoiled DNA. Strand breaks cause supercoiled DNA to relax, allowing loops of DNA to migrate toward the anode upon electrophoresis, forming a 'comet tail'. H_2O_2 (50 μM) treatment increased three-fold the tail moments in GM00637 cells versus GM00637/MT-III cells, indicating a three-fold increase in oxidative DNA damage (Fig. 2E).

4. Discussion

Oxidative stress is the result of an imbalance between the production of ROS and the ability of the cellular antioxidant defense systems. Cells have developed elaborate networks to deal with potentially damaging radicals, and these include enzymatic and non-enzymatic antioxidative systems. There is increasing evidence that MTs can function as effective antioxidants [5,6]. Therefore, we were interested to determine the effect of MT-III on oxidative stress in cells that constitutively express MT-III. Thus, we cloned the coding region of human MT-III and permanently transfected it into GM00637 cells. RT-PCR results demonstrated the specific expression of human MT-III mRNA in GM00637/MT-III cells; however, no transcript was found in reverse transcribed RNA samples of pcDNA3 plasmid transfected GM00637 cells (Fig. 1).

H_2O_2 is particularly attractive as a model oxidant because its cellular actions and its fate have been well studied [22]. H_2O_2 readily crosses the cellular membranes, and gives rise to the highly reactive hydroxyl radicals, which have the ability to react with macromolecules, including DNA, proteins, and lipids, and to ultimately damage a cell. Lipid peroxidation is considered to be an indirect measure of ROS generation. Previous studies have shown that cells overexpressing mouse MT-I were resistant to *tert*-butyl hydroperoxide and prevented lipid peroxidation by scavenging peroxyl radicals [10]. The present study demonstrates a lower production of TBARS in GM00637/MT-III cells than in GM00637 cells treated with H_2O_2, and shows that overexpressed MT-III inhibits H_2O_2-induced lipid peroxidation in GM00637/MT-III cells (Fig. 2C). Despite the obvious differences in TBARS, malondialdehyde generated from non-lipid sources and other endogenous aldehydes can interfere with the TBARS assay. Therefore, we determined DCF fluorescent changes after H_2O_2 treatment as an additional sensitive means of detecting intracellular ROS. It was observed that the levels of ROS in GM00637/MT-III cells were lower than in GM00637 cells after H_2O_2 exposure (Fig. 2D). Taken together, these results suggest a protective and an antioxidant role for MT-III in cells versus free radical toxicity.

DNA damage and its subsequent repair were assessed by the Comet assay (i.e. single-cell gel electrophoresis), a procedure that detects DNA strand breaks in individual cells resulting from events such as direct scission of the DNA backbone by free radical attack. As shown in Fig. 2E, H_2O_2 exposure induced a higher level of DNA damage in GM00637 cells than in GM00637/MT-III cells. These results suggest that the reduction in the DNA damage inflicted by H_2O_2 in GM00637/MT-III cells is more likely to be related to elevations in MT-III, which is able to react with and detoxify ROS directly.

MTs can be found in the cytoplasm and/or the nucleus and their subcellular distribution may be critical for determining their antioxidizing function [23]. The nuclear localization of MT-I/II in cells has been shown and is reported to be of importance in protecting DNA from oxidant-induced damage [8]. The present study shows that GM00637/MT-III cells are resistant to the cytotoxic (Fig. 2A–C) and DNA-damaging (Fig. 2E) effects of H_2O_2. Although we did not determine the cellular distribution of MT-III expression in the cell after gene transfer, we deduce that MT-III is present within both the nucleus and the cytoplasm from the protective effect shown by GM00637/MT-III cells against the cytotoxic effect and the DNA strand breakage caused by H_2O_2.

The mechanism of the antioxidant effects of the MTs remains unclear; however, several lines of evidence support the hypothesis that the increased expression of MTs provides protection against ROS [10,24]. It is suggested that MT-I/II can function as antioxidants by increasing zinc and copper levels in cells, which may activate antioxidant enzymes such as superoxide dismutase [25]. Since MTs have a high cysteine content, they can freely exchange metals with electrophiles, and thus MTs function as antioxidants. Indeed, metal-free MTs (thioneins) can then bind redox-active metals such as Fe or Cu, and prevent Fenton reactions, or react directly with H_2O_2 or hydroxyl radicals, and thus scavenge ROS before they can react with DNA. It has also been demonstrated that the in vitro oxidation of MT-I/II cysteines induces metal release [26], and that H_2O_2 can react directly with MT-I/II sulfhydryl groups [27]. Furthermore, in an in vitro study, the efficiency of the protective effect of MT-I/II against DNA degradation from oxidative damage was much higher than that of reduced glutathione [9]. The primary mode of free radical attack of MT-I/II could be via its sulfhydryl groups, although the detailed modes of action of MT-I/II, as an antioxidant, are not known. In the present study, when GM00637/MT-III cells were cultured in the presence of H_2O_2, MT-III was expressed at a high level in the cells, thus affording strong protection against oxidative stress. It is plausible that MT-III protects cells by sharing the mechanisms described above for MT-I/II. Moreover, the MT-III sequence can be aligned with those of MT-I/II so that the position of all 20 constituent cysteine residues is completely conserved [5,6].

Oxidative stress can damage both cells and tissues by generating free radicals. In the brain, oxygen free radicals are constantly produced and these can damage brain cells. There are several reports that MTs are associated with certain neurological diseases such as Alzheimer's disease and Parkinson's disease [12,28]. Moreover, it has been shown that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a neurotoxic agent that can cause Parkinson's disease, can generate free radicals and also induce MTs [28]. MT-III has many possible roles in brain neurological disease, for instance, MT-III is depleted in the cortical regions of individuals with Alzheimer's disease [12,13] and MT-III regulation has been studied in several animal models of brain damage [14,15]. Alterations in the expression, content, or function of MT-III could, therefore, be involved in some neurodegenerative situations and changes in the levels of MTs may be important in the pathology of several neurological diseases. The results reported here support this hypothesis

and suggest that the antioxidative role of MT-III underlies its neuroprotective effect. Further study is required to fully understand the exact role of MT-III in brain function, and the antioxidative characteristics of MT-III required to counteract the effect of free radicals. However, the present study provides, for the first time, evidence that MT-III can protect cells from the oxidative damage caused by H_2O_2, and demonstrates an antioxidant role for MT-III in mammalian cells. This finding further extends our understanding of the obvious neuroprotective implications of brain specific MT-III.

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References

[1] Brown, R.K., Mcburney, A., Lunec, J. and Kelly, F.J. (1995) Free Radical Biol. Med. 18, 801–806.
[2] Gotz, M.E., Kunig, G., Riederer, P. and Youdim, M.B.H. (1994) Pharmacol. Ther. 63, 37–122.
[3] Michiels, C., Raes, M., Toussaint, O. and Remacle, J. (1994) Free Radical Biol. Med. 17, 235–248.
[4] Rhee, S.G., Kim, K.H., Chae, H.Z., Yim, M.B., Uchida, K., Netto, L.E. and Stadtman, E.R. (1994) Ann. NY Acad. Sci. 738, 86–92.
[5] Vasak, M. and Hasler, D.W. (2000) Curr. Opin. Chem. Biol. 4, 177–183.
[6] Palmiter, R.D. (1998) Proc. Natl. Acad. Sci. USA 95, 8428–8430.
[7] Abel, J. and de Reuiter, N. (1989) Toxicol. Lett. 47, 191–196.
[8] Chubatsu, L.S. and Meneghini, R. (1993) Biochem. J. 291, 193–198.
[9] Miura, T., Muraoka, S. and Ogiso, T. (1997) Life Sci. 60, 301–309.
[10] Schwarz, M.A., Lazo, J.S., Yalowich, J.C., Reynolds, I., Kagan, V.E., Tyurin, V., Kim, Y.-M., Watkins, S.C. and Pitt, B.R. (1994) J. Biol. Chem. 269, 15238–15243.
[11] Lazo, J.S., Kondo, Y., Dellapiazza, D., Michalska, A.E. and Choo, K.H.A. (1995) J. Biol. Chem. 270, 5506–5510.
[12] Uchida, Y., Takio, K., Titani, K., Ihara, Y. and Tomonaga, M. (1991) Neuron 7, 337–347.
[13] Yu, W.H., Lukiw, W.J., Bergeron, C., Niznik, H.B. and Fraser, P.E. (2001) Brain Res. 894, 37–45.
[14] Yuguchi, T., Kohmura, E., Yamada, K., Sakaki, T., Yamashita, T., Otsuki, H., Kataoka, K., Tsuji, S. and Hayakawa, T. (1995) J. Neurotrauma 12, 299–306.
[15] Acarin, L., Carrasco, J., Gonzalez, B., Hidalgo, J. and Castellano, B. (1999) J. Neuropathol. Exp. Neurol. 58, 389–397.
[16] Erickson, J.C., Hollopeter, G., Thomas, S.A., Froelick, G.J. and Palmiter, R.D. (1997) J. Neurosci. 17, 1271–1281.
[17] Palmiter, R.D., Findley, S.D., Whitmore, T.E. and Durnam, D.M. (1992) Proc. Natl. Acad. Sci. USA 89, 6333–6337.
[18] Amoureux, M.C., Wurch, T. and Pauwcls, P.J. (1995) Biochem. Biophys. Res. Commun. 214, 639–645.
[19] Sunderman, F.W., Marzouk, A., Hopfer, S.M., Zaharia, O. and Reid, M.C. (1985) Ann. Clin. Lab. Sci. 15, 229–236.
[20] Benov, L., Sztejnberg, L. and Fridovich, I. (1998) Free Radical Biol. Med. 25, 826–831.
[21] Johnson, M.K. and Loo, G. (2000) Mutat. Res. 459, 211–218.
[22] Halliwell, B., Clement, M.V. and Long, L.H. (2000) FEBS Lett. 486, 10–13.
[23] Cherian, M.G. (1994) Environ. Health Perspect. 102, 131–133.
[24] Aschner, M. (1998) Neurotoxicology 19, 653–660.
[25] Lazo, J.S. and Pitt, B.R. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 635–653.
[26] Maret, W. and Vallee, B. (1998) Proc. Natl. Acad. Sci. USA 95, 3478–3482.
[27] Quesada, A.R., Byrnes, R.W., Krezoski, S.O. and Petering, D.H. (1996) Arch. Biochem. Biophys. 334, 241–250.
[28] Ebadi, M., Hiramatsu, M., Kumari, M.V.R., Hao, R. and Pfeiffer, R.F. (1999) in: Metallothionein IV (Klaassen, C.D., Ed.), pp. 341–349, Birkhauser, Basel.