Cytotoxicity of Nitric Oxide Is Alleviated by Zinc-Mediated Expression of Antioxidant Genes

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Metallothioneins (MTs) are small, cysteine-rich zinc binding proteins that are powerful antioxidants. In this study, we investigated the interaction between zinc, MTs, and other components of the antioxidant defense system in HepG2 cells. Cells were preincubated with zinc and then exposed to sodium nitroprusside (SNP), a nitric oxide (NO) donor. Both zinc pretreatment and SNP exposure separately induced transcription of MT genes (MT1A, MT2A, MT1E, MT1X), as measured using real time–polymerase chain reaction (PCR) after reverse transcription (RT). Pretreatment of HepG2 cells with zinc sulfate (ZnSO$_4$) followed by SNP exposure caused MT and glucose-6-phosphate dehydrogenase (G6PD) mRNA levels to increase more than in cells only exposed to SNP. However, when cells were incubated with N,N,N′,N′-tetrakis(2-pyridylmethyl)ethyl-enediamine (TPEN), a membrane-permeant Zn^{2+} chelator, the stimulation of MT transcription by SNP was blocked, suggesting that SNP-induced upregulation of these genes is zinc-dependent. Human glutathione-S-transferase (hGSTA1) and G6PD mRNA levels in the cells treated with 5 μM TPEN decreased. Additionally, the induction of MT by SNP after zinc pretreatment appears to be mediated by metal-activated transcription factor-1 (MTF-1), which is induced by labile zinc in the cytosol. SNP cytotoxicity was inhibited by preincubation with zinc. Taken together, these results suggest that NO plays an important role in regulation of cellular zinc homeostasis and that NO-mediated release of protein-bound Zn^{2+} may be an important signal in antioxidant defense. Exp Biol Med 231:1555–1563, 2006

Key words: metallothioneins; antioxidant gene; zinc; sodium nitroprusside; N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine

Introduction

Cells utilize a variety of strategies to ensure survival under adverse conditions. One of these strategies involves immediate upregulation of genes that encode antioxidant proteins (1) in response to exposure to reactive oxygen species (ROS). For example, the metallothioneins (MTs), a family of proteins with antioxidant activity (1), are upregulated in response to zinc and oxidative stress (e.g. elevated hydrogen peroxide [H$_2$O$_2$]).

In their reduced forms, the sulfhydryl-rich MTs form a complex with Zn^{2+}, which is released when MTs are oxidized. Zinc induces MT transcription by binding to metal-activated transcription factor-1 (MTF-1). MTF-1 bound to labile intracellular Zn^{2+} interacts with metal responsive elements (MRE) in the 5' regulatory region of MT genes (2, 3). However, MTF-1 plays a wider role than just mediating transcription of MT. Among the confirmed MTF-1 target genes are MTF-1 itself, ZnT-1 (a Zn^{2+} transporter also known as slc30A1), glutathione-S-trans-ferase (GST), and glucose-6-phosphate dehydrogenase (G6PD) (4–8). GSTs represent a major group of detox-ification enzymes (9). In humans, hGSTA1 is one of the most important detoxification proteins (10), and is the most abundant GST isoenzyme in liver and kidney (11). G6PD is essential to the maintenance of a supply of reducing power in the form of nicotinamide adenine dinucleotide phosphate (NADPH) (12), which, as the reducing equivalent for all major ROS-producing and detoxifying pathways, plays a central role in cellular metabolism of ROS (13, 14). NADPH is also required for the enzymatic restoration of reduced glutathione (GSH) by GSH reductase (15). GSH, in turn, is used by GSH peroxidases to convert H$_2$O$_2$ and organic peroxides into water or alcohols. In addition to GST and G6DP, a large number of other genes with antioxidant functions possess multiple MREs in their 5' flanking regions (2, 4).[1] Thus, it is possible that the well-known protective

[1] N. Hill, P. Cunningham, C. Hogstrand and P. Kille, unpublished data, October 3, 2003.

effect of zinc against oxidative damage is largely a result of zinc-stimulated gene expression.

Nitric oxide (NO) is a free radical species that can react with superoxide anion ($O_2^{\cdot-}$) to generate peroxynitrite ($ONOO^-$), which induces apoptosis by increasing cellular oxidative stress, mitochondrial cytochrome-c release, and DNA damage (16). NO nitrosates thiol groups of MTs *in vitro* (17), and release of high levels of NO is associated with cell death (18). The damaging effects of NO are countered by NO-induced release of bound zinc (Zn) (19) or cadmium (Cd) (20) ions from MTs and perhaps other intracellular stores. The mobilization of zinc by NO has been described by Katakai *et al.* (19) and their data indicate that MT-1 levels may increase inside the cell following zinc release.

In the present study, we examined the interaction between zinc and the antioxidant defense system in HepG2 human hepatoma cells, using sodium nitroprusside (SNP) as a slow-release NO donor. The objectives of this research were 3-fold: (i) to establish whether key antioxidant genes are induced by zinc and SNP, (ii) to determine whether ROS-responsive mammalian antioxidant genes with multiple MREs are dependent on zinc and MTF-1 for activation by ROS exposure, and (iii) to assess if induction of antioxidant genes by zinc offers protection against oxidative stress. The overall aim of this work is to provide evidence for a role of zinc as an intracellular signal for oxidative stress.

Materials and Methods

Chemicals. Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), liquid gentamicin reagent solution, penicillin and streptomycin (PEST), and trypsin (T)-EDTA were purchased from Join Bio-Innovation (Seoul, Korea). Falcon tissue culture plates were obtained from Becton Dickinson (Franklin Lakes, NJ). FluoZin-3 AM was obtained from Molecular Probes, Inc. (Eugene, OR). Powerscript Reverse Transcriptase was obtained from Clontech (Palo Alto, CA). An oligo(dT)$_{15}$ primer and random hexamers were obtained from Promega (Madison, WI). Most other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

The membrane permeable zinc chelator N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and SNP were purchased from Sigma Chemical. TPEN was stored as a 25 mM stock solution in dimethyl sulfoxide (DMSO) at −20°C and added to cultures at the appropriate dilution. SNP was diluted in DMEM immediately before use.

Cell Culture. Human hepatoma cells (HepG2) were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were seeded in 6-well Falcon plates at 1×10^6 cells/ml in DMEM supplemented with 10% FBS, 1% liquid gentamicin reagent solution, and 1% PEST. They were cultured at 37°C in a humid atmosphere containing 5% CO_2

until 60%–80% confluent and then used in RT-PCR or real time PCR assays.

HepG2 cells were seeded in 24-well Falcon plates at 1 × 10^4 cells/ml and grown for 48 hrs and then used in the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Culture medium was replaced on alternate days, and cells were kept in medium free of serum and antibiotics during treatment.

Treatments. In experiments in which the effects of zinc and the membrane-permeant zinc chelator TPEN on cell viability were examined using the MTT assay, HepG2 cells were incubated in fresh DMEM with or without experimental additives. Cells were exposed to zinc sulfate (ZnSO$_4$; 0, 50, 100, and 200 μM) alone, to TPEN (0 and 50 μM) alone for 24 hrs, to DMEM containing SNP (0, 2, and 4 mM) after pretreatment with ZnSO$_4$ (0–200 μM) for 24 hrs, or TPEN (0 and 5 μM) and SNP (0, 2, and 4 mM) together for 24 hrs.

In cultures used in reverse transcriptase–polymerase chain reaction (RT-PCR) or real time PCR assays, HepG2 cells were pretreated with 100 μM ZnSO$_4$ in DMEM medium for 24 hrs. The zinc-containing medium was then removed, and cells were exposed to fresh DMEM containing 2 mM SNP (for 12 hrs). Cells in 2 mM SNP were incubated for an additional 12 hrs. HepG2 cells were treated with 2 mM ZnSO$_4$ together with 5 μM TPEN for 12 hrs. Controls consisted of (i) untreated cells, (ii) cells treated with ZnSO$_4$ only, and (iii) cells treated with SNP only.

RNA Extraction and RT-PCR or Real Time PCR. Total RNA was extracted from cells using the TRI REAGENT LS kit (Sigma Chemical) according to the manufacturer's protocol. Total RNA (2 μg) was reverse-transcribed into cDNA using PowerScript Reverse Transcriptase (Clontech) according to the manufacturer's protocol, using an oligo(dT)$_{15}$ primer and random hexamers provided by Promega. The resulting volume of cDNA was 20 μl.

PCR primers were designed based on published nucleotide sequences and are listed in Table 1. DNA sequences for MT-1A, MT-2A, MT-1E, MT-1X, MTF-1, ZnT-1, and 18S rRNA are from Hasumi *et al.* (21), and sequences for hGSTA1 and G6PD are from Xiang *et al.* (11) and Riganti *et al.* (15), respectively. RT-PCR was performed according to the method described by Chung *et al.* (22). PCR with G6PD and GSTA1 as templates began with incubation at 95°C for 15 mins, followed by 25 cycles at 94°C for 30 secs and 60°C for 30 secs, and final extension at 72°C for 30 secs. PCR using 18S rRNA templates began with incubation for 10 mins at 95°C, followed by 10 cycles of 30 secs at 94°C, 30 secs at 60°C, and 30 secs at 72°C. Final extension was carried out for 5 mins at 72°C.

PCR products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide under UV light. The intensities of the bands were analyzed and quantified by SigmaGel software (Jandel Scientific, San Rafael, CA). The intensities of 18S rRNA transcripts were used as internal

Table 1. Sequences of PCR Primers

Gene	Primer	Sequence[a]
MT1A	forward	CTCGAAATGGACCCCAACT
	reverse	ATATCTTCGAGCAGGGCTGTC
MT2A	forward	CCGACTCTAGCCGCCTCTT
	reverse	GTGGAAGTCGCGTTCTTTACA
MT1E	forward	GCTTGTTCGTCTCACTGGTG
	reverse	CAGGTTGTGCAGGTTGTTCTA
MT1X	forward	TCTCCTTGCCTCGAAATGGAC
	reverse	GGGCACACTTGGCACAGC
ZnT-1	forward	AATACCAGCAACTCCAACGG
	reverse	ATCACTGAACCCAAGGCATC
MTF-1	forward	CTTTCTGTTGTTGCTGGGGC
	reverse	ATGCCTCTTCTTGTTTGATGATG
hGSTA1	forward	TCCATATGGCAGAGAAGCCCAAGC
	reverse	CCGAATTCTCCATGACTGCGTTATTA
G6PD	forward	CCGGATCGACCACTACCTGGGCAAG
	reverse	GTTCCCCACGTACTGGCCCAGGACCA
18S rRNA	forward	CGGCTACCACATCCAAGGAA
	reverse	GCTGGAATTACCGCGGCTGC

[a] Primers are written $5' \rightarrow 3'$.

controls. The identities of PCR products were verified by DNA sequencing.

Real-time PCR was performed with 12.5 μl iQ SYBR Green Supermix from Bio-Rad (Hercules, CA), 0.5 μl each primer (15 μM), and 1 μl cDNA. The final volume of the reaction mixture was 25 μl. Real-time PCR using the MT1A, MT2A, MT1E, and MT1X templates was performed in one cycle of 3 mins at 95°C followed by 40 cycles of 30 secs at 94°C, 30 secs at 60°C, and 30 secs at 72°C. A final extension was carried out for 5 mins at 72°C. Following amplification, a melting curve of amplified DNA was analyzed at temperatures between 54°C and 95°C. All real-time PCRs were performed in an iCycler iQ (Bio-Rad). During the primer extension step, the increase in fluorescence from the amplified DNA was recorded using the SYBR Green optic channel set at a wavelength of 490 nm.

Fluorescence Imaging of Free Intracellular Zinc by FluoZin-3 AM Staining. Cells were seeded at 1×10^5 cells/ml in 6-well Falcon plates (2 ml/well) and grown for 1 or 7 days. Cells were serum-starved for 6 hrs and then treated with the cell-permeant Zn^{2+}-selective probe FluoZin-3 AM (Molecular Probes; 4 μM) at 37°C for 30 mins in DMEM (23). Cells were washed in DMEM to remove nonspecifically bound FluoZin-3 and then incubated for an additional 40 mins to complete the intracellular cleavage of the acetoxymethyl (AM)-ester. During this time, ZnSO$_4$ or ZnSO$_4$ plus SNP were added. Samples were observed under a Zeiss fluorescence microscope (Carl Zeiss, Munchen-Hallbergmoos, Germany) with excitation at 494 nm and emission at 516 nm. Images were obtained at ×20 magnification with a Zeiss digital camera.

DNA Fragmentation Assay. DNA isolation was performed according to the TRI REAGENT LS (Sigma

Chemical) protocol. The extracted DNA content was measured spectrophotometrically. Isolated DNA samples were analyzed electrophoretically on a 1.2% agarose gel containing 0.2 mg/ml ethidium bromide at 95 V for 1 hr. Bands were visualized under UV light.

Cell Viability Measurements. Cell viability was assessed with the MTT assay according to the method described in Chung *et al.* (22). In brief, mitochondrial dehydrogenase activity was estimated using MTT as a substrate. Data are expressed as a percentage, with untreated cells equal to 100%.

Statistical Analyses. Each experiment was performed a minimum of three times. One-way analysis of variance (ANOVA) followed by Tukey's test was used to compare the results from different treatments. A Student's t test was used for comparisons between two groups. Statistical significance was set at $P < 0.05$. Data are reported as the mean $\pm$ SD.

Results

Effect of Zinc and TPEN on Expression of Antioxidant Genes (MT1A, MT2A, MT1E, MT1X, hGSTA1, and G6PD) in Response to Oxidative Stress. When HepG2 cells were treated for 12 hrs with 2 mM SNP or pretreated for 24 hrs with 100 μM ZnSO$_4$, a significant increase in the levels of MT1A, MT2A, MT1E, and MT1X mRNA was observed ($n = 3$, $P < 0.05$) (Fig. 1). Treatment with 100 μM ZnSO$_4$ also increased the abundance of G6PD mRNA by 1.6-fold above the control (results not shown). Treatment with ZnSO$_4$ prior to exposure to 2 mM SNP for 12 hrs resulted in significantly higher levels of MT1A, MT2A, MT1E, and MT1X mRNA (Fig. 1) and G6PD mRNA (results not shown) than with ZnSO$_4$ or SNP treatment alone. Neither SNP nor zinc separately or in combination changed expression of hGSTA1 mRNA (results not shown). However, treatment with the zinc chelator, TPEN, drastically reduced basal transcript levels for hGST1 (Fig. 2). TPEN also completely abolished the induction of G6PD (Fig. 2) and MTs (Fig. 3) in response to SNP, suggesting that this is a zinc-dependent effect.

NO Leads to an Increase in Labile Intracellular Zinc. Because the above results suggested that NO leads to mobilization of intracellular zinc, labile intracellular zinc was measured using fluorescence imaging with the cell-permeant fluorescent zinc probe, FluoZin-3 AM (23) after 1 and 7 days in cultured cells. After loading with 4 μM FluoZin-3 AM, untreated cells exhibited no fluorescence (Fig. 4, A-1 and B-1), whereas cells treated with 100 μM ZnSO$_4$ exhibited bright fluorescence at days 1 and 7 (Fig. 4, A-2 and B-2). Treatment with 100 μM ZnSO$_4$ plus 2 mM SNP caused an increase in fluorescence at days 1 and 7 (Fig. 4, A-3 and B-3). Thus, SNP exposure caused release of zinc from the intracellular pools.

Induction of MTF-1 Transcription by SNP and Zinc. To investigate the role of MTF-1 in zinc-mediated
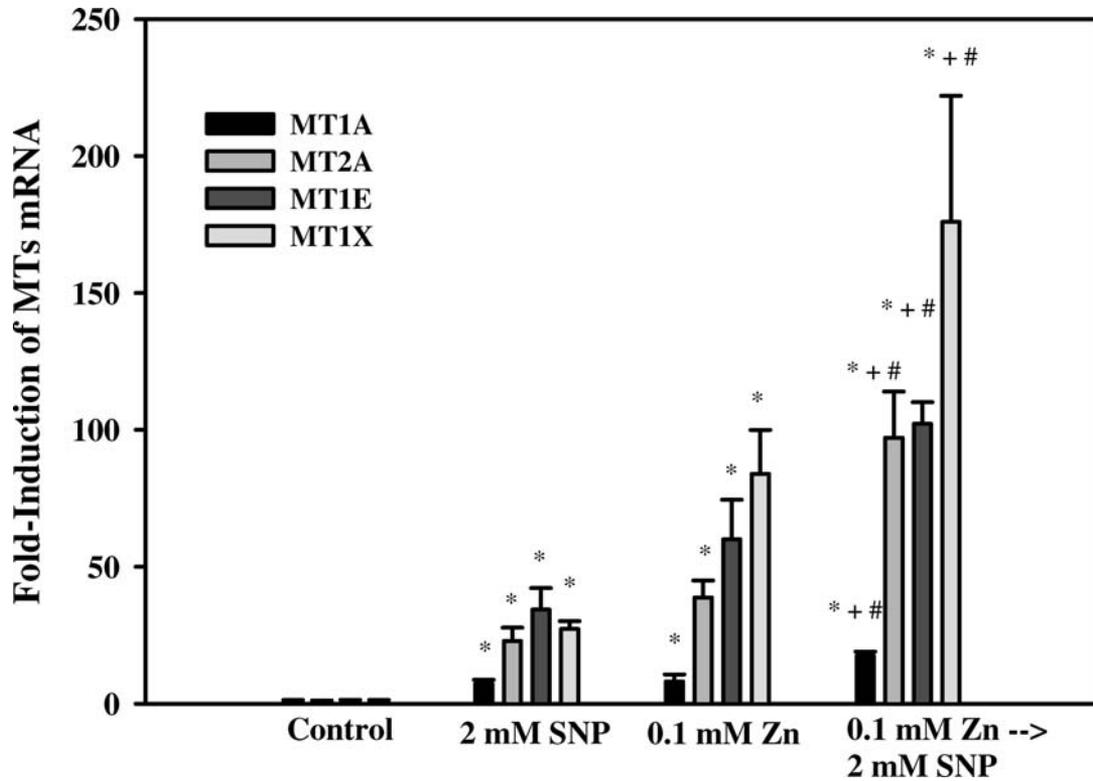
Figure 1. Effect of ZnSO$_4$ on SNP-induced activation of the MT genes MT1A, MT2A, MT1E, and MT1X. HepG2 cells were preincubated with medium with or without ZnSO$_4$ at the indicated concentrations for 24 hrs. Medium was then removed and replaced with medium with or without SNP at the indicated concentrations for 12 hrs. Arrows ($\rightarrow$) indicate 24-hr preincubation with ZnSO$_4$, followed by 12-hr exposure to SNP without ZnSO$_4$. Results are from three experiments and are expressed as the mean $\pm$ SD ($n = 3$). *$P < 0.05$ compared to control; $^+P < 0.05$ compared to 2 mM SNP; $^\#P < 0.05$ compared to 100 μM ZnSO$_4$. The quantity of MT mRNA in each sample was normalized to the quantity of 18S rRNA (18S). "Fold-induction" of each mRNA species was calculated as follows: d Threshold cycle (dCt) $=$ (Ct of MT mRNA) $-$ (Ct of 18S mRNA); ddCt $=$ (dCt of mRNA in treated cells) $-$ (dCt of mRNA in untreated cells); fold-induction $= 2^{-ddCt}$.
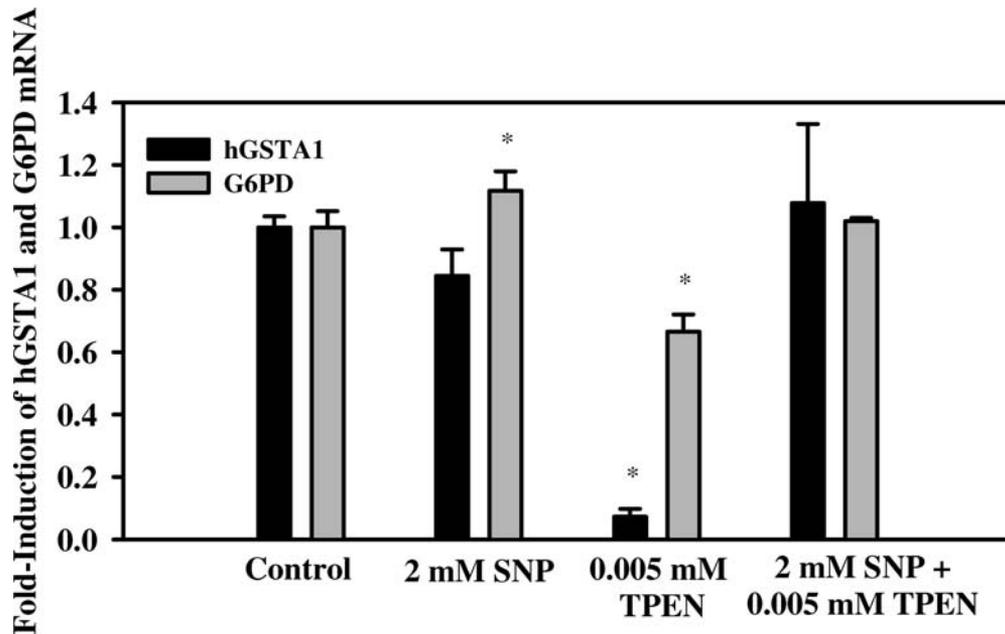

Figure 2. Effect of TPEN on SNP-induced activation of hGST1A and G6PD gene expression. HepG2 cells were incubated with 2 mM SNP or 5 μM TPEN for 12 hrs, as indicated, or with 2 mM SNP $+$ 5 μM TPEN together for 12 hrs. Results are from three experiments and are expressed as the mean $\pm$ SD ($n = 3$). *$P < 0.05$ compared to control. The quantity of hGST1A and G6PD mRNA in each sample was normalized to the quantity of 18S rRNA (18S). "Fold-induction" of each mRNA species was calculated as the ratio of the level of that mRNA in treated cells to that of the corresponding mean value in control cells.
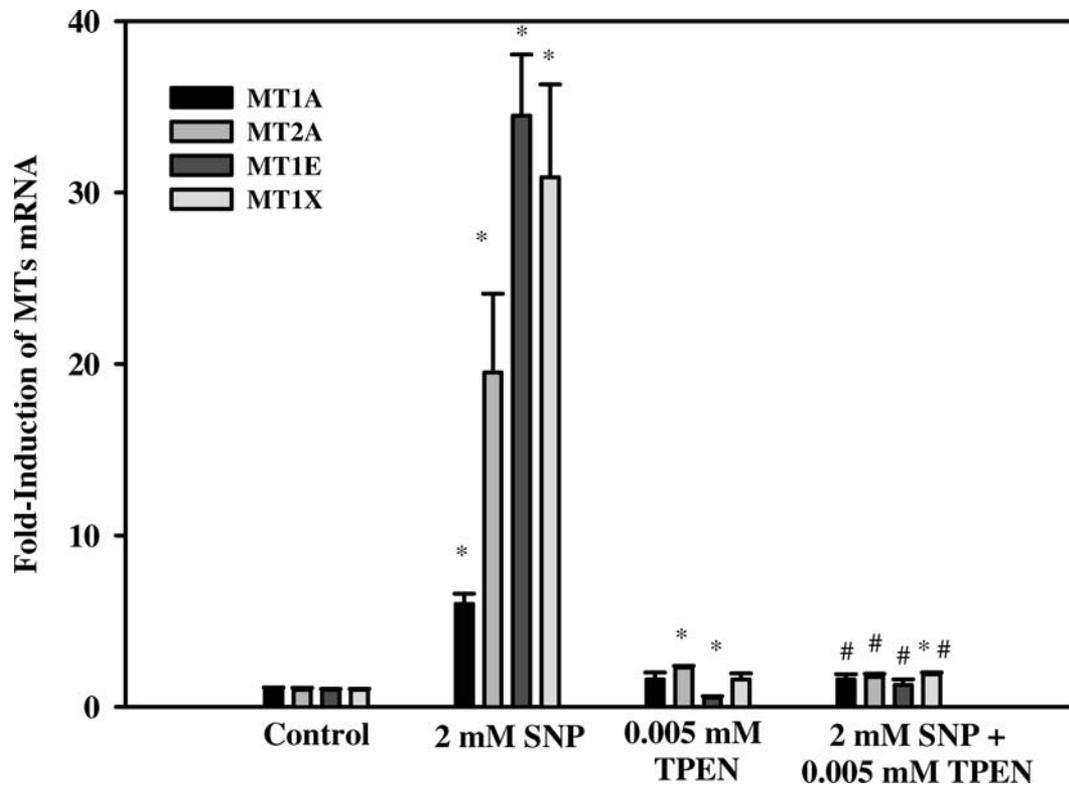
Figure 3. Effect of TPEN on SNP-induced activation of MT1A, MT2A, MT1E, and MT1X genes. HepG2 cells were incubated in medium alone or in medium containing the indicated concentrations of SNP alone, TPEN alone, or SNP + TPEN together for 12 hrs. Results are from three experiments and are expressed as the mean ± SD. *$P < 0.05$ compared to control; #$P < 0.05$ compared to 2 mM SNP. The quantity of MT mRNA in each sample was normalized to the quantity of 18S rRNA (18S). "Fold-induction" of each mRNA species was calculated as follows: d Threshold cycle (dCt) = (Ct of MTs mRNA) − (Ct of 18S mRNA); ddCt = (dCt of mRNA in treated cells) − (dCt of mRNA in untreated cells); fold-induction = 2^{-ddCt}.

enhancement of SNP-induced activation of MT gene transcription, we determined the level of the MTF-1 transcript by RT-real time PCR. Cells were treated with 2 mM SNP or 100 μM ZnSO$_4$, or were pretreated with 100 μM ZnSO$_4$ and then treated with 2 mM SNP. MTF-1 mRNA increased in response to a 12-hr exposure to either of the individual treatments (SNP or ZnSO$_4$). An even greater increase was observed in cells that were exposed to 2 mM SNP for 12 hrs after zinc pretreatment (Fig. 4B). The stimulatory effect of SNP on MTF-1 mRNA transcription was reduced by TPEN (Fig. 4B), suggesting that this stimulation is a result of NO-induced zinc mobilization.

Effect of Zn^{2+} Ions on ZnT-1 Gene Expression. To further test the effect of NO on expression of MTF-1 regulated genes, we measured the abundance of ZnT-1 mRNA in response to NO and zinc. HepG2 cells were treated with 100 μM ZnSO$_4$, 2 mM SNP, or 2 mM SNP for 12 hrs after zinc pretreatment. The levels of ZnT-1 transcripts increased after exposure to zinc or SNP, and zinc pretreatment enhanced the transcription-activating effect of subsequent SNP exposure ($n = 3$, $P < 0.05$) (Fig. 5). To test the effect of zinc depletion on transcription of ZnT-1, we exposed HepG2 cells to 2 mM SNP alone, 5 μM TPEN alone, or SNP and TPEN together, for 12 hrs. In cells treated

with 5 μM TPEN together with 2 mM SNP, ZnT-1 mRNA levels were reduced to 19% of that observed in cells exposed to 2 mM SNP alone (Fig. 5). These results demonstrate that the interaction between NO- and MTF-1–mediated gene expression in HepG2 cells.

Zinc Pretreatment Reduces the Cytotoxic Effects of Nitrosative Stress. Cells were treated with 2 mM SNP or 2 mM SNP after 100 μM ZnSO$_4$ pretreatment, and genomic DNA extracted from treated and untreated cells was analyzed. DNA fragmentation was not obvious in cells treated with ZnSO$_4$ alone, and pretreatment with 100 μM ZnSO$_4$ suppressed the DNA fragmentation caused by exposure to 2 mM SNP.

Cells were treated with various concentrations of ZnSO$_4$ (Fig. 6B) or SNP (Fig. 6B), and cell viability was quantified using MTT assays. Although high concentrations of zinc have been reported to be cytotoxic (24, 25), the exposure of HepG2 cells to ZnSO$_4$ (at up to 200 μM) for 24 hrs had no detectable cytotoxic effect in our experiments (Fig. 6B). In contrast, chelation of intracellular zinc with 5 μM TPEN reduced cell viability to 60% of the control (Fig. 6B).

Exposure to 2 mM or 4 mM SNP for 24 hrs caused a significant, dose-dependent reduction in cell viability. Zinc
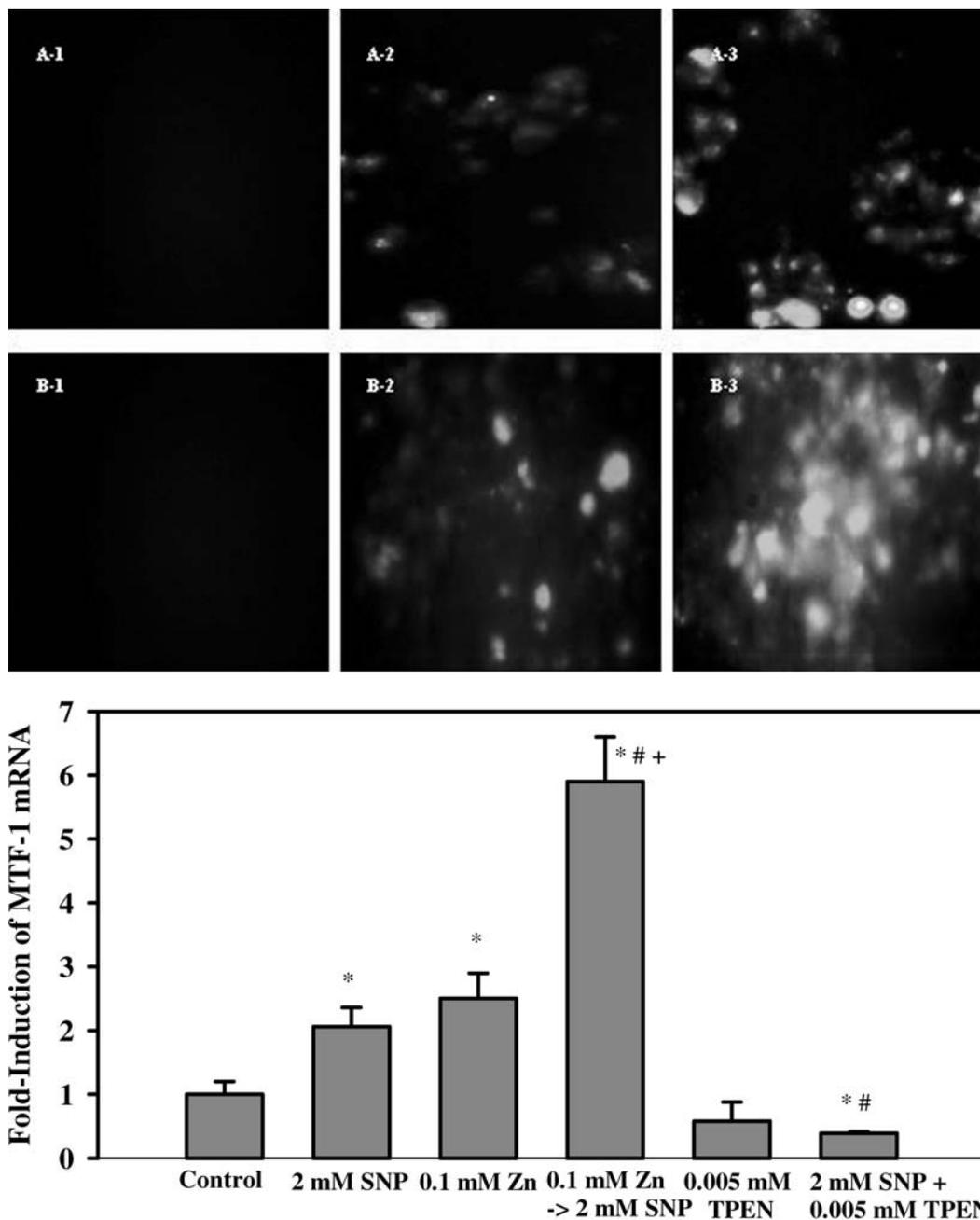
Figure 4. (A) Cellular Zn^{2+} in HepG2 cells detected by fluorescence microscopy using FluoZin-3 AM and (B) effect of $ZnSO_4$ and TPEN on SNP-induced activation of the MTF-1 gene. HepG2 cells were incubated in serum-free media for 6 hrs prior to the experiment. (A-1–3) 1-day culture. (B-1–3) 7-day culture. (A-1 and B-1) Untreated cells. (A-2 and B-2) Cells treated with 100 μM $ZnSO_4$. (A-3 and B-3) Cells treated with 100 μM $ZnSO_4$ and 2 mM SNP. HepG2 cells were incubated with 2 mM SNP for 12 hrs, as indicated, or were preincubated with 100 μM $ZnSO_4$ for 24 hrs followed by a 12-hr incubation in medium with or without SNP, as indicated by arrows ($\rightarrow$). Results are from three experiments and are expressed as the mean $\pm$ SD ($n = 3$). *$P < 0.05$ compared to controls; #$P < 0.05$ compared to 2 mM SNP; +$P < 0.05$ compared to 100 μM $ZnSO_4$. The quantity of MTF mRNA in each sample was normalized to the quantity of 18S rRNA (18s). "Fold-induction" of each mRNA species was calculated as follows: d Threshold cycle (dCt) = (Ct of MTs mRNA) − (Ct of 18S mRNA); ddCt = (dCt of mRNA in treated cells) − (dCt of mRNA in untreated cells); fold-induction = 2^{-ddCt}.

pretreatment (50 μM or 100 μM) provided significant protection against nitrosative cytotoxicity (Fig. 6B). Exposure to 4 mM SNP alone for 24 hrs produced about a 30% loss of viability, and simultaneous treatment with 4 mM SNP and 5 μM TPEN for 24 hrs exacerbated cell death (Fig. 6B).

Discussion

There is an increasing appreciation for the role of zinc in protection against oxidative stress (3, 22, 39). It has previously been shown that NO or H_2O_2 can release zinc from zinc-sulfur clusters and that the resulting increase in intracellular labile zinc can stimulate MT transcription
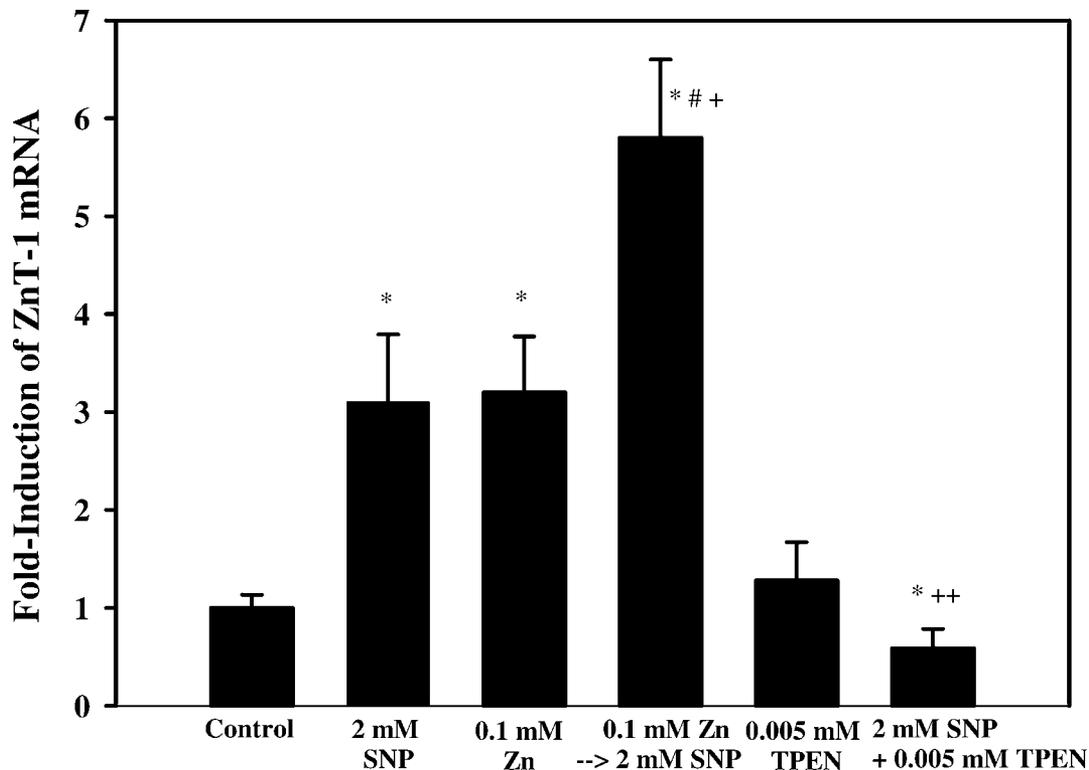
Figure 5. Effect of ZnSO$_4$ and TPEN on SNP-induced activation of the ZnT-1 gene. Cells were incubated with the indicated concentrations of SNP only or TPEN only, or were preincubated with 100 mM ZnSO$_4$ for 24 hrs followed by incubation in media with or without SNP for 12 hrs, as indicated by arrows ($\rightarrow$), or were exposed to SNP + TPEN for 12 hrs. Results are from three experiments and are expressed as the mean $\pm$ SD ($n = 3$). *$P < 0.05$ compared to control; #$P < 0.05$ compared to 2 mM SNP; +$P < 0.05$ compared to 100 μM ZnSO$_4$. The quantity of ZnT-1 mRNA in each sample was normalized to the quantity of 18S rRNA (18S). "Fold-induction" of each mRNA species was calculated as follows: d Threshold cycle (dCt) = (Ct of MTs mRNA) − (Ct of 18S mRNA); ddCt = (dCt of mRNA in treated cells) − (dCt of mRNA in untreated cells); fold-induction = 2^{-ddCt}.

through MTF-1 activation (5, 17, 19–22, 26). In rainbow trout gill cells, treatment with zinc before exposure to H$_2$O$_2$ protects against oxidative damage through production of antioxidant proteins, including MT (22). The results of the present study suggest that the same mechanism of zinc-mediated protection against oxidative stress is present in human cells and support a role for zinc as a signal that communicates between oxidative stress and mobilization of antioxidant mechanisms. NO seems to regulate the concentration of labile intracellular zinc by controlling the release of zinc from sulfhydryl groups. This may serve as a signal for oxidative stress, but can also be involved in other cell functions, including hypoxic vasoconstriction (27). The pathway of zinc mobilization is regulated by negative feedback, because there is a coordinated expression of MT, ZnT-1, and MTF-1 in response to elevation of labile zinc. In addition to its function in restoring intracellular zinc balance, the increase in MT expression enhances protection against oxidative damage (28–30).

Several studies have found an association between antioxidant activities and the state of oxidative stress in cells (22, 31). An important aspect of a model in which zinc mediates the antioxidant response is that a range of antioxidant genes seems to be MTF-1 regulated. Transcrip-

tional activation of GST seems to be linked to Zn^{2+} through MREs and MTF-1 (22, 32). Lichtlen *et al.* (33) used a combination of bioinformatics and experimental approaches to identify six mammalian antioxidant genes (in addition to MT) that are potentially regulated by MTF-1. Through global mapping of MRE distribution in the compact genome of the fugu puffer fish (*Takifugu rubripes*), we found 28 genes with antioxidant ontology annotations that possessed multiple MRE copies within 2 kb of the open reading frame.[2] This includes genes with key roles in antioxidant defense, such as G6PD, GST, glutathione peroxidase-1 (GPX1), Cu/Zn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), and catalase (CATA). We repeated the search for MREs in the human genome and discovered 20 key antioxidant genes in addition to the MTs. Thus, considering the large number of antioxidant genes with multiple MREs in their 5' flanking region and that MTF-1 target genes are clearly activated through mobilization of zinc during oxidative stress, a role for zinc in signaling of oxidative stress seems plausible.

Small changes in the distribution of intracellular Zn^{2+} between labile and bound pools regulate MTF-1 activity

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[2] N. Hill, P. Cunningham, C. Hogstrand and P. Kille, unpublished data, October 3, 2003.
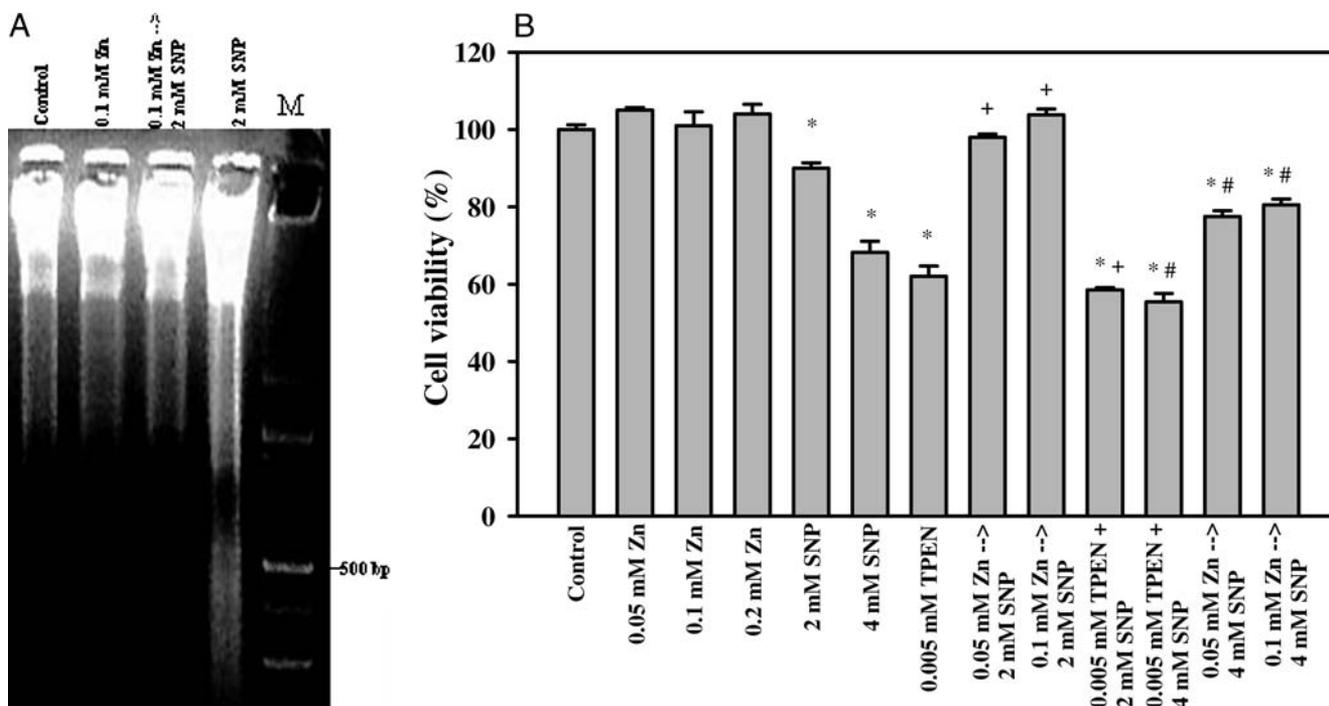
Figure 6. (A) Influence of zinc on SNP-induced DNA fragmentation in HepG2 cells and (B) effect of ZnSO$_4$ and TPEN on SNP cytotoxicity by MTT assay. Cells were incubated with the indicated concentrations of SNP only or TPEN only, or were preincubated with 100 mM ZnSO$_4$ for 24 hrs followed by incubation in media with or without SNP for 24 hrs, as indicated by arrows ($\rightarrow$), or were exposed to SNP + TPEN for 24 hrs. Cell viability was determined by the MTT assay as described in the Materials and Methods section. Results are expressed as percentage viability, with 100% viability for untreated cells. Results are from four experiments and are expressed as the mean $\pm$ SD ($n=4$). *$P < 0.05$ compared to control; $^+P < 0.05$ compared to 2 mM SNP; $^\#P < 0.05$ compared to 4 mM SNP.

(34). In the present study we found that treatment with SNP and zinc, separately or in combination, activates MTF-1 transcription, and that in either case MTF-1 activation is zinc-dependent. Previous research has shown that labile zinc can control MT gene expression by interacting with MTF-1, that aerobic NO can nitrosate protein sulfhydryl groups, and that MT-bound Cd^{2+} and Zn^{2+} are released from MT upon NO exposure (5, 19, 20).

We observed induction of ZnT-1 gene expression in HepG2 cells upon exposure to SNP alone, pretreatment with zinc alone, and by SNP exposure after zinc pretreatment. The stimulating effect of zinc supplementation on ZnT-1 gene induction appears to be general to vertebrates and seems to be mediated by MTF-1 (6–8, 24). Since ZnT-1 is a zinc efflux transporter, induction of ZnT-1 gene expression has been proposed to provide protection against high intracellular zinc levels (7, 35, 36).

An antioxidant role for MTs has been proposed based on observations that animals or cells overexpressing MTs, due to chemical induction or zinc supplementation, are resistant to several forms of oxidative injury (22, 25, 37–39). In addition, animals or cells with decreased MT levels demonstrate enhanced sensitivity to oxidative injury (22, 40). Preincubation with zinc provides cells with some protection against SNP-induced death, probably through the induction of antioxidant genes, such as MT. Zinc-mediated inhibition of cell death by induction of MT, GST, and G6PD

has been reported in rainbow trout gill cells exposed to H$_2$O$_2$ (22).

In conclusion, induction of antioxidant genes is significantly greater when cells are exposed to SNP after zinc pretreatment than when they are exposed to SNP or zinc alone. This induction likely occurs through activation of MTF-1. The increase in labile zinc may be responsible for the observed increase in the level of ZnT-1 mRNA. Preincubation with zinc protects HepG2 cells against SNP toxicity and these results support a model in which zinc functions as a messenger of oxidative stress.

1. Sato M, Bremner I. Oxygen free radicals and metallothionein. Free Radic Biol Med 14:325–337, 1993.

2. Wimmer U, Wang Y, Georgiev O, Schaffner W. Two major branches of anti-cadmium defense in the mouse: MTF-1/metallothioneins and glutathione. Nucleic Acids Res 33:5715–5727, 2005.

3. Searle PF. Zinc dependent binding of a liver nuclear factor to metal response element MRE-a of the mouse metallothionein-I gene and variant sequences. Nucleic Acids Res 18:4683–4690, 1990.

4. Andrews GK. Cellular zinc sensors: MTF-1 regulation of gene expression. Biometals 14:223–237, 2001.

5. Zhang B, Georgiev O, Hagmann M, Günes C, Cramer M, Faller P, Vasák M, Schaffner W. Activity of metal-responsive transcription

factor 1 by toxic heavy metals and H_2O_2 *in vitro* is modulated by metallothionein. Mol Cell Biol 23:8471–8485, 2003.

6. Langmade SJ, Ravindra R, Daniels PJ, Andrews GK. The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. J Biol Chem 275:34803–34809, 2000.

7. Balesaria S, Hogstrand C. Identification, cloning and characterization of a plasma membrane zinc efflux transporter, TrZnT-1 from fugu pufferfish (*Takifugu rubripes*). Biochem J 394:485–493, 2006.

8. Liuzzi JP, Blanchard RK, Cousins RJ. Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. J Nutr 131:46–52, 2001.

9. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 30: 445–600, 1995.

10. Board PG, Webb GC. Isolation of a cDNA clone and localization of human glutathione S-transferase 2 genes to chromosome band 6p12. Proc Natl Acad Sci U S A 84:2377–2381, 1987.

11. Xiang H, Wei W, Zhang Y, Tan H. Human glutathione-S-transferase: cloning and expression in *Lactococcus lactis*. Biomol Eng 16:207–209, 2000.

12. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. Free Radic Biol Med 8:583–599, 1990.

13. Egglestion LV, Krebs HA. Regulation of the pentose phosphate cycle. Biochem J 138:425–435, 1974.

14. Splolarics Z. Endotoxemia, pentose cycle, and the oxidant/antioxidant balance in the hepatic sinusoid. J Leukoc Biol 63:534–541, 1998.

15. Riganti C, Aldieri E, Bergandi L, Fenoglio I, Costamagma C, Fubini B, Bosia A, Ghigo D. Crocidolite asbestos inhibits pentose phosphate oxidative pathway and glucose 6-phosphate dehydrogenase activity in human lung epithelial cells. Free Radic Biol Med 32:938–949, 2002.

16. Razavi HM, Hamilton JA, Feng Q. Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure. Pharmacol Ther 106:147–162, 2005.

17. Kröncke KD, Fehsel K, Schmidt T, Zenke FT, Dasting I, Wesener JR, Bettermann H, Breuning KD, Kolbbachofen V. Nitric oxide destroys zinc-sulfur clusters inducing zinc release from metallothionein and inhibition of the zinc finger-type yeast transcription activator LAC9. Biochem Biophys Res Commun 200:1105–1110, 1994.

18. Olivier S, Fillet M, Malaise M, Piette J, Bours V, Merville M-P, Franchimont N. Sodium nitroprusside–induced osteoblast apoptosis is mediated by long chain ceramide and is decreased by raloxifene. Biochem Pharmacol 69:891–901, 2005.

19. Katakai K, Liu J, Nakajima K, Keefer LK, Waalkes MP. Nitric oxide induces metallothionein (MT) gene expression apparently by displacing zinc bound to MT. Toxicol Lett 119:103–108, 2001.

20. Misra RR, Hochadel JF, Smith GT, Cook JC, Waalkes MP, Wink DA. Evidence that nitric oxide enhances cadmium toxicity by displacing the metal from metallothionein. Chem Res Toxicol 9:326–332, 1996.

21. Hasumi M, Suzuki K, Matsui H, Koike H, Ito K, Yamanaka H. Regulation of metallothionein and zinc transport expression in human prostate cancer cells and tissues. Cancer Lett 200:187–195, 2003.

22. Chung MJ, Walker PA, Brown RW, Hogstrand C. Zinc-mediated gene expression offers protection against H_2O_2-induced cytotoxicity. Toxicol Appl Pharmacol 205:225–236, 2005.

23. Haase H, Maret W. Intracellular zinc fluctuations modulate protein tyrosine phosphatase activity in insulin/insulin-like growth factor-1 signaling. Exp Cell Res 291:289–298, 2003.

24. Devergnas S, Chimienti F, Naud N, Pennequin A, Coquerel Y, Chantegrel J, Favier A, Seve M. Differential regulation of zinc efflux transporters ZnT-1, ZnT-5 and ZnT-7 gene expression by zinc levels: a real-time RT-PCR study. Biochem Pharmacol 68:699–709, 2004.

25. Chimienti F, Jourdan E, Favier A, Seve M. Zinc resistance impairs sensitivity to oxidative stress in HeLa cells: protection through metallothioneins expression. Free Radic Biol Med 31:1179–1190, 2001.

26. Andrew GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem Pharmacol 59:95–104, 2000.

27. St Croix CM, Leelavaninchkul K, Watkins SC, Kagan VE, Pitt BR. Nitric oxide and zinc homeostasis in acute lung injury. Proc Am Thorac Soc 2:236–242, 2005.

28. Davis SR, Cousins RJ. Metallothionein expression in animals: a physiological perspective on function. J Nutr 130:1085–1088, 2000.

29. Kumari MV, Hiramatsu M, Ebadi M. Free radical scavenging actions of metallothionein isoforms I and II. Free Radic Res 29:93–101, 1998.

30. Zangger K, Oz G, Haslinger E, Kunert O, Armitage IM. Nitric oxide selectively releases metals from the amino-terminal domain of metal-lothioneins: potential role at inflammatory sites. FASEB J 15:1303–1305, 2001.

31. Maulik N, Wei Z, Liu X, Engelman RM, Rousou JA, Das DK. Improved postischemic ventricular functional recovery by amphet-amine is linked with its ability to induce heat shock. Mol Cell Biochem 137:17–24, 1994.

32. Hogstrand C, Balesaria S, Glover CN. Application of genomics and proteomics for study of the integrated response to zinc exposure in a non-model fish species, the rainbow trout. Comp Biochem Physiol 133B:523–535, 2002.

33. Lichtlen P, Wang Y, Belser T, Georgiev O, Certa U, Sack R, Schaffner W. Target gene search for the metal-responsive transcription factor MTF-1. Nucl Acids Res 29:1514–1523, 2001.

34. Dalton TP, Bittel D, Andrews GK. Reversible activation of mouse metal response element–binding transcription factor 1 DNA binding involves zinc interaction with the zinc finger domain. Mol Cell Biol 17: 2781–2789, 1997.

35. Palmiter RD, Findley SD. Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J 14: 639–649, 1995.

36. Palmiter RD, Cole TB, Findley SD. ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. EMBO J 15:1784–1791, 1996.

37. Schwarz MA, Lazo JS, Yalowich JC, Reynolds I, Kagan VE, Tyurin V, Kim YM, Watkins SC, Pitt BR. Cytoplasmic metallothionein over-expression protects NIH 3T3 cells from tert-butyl hydroperoxide toxicity. J Biol Chem 269:15238–15243, 1994.

38. Schwarz MA, Lazo JS, Yalowich JC, Allen WP, Whitmore M, Bergonia HA, Tzeng E, Billar TR, Robbins PD, Lancaster JR Jr, Pitt BR. Metallothionein protects against the cytotoxic and DNA-damage effects of nitric oxide. Proc Natl Acad Sci U S A 92:4452–4456, 1995.

39. Perez MJ, Cederbaum AI. Metallothionein 2A induction by zinc protects HepG2 cells against CYP2E1-dependent toxicity. Free Radic Biol Med 34:443–455, 2003.

40. Lazo JS, Kondo Y, Dellapiazza D, Michalska AE, Choo KH, Pitt BR. Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. J Biol Chem 270:5506–5510, 1995.