Upregulation of haeme oxygenase-1 by zinc in HCT-116 cells

By: Abigail F. Smith, George Loo

Made available courtesy of Informa Healthcare:
http://www.dx.doi.org/10.3109/10715762.2012.690872

Abstract:

Haeme oxygenase-1 (HO-1) is often viewed as a cytoprotective gene. Toxic heavy metals induce HO-1, but it is unclear whether particular metal micronutrients also induce HO-1. Hence, the ability of exogenously-added copper, iron and zinc to influence HO-1 expression in HCT-116 cells was evaluated. Under the chosen experimental conditions, only zinc noticeably increased the expression of HO-1 mRNA and protein. Concurrently, zinc decreased non-protein thiol levels to a certain extent, but zinc did not increase the production of reactive oxygen species (ROS). Moreover, ascorbate and Trolox did not inhibit zinc-induced HO-1 upregulation. In contrast, deferoxamine blunted the induction of HO-1 mRNA, protein, and enzymatic activity caused by zinc. Additionally, N-acetylcysteine and Tiron inhibited zinc-induced HO-1 upregulation and also nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2). Collectively, these findings suggest that zinc at above normal levels upregulates HO-1 expression in HCT-116 cells in a ROS-independent manner.

Keywords: N-acetylcysteine | deferoxamine | metals | oxidative stress | Tiron

Article:

Introduction

Cells in the body are exposed to metallic elements that can have either beneficial or adverse effects depending on the concentrations of the metallic elements. For example, copper, iron and zinc at proper levels are needed to facilitate many important biochemical reactions and other cellular processes. Thus, copper, iron and zinc are often recognised as essential trace elements although they can be toxic at above normal levels [1]. In contrast, other metallic elements such as arsenic, cadmium and chromium are usually regarded as toxic heavy metals from the environment, including the plant food chain [2].

Multiple lines of defence enable cells to deal with potentially harmful metallic elements. One defensive mechanism is the expression of cytoprotective genes that helps safeguard against

heavy metal toxicity. An important cytoprotective gene is haeme oxygenase-1 (HO-1). HO-1 codes for a protein that functions as an enzyme in catalysing the degradation of haeme to biliverdin, ferrous iron and carbon monoxide [3]. Furthermore, HO-1 is a redox-sensitive gene [4]. Upregulation of HO-1 expression is an adaptive cellular response to oxidative stress [5], which can be an early consequence of exposure to certain metallic elements. It is known that HO-1 expression is upregulated by arsenic, cadmium and chromium [6]. Whereas zinc is redox-inactive, both copper and iron are redox-active and can induce oxidative stress supposedly as the result of these two transition metals catalysing the Haber – Weiss reaction to generate reactive oxygen species (ROS) [7]. However, it is unclear whether exposing cells to exogenously-added copper, iron and zinc affects HO-1 expression. This uncertainty prompted the current study with a preconceived notion, as delineated below, that at the same concentration zinc would have a stronger effect than iron and copper in influencing HO-1.

When basically comparing copper, iron and zinc from an overall functional perspective, zinc can be considered more versatile at the cellular level. Zinc serves as a cofactor for hundreds of enzymes and allows for the structural formation of zinc fingers in multiple transcription factors [8]. Moreover, zinc can act indirectly as an antioxidant [9]. In particular, one indirect action of zinc is that it increases metallothionein gene expression [10].
Metallothionein scavenges ROS [11]. Also, metallothionein can bind zinc to protect cells against zinc toxicity [12]. Therefore, the upregulation of metallothionein expression caused by zinc can be viewed as a response to oxidative stress, since it is known that relatively high levels of zinc can paradoxically stimulate ROS production [13,14] and perturb glutathione redox homeostasis [15]. Under such stressful conditions, it is possible that other cytoprotective genes besides metallothionein are upregulated when cells are exposed to added zinc. Thus, the purpose of this present study was to determine if HO-1 is influenced by three metal micronutrients, namely, copper, iron and zinc, when added to the cellular environment. We hypothesized that HO-1 can be affected by zinc but not necessarily via ROS.

Materials and methods

Materials

HCT-116 human colon adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Goat HO-1 polyclonal antibody (C-20), rabbit Nrf2 polyclonal antibody (H-300), rabbit anti-goat IgG-horse radish peroxidase (HRP), goat anti-rabbit IgG-HRP and goat antimouse IgG-HRP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse β –actin monoclonal antibody (AC-15) was obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit IgG conjugated to Alexa Fluor 488 was obtained from Molecular Probes Inc. (Eugene, OR).

Cell culture and treatment

HCT-116 cells were propagated in McCoy's 5A medium supplemented with 100 ml/L fetal bovine serum, 0.54 mol/L amphotericin B, 100,000 units/L penicillin and 100 mg/L streptomycin. Upon reaching 70 – 80% confluency, cell samples (i.e. cells grown in petri dishes,

multi-well microplates or chamber slides) were incubated with copper (cupric sulphate), iron (ferrous sulphate) and zinc (zinc sulphate), but also with the reference compounds, tert-butyl hydroperoxide (TBHP) and ethacrynic acid, for comparison. In subsequent experiments, HCT-116 cells were pre-treated with ascorbate, 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid (Trolox), N-acetylcysteine (NAC), 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron) or deferoxamine mesylate (DFO) before subsequently exposing the cells to zinc.

Assessment of oxidative stress and determination of non-protein thiol levels

Overall cellular ROS production and mitochondrial superoxide production were both assessed by fluorescence microscopy using the Image-iT LIVE Green ROS detection reagent and the MitoSOX Red mitochondrial superoxide indicator reagent (Molecular Probes, Eugene, OR), respectively. Cellular levels of non-protein thiols were determined spectrophotometrically, using Ellman's reagent along with glutathione as the standard [16].

Determination of HO-1 mRNA expression

After isolating total RNA from cells with an RNeasy Mini kit (Qiagen Inc., Valencia, CA), HO-1 mRNA expression was determined by multiplex relative RT-PCR analysis using a Qiagen OneStep RT-PCR kit. The primer sequences for HO-1 (NIH, NCBI, UniSTS: 92045, RH27826) were: forward, TGTGGCAGCTGTCTCAAACCTCCA; reverse, TTGAGGCTGAGCCAGGAACAGAGT. To normalize the data, a β -actin/competimer pair (Ambion Inc., Austin, TX) was used as the internal standard. The RT – PCR conditions were 30 minutes at 50 ° C followed by 15 minutes at 95 ° C (RT), then 0.5 minutes at 95 ° C, 0.5 minutes at 60 ° C and 1 minutes at 72 ° C (PCR) for 25 cycles. The PCR product sizes were 175 bp (HO-1) and 294 bp (β -actin). A Kodak 440 CF image station was used to attain the images and densitometric analysis (HO-1/ β -actin).

Determination of HO-1 protein expression

Harvested cells were lysed with ice-cold buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and Roche Complete Protease Inhibitor Cocktail, pH 7.4). Whole lysate aliquots were electrophoresed using 4 – 12% Bis-Tris NuPAGE mini-gels (Invitrogen, Carlsbad, CA), before electroblotting to nitrocellulose membrane and blocking with 5% skim milk powder in TBST (20 mM Tris – HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.4). The blocked membrane was incubated overnight at 4 ° C with goat HO-1 polyclonal antibody (1:1000 dilution) and eventually for 2 hours at 25 ° C with anti-goat IgG-HRP (1:100,000 dilution). Images were obtained after processing the membrane with SuperSignal WestFemto Maximum Sensitivity Kit reagents (Pierce, Rockford, IL) for subsequent image capture. After stripping, the membrane was re-probed with mouse β -actin monoclonal antibody (1:40,000 dilution) along anti-mouse IgG-HRP (1:200,000 dilution) before re-imaging.

Assay of haeme oxygenase activity

The haeme oxygenase assay was essentially performed as described [17]. Cells were sonicated in ice-cold 0.1 M potassium phosphate buff er (pH 7.4) containing 2 mM MgCl 2 and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN), before centrifugation at 18,000 g for 20 minutes (4 ° C). The supernate (700 μ g protein) was added to a reaction mixture containing 1 mM NADPH, 2 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, 25 μ M haemin and rat liver cytosol (2 mg protein) in a total volume of 1 ml. After incubation at 37 ° C for 1 hour in the dark, 1 ml chloroform was added to the samples and then rapidly vortexed. The bottom chloroform phase containing the product, bilirubin, was analysed spectrophotometrically (difference in absorbance between 464 and 530 nm). Using an extinction coeffi cient of 40 mM_ 1 cm _ 1 , haeme oxygenase activity was calculated and expressed as pmol/h/mg protein.

Determination of nuclear Nrf2 expression

Nuclear extracts were prepared as described [18]. Determination of Nrf2 protein expression in nuclear extracts was done by Western-immunoblotting analysis as described above, but using rabbit Nrf2 polyclonal primary antibody and goat anti-rabbit IgG-HRP secondary antibody. Additionally, nuclear Nrf2 expression was assessed by immunocytofl uorescence microscopy. Cells grown and treated in chamber slides were fixed with 4% formaldehyde *Upregulation of haeme oxygenase-1 by zinc* 1101 and permeabilised with 0.2% Triton X-100. The slides were blocked with 1% bovine serum albumin, before adding rabbit Nrf2 polyclonal antibody (1:500 dilution) for incubation overnight at 4 ° C. This step was eventually followed by incubation with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:500 dilution). Counterstaining was with 4′ ,6-diamidino-2-phenylindole (DAPI) to visualise nuclei. The NIH ImageJ image processing program was used to capture and quantify the green fluorescence intensities of cell nuclei, reflecting nuclear Nrf2 expression.

Results

Effect of copper, iron and zinc on HO-1

To determine whether some essential trace elements at a certain concentration infl uence HO-1, HCT-116 cells were incubated with 200 μ M cuprous sulphate (copper), ferrous sulphate (iron) or zinc sulphate (zinc). For comparison, cells were also incubated with the reference compound, tert-butyl hydroperoxide (TBHP), which is a well-known inducer of oxidative stress in cells. The outcome of this experiment is shown in Figure 1A. Of the three trace elements tested, only zinc distinctly increased the expression of HO-1 mRNA and HO-1 protein. Under the chosen experimental conditions, TBHP increased the expression of HO-1 mRNA, but not HO-1 protein.

To further assess the upregulatory effect of zinc on HO-1 mRNA and protein expression, HCT-116 cells were incubated with incremental concentrations of zinc for a fixed time period (Figure 1B) and also for incremental time periods with a fixed concentration of zinc (Figure 1C). Based on these particular positive data, cells were incubated with 150 μ M zinc for 5 – 6 hours in subsequent experiments examining HO-1 expression.

Effect of copper, iron and zinc on cellular ROS production

To determine if copper, iron or zinc induce oxidative stress that could be either negatively or positively related to HO-1 expression, ROS production was assessed in HCT-116 cells incubated with the three individual trace elements. The overall assessment was done by visually detecting the oxidation of a carboxydichlorodihydrofluorescein diacetate probe to a product emitting green fluorescence (Figure 2A). For a positive control, HCT-116 cells were incubated with TBHP. As can be seen, the cellular production of ROS was not increased by 150 μM copper, iron or zinc after 3 hours of incubation. In the case of zinc-treated cells, further examination revealed no evidence of greater production of ROS at earlier time points (Figure 2B). Compartmentally, zinc did not increase mitochondrial superoxide production, as assessed with MitoSOX Red fluorescent probe and using deoxycholate-treated HCT-116 cells as a positive control (Figure 2C).
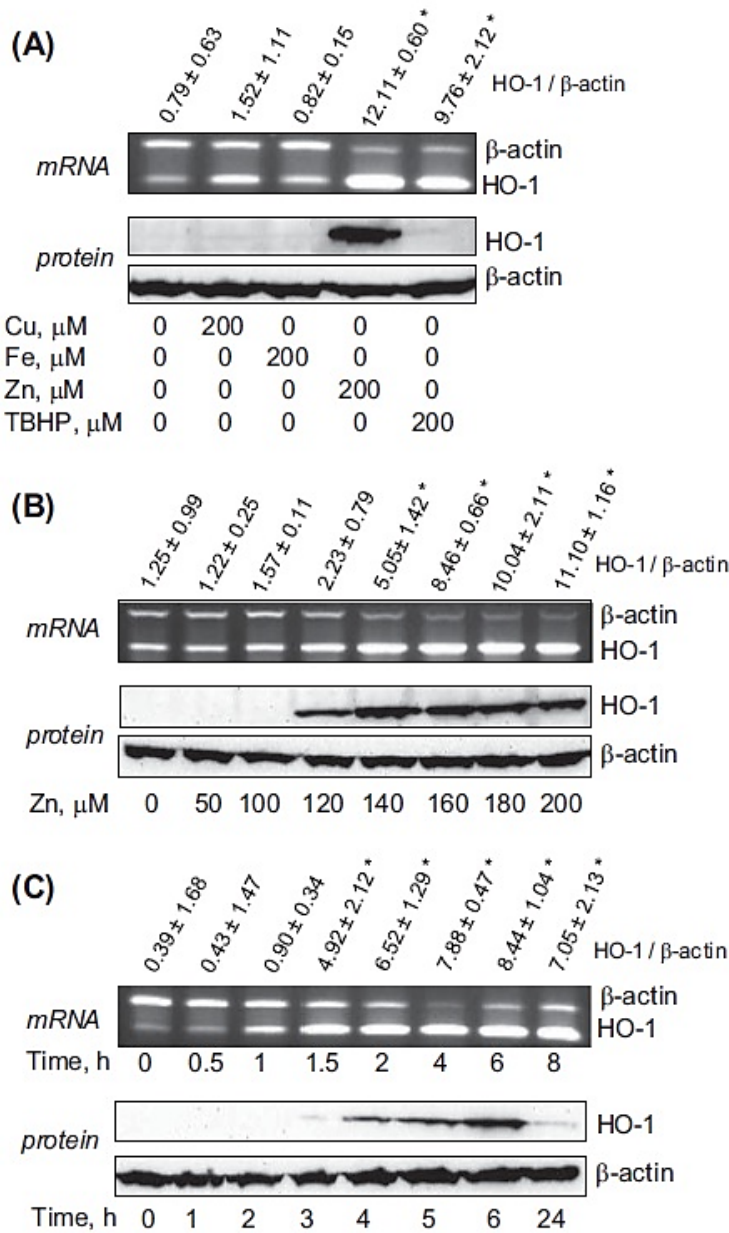
Figure 1. Upregulation of HO-1 expression by zinc but not by copper and iron. HCT-116 cells were incubated for 5 – 6 hours with 200 μ M of cuprous sulphate (Cu), ferrous sulphate (Fe) or zinc sulphate (Zn), and with the standard oxidant, tert-butylhydroperoxide (TBHP), for comparison (A). Additionally, cells were incubated for 5 – 6 hours with an increasing concentration range of zinc (B) or with the same zinc concentration (150 μ M) for diff erent time periods (C). Afterwards, the expression of HO-1 mRNA and HO-1 protein was evaluated by multiplex relative RT-PCR analysis and Westernimmunoblotting analysis, respectively. The results are representative of three diff erent experiments (* $p < 0.05$ compared to control, for mRNA images).

Effect of zinc on cellular non-protein thiol levels

To determine if zinc aff ects cellular sulfhydryl compounds, of which glutathione is the primary one and also the major intracellular antioxidant, levels of non-protein thiols were determined in HCT-116 cells incubated with 150 μ M zinc. A small but evident drop in non-protein thiols was found after 3 – 4 hours of incubation (Figure 3A). For comparison with zinc, TBHP and ethacrynic acid were also evaluated (Figure 3B). Under the chosen experimental conditions, TBHP did not lower non-protein thiol levels. But, ethacrynic acid, which is a known to conjugate or form a covalent adduct with glutathione [19], did lower the non-protein thiol levels. Interestingly, zinc and ethacrynic acid did not increase ROS production whereas TBHP did (Figure 3C).
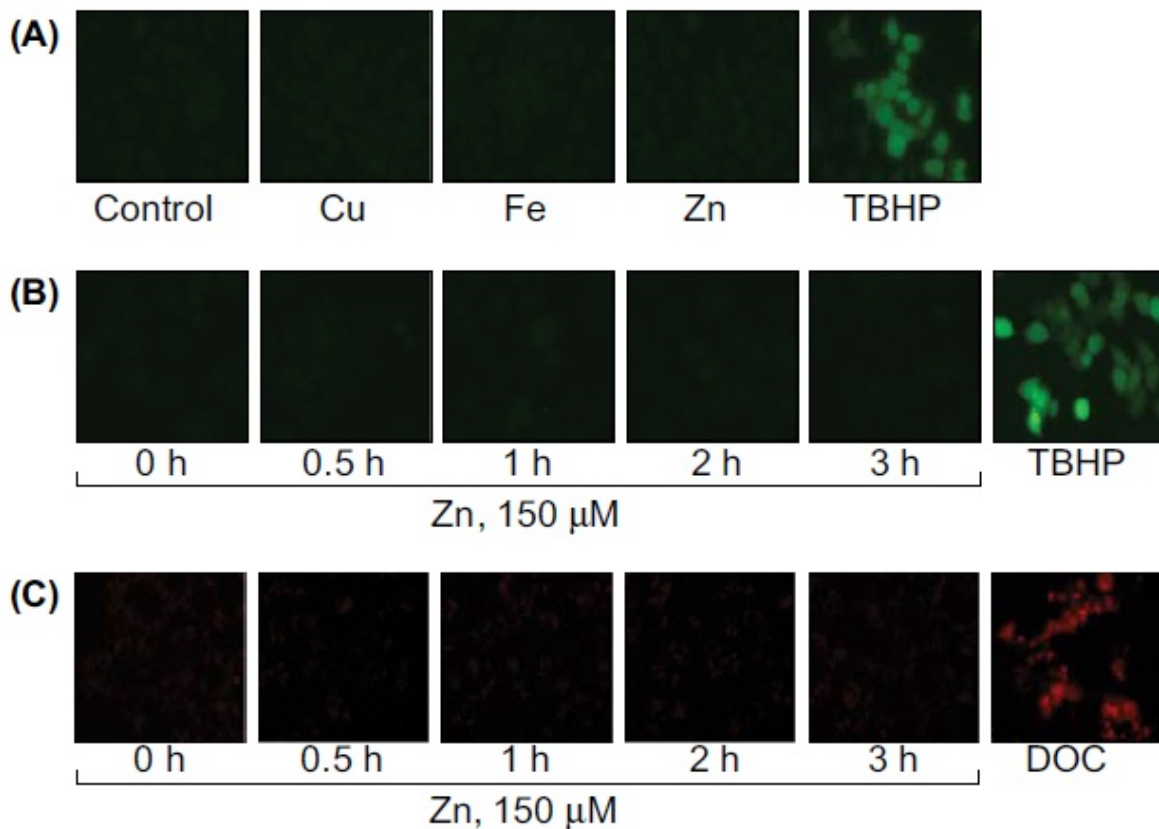
Figure 2. No stimulatory eff ect of copper, iron and zinc on production of ROS. HCT-116 cells were incubated for 1 hour with 200 µ M of cuprous sulphate (Cu), ferrous sulphate (Fe) or zinc sulphate (Zn), and also with the standard oxidant, tert-butylhydroperoxide (TBHP), for comparison (A). Additionally, cells were incubated for different time periods with 150 µ M zinc sulphate (Zn), but also with TBHP and deoxycholate (DOC) as positive controls (B and C). Afterwards, the general production of ROS (B) and specifi c production of mitochondrial superoxide (C) were assessed by fluorescence microscopy. The results are representative of 3 – 4 different experiments.

Effect of antioxidants on zinc-induced upregulation of HO-1

To further determine whether ROS are involved in zincinduced HO-1 upregulation in HCT-116 cells, several common antioxidants were evaluated for effectiveness in inhibiting zinc. Unless otherwise stated, cells were pretreated with the specified antioxidants for 1 hour, and then without changing the culture media, treated with zinc for 5 – 6 hours. In initial experiments, neither ascorbate nor Trolox, which is a cell-permeable water-soluble derivative of vitamin E, inhibited the induction of HO-1 protein caused by zinc (Figure 4A).

The iron chelator known as deferoxamine (DFO) has been reported to have antioxidant properties but not because of its ability to chelate iron [20]. Thus, the effect of DFO on the induction of HO-1 caused by zinc was next assessed. Pre-treating cells with DFO for 1 hour did not have any striking effect on zinc-induced upregulation of HO-1 mRNA and HO-1 protein (Figure 4B). A longer pre-treatment time of 20 hours with DFO before subsequent zinc treatment led to only slight inhibition of the HO-1 mRNA upregulation caused by zinc (Figure 4C), but zinc-induced HO-1 protein upregulation was inhibited by the 20-hour DFO pre-treatment (Figure 4D). In accord with this last finding, zinc increased haeme oxygenase enzymatic activity in HCT-116 cells (Figure 4E), but this effect was attenuated in cells pre-treated with DFO for 20 hours before next being exposed to zinc. To determine if the effect of DFO was due to chelation and depletion of cellular iron, HCT-116 cells that had been pre-treated with DFO were 'rescued' with a dose of iron (ferrous sulphate) and then treated with zinc. Zinc-induced HO-1 upregulation was partially restored in the iron-rescued cells (Figure 4F).

Two other well-known antioxidants were evaluated similarly. That is, cells were pre-treated with the test antioxidants (NAC and Tiron) for 1 hour, and then without changing the culture media, treated with zinc. NAC, which is often regarded as a thiol antioxidant, inhibited the increases in HO-1 mRNA and HO-1 protein caused by zinc (Figure 5A). Tiron, which is often regarded as a scavenger of superoxide, inhibited zinc-induced upregulation of HO-1 mRNA and HO-1 protein as well (Figure 5B). To expand the experiments with NAC and Tiron, cells were pretreated with either NAC or Tiron for 1 hour, but then, the culture media was removed and replaced with fresh media (containing no NAC and Tiron) before treating the cells with zinc for 5 – 6 hours. Under these particular experimental conditions, the outcome (Figure 5C) was somewhat different than before. At a concentration of 1 mM, neither NAC nor Tiron prevented zinc-induced HO-1 mRNA upregulation (mRNA gel lanes 4 and 8, compared to lane 2). At 10 mM, NAC still did not have a noticeable inhibitory effect, but Tiron did (mRNA gel lanes 5 and 9, compared to lane 2). On the other hand, zinc-induced HO-1 protein upregulation was inhibited

by 1 mM and 10 mM NAC (protein gel lanes 4 and 5, compared to lane 2). Tiron did not have an inhibitory eff ect at 1 mM but did at 10 mM (protein gel lanes 8 and 9, compared to lane 2). Therefore, taken together, the data would suggest that NAC and Tiron work partly by reacting with some of the zinc in the extracellular compartment (i.e. in the cell culture medium), thereby reducing the amount of zinc available for entry into the cells.

To compare the eff ect of NAC and Tiron on HO-1 upregulation caused by zinc with that caused either by the glutathione depleter, ethacrynic acid or the oxidant, TBHP, HCT-116 cells were pre-treated with either NAC or Tiron for 1 hour and then treated with either zinc, ethacrynic acid or TBHP. As shown in Figure 5D, NAC and Tiron once again prevented zinc-induced upregulation of HO-1 mRNA and HO-1 protein (mRNA and protein gel lanes 3 and 4, compared to lane 2). NAC prevented ethacrynic acid-induced upregulation of HO-1 mRNA and HO-1 protein expression (mRNA and protein gel lane 6, compared to lane 5), whereas Tiron had no such inhibitory effect (mRNA and protein gel lane 7, compared to lane 5). Regarding TBHP-induced upregulation of HO-1 mRNA (mRNA gel lane 8), neither NAC nor Tiron positively inhibited the upregulation (mRNA gel lanes 9 and 10).
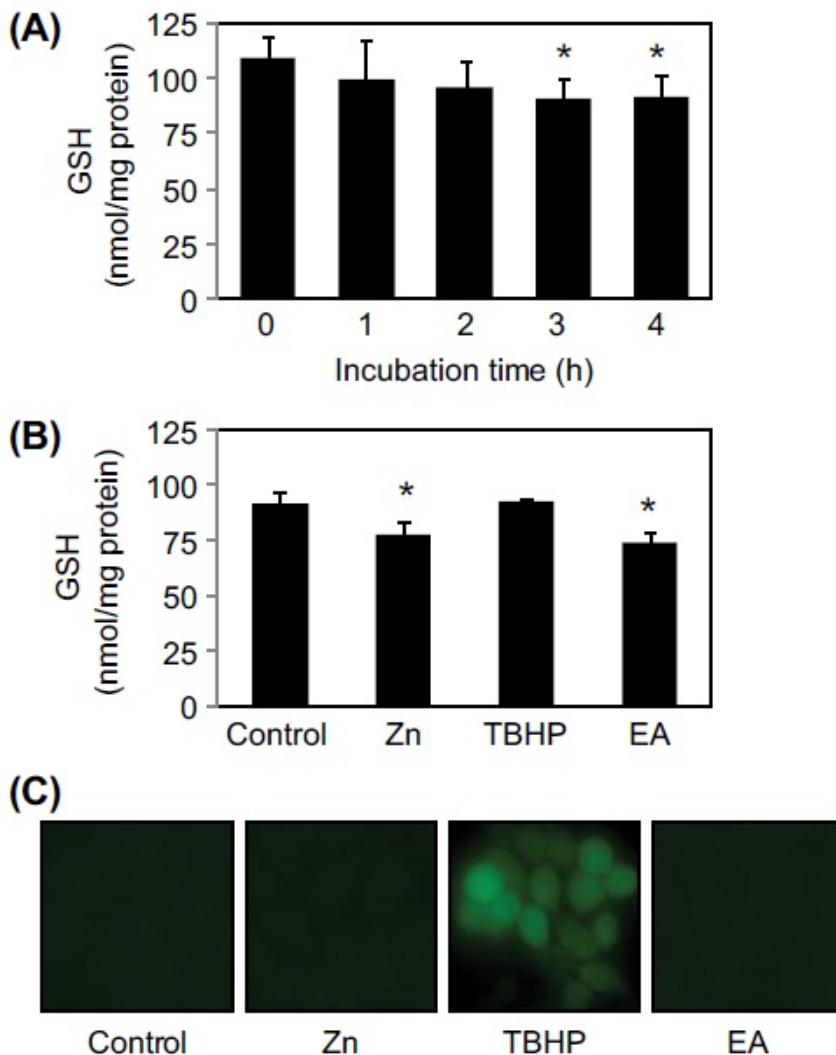
Figure 3. Lowering of non-protein thiols by zinc in relation to ROS production. HCT-116 cells were incubated with 150 μ M zinc sulphate (Zn) for a time range of 0 – 4 hours (A). Additionally, cells were incubated for 3 hours with 150 μ M Zn, and also with 200 μ M tert-butylhydroperoxide (TBHP) and 50 μ M ethacrynic acid (EA) for comparison (B and C). Afterwards, the levels of non-protein thiols were determined by spectrophotometry using glutathione (GSH) as the standard (A and B). The values are the average _ SD obtained from four different experiments (* $p < 0.05$ compared to control). The production of ROS was assessed by fluorescence microscopy (C). The images are representative of three different experiments.

Effect of zinc, in the absence and presence of Tiron and NAC, on nuclear Nrf2 expression

Nrf2 is a transcription factor that can activate antioxidant genes [21], such as HO-1. Nrf2 is normally localised in the cytoplasm, but translocates into the nucleus in activating the HO-1 gene. To determine if zinc stimulates Nrf2 translocation, HCT-116 cells were incubated with zinc and then examined by immunocytofluorescence microscopy (Figure 6A). As can be seen, zinc increased Nrf2 in nuclei, as indicated by the more intense green fluorescence in nuclei that have been counter-stained with DAPI to emit blue fluorescence. Moreover, Tiron and NAC each inhibited this apparent zinc-induced Nrf2 translocation. Relative differences in fluorescence intensities among the cell samples are shown in Figure 6B. To confirm these results, Nrf2 was next examined by Western-immunoblotting analysis. Nrf2 protein expression was found to be increased in nuclear extracts prepared from zinc-treated cells, and this effect of zinc was largely negated by either Tiron or NAC (Figure 6C).
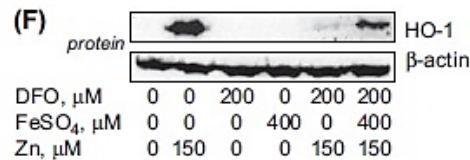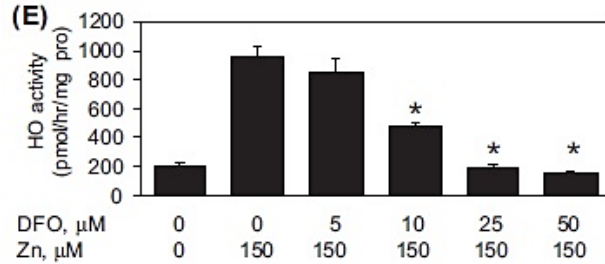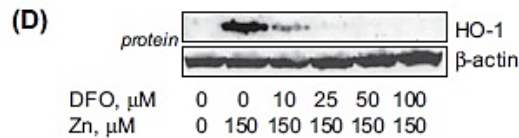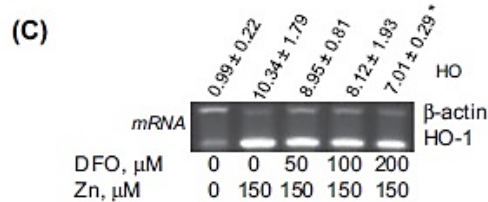
(A)

protein — HO-1 / β-actin

Ascorbate, μM	0	0	200	0	200	0
Trolox, μM	0	0	0	200	0	200
Zn, μM	0	150	150	150	0	0

(B)

0.89 ± 0.06 9.17 ± 1.31 0.55 ± 0.41 7.82 ± 1.29 HO-1 / β-actin

mRNA — β-actin, HO-1

protein — HO-1, β-actin

| DFO, μM | 0 | 0 | 200 | 200 |
| Zn, μM | 0 | 150 | 0 | 150 |

(C)

0.99 ± 0.22 10.34 ± 1.79 8.95 ± 0.81 8.12 ± 1.93 7.01 ± 0.29 * HO

mRNA — β-actin, HO-1

| DFO, μM | 0 | 0 | 50 | 100 | 200 |
| Zn, μM | 0 | 150 | 150 | 150 | 150 |

(D)

protein — HO-1, β-actin

| DFO, μM | 0 | 0 | 10 | 25 | 50 | 100 |
| Zn, μM | 0 | 150 | 150 | 150 | 150 | 150 |

(E)

HO activity (pmol/hr/mg pro)

| DFO, μM | 0 | 0 | 5 | 10 | 25 | 50 |
| Zn, μM | 0 | 0 | 150 | 150 | 150 | 150 |

(F)

protein — HO-1, β-actin

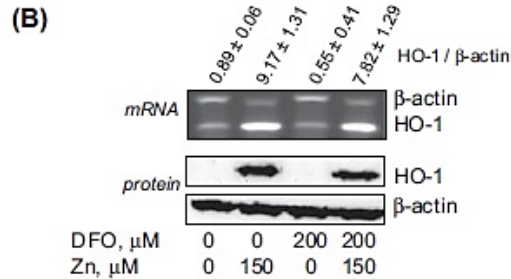| DFO, μM | 0 | 0 | 200 | 0 | 200 | 200 |
| FeSO$_4$, μM | 0 | 0 | 0 | 400 | 0 | 400 |
| Zn, μM | 0 | 150 | 0 | 0 | 150 | 150 |

Figure 4. Attenuation by deferoxamine (DFO), but not by ascorbate and trolox, of zinc-induced HO-1 upregulation. HCT-116 cells were pre-treated with either ascorbate or trolox (A) and also with DFO (B) for 1 hour, and then, treated with zinc sulphate (Zn) for 5 – 6 hours. Also, cells were pre-treated with DFO for 20 hours before Zn treatment (C – E), but some of these DFO-pre-treated cells were 'rescued' with ferrous sulphate (FeSO 4) for 4 hours before Zn treatment (F). Afterwards, the expression of HO-1 mRNA and HO-1 protein (A – D and F) was evaluated by multiplex relative RT-PCR analysis and Western-immunoblotting analysis, respectively. The results are representative of three diff erent experiments (* $p < 0.05$ compared to Zn-treated, for mRNA images). The enzymatic activity of haeme oxygenase (E) was assayed

spectrophotometrically, and the values are the average _ SD obtained from four diff erent experiments (* $p < 0.05$ compared to Zn-treated).

(A)

HO-1 / β-actin values: 0.75±0.09, 8.82±1.06, 5.45±1.11, 2.97±0.33*, 0.93±0.99*, 0.56±0.23*

mRNA: β-actin, HO-1
protein: HO-1, β-actin

NAC, mM	0	0	0.25	0.5	1	2
Zn, µM	0	150	150	150	150	150

(B)

HO-1 / β-actin values: 0.63±0.61, 12.92±2.00, 13.44±1.87, 10.33±2.71, 1.03±0.99*, 0.56±0.11*, 0.57±0.42*, 0.51±0.41*

mRNA: β-actin, HO-1
protein: HO-1, β-actin

Tiron, mM	0	0	0.1	0.2	0.5	1	2	5
Zn, µM	0	150	150	150	150	150	150	150

(C)

HO-1 / β-actin values: 0.69±0.66, 8.13±1.77, 0.70±0.14*, 6.92±2.12, 6.52±1.29, 0.59±0.16, 0.57±0.04*, 7.55±0.41, 1.52±1.29*, 0.53±0.29

mRNA: β-actin, HO-1
protein: HO-1, β-actin

NAC, mM	0	0	1	1ª	10ª	10	0	0	0	0
Tiron, mM	0	0	0	0	0	0	1	1ª	10ª	10
Zn, µM	0	150	150	150	150	0	150	150	150	0

ª1h pre-treatment, then removed and treated with zinc for 5-6h

(D)

HO-1 / β-actin values: 0.88±0.06, 10.99±0.07, 0.79±0.18*, 0.95±1.12*, 11.32±2.09, 1.12±0.44**, 9.73±0.54, 8.08±1.41, 4.12±0.39***, 8.23±1.87

mRNA: β-actin, HO-1
protein: HO-1, β-actin

NAC, mM	0	0	1	0	0	1	0	0	1	0
Tiron, mM	0	0	0	1	0	0	1	0	0	1
Zn, µM	0	150	150	150	0	0	0	0	0	0
EA, µM	0	0	0	0	50	50	50	0	0	0
TBHP, µM	0	0	0	0	0	0	0	150	150	150

Figure 5. Attenuation by N-acetylcysteine (NAC) and Tiron of the induction of HO-1 by zinc and other stress agents. HCT-116 cells were pre-treated with either NAC (A) or Tiron (B) for 1 hour, and then without changing the culture media, treated for 5 – 6 hours with zinc sulphate (Zn). Moreover, cells were pre-treated with either NAC or Tiron, but then, the NAC-containing and Tiron-containing culture media were replaced with new media before the Zn treatment (C). Additionally, cells were pre-treated with either NAC or Tiron for 1 hour, and then, treated for 5 – 6 hours with Zn, ethacrynic acid (EA) and tert-butylhydroperoxide (TBHP) for comparison (D). Afterwards, the expression of HO-1 mRNA and HO-1 protein was evaluated by multiplex relative RT-PCR analysis and Westernimmunoblotting analysis, respectively. The results are representative of three different experiments (* $p < 0.05$ compared to Zn-treated, for mRNA images in A – C; * $p < 0.05$ compared to Zn-treated, * * $p < 0.05$ compared to EA-treated and * * * $p < 0.05$ compared to TBHP-treated, for mRNA images in D).
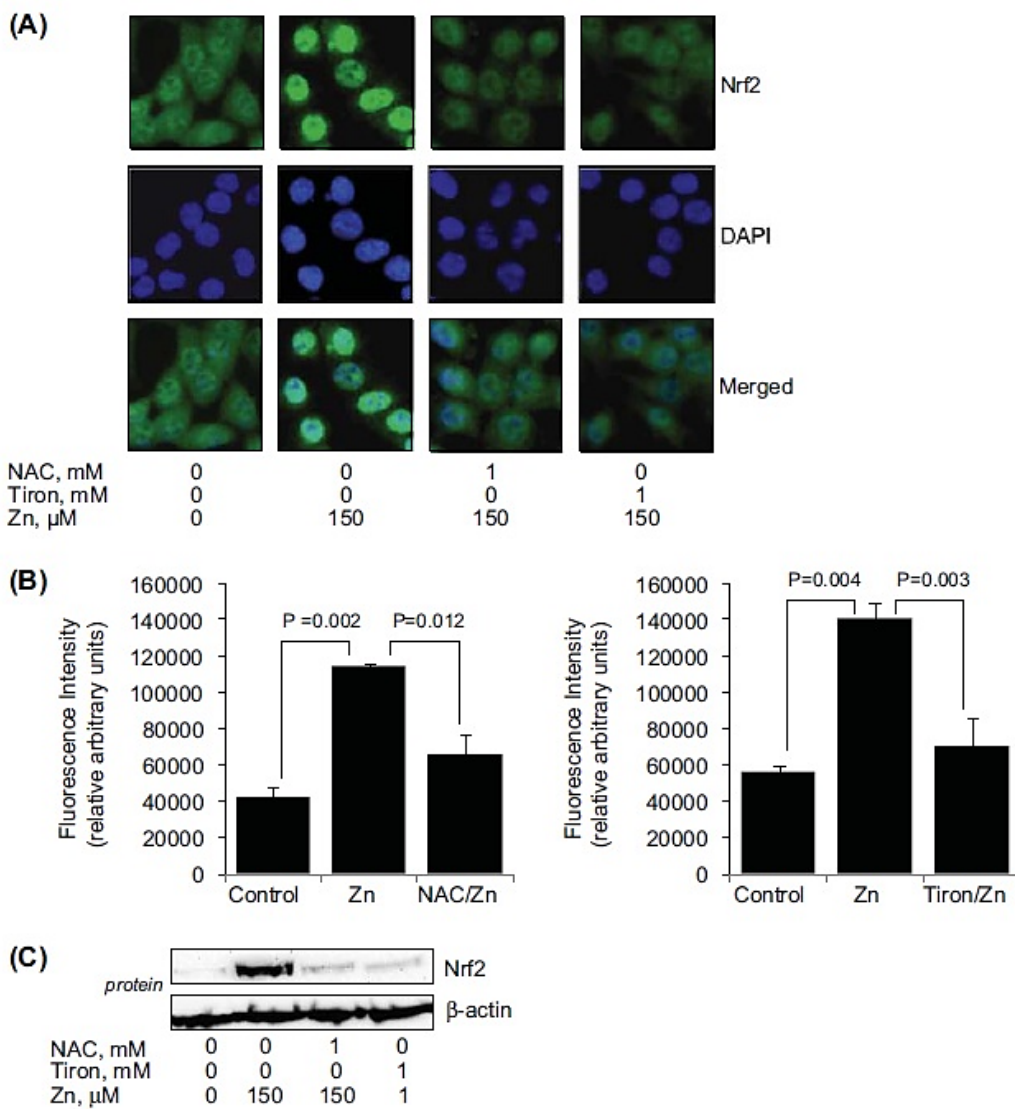

Figure 6. Zinc increases nuclear Nrf2 expression that is attenuated by NAC and Tiron. HCT-116 cells were pre-treated with either NAC or Tiron for 1 hour, and then treated with zinc sulphate

(Zn) for 4 hours. The cells were then visualised by fl uorescence microscopy (A), with subsequent imaging analysis of the green fl uorescence intensity in nuclei refl ecting Nrf2 (B). Additionally, nuclear extracts of cells were prepared for Western-immunoblotting analysis (C). The results are representative of 3 – 4 diff erent experiments.

Discussion

At the outset, it was found that exogenously-added zinc, but neither copper nor iron, increased HO-1 expression in HCT-116 cells. As a micronutrient, zinc is a metallic element that all cells require for their viability. Uptake of exogenously-added zinc by cells occurs within an hour [22] via ZIP zinc-uptake proteins [23] that permit non-saturable passive electrodiff usion of zinc ions [24]. It has been approximated that total intracellular concentrations of zinc fall in the range of 0.1 – 0.5 mM, but the actual free zinc levels are unclear [25]. However, beyond certain concentrations, zinc can become a toxic metal to cells, such as neurons [26] and lung cells [27] for example. To help protect cells against the adverse eff ects of too much zinc, increased expression of certain cytoprotective or stress genes including HO-1 could be critical. HO-1 is the inducible isoform of haeme oxygenase, which contributes to cytoprotection in the body based on genetic studies [28,29] showing that susceptibility to oxidative stress and inflammation is increased in human cases of HO-1 deficiency. Apparently, upregulation of HO-1 expression by zinc occurs sooner than upregulation of another zinc-inducible cytoprotective gene, namely, metallothionein. Zinc increased metallothionein expression in HCT-116 cells, but only after the cells were incubated with 250 µ M zinc for at least 24 – 72 hours [30]. Yet, in the present study, HO-1 expression was increased in HCT-116 cells when incubated with 150 µ M zinc for 5 – 6 hours overall. Hence, HO-1 appears to be an earlier response gene than metallothionein when HCT-116 cells are confronted with added zinc.

As an inducer of HO-1 expression, zinc elicits a cellular response that has been produced by other stress agents, which are thought to be either undesirable or desirable. Strong oxidants [31], UV radiation [32], chemical carcinogens [33] and toxic heavy metals [6] increase HO-1 expression as well. These particular kinds of HO-1 inducers are considered undesirable because they also damage cellular components and disrupt multiple cellular processes. However, other kinds of HO-1 inducers, such as certain phytochemicals found in edible plant products [34,35] are considered desirable because they do not pose a serious threat to the integrity of cells. Instead, by inducing HO-1 in a non-threatening manner, phytochemicals are thought to help protect cells against more stressful situations according to the concept of hormesis [36]. Since phytochemicals and zinc are similar in that both are bioactive food components, zinc might be seen from a different perspective in the same hormetic limelight as phytochemicals. Similar to known hormetins, zinc has contrasting effects depending on the circumstances or experimental conditions. Zinc can stimulate ROS production [13,14] and cause apoptosis [37], but paradoxically, zinc can also act as an antioxidant [9] and protect against apoptosis [38].

In explaining the upregulation of HO-1 expression caused by zinc, it is possible that ROS could play a role since ROS can be involved in the molecular pathway leading to HO-1 gene activation [4]. Zinc at relatively high concentrations has been reported to stimulate ROS production in neurons and astrocytes [13] and prostate cells [14], but the exact mechanism is unclear. Also, it has been reported that incubating HCT-116 cells with 250 µ M zinc increased ROS production

after 24 hours, although higher production was not noticeable after 12 hours of zinc exposure [30]. In agreement, greater production of ROS was not found in HCT-116 cells that were incubated with only 150 μ M zinc for time periods up to only 3 hours in the present study. Yet, increased HO-1 mRNA expression was detected before 3 hours. Moreover, the two standard antioxidants, ascorbate and trolox, did not prevent the upregulation of HO-1 caused by zinc. Taken together, these particular current fi ndings would suggest that ROS are not involved in the zinc-induced HO-1 upregulation ascertained in HCT-116 cells.

Despite no unequivocal evidence of oxidative stress in the zinc-treated HCT-116 cells, it was somewhat unexpected to find that other well-recognised antioxidative compounds influence the ability of zinc to upregulate HO-1 expression in HCT-116 cells. NAC and Tiron each inhibited zinc-induced HO-1 upregulation. However, Tiron and NAC have another important property in common outside of being capable of scavenging ROS. In particular, NAC and Tiron are general chelators of metal ions [39,40]. As such, in inhibiting zinc-induced HO-1 upregulation, NAC and Tiron possibly act by chelating the added zinc. Regarding the standard high-affi nity iron chelator, DFO, it was found that pre-treating HCT-116 cells with DFO for the same time period (1 hour) as for NAC and Tiron had no inhibitory effect on the HO-1 upregulation caused by zinc. On the other hand, zinc-induced HO-1 upregulation was blunted in cells that were pre-treated with DFO for a longer time period (20 hours). This blunting effect of DFO is probably not the result of DFO chelating the added zinc but more likely due to DFO chelating intracellular iron. Seemingly, adequate amounts of iron must be available in HCT-116 cells to somehow enable zinc to upregulate HO-1.

Besides ROS, non-protein thiols can influence the expression of redox-sensitive genes [41]. Because HO-1 is a redox-sensitive gene [31], zinc could conceivably upregulate HO-1 in HCT-116 cells by affecting the levels of non-protein thiols, such as glutathione, which is known to be the most abundant thiol antioxidant in cells. In support of this possibility, lowering non-protein thiol levels in HCT-116 cells with ethacrynic acid was associated with HO-1 upregulation in the current study. More notable, zinc also lowered non-protein thiol levels in the cells in association with HO-1 upregulation. Interestingly, in upregulating HO-1 in HCT-116 cells, zinc and ethacrynic acid seem to act similarly in that each lowered the nonprotein thiol levels while not increasing ROS production. Hence, stimulation of ROS production may not be an absolute requirement for HO-1 upregulation.

The molecular pathway leading to HO-1 upregulation may involve the transcription factor, Nrf2, which is known to upregulate the expression of the HO-1 gene [42] specifically by binding to characteristic stress response elements [43]. Activation of Nrf2 is thought to be a cytoprotective response to environmental toxins [44], including potentially toxic metals. Supporting this view, various metallic elements including zinc stimulated the activity of an Nrf2-specifi c reporter gene construct containing stress response elements that was stably transfected into cells [45]. For Nrf2-mediated transcriptional activation of the HO-1 gene to occur [46], Nrf2 is activated by being dissociated from the cysteine-rich protein, Kelchlike ECH-associated protein 1 (Keap1), which under nonstressful conditions binds and retains Nrf-2 in the cytoplasm. Evidently, Keap1 is disabled by stressors such as various types of oxidants and electrophilic compounds that react with cysteine residues on Keap1 [47,48], thereby preventing Keap1 from binding and targeting Nrf2 for ubiquitination and consequent proteosomal degradation.

As such, preservation of Nrf2 allows it to translocate into the nucleus. Consistent with this sequence of events, there was a lower presence in cytoplasm and a higher presence in the nucleus of Nrf2 after HCT-116 cells had been treated with zinc, which was largely negated by either NAC or Tiron.

How added zinc is able to stimulate nuclear translocation of Nrf2 in HCT-116 cells is unclear. The zinc lowered cellular levels of non-protein thiols, likely reflecting to a large extent the chemical reactivity of zinc ions with the free sulfhydryl group of glutathione molecules. On the other hand, it is highly conceivable that the added zinc also reacts with free sulfhydryl groups of protein thiols. As such, one plausible explanation for the stimulatory effect of added zinc on nuclear translocation of Nrf2 concerns the electrophilic behaviour of the added zinc ions towards the cysteine residues of Keap1. Since ROS production was not increased in zinc-treated HCT-116 cells, it can be inferred that the cysteine residues of Keap1 were not subjected to possible oxidative attack. Instead, displaying electrophilic behaviour and thiol-reactivity, the added zinc ions possibly reacted with particular cysteine sulfhydryl groups of Keap1, resulting in disabling of Keap1 and subsequent liberation of Nrf2. It has been discovered that Keap1 has zinc-binding capacity [49]. Moreover, it has been shown that zinc can inhibit sulfhydryl proteins involved in the activation of redox-sensitive transcription factors [50]. Nevertheless, it cannot be ruled out that other redox-sensitive transcription factors play a role in zinc-induced HO-1 upregulation. The promoter region of the HO-1 gene contains sites for binding of NF-kappa B, AP-1 and AP-2 [51].

In conclusion, the collective findings suggest that zinc is a metal micronutrient that at concentrations above normal in HCT-116 cells can upregulate HO-1 expression. Moreover, ROS do not appear to be involved in the zincinduced HO-1 upregulation.

Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Goldhaber SB. Trace element risk assessment: essentiality vs. toxicity. Regul Toxicol Pharmacol 2003;38:232 – 242.

[2] Peralta-Videa JR, Lopez ML, Narayan M, Saupe G, Gardea-Torresdey J. The biochemistry of environmental heavy metal uptake by plants: implications for the food chain. Int J Biochem Cell Biol 2009;41:1665 – 1677.

[3] Kikuchi G, Yoshida T, Noguchi M. Heme oxygenase and heme degradation. Biochem Biophys Res Commun 2005;338: 558 – 567.

[4] Camhi SL, Alam J, Wiegand GW, Chin BY, Choi AM. Transcriptional activation of the HO-1 gene by lipopolysaccharide is mediated by 5 ' distal enhancers: role of reactive oxygen intermediates and AP-1. Am J Respir Cell Mol Biol 1998;18: 226 – 234.

[5] Vile GF, Basu-Modak S, Waltner C, Tyrrell RM. Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fi broblasts. Proc Natl Acad Sci USA 1994;91:2607–2610.

[6] Elbekai RH, El-Kadi AO. Modulation of aryl hydrocarbon receptor-regulated gene expression by arsenite, cadmium, and chromium. Toxicology 2004;202:249 – 269.

[7] Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990;186:1 – 85.

[8] Coleman JE. Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu Rev Biochem 1992;61:897 – 946.

[9] Powell SR. The antioxidant properties of zinc. J Nutr 2000;130: 1447S – 1454S.

[10] Perez MJ, Cederbaum AI. Metallothionein 2A induction by zinc protects HEPG2 cells against CYP2E1-dependent toxicity. Free Radic Biol Med 2003;34:443 – 455.

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

[12] Palmiter RD. Protection against zinc toxicity by metallothionein and zinc transporter 1. Proc Natl Acad Sci USA 2004;101: 4918 – 4923.

[13] Noh KM, Koh JY. Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. J Neurosci 2000;20:1 – 5.

[14] Park SE, Park JW, Cho YS, Ryu JH, Paick JS, Chun YS. HIF-1alpha promotes survival of prostate cells at a high zinc environment. Prostate 2007;67:1514 – 1523.

[15] Wilhelm B, Walther UI, Fichtl B. Eff ects of zinc chloride on glutathione and glutathione synthesis rates in various lung cell lines. Arch Toxicol 2001;75:388 – 394.

[16] Mazza F, Goodman A, Lombardo G, Vanella A, Abraham NG. Heme oxygenase-1 gene expression attenuates angiotensin IImediated DNA damage in endothelial cells. Exp Biol Med 2003;228:576 – 583.

[17] Polte T, Abate A, Dennery PA, Schr o der H. Heme oxygenase-1 is a cGMP-inducible endothelial protein and mediates the cytoprotective action of nitric oxide. Arterioscler Thromb Vasc Biol 2000;20:1209 – 1215.

[18] Ye J, Zhang X, Young HA, Mao Y, Shi X. Chromium(VI)- induced nuclear factor-kappa B activation in intact cells via free radical reactions. Carcinogenesis 1995;16:2401 – 2405.

[19] Tirona RG, Pang KS. Bimolecular glutathione conjugation kinetics of ethacrynic acid in rat liver: in vitro and perfusion studies. J Pharmacol Exp Ther 1999;290:1230 – 1241.

[20] Shimoni E, Armon R, Neeman I. Antioxidant properties of deferoxamine. J Am Oil Chem Soc 1994;71:641 – 644.

[21] Kobayashi M, Yamamoto M. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. Antioxid Redox Signal 2005;7:385 – 394.

[22] Cortese MM, Suschek CV, Wetzel W, Kr o ncke KD, Kolb-Bachofen V. Zinc protects endothelial cells from hydrogen peroxide via Nrf2-dependent stimulation of glutathione biosynthesis. Free Radic Biol Med 2008; 44 :2002 – 2012.

[23] Liuzzi JP, Cousins RJ. Mammalian zinc transporters. Annu Rev Nutr 2004; 24 :151 – 172.

[24] Lin W, Chai J, Love J, Fu D. Selective electrodiff usion of zinc ions in a Zrt-, Irt-like protein, ZIPB. J Biol Chem 2010; 285 : 39013 – 39020.

[25] Eide DJ. Zinc transporters and the cellular traffi cking of zinc. Biochim Biophys Acta 2006;1763:711 – 722.

[26] Chen CJ, Liao SL. Zinc toxicity on neonatal cortical neurons: involvement of glutathione chelation. J Neurochem 2003;85: 443 – 453.

[27] Walther UI, M u ckter H, Fichtl B, Forth W. Infl uence of glutathione on zinc-mediated cellular toxicity. Biol Trace Elem Res 1999;67:97 – 107.

[28] Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, et al. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 defi ciency. J Clin Invest 1999;103:129 – 135.

[29] Kawashima A, Oda Y, Yachie A, Koizumi S, Nakanishi I. Heme oxygenase-1 defi ciency: the fi rst autopsy case. Hum Pathol 2002;33:125– 130.

[30] John S, Briatka T, Rudolf E. Diverse sensitivity of cells representing various stages of colon carcinogenesis to increased extracellular zinc: implications for zinc chemoprevention. Oncol Rep 2011;25:769 – 780.

[31] Li MH, Cha YN, Surh YJ. Peroxynitrite induces HO-1 expression via PI3K/Akt-dependent activation of NF-E2-related factor 2 in PC12 cells. Free Radic Biol Med 2006;41:1079 – 1091.

[32] Tyrrell RM. Solar ultraviolet A radiation: an oxidizing skin carcinogen that activates heme oxygenase-1. Antioxid Redox Signal 2004;6:835 – 840.

[33] Chin BY, Trush MA, Choi AM, Risby TH. Transcriptional regulation of the HO-1 gene in cultured macrophages exposed to model airborne particulate matter. Am J Physiol Lung Cell Mol Physiol 2003;284:L473 – L480.

[34] Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, et al. Curcumin activates the haeme oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochem J 2003;37:887 – 895.

[35] Chen CY, Jang JH, Li MH, Surh YJ. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. Biochem Biophys Res Commun 2005;331:993 – 1000.

[36] Son TG, Camandola S, Mattson MP. Hormetic dietary phytochemicals. Neuromolecular Med 2008;10:236 – 246.

[37] Rudolf E, Rudolf K, Cervinka M. Zinc induced apoptosis in HEP-2 cancer cells: the role of oxidative stress and mitochondria. Biofactors 2005;23:107– 120.

[38] Szuster-Ciesielska A, Plewka K, Daniluk J, Kandefer-Szerszeń M. Zinc inhibits ethanol-induced HepG2 cell apoptosis. Toxicol Appl Pharmacol 2008;229:1 – 9.

[39] Banner W Jr, Koch M, Capin DM, Hopf SB, Chang S, Tong TG. Experimental chelation therapy in chromium, lead, and boron intoxication with N-acetylcysteine and other compounds. Toxicol Appl Pharmacol 1986;83:142 – 147.

[40] Sharma P, Johri S, Shukla S. Beryllium-induced toxicity and its prevention by treatment with chelating agents. J Appl Toxicol 2000;20:313 – 318.

[41] Tanaka C, Kamata H, Takeshita H, Yagisawa H, Hirata H. Redox regulation of lipopolysaccharide (LPS)-induced interleukin-8 (IL-8) gene expression mediated by NF kappa B and AP-1 in human astrocytoma U373 cells. Biochem Biophys Res Commun 1997;232:568 – 773.

[42] Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 1999;274: 26071 – 26078.
[43] Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 2003;9:2499 – 2511.

[44] Osburn WO, Kensler TW. Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. Mutat Res 2008;659:31 – 39.

[45] Simmons SO, Fan CY, Yeoman K, Wakefi eld J, Ramabhadran R. NRF2 Oxidative Stress Induced by Heavy Metals is Cell Type Dependent. Curr Chem Genomics 2011;5:1 – 12.

[46] Itoh K, Tong KI, Yamamoto M. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radic Biol Med 2004;36:1208 – 1213.

[47] Zhang DD, Hannink M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 2003;23:8137 – 8151.

[48] Fourquet S, Guerois R, Biard D, Toledano MB. Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfi de formation. J Biol Chem 2010;285:8463 – 8471.

[49] Dinkova-Kostova AT, Holtzclaw WD, Wakabayashi N. Keap1, the sensor for electrophiles and oxidants that regulates the phase 2 response, is a zinc metalloprotein. Biochemistry 2005;44: 6889 – 6899.

[50] Jeon KI, Jeong JY, Jue DM. Thiol-reactive metal compounds inhibit NF-kappa B activation by blocking I kappa B kinase. J Immunol 2000;164:5981 – 5989.

[51] Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, Abraham NG. Identifi cation of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. Proc Natl Acad Sci USA 1994;91: 5987– 5991.