Accumulation of manganese superoxide dismutase under metal-depleted conditions: proposed role for zinc ions in cellular redox balance

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A diet low in copper results in increased levels of MnSOD (manganese superoxide dismutase), a critical antioxidative enzyme conferring protection against oxidative stress, in rat liver mitochondria. The mechanism for this was investigated using cultured HepG2 cells, a human hepatocellular carcinoma-derived line. MnSOD activity increased 5–7-fold during incubation in a medium supplemented with metal-depleted fetal bovine serum, with a corresponding elevation of its mRNA levels. Metal depletion also decreased CuZnSOD and glutathione peroxidase levels to approx. 70–80 % of baseline. When zinc ions were added to the medium at micromolar levels, MnSOD accumulation was suppressed; however, copper ions had essentially no effect on MnSOD expression. Since the intracellular redox status was shifted to a more oxidized state by metal depletion, we examined the DNA-binding activity of NF-κB (nuclear factor-κB), an oxidative stress-sensitive transactivating factor that plays a primary role in MnSOD induction. A gel shift assay indicated that the DNA-binding activity of NF-κB was increased in cells maintained in metal-depleted culture, suggesting the involvement of the transactivating function of NF-κB in this induction. This was further supported by the observation that curcumin suppressed both the DNA-binding activity of NF-κB and the induction of MnSOD mRNA in cells cultivated under metal-depleted conditions. These results suggest that the level of zinc, rather than copper, is a critical regulatory factor in MnSOD expression. It is possible that a deficiency of zinc in the low-copper diet may be primarily involved in MnSOD induction.

Key words: copper ion, glutathione peroxidase, metal depletion, reactive oxygen species, superoxide dismutase, zinc ion.

INTRODUCTION

SODs (superoxide dismutases) catalyse the dismutation of superoxide radicals to hydrogen peroxide and serves to protect cells against ROS (reactive oxygen species) [1]. Although CuZnSOD and MnSOD, encoded by the *SOD1* and *SOD2* genes respectively, represent a major superoxide-scavenging system, their contribution varies depending on the type of cells being investigated.

Despite the fact that a mutation in *SOD1* is a cause of familial amyotrophic lateral sclerosis, *SOD1*-knockout mice grow normally and live healthily under conventional breeding conditions [2]. Intervention, however, frequently impairs knockout mice more severely than their wild-type counterparts [3,4]. *SOD2*-null knockout mice, on the other hand, show dilated cardiomyopathy and die during the neonatal stage [5]. An increase in oxidative damage has been reported even in heterozygous *SOD2*-knockout mice [6,7].

SOD2 expression is regulated by many agents, including tumour necrosis factor, interleukin-1, interleukin-6, lipopolysaccharide, phorbol esters and inhibitors of protein synthesis [8–11]. Constitutive *SOD1* expression occurs commonly in most tissues, except for keratinocytes and glomerular mesangial cells, in which nitrosoglutathione induces *SOD1* gene expression [12,13]. The promoter and regulatory elements of *SOD2* have been extensively characterized, and several transactivating factors for the gene have been identified [14–17]. ROS, which are elevated by many stimuli, induce *SOD2* expression by activating redox-sensitive transactivating factors, such as NF-κB (nuclear factor-κB) and AP-1 (activator protein-1) [18–21].

Copper and manganese constitute the active centres of CuZnSOD and MnSOD respectively [1]. Hence a deficiency of these metal ions would be expected to lead to a decrease in the activity of these enzymes. A copper-deficient diet actually results in a decrease in CuZnSOD activity, but a concomitant increase in MnSOD activity occurs in rat liver cells [22]. This increase in MnSOD activity appears to be due to the transcriptional activation of *SOD2* under copper-deficient conditions [23]. *SOD2* expression in the liver is up-regulated by copper chelation with diethyldithiocarbamate and, in turn, down-regulated by iron chelation with desferrioxamine or by treatment with *N*-acetylcysteine, an antioxidant [24]. While replenishment with iron enhances lipopolysaccharide-induced *SOD2* induction, iron chelation by desferrioxamine suppresses it in cultivated hepatocytes and hepatocellular carcinoma cells [25]. Thus oxidative stress resulting from the down-regulation of CuZnSOD or the accumulation of iron is the suspected cause of *SOD2* induction under copper-deficient conditions.

In the present study, we report on an investigation of the effects of metal depletion of FBS (fetal bovine serum) on the expression of antioxidative enzymes in HepG2 cells. Our results differ from some earlier findings, i.e. MnSOD accumulation is caused by augmented ROS via the activation of redox-sensitive transactivating factors, and does not appear to be necessarily associated with a decrease in CuZnSOD activity. Moreover, a physiological level of zinc ions had an enhancing effect on the cellular

Abbreviations used: DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified minimum essential medium; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; LDH, lactate dehydrogenase; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; SOD, superoxide dismutase.

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redox state, returning it to normal levels, and also suppressed MnSOD induction.

EXPERIMENTAL

Cell culture

HepG2 cells were maintained in DMEM (Dulbecco's modified minimum essential medium; Sigma) containing 100 units/ml penicillin and 100 μg/ml streptomycin supplemented with 10 % (v/v) FBS (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere with 5 % CO_2. For some experiments, cells were cultivated under the same conditions except that metal-depleted FBS was substituted for the conventional medium. Metal depletion of FBS was carried out as described by Tong et al. [26]. Briefly, FBS (0.4 litres) was dialysed at 4 °C against three changes of 30 mM triethylenetetramine (1.6 litres) (Wako, Osaka, Japan) for 12 h each in PBS. To remove residual triethylenetetramine, FBS was dialysed further against nine changes of PBS (1.6 litres).

Measurement of enzyme activities

After washing once with PBS, cells were scraped from the culture dishes and collected by centrifugation at 900 ***g*** for 4 min. Cells were suspended in an appropriate volume of 10 mM Tris/HCl, pH 7.4, and disrupted twice by means of a Bioruptor (Cosmo Bio, Tokyo, Japan) at 200 W for 30 s each with cooling in ice-cold water. The supernatant was collected after centrifugation at 17 000 ***g*** for 15 min and was used for the assay of enzyme activities.

SOD activity was determined essentially using the method described by Beauchamp and Fridovich [27], with minor modifications. WST-1 (Wako, Osaka, Japan) instead of Nitroblue Tetrazolium was used for detection of the superoxide anions generated. Briefly, the reaction mixture contained an appropriate amount of diluted xanthine oxidase (Roche), 0.1 mM xanthine, 0.025 mM WST-1, 0.1 mM EDTA and 50 mM $NaHCO_3$, pH 10.2, in a total volume of 3 ml. The increase in absorbance at 438 nm was monitored at 25 °C for 1 min. One unit is defined as the amount of enzyme required to inhibit 50 % of the absorbance change of 0.060/min. This unit of enzyme activity was equivalent to that determined by the standard procedure using Nitroblue Tetrazolium. CuZnSOD and MnSOD activities were defined as activities that were respectively inhibited by and resistant to 2 mM NaCN.

GPX (glutathione peroxidase) activity was determined as described previously [28]. One unit is defined as the amount of enzyme required to oxidize 1 μmol of NADPH (corresponding to 2 μmol of GSH)

The cytochrome oxidase assay involved measurement of the oxidation of cytochrome *c* [29]. The reaction mixture consisted of 50 μM reduced cytochrome *c*, 0.1 % Brij 58, 10 mM sodium phosphate buffer, pH 7.1, and 100 μl (0.2 mg of protein) of whole-cell homogenate in a total volume of 1.0 ml. The reactions were monitored at 500 nm at room temperature by means of a spectrophotometer (U-2000; Hitachi, Tokyo, Japan).

SDS/PAGE and immunoblot analysis

Protein samples were subjected to SDS/12 %-PAGE [30] and then transferred to a Hybond-P membrane (Amersham Pharmacia) under semi-dry conditions with the use of a Transfer-blot SD Semi-dry transfer cell (Bio-Rad). The membrane was then blocked by incubation with 5 % (w/v) skimmed milk in TBS (150 mM NaCl and 20 mM Tris/HCl, pH 7.6) for 2 h at room temperature. The membranes were incubated with a rabbit anti-(human MnSOD) Ig (1:1000 dilution) or a goat anti-(human Cu,ZnSOD) Ig (1:1000 dilution) (gifts from Dr N. Taniguchi, Osaka University, Japan) for 16 h at 4 °C. After washing with TBS containing 0.1 % Tween 20, the membrane was incubated with 1:1000 diluted peroxidase-conjugated goat anti-(rabbit IgG) Ig or donkey anti-(goat IgG) Ig (Santa Cruz Biotechnology) for 1 h at room temperature. After washing, peroxidase activity was detected by a chemiluminescence method using an ECL Plus® kit (Amersham Pharmacia).

Northern blot analysis

Total cellular RNA was prepared from the cells in triplicate using Isogen (Wako). RNA (10 μg) was electrophoresed on a 1 % (w/v) agarose gel containing 2.2 M formaldehyde. The size-fractionated RNA was transferred by a PosiBlot pressure blotter (Stratagene) on to a Hybond N^+ membrane (Amersham Pharmacia) and cross-linked to the membrane by a UV cross-linker (Amersham Pharmacia). After hybridization with a ^{32}P-labelled human *SOD2* [10], a human *SOD1* [10] or a rat *GPX1* [28] probe at 42 °C in the presence of 50 % formamide, the membrane was washed twice at 55 °C in 0.2 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0) and 0.1 % SDS for 30 min. The radioactivity on the membrane was visualized and quantified by a Fujix Bioimaging Analyzer BAS 2000 (Fuji Photo Film, Tokyo, Japan). After deprobing, the membranes were re-hybridized with a rat GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA probe. The mean mRNA level for each enzyme prepared from conventional culture is represented as 1.0.

Flow cytometric analysis of intracellular redox status

The intracellular redox status of HepG2 cells was analysed by flow cytometry utilizing DCFH-DA (2′,7′-dichlorofluorescein diacetate; Molecular Probes) as a probe. The cells were trypsinized prior to loading with DCFH-DA. The reaction was terminated by the addition of an excess of soybean trypsin inhibitor (Type II-S; Sigma). After washing once with PBS, the cells were loaded with 5 μM DCFH-DA in PBS at 37 °C for 5 min, and then washed once with PBS. DCF (2′,7′-dichlorofluorescein) fluorescence of individual cells was analysed by FACSCalibur flow cytometry (Becton Dickinson) interfaced with an argon laser with excitation at 488 nm. DCF fluorescence was collected through a 530/30-nm filter (FL1), and analysed using the CellQuest software program.

Measurement of the cytotoxic effects of superoxide and hydrogen peroxide

LDH (lactate dehydrogenase) from cells was measured, in order to assess the sensitivity of the cells to superoxide and hydrogen peroxide. Cells were seeded into 24-well plates and cultured for 8 days in DMEM supplemented with 10 % (v/v) metal-depleted FBS. Some cells were cultured for 8 days in 10 % (v/v) metal-depleted FBS with replenishment of 10 μM $ZnSO_4$. The cells cultured under conventional conditions grew faster and, as a result, were seeded 16 h before the experiment. A flux of superoxide was generated at 37 °C for 2 h using 4 mM hypoxanthine and various concentrations of xanthine oxidase (0–80 m-units/ml) in DMEM. After washing twice with PBS, the cells were cultured for 21 h in the culture medium. Hydrogen peroxide treatment of the cells was performed with the indicated concentrations of reagents in DMEM for 23 h. At the end of the culture period, portions of the medium were collected for measurement of LDH activity.

The cells were disrupted by brief sonication in PBS containing 0.1 % Tween 20. Cellular extracts, free of debris, were prepared by centrifugation at 17 000 ***g*** for 10 min. The assay of LDH activity was performed using an LDH CII kit (Wako). The viability of the cells was calculated by expressing the LDH activity recovered in the cell extract as a percentage of the total (cell extract plus medium) activity recovered.

EMSA (electrophoretic mobility-shift assay) of NF-κB

HepG2 cells that had been cultured under metal-depleted or zinc-supplemented conditions for various periods were washed once with ice-cold PBS. Cells were scraped off, collected by centrifugation (900 ***g***), and resuspended by pipetting in 0.4 ml of buffer A (10 mM Hepes/KOH, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 20 μM APMSF (*p*-amidinophenylmethanesulphonyl fluoride), 2 μg/ml aprotinin, 2 μg/ml pepstatin A and 2 μg/ml leupeptin). After incubation on ice for 15 min, cells were lysed by addition of 25 μl of 10 % (v/v) Nonidet P40 with vigorous vortexing. Then the cell lysate was left on ice for 5 min. Nuclei were sedimented by centrifugation at 12500 ***g*** for 30 s, followed by extraction for 20 min on ice in buffer C [20 mM Hepes/KOH, pH 7.8, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 10 % (v/v) glycerol]. Supernatants containing nuclear proteins were harvested by centrifugation for 15 min at 15 000 ***g*** at 4 °C. The protein concentration was determined by using the BCA (bicinchoninic acid) protein assay reagent (Pierce), and aliquots were stored at − 80 °C until used.

The binding activity of NF-κB in nuclear extracts was evaluated by EMSA using a ^{32}P-labelled oligonucleotide probe, 5′-GAGACTGGGGAATACCCCAGT-3′ (NF-κB binding sequences are underlined), which corresponds to human *SOD2* regulatory sequences according to Kiningham et al. [21]. The nuclear extract and the labelled oligonucleotide were incubated in a binding buffer at room temperature for 30 min, and then subjected to electrophoresis on 4 % non-denaturing PAGE. For competition experiments, nuclear extracts were incubated for 10 min at room temperature with competing oligonucleotide (100-fold excess) prior to the addition of the labelled probe. For supershift assays, the reaction mixture contained 2 μg/tube of anti-p50 and anti-p65 polyclonal antibodies (Santa Cruz Biotechnology) and was incubated for 30 min at room temperature. After drying under vacuum with heat, the gels were exposed to Kodak X-AR film overnight.

Determination of metals in culture media

The metal content of the culture medium was determined by a flame atomic absorption method using a Spectro AA-55 instrument (Varian).

Statistics

Data are presented as means ± S.D., with the number of experiments in parentheses. Student's *t* test was used to compare the significance of the differences between data. Values of $P < 0.05$ were considered significant.

RESULTS

MnSOD levels are increased under metal-depleted culture conditions

To investigate the underlying mechanism of MnSOD accumulation in the liver following consumption of a copper-deficient diet, we cultured HepG2 cells in medium supplemented with metal-depleted FBS that had been dialysed against the metal chelator triethylenetetramine (Figure 1A). MnSOD activity increased gradually after 2 days of incubation, and had increased approx. 5-fold compared with the baseline level at 1 week, while CuZnSOD activity decreased by about 70 %. Replenishment of $CuSO_4$ at a level of 10 μM resulted in increased CuZnSOD activity (approx. 1.4-fold compared with baseline level), but had only a slight effect on the elevation of MnSOD activity. Thus the increase in MnSOD activity was not related to the levels of CnZnSOD activity. Western blots using an anti-MnSOD antibody demonstrated that the elevation in MnSOD activity could be accounted for by increased levels of MnSOD protein (Figure 1B).

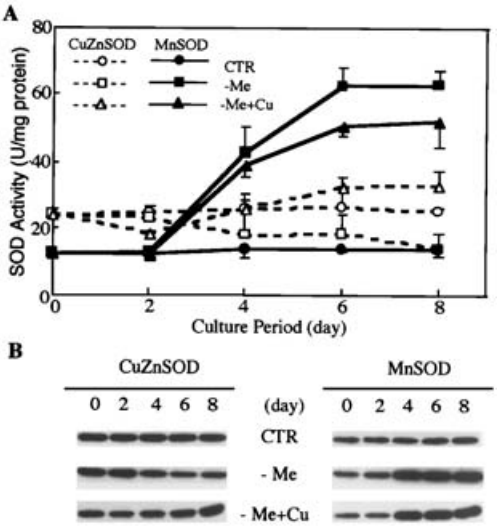

Figure 1 MnSOD accumulates in HepG2 cells cultivated in medium containing metal-depleted FBS

(**A**) HepG2 cells were cultivated for the indicated numbers of days in medium containing 10 % (v/v) conventional FBS (CTR), metal-depleted FBS (− Me) or metal-depleted FBS with replenishment of 10 μM $CuSO_4$ (− Me + Cu). After sonication on ice, the soluble fraction was obtained by centrifugation at 17 000 ***g*** for 15 min. CuZnSOD activity was calculated by subtracting MnSOD activity (which was measured in the presence of 2 mM NaCN) from total SOD activity. Values are means ± S.D. ($n = 3$). (**B**) Western blot analyses were carried out using samples (1 μg of protein per lane) obtained at the indicated times, using anti-MnSOD or anti-CuZnSOD antibodies. Data typical of several experiments are presented.

Suppression of MnSOD accumulation by zinc

We then examined the effects of replenishment of the medium containing metal-depleted FBS with various metal ions (Figure 2). The elevation in MnSOD activity was markedly suppressed by $ZnSO_4$ at 10 μM; the effects of other metal ions were considerably less. Because $ZnCl_2$ exhibited the same suppressive effect (results not shown), the participation of anion species was excluded. We also examined the dose-dependent effects of $ZnSO_4$ on MnSOD and CuZnSOD activities (Figure 2B). The findings indicate that even submicromolar concentrations of zinc ions inhibited MnSOD accumulation. Table 1 shows the concentrations of metals in the medium. The culture medium for control cells contained 240 μg/l zinc, but the medium supplemented with metal-depleted FBS contained less than 100 μg/l. The copper content also fell below detectable levels (< 50 μg/l) in metal-depleted medium. Since Fe is tightly associated with transferrin in serum, it would be barely chelated by triethylenetetramine. Mn would also be partly unchelatable due to a tight association with

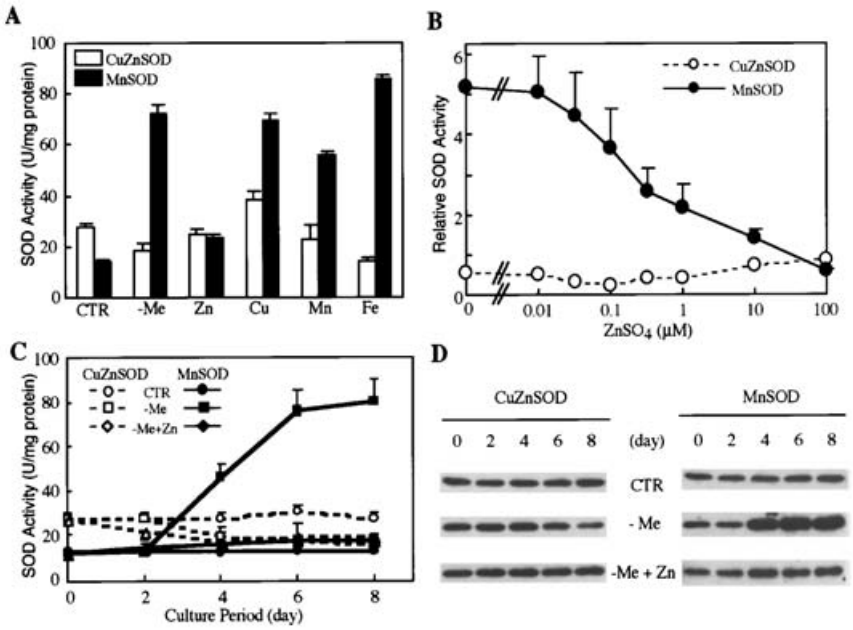

Figure 2 Suppression of MnSOD accumulation by metal ions

(**A**) HepG2 cells were cultivated in medium containing 10 % (v/v) metal-depleted FBS (− Me), with supplementation with $CuSO_4$ (Cu), $ZnSO_4$ (Zn), $MnCl_2$ (Mn) or $FeCl_3$ (Fe) at 10 μM. After 8 days in culture, CuZnSOD (□) and MnSOD (■) activities were determined as described in the legend to Figure 1. Values are means ± S.D. ($n = 3$). (**B**) Cells were cultivated in medium containing metal-depleted FBS with supplementation with various concentrations of $ZnSO_4$. After 8 days in culture, CuZnSOD (○) and MnSOD (●) activities were determined as described above. Values are means ± S.D. ($n = 3$). (**C**) Cells were cultivated for the indicated numbers of days in medium containing 10 % (v/v) conventional FBS (CTR), metal-depleted FBS (− Me) or metal-depleted FBS with replenishment with 10 μM $ZnSO_4$ (− Me + Zn). Values are means ± S.D. ($n = 3$). (**D**) Western blot analyses were carried out using samples (1 μg of protein per lane) obtained at the indicated times, with anti-MnSOD or anti-CuZnSOD antibodies. Data typical of several experiments are presented.

Table 1 Metal ion content of media used in the culture experiments

Control, 10 % (v/v) conventional FBS; − Metal, 10 % (v/v) metal-depleted FBS; − Metal + Zn, 10 % (v/v) metal-depleted FBS with replenishment with 10 μM $ZnSO_4$. Values are means ± S.D. ($n = 3$). ND, not detectable (< 100 μg/l Zn or < 50 μg/l Cu).

	Metal (μg/l)			
Conditions	Zn	Cu	Fe	Mn
Control	233 ± 9	137 ± 15	180 ± 7	3.3 ± 0.2
− Metal	ND	ND	185 ± 37	2.6 ± 0.0
− Metal + Zn	750 ± 10	ND	166 ± 4	2.8 ± 0.5

proteins, although the form of Mn present in serum has not been fully elucidated.

The growth rate of cells in medium containing metal-depleted FBS slowed down after 2 days of culture, and was almost zero by day 4 (Figure 3). Replenishment of the medium with 10 μM $ZnSO_4$ alone increased cell growth back to around control levels. When the morphology of the cells was examined by phase-contrast microscopy, cells cultured in medium supplemented with metal-depleted FBS had a flat, fibroblast-like shape (results not shown). Replenishment with zinc resulted in a morphological change to a near-normal shape, smiliar to that of cells cultivated in medium containing conventional FBS.

MnSOD induction under metal-depleted conditions and correlation of expression with intracellular redox state

Next we investigated intracellular redox status using an oxidation-sensitive dye, DCFH-DA. Cells cultured under metal-depleted

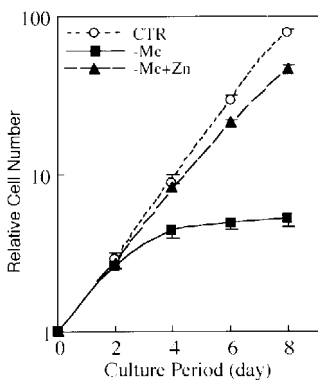

Figure 3 Cultured cells under metal-depleted conditions show growth retardation

Growth curves are shown for cells cultivated in medium containing 10 % (v/v) conventional FBS (CTR), metal-depleted FBS (− Me) or metal-depleted FBS with replenishment with 10 μM $ZnSO_4$ (− Me + Zn). Values are means ± S.D. ($n = 3$).

conditions were in a more highly oxidized state than those cultured under conventional conditions (Figure 4). The addition of 10 μM zinc to the medium resulted in a less oxidized state, comparable with that of cells grown in conventional medium. We then examined the activity of GPX, a major peroxide-scavenging system, as well as MnSOD and CuZnSOD activities (Table 2). MnSOD activity reached levels approximately double control levels following replenishment with zinc ions, while CuZnSOD activity returned to control levels. GPX activity, however, was decreased by around half by the presence of zinc ions. The MnSOD level was correlated with the change in the intracellular

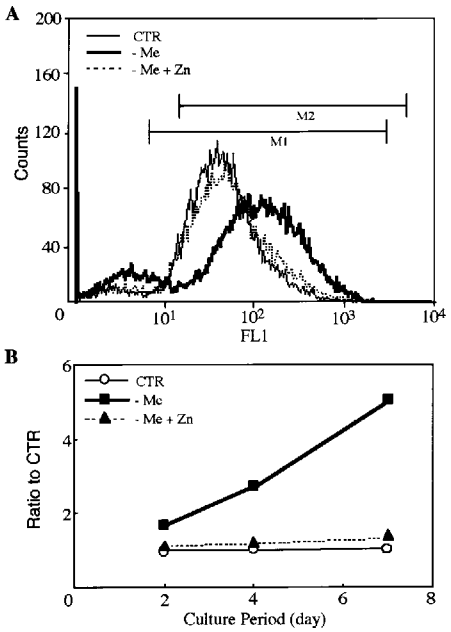

Figure 4 Redox state of cells cultivated in metal-depleted medium

Cells cultivated for 2, 4 or 7 days in medium containing 10 % (v/v) conventional FBS (CTR), metal-depleted FBS (− Me) or metal-depleted FBS with replenishment with 10 μM $ZnSO_4$ (− Me + Zn) were loaded with 5 μM DCFH-DA for 5 min. Flow cytometric analysis was carried out by detecting the oxidized fluorescent form, DCF, in the cells. Data typical of several experiments are presented. (**A**) Typical data after 4 days of cultivation. (**B**) Means of the areas marked M1 for CTR and − Me + Zn and M2 for − Me were calculated. Means of duplicate experiments are indicated for each data point.

Table 2 MnSOD, CuZnSOD and GPX activities in cells cultured under the indicated conditions

Control, 10 % (v/v) conventional FBS; − Metal, 10 % (v/v) metal-depleted FBS; − Metal + Zn, 10 % (v/v) metal-depleted FBS with replenishment with 10 μM $ZnSO_4$. Values are means ± S.D. ($n = 3$).

	Enzyme activity (units/mg of protein)		
Conditions	MnSOD	CuZnSOD	GPX
Control	12.2 ± 1.0	30.1 ± 2.0	0.015 ± 0.001
− Metal	72.4 ± 4.5	22.1 ± 5.2	0.013 ± 0.002
− Metal + Zn	26.5 ± 1.3	31.2 ± 2.5	0.007 ± 0.001

redox state. Such an alteration in the MnSOD level is probably due to the induced expression of SOD2 mRNA, as indicated by Northern blot analysis of total RNA prepared from cells at day 4 (Figure 5).

Increase in the DNA-binding activity of NF-κB under metal-depleted conditions

Since NF-κB is a key transactivating factor for *SOD2* induction in response to oxidative stress [19,21], we evaluated the possible involvement of NF-κB in the induction of *SOD2* by EMSA using an oligonucleotide probe specific for human *SOD2* (Figure 6). The human *SOD2* probe detected several bands on the gel. The lower bands (marked with asterisks in Figure 6) were non-specific, since the lowest band remained unchanged in lanes containing excess amounts of wild-type oligonucleotide, and the second lowest band was diminished by mutant oligonucleotide. The two upper

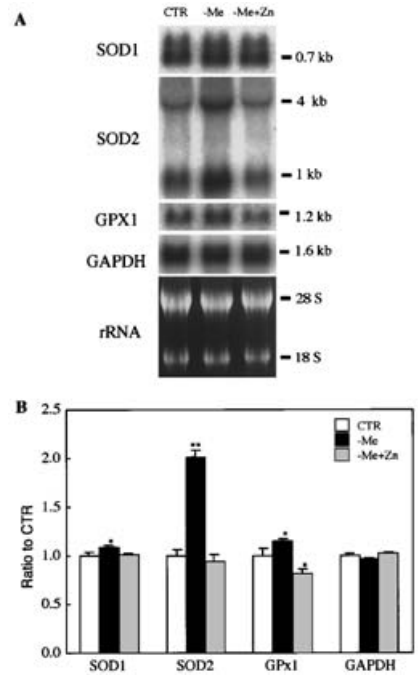

Figure 5 Levels of SOD1, SOD2 and GPX1 mRNAs in cells cultivated under metal-depleted conditions

(**A**) Samples of total RNA (10 μg) from cells cultivated in medium containing conventional FBS (CTR), metal-depleted FBS (− Me) or metal-depleted FBS with replenishment with 10 μM $ZnSO_4$ (− Me + Zn) for 4 days were separated on a 1 % (w/v) agarose gel. The blots were hybridized with human *SOD1*, human *SOD2* and rat *GPX1* cDNA probes. After stripping the probe, the same membranes were reprobed with a GAPDH probe. Typical results from triplicate experiments are shown. (**B**) After quantification of the radioactivity in each band, the relative intensities of the mRNAs for SOD1, SOD2, GPX1 and GAPDH were calculated. Values are means ± S.D. ($n = 3$); $^*P < 0.05$, $^{**}P < 0.001$ compared with control experiment.

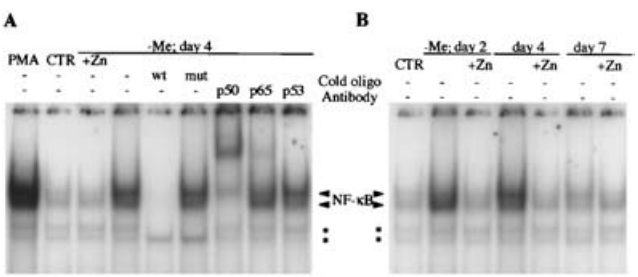

Figure 6 EMSA for NF-κB

(**A**) Nuclear proteins were extracted from HepG2 cells cultured in medium containing conventional FBS (CTR), metal-depleted FBS (− Me) or metal-depleted FBS with replenishment with 10 μM $ZnSO_4$ (− Me + Zn) for the indicated times and incubated with a probe for NF-κB. Nuclear extracts from cells treated with 160 nM phorbol ester (PMA) for 90 min were used as a positive control. EMSA was performed using an oligonucleotide probe which was established in human SOD2 for NF-κB binding [21]. The subunit composition of NF-κB was examined by supershift using specific antibodies to the p50 and p65 subunits. Anti-p53 was used as a non-specific antibody. wt, wild-type oligonucleotide; mut, mutant oligonucleotide. The positions of bands specific for NF-κB are indicated by arrowheads; asterisks indicate non-specific bands. Data typical of several experiments are presented. (**B**) EMSA was performed using nuclear extracts prepared from cells cultivated for 0, 2, 4 and 7 days.

bands (arrowheads in Figure 6) were considered to be NF-κB specific. A supershift experiment using anti-p50 and anti-p65 antibodies indicated that p50 was a major constituent. The supershift band obtained with the anti-p65 antibody was faint, suggesting that the involvement of p65 in the DNA-binding activity of NF-κB

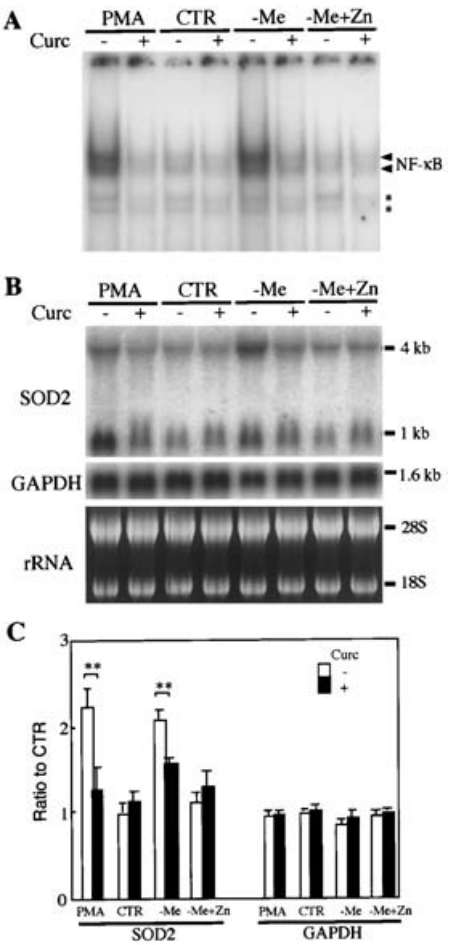

Figure 7 Suppression by curcumin of both NF-κB activation and SOD2 mRNA induction under metal-depleted conditions

(**A**) EMSA for NF-κB was performed using nuclear extracts from cells as described in the legend to Figure 6, except for the addition of 50 μM curcumin (Curc) 17 h prior to nuclear extraction. The positions of bands specific for NF-κB are indicated by arrowheads; asterisks indicate non-specific bands. Data typical of several experiments are presented. (**B**) Northern blot analysis was performed for total RNA (10 μg) from cells cultivated under the same conditions as in (**A**). The blots were hybridized with human *SOD2*. After stripping the probe, the same membranes were reprobed with GAPDH. Typical data from triplicate experiments are shown. (**C**) After quantification of the radioactivity in each band from triplicate experiments as in (**B**), the relative intensities of the mRNAs for SOD2 and GAPDH compared with the control (CTR) were calculated. Values are means $\pm$ S.D. ($n = 3$); $^{**}P < 0.01$ compared with control experiment.

was low in HepG2 cells (Figure 6A). Faint bands were seen at a corresponding position to NF-κB in control cells. This suggested an increase in the DNA-binding activity of NF-κB in cultivated cells, presumably due to higher oxygen concentrations than are found *in vivo*. The DNA-binding activity of NF-κB increased from day 2 to day 4 under metal-depleted conditions, but became lower subsequently (Figure 6B). These results are consistent with a scenario in which MnSOD induction by metal depletion involves an increase in the DNA-binding activity of NF-κB.

To obtain further evidence that increased DNA-binding activity of NF-κB was the cause of *SOD2* induction, we examined the effects of curcumin, an inhibitory agent for NF-κB, on the DNA-binding activity of NF-κB and the levels of SOD2 mRNA (Figure 7). Curcumin at 50 μM effectively suppressed the DNA-binding activity of NF-κB in cells that were cultivated for 4 days under metal-depleted conditions, as well as in cells

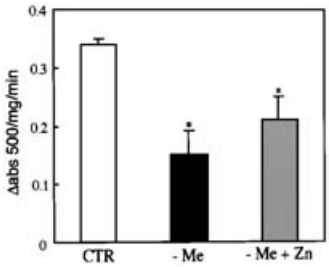

Figure 8 Cytochrome *c* oxidase activity in cells cultivated under metal-depleted conditions

After sonication of cells cultivated in medium containing 10 % (v/v) conventional FBS (CTR), metal-depleted FBS ($-$ Me), or metal-depleted FBS with replenishment with 10 μM $ZnSO_4$ ($-$ Me $+$ Zn) for 8 days, cytochrome *c* oxidase activity was measured. Values are means $\pm$ S.D. ($n = 3$); $^{*}P < 0.05$ compared with control experiment.

stimulated with PMA. Significant suppression of SOD2 mRNA induction was also observed following treatment of these cells with curcumin. This supports the notion that the increase in the DNA-binding activity of NF-κB was, at least in part, a direct cause of *SOD2* induction.

Decrease in cytochrome *c* oxidase activity following metal depletion

Since the mitochondrial content was shown to increase in the hearts of rat fed a copper-deficient diet [31], the content of mitochondria in cultured HepG2 cells was measured. Analysis by flow cytometry indicated that the mitochondrial content of the cells appeared to be increased under metal-depleted conditions, but changes were not significant (results not shown). Replenishment with 10 μM $ZnSO_4$, which suppressed *SOD2* induction, had no effect on mitochondrial content. Cytochrome *c* oxidase activity, an activity unique to mitochondria, was decreased in the metal-depleted culture, and was not recovered following replenishment with zinc (Figure 8).

Protective effect of augmented MnSOD activity against superoxide and hydrogen peroxide

To evaluate the physiological significance of the accumulation of MnSOD, we examined the viability of cells in the presence of the cytotoxic effects of superoxide, generated by a xanthine/xanthine oxidase system, and hydrogen peroxide (Figure 9). Cells that had been cultivated under metal-depleted conditions were more resistant to these agents. Thus MnSOD that accumulated under metal-depleted conditions appeared to actually serve as a form of protection against ROS. Supplementation of culture media with zinc also increased the resistance of cells to the stimuli. Since suppression of MnSOD accumulation by zinc was not complete, resulting in a level about twice that in control cells (see Table 2), this elevation might effectively protect cells from weak oxidative stress, caused by low concentrations of xanthine oxidase. However, at higher concentrations, cells cultivated in metal-depleted medium with replenishment of 10 μM $ZnSO_4$ became more susceptible to xanthine oxidase than those cultivated in metal-depleted medium.

DISCUSSION

The accumulation of MnSOD protein and mRNA was observed in HepG2 cells cultivated in medium supplemented with metal-depleted FBS (Figure 1). In addition, the elevation in MnSOD levels was caused, in part, by an increase in the DNA-binding

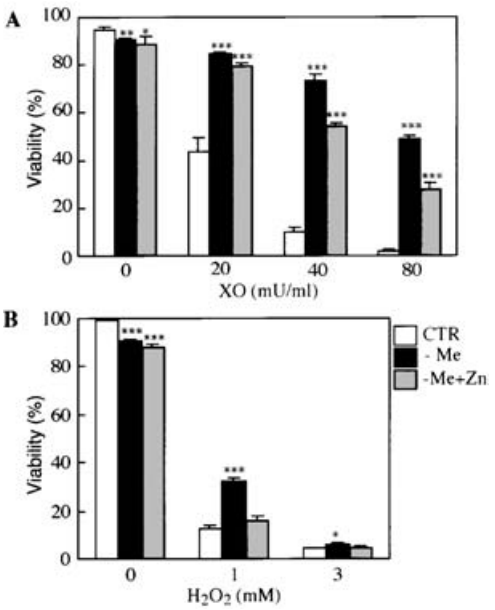

Figure 9 Cells cultivated under metal-depleted conditions are resistant to superoxide and hydrogen peroxide

Cells were cultivated for 8 days in medium containing 10 % (v/v) conventional FBS (CTR), metal-depleted FBS (− Me), or metal-depleted FBS with replenishment with 10 μM $ZnSO_4$ (− Me + Zn). They were then exposed to a xanthine/xanthine oxidase (XO) system for 2 h and incubated for another 21 h in the same culture medium (**A**), or exposed to hydrogen peroxide for 23 h (**B**). The viability of the cells was assessed by measuring LDH activity. Values are means ± S.D. ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control experiment.

activity of NF-κB due to augmented oxidative stress under metal-depleted culture conditions. Furthermore, the fact that zinc, among the metal ions examined, suppressed the enhanced MnSOD accumulation most efficiently (Figure 2) suggests that this metal is a critical regulatory factor in MnSOD expression.

It is known that dietary copper depletion leads to an increase in mitochondrial volume in cardiac cells [31]. However, the activity of cytochrome *c* oxidase, an enzyme unique to mitochondria, was decreased under the metal-depleted culture conditions, as reported previously [31], and was unaffected by zinc levels. In terms of MnSOD, zinc ions markedly suppressed the accumulation of MnSOD observed under metal-depleted conditions, while it had no effect on mitochondrial volume under these conditions. Thus an increase in mitochondrial volume did not necessarily accompany the increase in mitochondrial protein. Rather, the hypertrophic change in mitochondria appears to reflect an adaptive change as a result of impaired mitochondrial function such as oxidative phosphorylation.

The observed increase in MnSOD activity in the livers of rats fed on a copper-deficient diet appears to be caused by the transcriptional activation of *SOD2* [23]. Experiments using metal chelators and antioxidants indicated that oxidative stress resulting from the down-regulation of CuZnSOD or iron accumulation is a principal cause of *SOD2* induction [24,25]. Since some electron transport components contain metal ions as an essential cofactor, an inadequate supply of metal would impair the transfer of electrons, resulting in ROS production. In fact, we observed augmented ROS levels in cells cultivated under metal-depleted conditions (Figure 4). Hence the activation of redox-sensitive transactivating factors is a candidate for explaining the observed induction of *SOD2*. Human *SOD2* has been extensively characterized, and some *cis*-elements that are responsive to them have been identified. Several transactivating factors, including NF-κB and p53, are known to be associated with *SOD2* expression [18,21,32], and NF-κB plays a primary role in *SOD2* induction in response to various stimuli. The active form of NF-κB was elevated in cells grown in metal-depleted culture (Figure 6), which suggests that DNA-binding activity of NF-κB is increased by elevated levels of ROS, resulting in the induction of the *SOD2* expression. Since replenishment with zinc ions decreased both ROS accumulation and NF-κB DNA-binding activity, zinc ions appear to suppress MnSOD accumulation by decreasing ROS production via an unknown mechanism. The cells shown in Figure 9 were prepared after 8 days in metal-depleted culture, corresponding to the time when MnSOD was maximally elevated. The increase in the DNA-binding activity of NF-κB was marked at day 2 and 4, but less at day 7 (see Figure 6B). ROS production actually increased during incubation under metal-depleted conditions (Figure 4). This can explain the observation that cells cultivated under metal-depleted conditions produce more ROS, and hence more MnSOD as a defence system. There is some inconsistency between the levels of ROS and NF-κB DNA-binding activity. ROS levels were high on day 7, at which time the DNA-binding activity of NF-κB had returned to approximately control levels. This may be explained by the down-regulation of NF-κB DNA-binding activity under conditions of sustained high oxidative stress [33].

When the effects of various metal ions (zinc, copper, iron and manganese) on MnSOD accumulation in HepG2 cells cultivated under metal-depleted conditions were examined, zinc suppressed the accumulation of MnSOD most efficiently (Figure 2). Northern blot analysis indicated that this suppression occurred at the transcriptional stage, because a decrease in SOD2 mRNA was observed following supplementation with Zn (Figure 5). Collectively, the presented data suggest that induction of *SOD2* was attributed, at least partly, to the increase in DNA-binding activity of NF-κB induced by increased ROS under metal-depleted conditions. Zn, in turn, decreased the levels of ROS (Figure 4), which resulted in a decrease in NF-κB DNA-binding activity (Figure 6). Thus the decrease in SOD2 mRNA following Zn supplementation appears to be due to a decrease in NF-κB DNA-binding activity following suppression of ROS generation, although the mechanism by which Zn may suppress ROS levels is not clear. Since some transcription factors contain a zinc-finger motif, the expression of many genes may be affected by zinc depletion.

We conclude that metal deficiency elevated intracellular ROS levels, which increased NF-κB DNA-binding activity. *SOD2* expression was enhanced concomitantly via this increase in the DNA-binding activity of NF-κB in cells, thus mediating protection from the toxicity of ROS. Among the metal ions investigated, zinc appeared to play a key role in maintaining the redox status of the cells, although the precise mechanism of this effect is largely unknown. The phenomena observed here may provide a clue in our understanding of the novel function of zinc in regulating redox balance within mammalian cells.

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