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Magnesium deficiency enhances hydrogen peroxide production and oxidative damage in chick embryo hepatocyte *in vitro*

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Abstract

Magnesium deficiency and oxidative stress have been identified as correlative factors in many diseases. The origin of free radicals correlated with oxidative damage resulting from Mg-deficiency is unclear at the cellular level. To investigate whether hydrogen peroxide (H_2O_2) is associated in the oxidative stress induced by Mg-deficiency, the effect of Mg^{2+} deficiency (0, 0.4, 0.7 mM) on the metabolism of H_2O_2 was investigated in cultured chick embryo hepatocytes. After being cultured in the media with various concentrations of Mg^{2+} for 1, 2, 4, 6 and 10 days, parameters of H_2O_2 production, catalase activity, lipid peroxidation, intracellular total Mg and cell viability were analyzed. Results demonstrated that long-term incubation of chick embryo hepatocyte in extracellular Mg^{2+}-deprivative and Mg^{2+}-deficient (0.4 mM) states significantly enhanced the production of H_2O_2 (approximately twofold, respectively) and lipid peroxidation in the cell cultures, while decreasing the cell viability. Additionally, the reversing action of Mg^{2+} re-added to 1.0 mM and the partial reversing action of dimethylthiourea suggested that (i) $[Mg^{2+}]_e$ deficiency induced the increase of H_2O_2 production, (ii) $[Mg^{2+}]_e$ deficiency decreased catalase activity in chick embryo hepatocyte *in vitro*, subsequently causing oxidative stress and cell peroxidative damage.

Introduction

Magnesium (Mg) exerts and affects many cellular functions, including modulating signal transduction, energy metabolism and cell proliferation (Vernon 1988; Zhou & Kummerow1995; Wolf & Cittadini 1999; Yang *et al.* 2000; Egerbacher *et al.* 2001). Several studies have tried to identify the origin and characterize the increased oxidative stress response associated with Mg deficiency, which were accompanied by inflammatory response and subsequent tissue injury (Freedman *et al.* 1991; Weglicki et al. 1996; Malpuech-Brugere *et al.* 2000). Mg deficient animals were shown to be less tolerant to stress with a decreased lifespan (Rayssiguier *et al.* 1993). *In vivo* studies demonstrated that Mg deficiency enhanced oxidative damage in the heart, increased the plasma levels of lipoproteins and also imposed oxidative damage to erythrocytes, endothelial and neuronal cells (Freedman *et al.* 1991; Dickens *et al.* 1992; Gueux *et al.* 1995; Regan *et al.* 2000), whereas various antioxidant drugs and nutritional states displayed protective effects on the injury induced by Mg

deficiency. These findings also suggested that free radicals are involved in the injury process (Rayssiguier *et al.* 1993). Additionally, several lines of research demonstrated that Mg deficiency affected variables related to lipid peroxidation, such as microsomes from the liver of Mg-starved animals were more susceptible to lipid peroxidation induced *in vitro* (Wolf *et al.* 2003). Previous cytochemical studies also showed that low excellular Mg^{2+}, $[Mg^{2+}]_e$, affected the anti-oxidation ability of cells. In contrast, controlled studies have shown the protective characteristics that Mg supplementation displayed in cellular oxidative damage (Morrill *et al.* 1998; Garcia *et al.* 1998; Cernak *et al.* 2000; Mak *et al.* 2000; Manuel y Keenoy *et al.* 2000; Maulik *et al.* 2001). The evidence from these studies suggests that reactive oxygen species disorder induced by Mg-deficiency is involved in oxidative damage. In spite of these discoveries, the cellular origin of free radicals that may be producing the Mg-deficiency lesion in cells and tissues remains unknown.

Hydrogen peroxide (H_2O_2), one of reactive oxygen species produced during the metabolism, has been implicated in many stress conditions. H_2O_2 is continuously produced *in vivo* and remains in a quasi steady level under conditions where cellular homeostasis is not disrupted. Cell pathological processes are associated with disrupted H_2O_2 hemeotasis because reactive oxygen species such as H_2O_2 can be produced in large amounts, exceeding endogenous antioxidant defenses and promoting tissue injury (Rose *et al.* 2002). To date, it is not clear whether cell or tissue oxidative damage induced by Mg deficiency is mediated by disordered H_2O_2 metabolism. In this study, chick embryo hepatocyte cells exposed to various concentrations of Mg^{2+} and dimethylthiourea (DMTU), one of H_2O_2 scavenges, were used to clarify whether the oxidative damage induced by low levels of Mg^{2+} was mediated by interfering the metabolism of H_2O_2.

Materials and methods

Chemicals

Homovanillic acid, catalase and dimethylthiourea (DMTU) were obtained from Sigma. All culture media, fetal bovine serum, and penicillin–streptomycin were purchased from Invitrogen Corporation. Other chemicals were obtained from Sigma, unless otherwise stated.

Cell culture and treatment with various concentrations of Mg^{2+} in culture media.

12-day-old White leghorn fertile chick embryos, obtained from Institute of Animal Science, Chinese Academy of Agriculture Sciences, had been incubated in an electric forced incubator at 37.8 °C and 60% relative humidity. Primary cultures of hepatocytes were obtained from 12-day-old chick embryos and maintained as described previously by Hahn *et al.* (1997). Livers were isolated, and immediately washed in the D-Hanks buffer (pH 7.4), minced into pieces and dissociated in trypsin/EDTA (0.2% trypsin; 0.25% EDTA) for 15 min at room temperature, thereafter gently pipetted to produce a single-cell suspension. The cell suspension was filtered through a 200 mesh screen, washed twice by gentle centrifugation (800×*g*, 10 min) and resuspended in RPMI-1640 medium. Cell viability was estimated by Trypan blue exclusion and viable hepatocytes at the density of 5×10^4–1×10^5 cells/ml were seeded in 12-well-plates and cultured in RPMI 1640 (1.0 mM Mg^{2+}) replenished with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in the medium at 39 °C in a humidified atmosphere containing 5% CO_2. Intact hepatocytes adhered to the culture plates, and the cell debris and blood cells were sucked off during the media being replaced after plating for 10 h. After further incubation for 14 h, Trypan blue exclusion assay was used to assess cell viability and only cells with viability >98% were used in the experiment, then the media was replaced with RPMI 1640 (10% fetal bovine serum) containing 0, 0.4, 0.7, 1.0 mM $MgSO_4$ and 0 mM $MgSO_4$ + 20 mM DMTU respectively. Subsequently, the media was replaced every 48 h. On the day 4, Mg^{2+} with 0 and 0.4 mM in the media for two extra treatments was re-added to 1.0 mM, The cells cultured in the RPMI 1640 medium containing 1.0 mM Mg^{2+} were used as control, which is generally considered the concentration of Mg^{2+} in physiological condition (Seelig 1994).

H_2O_2 determination

Cells treated with various concentrations of Mg for desired time course were collected and the production of H_2O_2 in cell culture was assayed using a modification of homovanillic acid fluorescence assay (Raspe *et al*. 1991). This fluorometric method is based on the conversion of homovanillic acid, a substituted phenol, to its fluorescent dimer in the presence of H_2O_2 and horseradish peroxidase. Briefly, the media was removed and the plates were washed three times with cold phosphate-buffered saline (PBS) to rinse away the dead cells, and the primary chick embryo hepatocytes were scraped off the plates in cold PBS, and quickly washed twice in a HEPES buffer of the following composition: 120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO4, 2.5 mM $NaHCO_3$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES and 0.01% BSA, pH 7.4. The cells were then suspended in 0.85 ml/well HEPES-buffered medium (see above) supplemented with 1.0 mM 1,4-dithiothreitol (DTT), 16.0 mg/l phenylmethylsulfonyl fluoride (PMSF), 440 mM homovanillic acid, 0.5 sigma U/ml horseradish peroxidase type II and 0.01% NaN_3 were added to the media. DTT and PMSF were added to inhibit the activity of catalase. After incubation at 39 °C for 30 min, they were quickly chilled on ice to terminate the reaction. The fluorescence of the medium was measured using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi Co. Ltd). The excitation wavelength and the emission wavelength were set at 315, 425 nm, respectively. A standard curve was obtained from measurements of buffer samples with known amounts of H_2O_2. 0.01% catalase was added into the medium to determine the sensitivity of H_2O_2 to catalase, certifying that it was H_2O_2 that oxidized homovanillic acid to its fluorescent dimer. H_2O_2 production in chick embryo hepatocytes was also determined before cells were cultured. Results are expressed as nmol H_2O_2 per minute and milligram of soluble protein (nmol/min·mg protein). The concentration of protein in the cell extracts was determined by the method of Lowry *et al* (1951).

Assay for the activity of catalase

At the appropriate time points after treatment and dead cells were rinsed off, the primary chick embryo hepatocytes in cold PBS were scraped off the plates, followed by re-suspension in cold PBS. Catalase activity was determined by the method described by Beers and Sizer (1952). Briefly, cell lysates, prepared by sonication on ice (20 sonication intervals of 2 s interspersed with 10 s intervals without sonication) in ice-cold 50 mM PBS buffer (pH 7.4), were centrifuged at 13,000 × *g* for 10 min at 4 °C, and the supernatants were used for the detection of catalase activity by monitoring the absorbance at 240 nm with Bio-Spec-1600 spectrophotometer (Shimadzu Ltd, Kyoto, Japan). The reaction rate was related to the amount of catalase present in the mixture. Catalase activity in chick embryo hepatocytes was also determined before cells were cultured. Results are expressed as international units per milligram of soluble protein in the cell extracts (u/mg protein).

Measurement of lipid peroxidation

After the primary chick embryo hepatocytes treated with various concentrations of Mg^{2+} for several periods were prepared as mentioned, oxidative damage to membranes was monitored by determining malondialdehyde, the main product of lipid peroxidation generation, using a modification of thiobarbituric acid-reactive substances method, as previously described (Gutteridge 1981). In brief, the hepatocyte suspension (0.5 ml) was added to a test tube containing 1.0 ml of trichloroacetic acid (10% w/v) and 50 μl antioxidant, dibutylhydroxytoluene (2% w/v). After heating at 90 °C for 10 min, the precipitate was removed by centrifugation. The supernatant was then added to 1.0 ml of thiobarbituric acid (1% w/v) and heated at 90 °C for 15 min. After cooling, absorbance was determined at 532 nm using a Bio-Spec-1600 spectrophotometer (Shimadzu Ltd, Kyoto, Japan). The content of malondialdehyde in chick embryo hepatocytes was also determined before cells were cultured. Results are expressed as nmol per milligram of soluble protein in the cell extracts (nmol/mg protein).

Determination of intracellular total Mg concentration

At the appropriate time points after treatment, the intracellular total concentration of Mg was assayed using a modified method as previously

described (Gunther *et al.* 1995). Briefly, media containing dead cells were removed and dishes were rinsed three times with cold PBS whose excess was carefully removed. To control the potential contamination by different amounts of extracellular Mg, parallel dishes were washed with PBS containing 0.5 mM EDTA in the first two washings and results were compared to samples obtained without EDTA. For the determination of the intracellular content of Mg, the cell sediments after centrifugation ($800 \times g$/min) were freeze-dried, powered and then incinerated twice, each incineration lasting 24 h at 560 °C. The cell ash was dissolved in 1.0 N HNO_3 for 24 h. The total Mg in cells was assayed in the presence of $LaCl_3$ by Atomic Absorption Spectrophotometer (Perkin–Elmer 272, Perkin–Elmer Corp., Norwalk, CT). Total Mg in chick embryo hepatocytes was also determined before cells were cultured. For each experiment, the content of protein was determined before the cells were ashed. Intracellular total Mg levels were expressed as nmol per milligram of soluble protein in the cell extracts (nmol/mg protein).

Assay for cell viability

The cell viability was accessed by WST-8 assay, which was performed using Cell Counting Kit-8 (Kumamoto Techno Research Park, Japan). After primary chick embryo hepatocytes (3.3×10^3/well) were seeded in 96-well microplates with various concentrations of Mg^{2+} media for 1, 2, 4 and 6 days, 10 μl of a solution of the Cell Counting Kit-8 was added to the wells, followed by a 3 h incubation. This solution contains novel water-soluble tetrazolium salt (WST-8), which is enzymatically converted into a highly water-soluble and noncytotoxic formazan dye in viable cells. Therefore the absorbance value is in direct proportion to cell viability. The absorbance of the media was measured at 450 nm using a GENios microplate reader (Tecan, USA) (Ishiyama *et al.* 1996). Each determination was done in triplicate. The absorbance in 1.0 mM treatment was designated as 100%.

Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A value of $p < 0.05$ was considered to be significant.

Results

Effect of $[Mg^{2+}]_e$ on cellular H_2O_2 levels

The amount of H_2O_2 accumulated in 30 min in chick embryo hepatocytes cultured with various concentrations of Mg^{2+} was examined using Homovanillic acid fluorescence assay method. In control cells incubated in 1.0 mM $[Mg^{2+}]_e$ medium, a steady and basal H_2O_2 formation, which is at the same level with that before cells were treated (0.53 ± 0.022 nmol/min·mg protein), was observed for the whole experiment period (Figure 1). No differences were observed among the cells treated with various concentration of Mg^{2+} on day 1, while H_2O_2 productions were much higher (approximately two-fold) in the presence of two low concentrations of $[Mg^{2+}]_e$ media on day 2 than those on day 1 ($p < 0.01$), increasing from 0.54 ± 0.040 to 1.29 ± 0.076 and from 0.54 ± 0.042 to 1.27 ± 0.093 nmol/min·mg protein respectively, and remained at the higher levels till the end of the experiment period, whereas the amounts of H_2O_2 in Mg-free + -DMTU culture, which were much lower than those in 0 mM Mg^{2+} media from the first day, were almost at the same level with the control treatment. Additionally, the amounts of H_2O_2 in 0 and 0.4 mM groups significantly decreased from 1.39 ± 0.113 to 0.55 ± 0.073 and 1.23 ± 0.094 to 0.53 ± 0.065 nmol/min·mg protein respectively on day 10, which displayed no significant differences compared to corresponding control, when Mg^{2+} was re-added to 1.0 mM from day 4.

All the fluorescence intensities in 0, 0.4, 0.7 1.0 mM Mg^{2+} cultures largely decreased after 0.01% catalase was added into the reaction system (data not shown), and there were no significant differences between the four treatments compared with the corresponding control respectively. The decrease of fluorescence values resulting from catalase added to the reaction systems verified it was H_2O_2 that oxidized homovanillic acid to its fluorescent dimer.

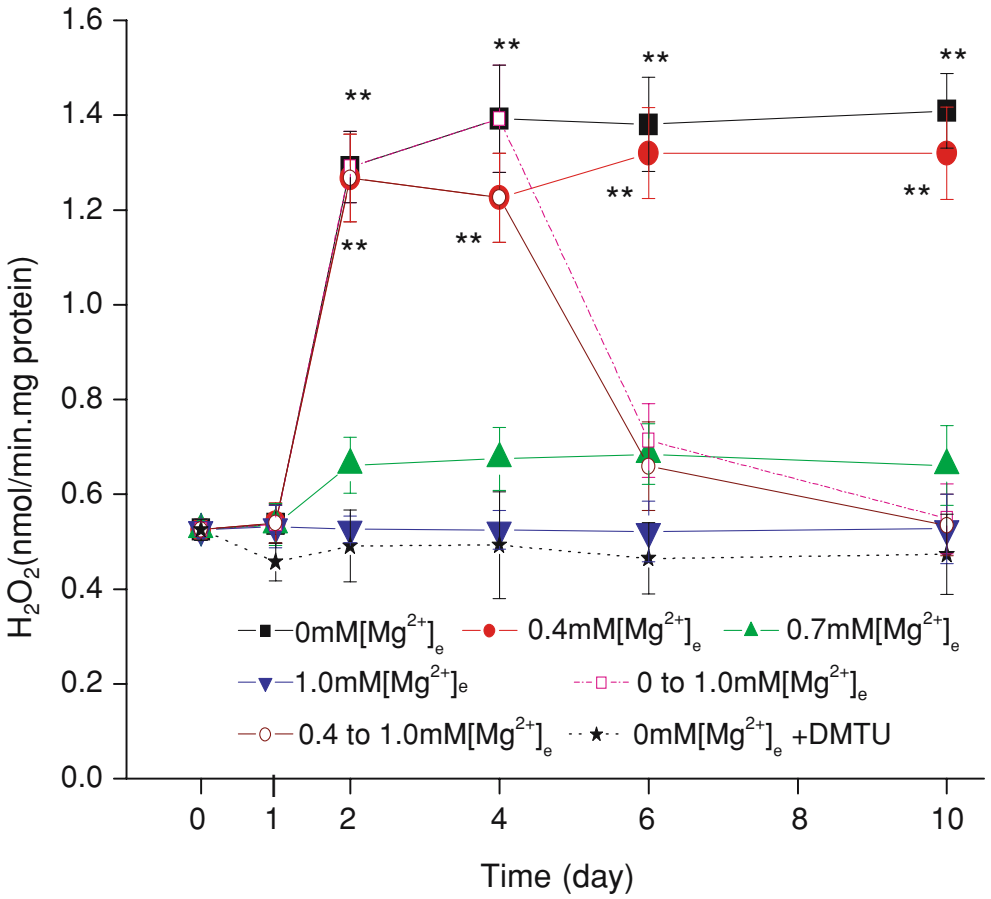

Figure 1. Time course of H_2O_2 production in chick embryo hepatocytes after cultured in the medium with 0, 0.4, 0.7 or 1.0 mM Mg^{2+}. $^*p<0.05$, $^{**}p<0.01$ *versus* corresponding control (1.0 mM $[Mg^{2+}]_e$ group). Values are the mean of six measurements, except 12 measurements before cells were cultured, and error bars indicate standard errors.

Ability of hepatocyte scavenging H_2O_2

H_2O_2 is mainly decomposed by catalase, which is one of the endogenous antioxidant enzymes. Therefore, catalase activity was assayed for identifying cell potential ability to scavenge H_2O_2 produced in chick embryo hepatocytes submitted to various $[Mg^{2+}]_e$ for several time courses. As shown in Figure 2, the activity of catalase from 1.0 mM Mg^{2+} group maintained at the same level as that from cells before treatment for the entire period of study (0.58 ± 0.034 u/mg protein). Interestingly, activities of catalase treated with 0, 0.4 and 0.7 mM Mg^{2+} displayed a bidirectional appearance, because catalase activities in Mg^{2+} free and 0.4 mM media (0.79 ± 0.066 and 0.77 ± 0.069 u/mg protein, respectively) were significantly enhanced compared with that in 1.0 mM Mg^{2+} (0.58 ± 0.050 u/mg protein) medium ($p<0.05$) on day 2, whereas on day 4, catalase activities decreased dramatically in the two low Mg^{2+} groups (0.30 ± 0.068 and 0.36 ± 0.049 u/mg protein, respectively) compared with that in the 1.0 mM Mg^{2+} group (0.58 ± 0.065 u/mg protein) ($p<0.01$), thereafter the state of lower catalase activity lasted to the end of the experiment. Activities of catalase in 0.7 and 1 mM groups were the same.

Cell oxidative damage

Since $[Mg^{2+}]_e$ levels influence cell H_2O_2 production, and malondialdehyde, a lipid peroxidation product generated during the oxidation of membrane lipids induced by reactive oxygen species, is a marker of lipid peroxidation, therefore the

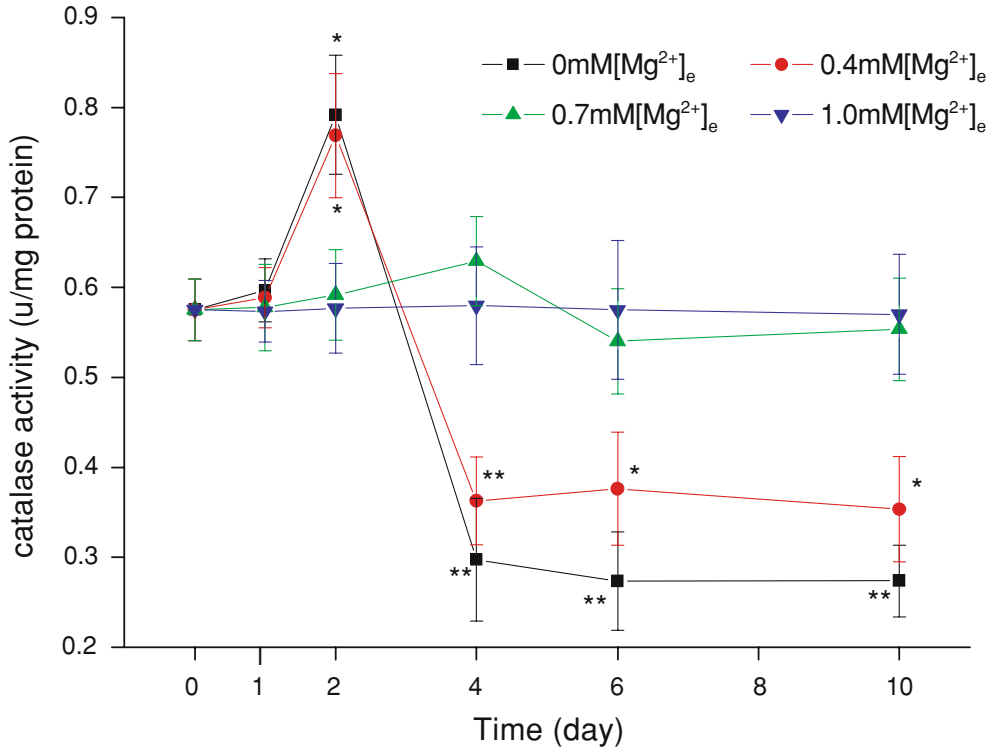

Figure 2. Catalase activity in chick embryo hepatocytes cells after treated with several concentrations of Mg^{2+} for various periods as indicated. $*p < 0.05$, $**p < 0.01$ *versus* corresponding control (1.0 mM $[Mg^{2+}]_e$ group). Values are the mean of six measurements, except 12 measurements before cells were cultured, and error bars indicate standard errors.

damage to lipids by monitoring malondialdehyde was investigated. The amounts of malondialdehyde in chick embryo hepatocytes exposed to various concentrations of Mg^{2+} for one day were at the same level with that of untreated cells (0.16 ± 0.012 nmol/mg protein). From day 2, the content of malondialdehyde showed a dose–dependent increase in respond to Mg^{2+} decreasing. The amounts of malondialdehyde were significantly higher in 0 and 0.4 mM Mg^{2+} groups than those in chick embryo hepatocytes exposed to 1.0 mM Mg^{2+} on each time point ($p < 0.01$) (Figure 3).

Intracellular total Mg concentration

Figure 4 represents time course of the intracellular total concentration of Mg in chick embryo hepatocytes cultured in various $[Mg^{2+}]_e$. For the entire period of study, chick embryo hepatocytes in 1.0 mM $[Mg^{2+}]_e$ did not change their intracellular total Mg compared with untreated cells (77.14 ± 0.335 nmol/mg protein), whereas the intracellular total Mg in 0, and 0.4 Mg^{2+} groups decreased on day 2 (75.05 ± 0.304 and 76.08 ± 0.399 nmol/mg protein, respectively) compared with that in 1.0 mM $[Mg^{2+}]_e$ (77.20 ± 0.421 nmol/mg protein) ($p < 0.05$), and reached their minimums on day 4 and 6, respectively, and then maintained at their own minimum levels respectively. The intracellular total Mg in Mg-free + DMTU culture had a similar time-course trend with that in 0 mM Mg^{2+} media. However, the intracellular total Mg in 0 and 0.4 mM groups quickly increased back from 74.61 ± 0.322 to 77.26 ± 0.566 and 75.10 ± 0.464 to 77.18 ± 0.426 nmol/mg protein respectively on day 6, which shows no significant differences compared with corresponding control, when Mg^{2+} was re-added to 1.0 mM from day 4, although the supplementation of DMTU didn't change intracellular total Mg in the 0 mM Mg^{2+} media. The intracellular total Mg in 0.7 mM group was significantly decreased compared with those in control on day 6 and 10 respectively ($p < 0.05$).

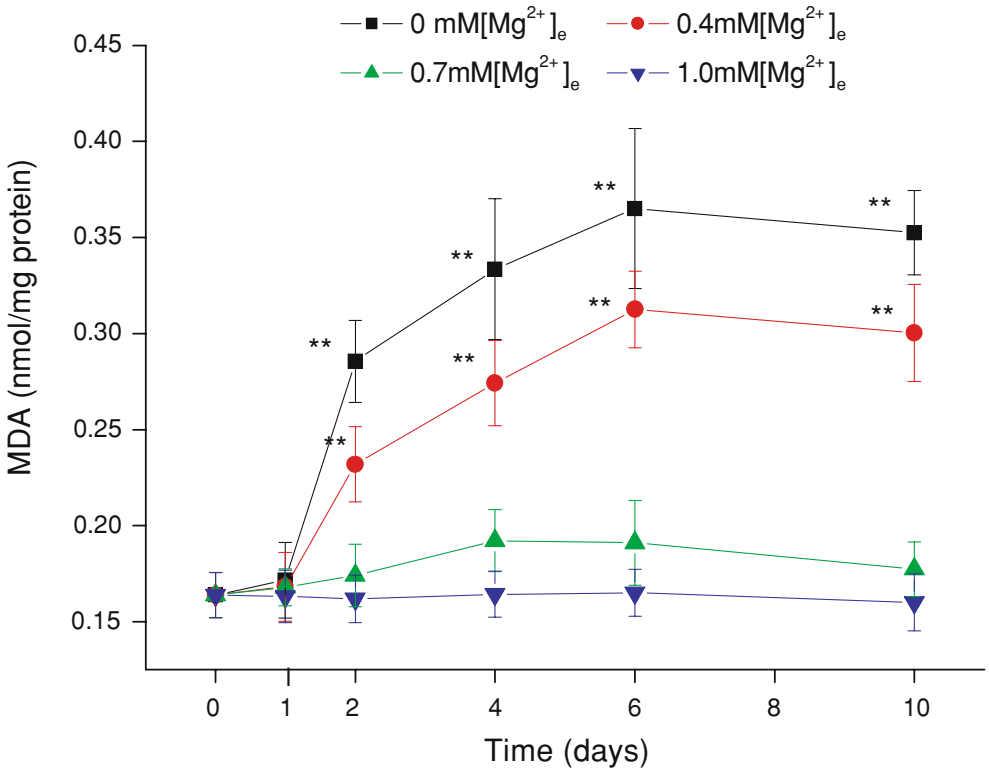

Figure 3. Malondialdehyde (MDA) formation in chick embryo hepatpcytes after treated with several concentrations of Mg^{2+} for various periods as indicated. *$p < 0.05$, **$p < 0.01$ *versus* corresponding control (1.0 mM $[Mg^{2+}]_e$ group). Values are the means of six measurements, except 12 measurements before cells were cultured, and error bars indicate standard errors.

Effects of extracellular Mg^{2+} on cell viability

The amount of formazan dye absorbance in viable cells is positively correlative with the amount of viable cells, so we can compare the effect of Mg^{2+} on cell viability indirectly by determine the absorbance of cell culture with different concentrations of Mg^{2+}. As shown in Figure 5, no differences on cell viability were observed between 1.0 and 0.7 mM groups on each time point. The cell viability in Mg^{2+} free group decreased significantly on day 2 ($65.9 \pm 5.49\%$) compared with that in control ($p < 0.01$), and maintained at a constant level after day 4 ($59.9 \pm 5.46\%$), However, the cell viability in the 0 mM Mg^{2+} + 20 mM DMTU considerably increased compared with those in the Mg^{2+}-free media, although DMTU didn't completely reverse it to the level of that in control. The 0.4 mM Mg^{2+} treatment had a significant lower cell viability on day 4 ($85.7 \pm 5.23\%$) compared with the control ($p < 0.05$), and then maintained at this level. Additionally, the cell viability in 0 and 0.4 mM groups significantly increased from 59.9 ± 5.46 to $94.4 \pm 6.37\%$ and 85.7 ± 5.23 to $96.0 \pm 5.25\%$ respectively on day 10 after Mg^{2+} was re-added to 1.0 mM from day 4, which shows no significant differences compared to corresponding control.

Discussion

Evidence has accumulated to suggest that extracellular Mg concentrations play a critical role in modulating cell activities (Zhang *et al.* 1993). It is clearly reported that Mg deficiency enhances free radical-induced cytotoxicity in several types of cells (Dickens *et al.* 1992; Gunther *et al.* 1995; Kumar & Shivakumar 1997). We wondered whether oxidative damage induced by low concentrations of Mg^{2+} could be associated with the disorder of H_2O_2. The morphological features of chick embryo hepatocytes cultured in the presence of physiological concentration of Mg^{2+} (1.0 mM)

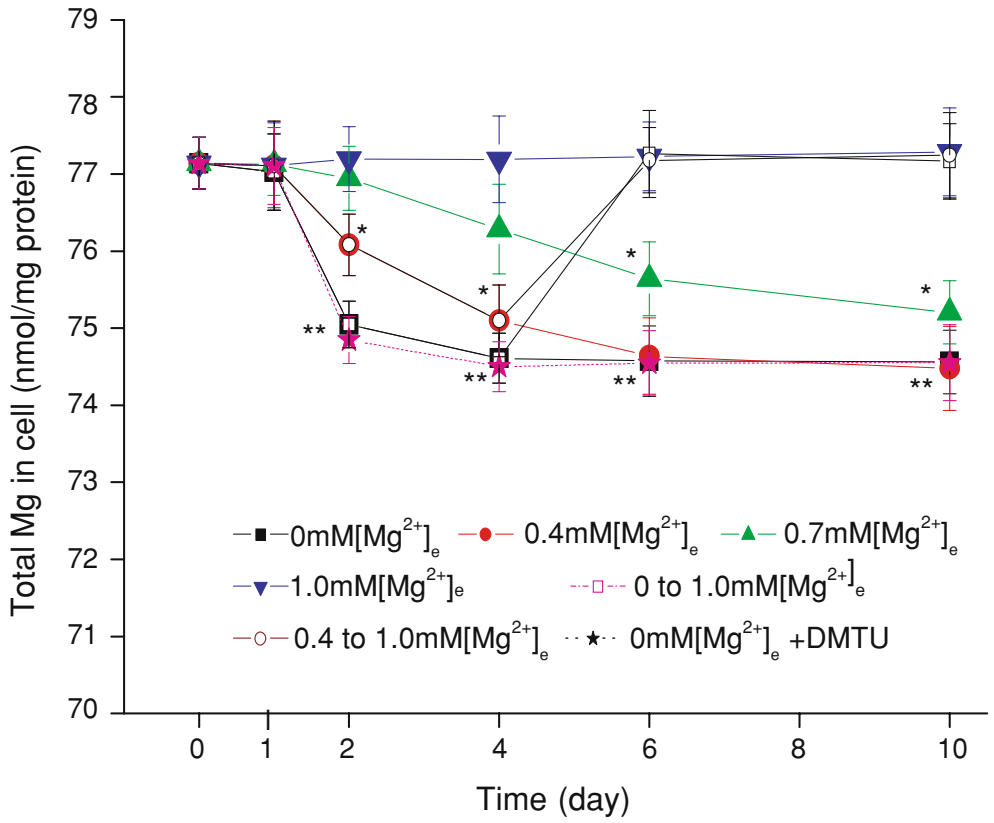

Figure 4. Intracellular total Mg in chick embryo hepatocytes after cultured in the medium with 0, 0.4, 0.7 and 1.0 mM Mg^{2+} for various periods as indicated. *$p < 0.05$, **$p < 0.01$ *versus* corresponding control (1.0 mM $[Mg^{2+}]_e$ group). Values are the means of six measurements, except 12 measurements before cells were cultured, and error bars indicate standard errors.

for 1, 2, 4, 6 and 10 days were similar to those previously reported rat hepatocytes cultured on a single layer of collagen (Richert *et al*. 2002). Furthermore, the total intracellular Mg, H_2O_2 production, catalase activity and the content of malondialdehyde in cells treated with 1.0 mM $[Mg^{2+}]_e$ maintained at their constant levels which are almost the same as those in cells before being cultured and the cell viabilities were always higher than 98%. These results support the previous finding that 1.0 mM Mg^{2+} is a favorable $[Mg^{2+}]_e$ condition for cell growth (Seelig 1994; Wolf *et al*. 2003).

A basal production of low level H_2O_2 exists in normal cells, originating from various sources, such as the mitochondria, cytochromes P_{450}, xantine oxidase, and the well-known NADPH-oxidase system of phagocytic cells (Halliwell & Gutteridge 1984). In general, cells produce $O_2^{\cdot}$ that is rapidly converted into H_2O_2 whose metabolism is modulated by the state of anti-oxidation and oxidation in cells. H_2O_2 can be catalyzed and excreted harmlessly as water and O_2 under condition of balanced anti-oxidation and oxidation, otherwise it can be converted to the highly damaging hydroxyl radical ($OH^{\cdot}$) if a substantial amount of H_2O_2 is accumulated. In the present experiment, the H_2O_2 concentration was determined in the presence of PMSF and DTT, which inhibit the activity of catalase, therefore the determined values indicated the amount of H_2O_2 produced by the cells. On the day 2 and thereafter, there was a significant dose-dependent increase on H_2O_2 amount in chick embryo hepatocytes incubated in the Mg-free or Mg-deficient media, whereas the content of H_2O_2 decreased a lot after restoring 0 or 0.4 mM Mg^{2+} incubation to 1 mM Mg^{2+} on day 4. It is likely that the modulation of H_2O_2 production by Mg is mediated by corresponding changes in intracellular Mg, which would directly or indirectly influence the process of H_2O_2 metabolism. To determine whether acute decreases

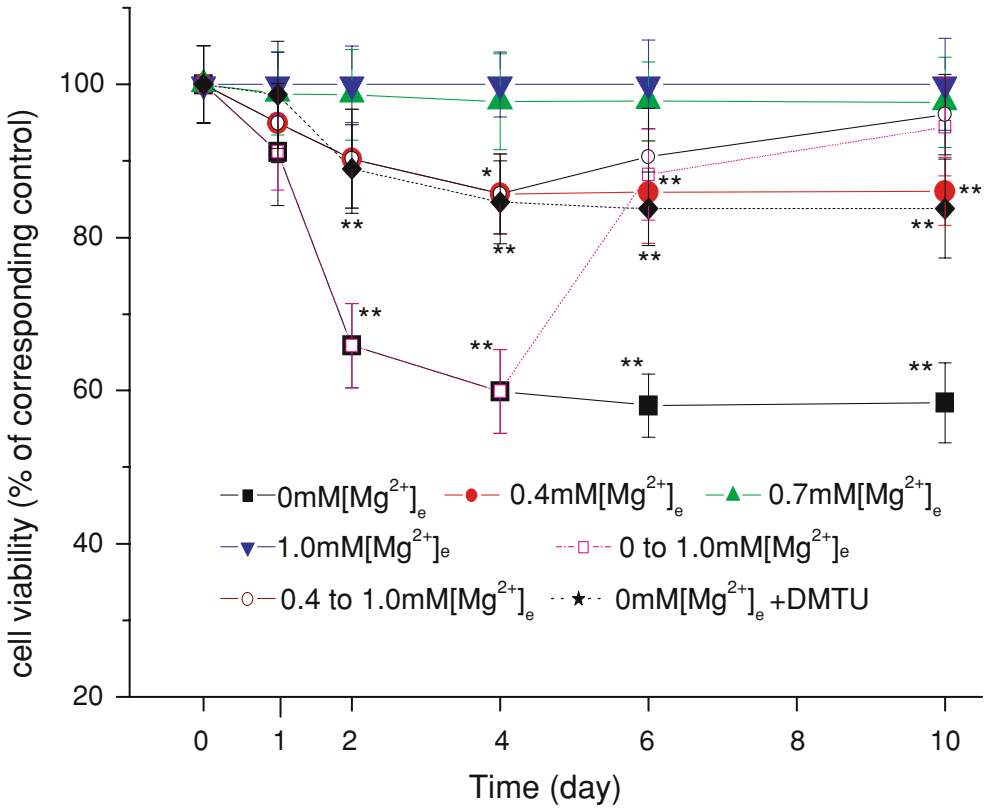

Figure 5. The cell viability of chick embryo hepatocytes after being cultured in the medium with 0, 0.4, 0.7 and 1.0 mM Mg^{2+} for various periods as indicated. $*p < 0.05$, $**p < 0.01$ *versus* corresponding control (1.0 mM $[Mg^{2+}]_e$ group). Values are presented as a percentage of corresponding controls.

in $[Mg^{2+}]_e$ were sufficient to induce the total intracellular Mg decrease and the relationship between intracellular total Mg level and H_2O_2 production, intracellular total Mg concentration was measured, though it should be recalled that intracellular Mg is not significantly modulated in rat hepatocytes chronically exposed to low concentrations of the cation (Martin *et al.* 2003). Decreases of intracellular total Mg and cell viability were positively associated with corresponding $[Mg^{2+}]_e$-deficiency from day 2 to 4, although total intracellular Mg dropped by only 1–2% during a 20% drop in the cell viability. We also showed that intracellular total Mg in 0 or 0.4 mM Mg^{2+} incubation restored to the level at which the cells hadn't been cultured in low concentrations of $[Mg^{2+}]_e$ after Mg^{2+} being re-added to 1.0 mM, which occurred in accordance with the responded H_2O_2 metabolism to $[Mg^{2+}]_e$. Similar to intracellular total Mg, the cell viabilities increased close to that of control after Mg^{2+} in the media being restored to 1.0 mM. Moreover, the supplementation of DMTU (Ohkuma *et al.* 1998; Foresti *et al.* 1999), a $OH\cdot$ and H_2O_2 scavenger that can permeate the cell membrane, decreased H_2O_2 amount in Mg^{2+}-free cells to a physiology level, but had no effect on intracellular Mg, suggesting that intracellular Mg drop surely can induce intracellular H_2O_2 accumulation that can be reduced by DMTU.

In a further assessment of the oxidative injury of Mg-deficiency on cell and organelle membranes, we investigated the thiobarbituric acid-reactive substances production, which is an indicator of lipid peroxidation involving free radical generation. Lower Mg^{2+} levels caused severe membrane damage and strong cytotoxicity, which is in agreement with earlier observations that Mg deficiency enhanced free radical-induced intracellular oxidation and cytotoxicity (Freedman *et al.* 1991;

Dickens *et al*. 1992; Vormann *et al*. 1995). However, the conformability of thiobarbituric acid-reactive substances production and cell viability to H_2O_2 increase and the $[Mg^{2+}]_e$-deficiency couldn't demonstrate that the cytotoxicity induced by $[Mg^{2+}]_e$ deficiency resulted from disturbed H_2O_2 metabolism. The issue can be explained by the supplementation of DMTU to the 0 mM Mg^{2+} group which improved the cell viabilities a lot compared to Mg free media, though DMTU didn't completely reverse the cell viability, suggesting that the decrease of cell viability induced by $[Mg^{2+}]_e$ deficiency definitely partly resulted from the increase of H_2O_2.

A substantial H_2O_2 accumulation may be a result from either enhancement of H_2O_2 generation or a decline of H_2O_2 scavenging due to decreased catalyzing activity of enzymes. To identify the ability of cells to metabolize H_2O_2, the catalase activity was tested. In the presence of low Mg^{2+}, catalase activities markedly increased for a short period (2 days) and then decreased significantly in a time and dose-dependent manner with the $[Mg^{2+}]_e$ level decreasing until the termination of the experiment (4–10 days). These results indicated that Mg^{2+} deficiency surely affected catalase activity. Previous studies had indicated that augmentation of catalase activity can be intrigued by unphysiological H_2O_2 concentration (Anuszewska *et al*. 1997; Duthie & Collins 1997). In this study, after a short period of incubation, Mg-deficiency resulted in the increase in production of H_2O_2, which stimulated the enhancement of catalase activity to scavenge redundant H_2O_2, maintaining the balance of oxidation–antioxidation. As the incubation time increased, the activity of catalase decreased for long-term Mg-deficiency, thus the ability for cell to metabolize H_2O_2 declined. Therefore the catalase activity decreasing accompanied by the increase of H_2O_2 production resulted in the accumulation of H_2O_2 in a large amount, which further promoted the damage resulted from Mg-deficiency. The relationship between Mg^{2+} deficiency and the activity of catalase is a controversial topic. A researcher demonstrated that hypomagnesemia in male mice induced liver catalase activity increase (Kuzniar *et al*. 2001), whereas other researchers revealed that Mg-deficient lowered the activity of catalase in rat heart (Kumar & Shivakumar 1997) and in the aorta of rat (Shivakumar & Kumar 1997). The bidirectional characteristic of catalase activity in respond to cellular Mg deficiency found in our study provides evidence for the conflict.

Given our data, the demonstration of the modulation of $[Mg^{2+}]_e$ level on H_2O_2 metabolism implies that Mg is concerned with balance of oxidation–antioxidation in chick embryo hepatocytes. These results provide a line of evidence in support of Mg modulating the oxidation–antioxidation of cells partly through affecting the metabolism of H_2O_2, which will represent a valuable tool for studies on the regulation of Mg homeostasis in cells and its alteration in cells on the oxidative stress state. It is intriguing to speculate on the possible salutary role of suitable levels of Mg to facilitate the catalase activity, to prevent membrane lipid peroxidation and depress the oxidative stress.

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