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Research Article

Cadmium Exposure Elevates Glutathione Concentration via the Increase of C-Myc that Contributes to Attenuate the Toxicity of the Cells

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ABSTRACT

Introduction: The expression of c-Myc proto-oncogene enhanced in cadmium (Cd)-treated HepG2 hepatic carcinoma cells due to an increase in the c-Myc mRNA stability. We investigated the role of the increased c-Myc in responding to Cd challenge in the cells.

Materials and Methods: HepG2 cells were treated with Cd. Cell viability was estimated by MTT assay. The subG1 fraction, which represents the proportion of apoptotic cells, was determined by flow cytometry. Depletion of c-Myc gene expression was conducted by siENA knockdown and the mRNA level was quantitated by qPCR. Cellular glutathione level was determined by fluorescence spectrophotometry..

Results: Cell viability reduced while the subG1 fraction, representing the proportion of apoptotic cells, increased with Cd treatment in a dose-dependent manner. Administration of PI3K, p38 or JNK inhibitor to Cd-treated cells reduces cell viability and increases the subG1 cell fraction. Depletion of c-Myc mRNA expression by siRNA markedly reduces the proliferation and increases the subG1 fraction of the cells with or without Cd treatment. Cd treatment increases the expression of metallothionein 2A (MT2A) and γ- glutamylcysteine synthetase heavy chain (γ-GCS_H) mRNAs and the level of cellular glutathione (GSH). Treating Cd-exposed HepG2 cells with PI3K, p38 or JNK inhibitor does not affect the expression of MT2A gene but reduces the γ-GCS_H mRNA and the GSH levels. Knockdown of c-Myc mRNA expression does not reduce MT2A mRNA expression but decreases the levels of γ-GCS_H mRNA and GSH in the cells. Administration of Cd under GSH depleted condition results in a severe damage of the HepG2 cells.

Conclusion: These results indicate that Cd increases the c-Myc level in HepG2 cells to enhance the synthesis of GSH. The increased GSH contributes partly to protect the HepG2 cells from Cd-induced cytotoxicity.

Keywords: Cadmium; c-Myc; Glutathione; γ-GCSH; Cytotoxicity

INTRODUCTION

Cadmium (Cd) is an environmental pollutant that frequently distributed via the release of industrial wastes and the application of phosphate fertilizers [1,2]. Exposure of Cd may cause protein dysfunction, elevation of oxidative stress and cell death [3,4]. Cd also activates the expression of several proto-oncogenes, such as c-fos, c-jun and c-Myc, which might increase the risk of tumor formation, migration and invasion [5, 6]. Therefore, Cd is recognized as an environmental carcinogen.

Cells have defensive mechanisms that are activated upon Cd exposure. Metallothionein (MT) and glutathione (GSH) are major factors to sequester Cd in the cells [7,8].GSH is a tripeptide (Glu-Cys-Gly) with a gamma peptide linkage between the carboxyl group of the glutamate side chain and the amine group of cysteine, followed by a regular peptide linkage with glycine. GSH is synthesized by γ-glutamylcysteine synthetase (γ-GCS) and Glutathione Synthetase (GS). γ-GCS is the rate-limiting enzyme that regulates the GSH synthesis [9]. It is a heterodimer composed of heavy (γ-GCS_H) and light chains (γ-GCS_L). The heavy chain is the catalytic unit while the light chain modulates the substrate binding affinity of the heavy chain [10]. GSH is biologically involves in heavy metal chelating, free radial scavenging, xenobiotics metabolism and oxi-reductive homeostasis, in addition to cysteine storage and transfer [11]. Cells in general have a high concentration of GSH. The thiol (-SH) group on GSH can effectively bind Cd. Thus, GSH is regarded as the first-line defense mechanism against Cd toxicity [12].

Besides GSH, MTs are critical cellular factors to reduce Cd toxicity. MTs are low molecular weight, cysteine-rich proteins that bind Cd with high affinity. MTs are ubiquitous in bio-organisms. The level of MT remains low in cells and is promptly induced upon Cd exposure [13]. The synthesis of MTs is regulated at the transcriptional level. A metal-responsive transcription factor (MTF-1) usually locates in the cytoplasm of vertebrate cells. Upon metal exposure or oxidative stress, MTF-1 translocates into the nucleus and binds onto the Metal Responsive Elements (MREs) at the promoter of the MT gene and initiates its transcription [14]. The synthesized protein binds Cd via cysteine residues with a tetrahedral coordination. Each MT protein can chelate maximally seven Cd ions [15]. Therefore, MT sequesters Cd with high capacity.

Cellular myelocytomatosis oncogene (c-Myc) plays roles in cell proliferation, differentiation, apoptosis and transformation. It belongs to the basic region/ helix-loop-helix/ leucine-zipper (b/HLH/Z) protein family, and forms dimer with the ubiquitously expressed protein Max for the binding of specific promoter sequence [CAC(G/A)TG or known as E-box] on various genes [16]. The quantity of cellular c-Myc is regulated at transcriptional, translational and post-translational levels [16-18]. Our recent study showed that Cd increased the amount of c-Myc in HepG2 cells [19]. However, this increase is not the result of elevated gene transcription. Instead, Cd coordinately activates PI3K, p38 and JNK to stimulate the Akt activity. Activation of Akt leads to the phosphorylation of the nuclear Foxo1, which then translocate to the cytoplasm. The phosphorylated Foxo1 enhances the c-Myc mRNA stability in the cytoplasm resulting in an increased accumulation of c-Myc in the cells [19].

Reportedly, the expression of γ-GCS subunit genes can be regulated by MTF-1 [20] and c-Myc [21]. Both MRE and E-box-like sequences have been identified in the promoter regions of the γ-GCS subunit genes. Upon Cd exposure, MTF-1 is activated and the c-Myc level increases in HepG2 cells [19]. Activation of MTF-1 elevates MT synthesis to attenuate Cd toxicity. Contradictorily, c-Myc can bind onto the promoters of the MT gene family and reduces MT gene expression [19,22]. Therefore, whether an increase in c-Myc expression plays a protective function in HepG2 hepatic carcinoma cells remains unclear. We investigate here the role of c-Myc in modulating Cd-induced toxicity in HepG2 cells.

MATERIALS AND METHODS:

Cell culture and chemicals

HepG2 cells (ATCC® HB-8065™) were cultured at 37 °C in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 2 mM L-glutamine, 3.7 g/l sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2/ 95% air and 100% humidity. Cell culture reagents were purchased from Invitrogen/GIBCO. Cadmium chloride and

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LY294002 were from Merck. SB202190 and SP600125 were obtained from AdooQ Bioscience. OPTI-MEM®I, c-Myc siRNAs (HSS106837, HSS106839 and HSS181389), Stealth RNAi™ siRNA Negative Control and Lipofectamin™ RNAiMAX were purchased from Invitrogen. Enzymes and reagents for reverse transcription were purchased from Fermentas. Other reagent grade chemicals were from Sigma unless specified.

Transfection of siRNA

HepG2 cells (5 x 10^5) were seeded in 6-well plates with 2.5 ml antibiotics-free medium overnight until the cells were 30 to 50% confluent. Cells were then transfected with 150 pmol of siRNA and Lipofectamine™ RNAiMAX according to the manufacturer's instructions. The transfected cells were incubated for another 48 h before various treatments.

Real-time Polymerase Chain Reaction (PCR)

Total RNA was extracted with TRIzol® Reagent (Invitrogen) following the manufacturer's instructions, and 1 µg RNA was reverse-transcribed with a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) to generate complementary DNA. The resulting DNA was used for Quantitative real-time PCR (qPCR) with SYBR® Green PCR Master Mix (Applied Biosystems). Reactions were performed on an Applied Biosystems StepOnePlus™ Real-Time PCR System. Primers for the reactions are listed in Table S1. The expression of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was quantified in each sample and used as an internal reference. The expression of γ-GCS_H or MT2A mRNA was compared on the basis of equivalent GAPDH transcripts.

Cell viability assay and cell counting

HepG2 cells (1.5 x 10^4) were seeded in 96-well plates for 24 h then treated with various concentrations of $CdCl_2$ and cultured for another 24 h. Twenty-five µl MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (2 mg/ml) was added 4 h before the end of culture. Medium was removed and cells were washed with phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) and 200 µl DMSO was added to each well. Plate were read with a ThermoMax microplate reader (Molecular Devices) at the wavelength of 550 nm.

For cell counting, HepG2 cells (3 x 10^5) were seeded in 35 mm plates for 24 h and subjected to various treatments. Cells were suspended by using 1 ml trypsin/ EDTA and then with the addition of 9 ml PBS. Cell numbers were determined with a hemocytometer.

Cell phase (subG1 fraction) analysis

Analyses of subG1 fractions were performed according to Ma et al. [23]. Briefly, HepG2 cells (1.2 x 10^5) were seeded in 24-well plates for 24 h. After various treatments, Cells were suspended with trypsin/ EDTA and collected by centrifugation, then washed once with PBS. Cell pellets were dispersed and fixed with 1 ml 75% ethanol for 12 h at -20 °C. The ethanol was removed and the cells were further incubated with 300 µl PBS containing 100 µg/ml RNAase A and 5 µg/ml Propidium Iodide (PI) for 30 min at 37 °C. Cells at various stage of the growth cycle were subsequently analyzed by flow cytometry (FACScalibur).

Determination of cellular glutathione (GSH)

GSH content was determined according to the method of Hissin et al [24]. HepG2 cells (6 x 10^5) were seeded in 35 mm plates for 24 h and harvested after treatments. Cells were trypsinized and collected by centrifugation (1,000 x g for 5 min) then resuspended into 100 µl deionized water. Half of the cells were lysed in water to quantify the proteins in the whole cell lysates. The remaining cells were mixed with 12.5 µl 25% HPO_3 and then removed by centrifugation (10,000 x g, 4 °C for 20 min). The supernatant (40µl) was incubated in the dark with an O-phthalaldehyde mixture (100 µl 0.1% ophthalaldehyde in methanol, 0.5 ml 0.1 M sodium phosphate buffer, and 2 ml H_20, pH 8.0) for 15 min at room temperature. The fluorescence of the samples was analyzed with a Wallac 1420 Victor2 Microplate Reader (Perkin Elmer) with excitation and emission wavelengths of 355 and 460 nm, respectively. The acquired values were normalized to that of the protein concentration of the whole cell lysates.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) and a Tukey post-hoc test for multiple comparisons (GraphPad Prism 7 software, La Jolla, CA, USA). All p values <0.05 are considered statistically significant.

RESULTS:

Cell viability assays were conducted to investigate the cytotoxicity of Cd in HepG2 cells. HepG2 cells were treated with various concentrations of Cd for 24 h and cell viability was analyzed by the MTT assay. As shown in Figure 1A, cell viability decreased in a dose-dependent manner with Cd treatment. Noticeable decrease was observed in samples treated with 5 µM Cd. Cells were then treated with and without 5 µM Cd for 24 or 48 h and total cell numbers were determined. A marked inhibition of cell growth was found in Cd-treated cells (Figure 1B). Since treating HepG2 cells with 5 µM Cd induces significant level of c-Myc expression [19] and has a more than 80% cell viability at 24 h (Figure 1A), thus 5 µM Cd was used for subsequent toxicological studies.

Reportedly, Cd induces cell apoptosis [25]. We therefore investigated whether apoptotic death occurred in Cd-treated hepatic cells. Cells were administered with various concentrations of Cd for 24 h, and the subG1 fraction from each sample, representing the apoptotic cells, was analyzed by flow cytometry. Figure 1C shows that the subG1 fraction increased dose dependently with Cd administration. The results indicate that Cd (5 µM or more) induces apoptosis in HepG2 cells when treated for 24 h.

Our recent study concludes that Cd activates multiple signal pathways that coordinately enhance the c-Myc level in HepG2 cells [19]. To investigate the participation of c-Myc in Cd-induced toxicity, inhibitors of the signaling pathways were used. Cells were treated with 5 µM Cd in the presence of PI3K (LY294002), p38 (SB202190) or JNK (SP600125) inhibitor for 24 h and cell viability was analyzed by the MTT assay. Figure 2A shows that administration of various inhibitors reduced the viability of Cd-induced cells. The reduction of cell viability was further analyzed by examining the subG1 fraction after treatments. Addition of the inhibitors enhanced the subG1 fraction in Cd-treated cells (Figure 2B).

Since inhibitor of signaling pathways that enhance the c-Myc level reduced the viability of Cd-treated cells, the role of c-Myc in protecting cells from Cd damage was examined. Cells were transfected with c-Myc siRNA in the presence or absence of Cd, and cell proliferation was determined by counting the total cell numbers after various treatments. As shown in Figure 3A, knockdown of c-Myc

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mRNA expression reduces cell proliferation. With Cd administration, the c-Myc depleted cells showed again a higher growth inhibition than cells given nonspecific (control) siRNA. This reduction in cell proliferation reflects the degree of cell death. Cells with or without c-Myc gene knockdown were treated with 5 μM Cd for 24 h and the subG1 fraction of the cells was analyzed. Figure 3B shows that the subG1 fraction increased in samples with c-Myc mRNA knockdown. Cd treatment also increased the subG1 fraction, and the proportion increased further with c-Myc gene knockdown.

MT and GSH are two major components in protecting cells from Cd toxicity [26]. For human MT gene families, MT2A gene has the greatest expression in response to Cd exposure. To investigate whether MT and GSH levels can be affected by Cd treatment in HepG2 cells, the expressions of MT2A and γ-GCS_H (the rate-limiting enzyme for GSH synthesis) genes were examined with qPCR. Cells were treated with 5 μM Cd for various time intervals and the mRNA levels of these genes were analyzed. As shown in Figure 4A, the MT mRNA increased 1 h after Cd exposure, and peaked at 8 h after treatment. The level of the MT2A mRNA remained high 24 h after Cd addition. For the γ-GCS_H mRNA, the transcripts reached the highest level within 4 to 8 h of Cd treatment (Figure 4B).

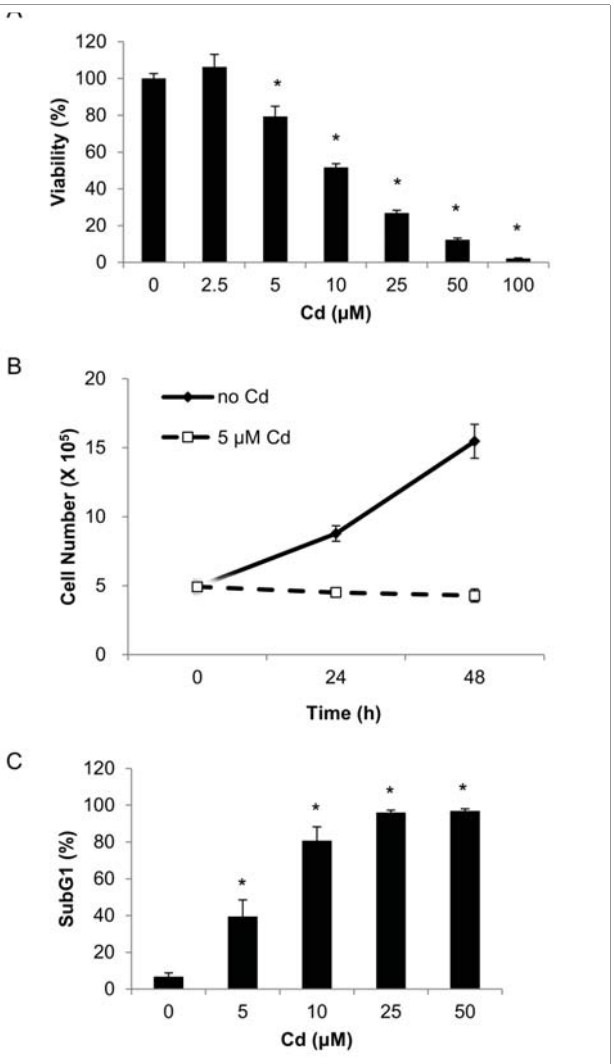

Figure 1: Effects of Cd on the viability, proliferation and apoptosis of HepG2 cells. (A) Cells were treated with various concentrations of Cd for 24 h and cell viability was analyzed by the MTT assay. (B) Cells were treated with 5 μM Cd for 24 or 48 h. Cell numbers were determined with a hemocytometer. (C) Cells were treated with various concentrations of Cd for 24 h and the subG1 fraction, which representing apoptotic cells, was analyzed by flow cytometry. Each value represents a mean ± standard deviation of three independent experiments. Asterisks indicate significant difference as compared to that of the untreated cells. *: $p < 0.05$ (A and C). Cell numbers are significantly different ($p < 0.05$) between the treatments at 24 and 48 h (B).

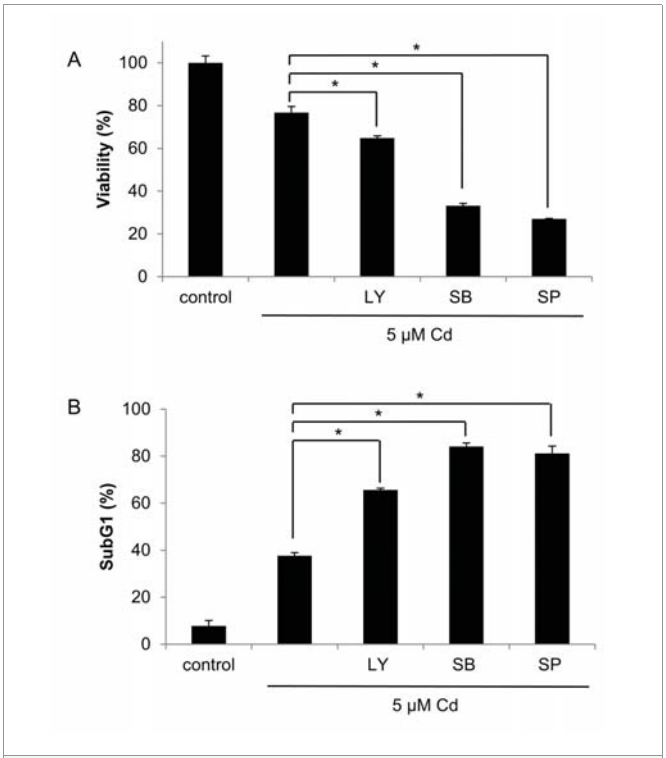

Figure 2: Effects of kinase inhibitor on the viability and the subG1 fraction of Cd-treated HepG2 cells. Cells were treated with 50 μM of LY294002 (LY, PI3K inhibitor), SB200190 (SB, p38 inhibitor) or SP600125 (SP, JNK inhibitor) 1 h prior to the addition of 5 μM Cd for 24 h. Cell viability (A) and subG1 fraction (B) were determined by MTT assay and flow cytometry, respectively. Each value represents a mean ± standard deviation of three independent experiments. Asterisks indicate significant difference between the paired samples. *: $p < 0.05$.

Since γ-GCS_H is the rate-limiting enzyme for GSH biosynthesis, the level of GSH was analyzed at various time intervals after Cd treatment. Figure 4C shows that cellular GSH level increased within 8 to 12 h after Cd exposure.

Cd administration increases MT2A and γ-GCS_H gene expressions. At the same time, the c-Myc level increased in the HepG2 cells. Hence, the role of c-Myc in regulating MT2A and γ-GCS_H gene expressions was examined. PI3K, p38 or JNK inhibitor can attenuate c-Myc accumulation in Cd-treated cells [19] and thus the inhibitors were added individually to the Cd-treated cells. Gene expressions were quantified by qPCR 4 h after 5 μM Cd exposure. The MT2A mRNA level did not alter significantly by any of the inhibitors (Figure 5A). However, the level of γ-GCS_H mRNA reduced markedly with the addition of inhibitor (Figure 5B). The GSH level in Cd-treated cells also decreased in the presence of the PI3K, p38 or JNK inhibitor (Figure 5C). The results imply that c-Myc affects the expression of γ-GCS_H and the accumulation of GSH, but has no effect on the gene expression of MT2A.

To investigate the role of Cd-induced elevation of c-Myc on

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gene expressions, siRNA was transfected to knockdown c-Myc expression. Cells were treated with Cd for 4 h and the c-Myc mRNA was quantified. Figure 6A shows that c-Myc mRNA dropped dramatically with the knockdown in the presence or absence of Cd. The MT2A mRNA level was then determined under the same experimental condition. As shown in Figure 6B, the MT2A mRNA level did not reduced in the c-Myc depleted cells with or without Cd treatment. Instead, the MT mRNA increased with c-Myc knockdown in either treatment. Analysis of the γ-GCS_H mRNA level otherwise showed a significant reduction of the transcripts with the c-Myc mRNA knockdown (Figure 6C). The reduction of γ-GCS_H mRNA expression led to a decrease in GSH level in cells exposed to Cd for 12 h (Figure 6D). These results indicate that Cd-induced elevation of c-Myc contributes mainly to increase γ-GCS_H expression and GSH accumulation in HepG2 cells.

Since GSH presents in high level in the cells, it is usually regarded as the first-line defense mechanism for Cd toxicity. We investigated how HepG2 cells response to Cd challenge under GSH depletion. Cells were treated with buthionine sulphoximine (BSO) for 24 h to deplete cellular GSH [27]. Cd was added to the cultures for another 24 h, and cell viability was examined by MTT assay. Figure 7 shows that depletion of GSH by BSO did not altered cell viability. Treating cells with 5 μM Cd for 24 h reduced the cell viability by less than 30%. Noticeably, treating GSH depleted cells with 5 μM Cd resulted in a severe lose in cell viability (by more than 90%). The results demonstrate the presence of GSH is required in HepG2 cells to form an integral defense network in response to Cd insults.

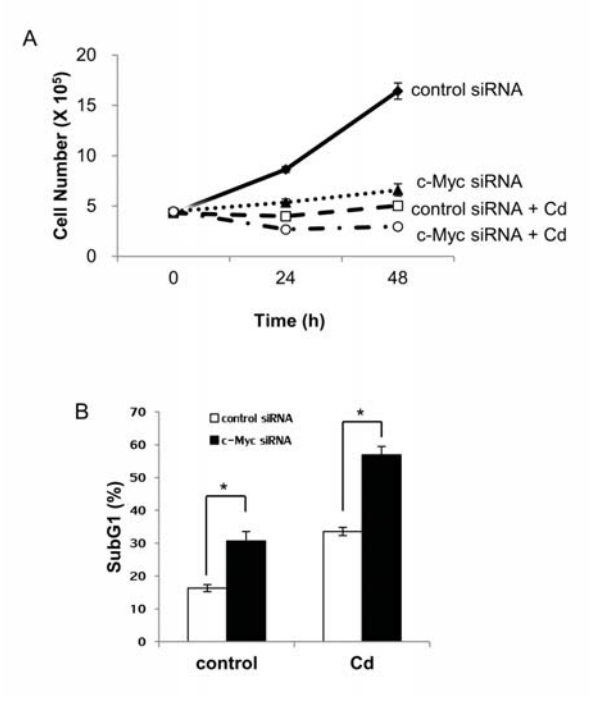

Figure 3: Effects of c-Myc mRNA knockdown on the proliferation and subG1 fraction of Cd-treated HepG2 cells. Cells were transfected with 50 nM of control or c-Myc siRNA for 48 h. (A) The transfected cells were treated with 5 μM Cd for 24 or 48 h. Cell numbers were determined with a hemocytometer. (B) The transfected cells were treated with 5 μM Cd for 24 h and the subG1 fractions were analyzed by flow cytometry. Each value represents a mean ± standard deviation of three independent experiments. Cell numbers are significantly different ($p < 0.05$) among the treatments at 24 and 48 h (A). Asterisks indicate significant difference between the paired samples. *: $p < 0.05$ (B).

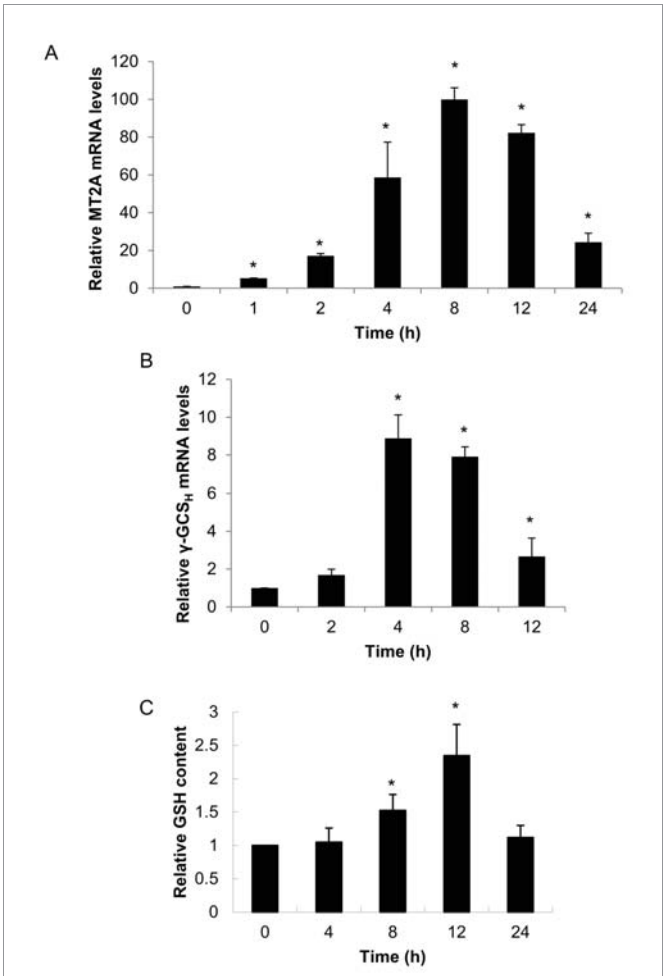

Figure 4: Effects of Cd on MT2A and γ-GCSH gene expressions and GSH level in HepG2 cells. Cells were treated with 5 μM Cd for various time intervals. MT2A (A) and γ-GCSH (B) mRNAs were analyzed by real-time PCR. The GSH content in the Cd-treated cells was analyzed by fluorospectrometry (C). Each value represents a mean ± standard deviation of three independent experiments. Asterisks indicate significant difference between the indicated samples and the untreated cells. *: $p < 0.05$.

DISCUSSION:

Recent study from our laboratory show that Cd activates the PI3K, the p38 and the JNK signal pathways in HepG2 cells coordinately to elevate the Akt activity that results in an increase of phosphorylated Foxo1 in the cytoplasm. The increase of cytoplasmic phospho-Foxo1 enhances the half-life of c-Myc mRNA and causes the increase of c-Myc in cells [19]. We investigated here the role of the increased c-Myc in responding to Cd challenge. MT and GSH are two major cellular factors to sequester Cd. Reportedly, c-Myc binds the promoter of MT and γ-GCS_H genes [2,19]. Whether an increase in c-Myc contributes to the expression of these genes in HepG2 cells remains unclear. We reveal in this study that Cd-induced c-Myc elevation plays a role in enhancing the GSH content in HepG2 cells. This effect contributes partly to the increase of Cd tolerance in HepG2 cells.

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transformation are indicated as the main functions of c-Myc [28]. Reduction of c-Myc level under various stresses may cause apoptotic cell death. Human colorectal carcinoma HCT116 cells treated with Dihydroartemisinin reduced c-Myc level and increased apoptotic cell death [29]. Human epithelial NA cells exposed to sodium nitroprusside also caused a decrease in c-Myc mRNA and an increase in apoptosis [30]. Similarly, reduction of c-Myc expression in human melanoma M14 cells increased the cells' sensitivity to cis-platin (cis-Pt) and melphalan that reduced the viability of the cells [31]. On the contrary, elevation of c-Myc expression can enhance cell viability under exogenous insults. Over-expression of c-Myc attenuates the sensitivity of cancer therapeutic drugs in various cells [32,33]. These results reveal that cellular c-Myc level is associated with drug tolerance. In this study, we found that c-Myc expression also modulates cellular tolerance to Cd toxicity. This effect is associated with an increase in γ-GCS_H expression and the subsequent GSH synthesis in HepG2 cells.

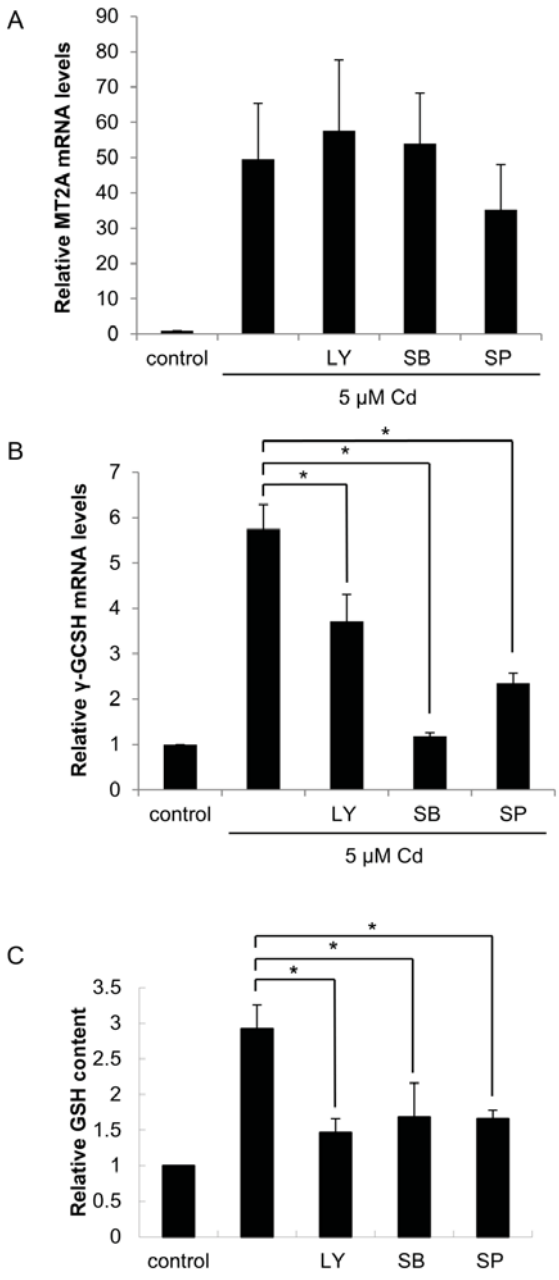

Figure 5: Effects of kinase inhibitors on MT2A and γ-GCS_H gene expression and GSH level in Cd-treated HepG2 cells. Cells were treated with 50 μM of LY294002 (LY, PI3K inhibitor), SB200190 (SB, p38 inhibitor) or SP600125 (SP, JNK inhibitor) 1 h prior to the addition of 5 μM Cd for 4 h. MT2A (A) and γ-GCS_H (B) mRNAs were analyzed by real-time PCR. The GSH content in cells treated with Cd for 8 h was analyzed by fluorospectrometry (C). Each value represents a mean ± standard deviation of three independent experiments. Asterisks indicate significant difference between the paired samples. *: $p < 0.05$.

We showed in this study that depletion of c-Myc reduced γ-GCS_H gene expression in HepG2 cells with Cd treatment (Figure 6C). However, MT2A mRNA increased under the same condition (Figure 6B). Reportedly, c-Myc binds onto the promoter of human MT2A gene [34]. Our result suggests a suppressive role of c-Myc in the expression of MT2A gene, as reported by Iizuka et al [22]. Possibly, the role of MT2A is partly replaced by the increase of GSH in Cd-treated HepG2 cells. A similar suppressive effect is noted in mouse cells where over-expression of c-Myc results in a decrease in basal MT gene expression [35].

MTF-1 is the most potent transcription factor to regulate the expression of MT genes [36]. Various kinases, such as PKC, PI3K, JNK and tyrosine protein kinase, have been implicated in reducing the MTF-1 activity [37,38]. However, Cd-induced MT2A gene expression was not affected by PI3K, JNK or P38 inhibitor in HepG2 cells (Fig 5A). Instead, these factors are involved in Cd-induced c-Myc expression [19]. Apparently, those Cd-activated signaling factors participate mainly in increasing the c-Myc level but not in modifying the MTF-1 activity.

BSO is a potent inhibitor of γ-GCS synthesis with consequent depletion of GSH in cells [21]. Reduction of GSH results in a marked sensitivity to Cd toxicity and severe damage in HepG2 cells (Figure 7). It has been speculated that GSH is a precursor or cysteine depot for MT synthesis since MT contains more than 30% of cysteine residues [39]. Previous study showed that MT level increased with zinc administration, but the increase was not affected by the depletion of hepatic GSH [40]. A similar result can be found in Cd-treated rat myoblast L6 cells; Cd-induced MT level was not affected under GSH depletion [41]. These results indicate that GSH is not a cysteine reservoir for MT synthesis but is required coordinately with MT to protect cells from Cd-induced toxicity.

Reportedly, Cd exposure increases oxidative stress and causes cell damages [42-44]. This increase is derived from enhancing ROS (reactive oxygen species) production and reducing the activity of anti-oxidative enzymes such as catalase, Glutathione Peroxidase (GPX) and superoxide dismutase [45,46]. Since liver is one of the main sites for Cd deposition when administered, inhibition of anti-oxidative enzymatic activity is commonly found in the livers of various animal species. For instance, dietary Cd uptake inhibits hepatic GPX activity in birds [47]. Long-term administration of Cd causes a reduction of GPX and catalase activities in rats [48]. Injection of Cd in fish decreases hepatic GPX mRNA expression [49]. These findings indicate that Cd exposure reduces the synthesis and/or activity of anti-oxidative enzymes and thus enhances the oxidative stress of cells. Since elevation of ROS may increase c-Myc level [50], we speculate that GSH level can be increased to compensate for the anti-oxidative activity.

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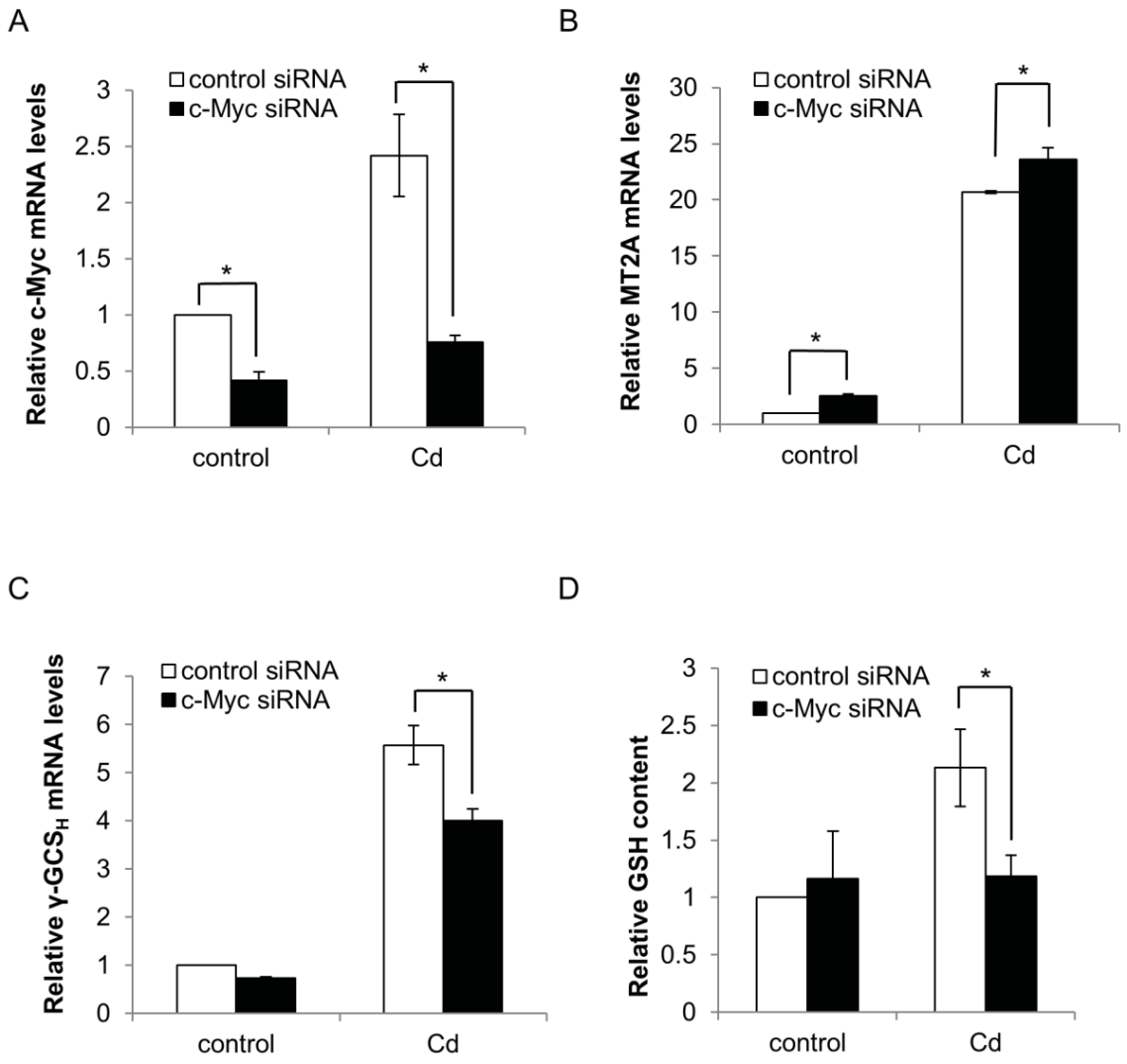

Figure 6: Effects of c-Myc mRNA knockdown on MT2A and γ-GCS$_H$ gene expression and GSH level in HepG2 cells. Cells were transfected with 50 nM of control or c-Myc siRNA for 48 h. The transfected cells were treated with 5 μM Cd for 4 h and c-Myc (A), MT2A (B) and γ-GCS$_H$ (C) mRNAs were analyzed by real-time PCR. (D) The transfected cells were treated with 5 μM Cd for 12 h and the GSH content in the cells was analyzed by fluorospectrometry. Each value represents a mean ± standard deviation of three independent experiments. Asterisks indicate significant difference between the paired samples. *: $p < 0.05$.

MT and GSH are the major cellular components to chelate cellular Cd. The thiol group of cysteine(s) on both factors is able to reduce the ROS level in cells [51,52]. Therefore, they can exert coordinative effect to reduce Cd-induced toxicity. MT is ubiquitously expressed in mammalian cells with Cd treatment. Reportedly, MT can be detected in HepG2 cells treated with 5 μM Cd for 6 h [53]. Since GSH content increased within 4 to 8 h after Cd treatment (Figure 4), MT and GSH can work together initially to protect cells from Cd toxicity. This protective role reduces with lengthening Cd exposure period due to the reduction of MT and γ-GCS$_H$ genes expression.

GSH depletion markedly enhances ROS production in cells and leads to apoptotic death [54]. We showed here that Cd exposure caused a severe cytotoxicity under GSH depletion (Figure 7). Possibly, over-production and ineffective removal of ROS in cells can be the key factors. Short-term depletion of GSH may have no significant effect on cell viability (Figure 7). However, synergistic damaging effect occurs with Cd exposure.

Besides metal chelating and ROS removal, GSH also participates in various cellular activities such as DNA synthesis, protein synthesis, transfer of amino acid, regulation of enzyme activity and chemical metabolism [55-57]. GSH is a ligand of Glutathione S-Transferase (GST), an isozymal family critical for cell metabolism. Administration of Cd inhibits total hepatic GST activities [58]. Under GSH depletion, GST activities are abolished and cell metabolism is disrupted with Cd treatment. Since GSH presents in high concentration in cells, it interacts immediately with Cd before the induction of MT synthesis [59, 47]. Under GSH depletion, Cd cannot be effectively sequestered before MTs are effectively synthesized. The free cellular Cd can facilitate ROS production resulting in severe cell damage [18].

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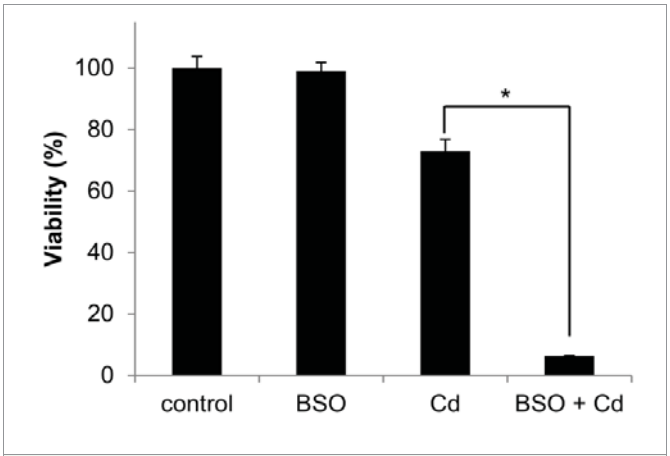

Figure 7: Effect of GSH depletion on the viability of Cd-treated HepG2 cells. Cells were treated with 50 mM BSO for 24 h to deplete GSH, followed by the addition of 5 μM Cd for 24 h. Cell viability was determined by the MTT assay. Each value represents a mean ± standard deviation of three independent experiments. Asterisks indicate significant difference between the paired samples. *: $p < 0.05$.

CONCLUSION:

In summary, we investigated the physiological role of the increased c-Myc upon Cd exposure in HepG2 cells. We found that both MT2A and γ-GCS_H gene expressions were activated with Cd treatment. However, blocking the signal transduction pathways or using siRNA knockdown to reduce c-Myc expression did not decrease the Cd-induced MT2A mRNA level. On the contrary, γ-GCS_H expression and GSH level decreased with the depletion of c-Myc expression. Cell viability was also reduced upon decreasing the cellular GSH content. Therefore, we conclude that Cd exposure evokes several signaling pathways to coordinately increase the phosphorylation of Foxo1. The activated Foxo1 enhances the stability of c-Myc mRNA and then the c-Myc level. The enhanced c-Myc stimulates GSH synthesis via γ-GCS_H expression and partly contributes to the reduction of Cd-induced cytotoxicity.

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