Cadmium Downregulates *NEMO* and *G-6-PD* mRNA Expression in Human Hepatocellular Carcinoma Cell Line (HepG2) Toxicity

Chatkul Techakitiroj, Wisit Tangkeangsirisin, and Palarp Sinhaseni

***Abstract*—** Cadmium is an ubiquitous environmental contaminants in food and herbal medicines. Previous studies reported that cadmium decreased the G-6-PD activity but the effect of cadmium on *G-6-PD* gene expression is not yet clearly understood. Furthermore the human NF-kappaB essential modulator (NEMO) gene is arranged head-to-head with the *G-6-PD* gene. We investigated the effects of dose and exposure time of cadmium on *NEMO* and *G-6-PD* mRNA expression in human hepatocellular carcinoma cell line (HepG2). We found that cadmium reduces the expression both of *NEMO* and *G-6-PD* mRNA in 24 hour but has no effect on *Beclin* mRNA expression. We propose that cadmium induced hepatocellular toxicity and cell death in a time and dose dependent manner without activation of autophagic cell death. HepG2 cell death is in accordance with *NEMO* and *G-6-PD* mRNA expression. These results bright about to better understanding of cadmium toxicity in hepatocellular injury related to *NEMO* and *G-6-PD* gene.

***Keywords*—** Cadmium, G-6-PD, HepG2, NEMO

I. INTRODUCTION

CADMIUM is a toxic transition metal of environmental concern [1]. Humans are susceptible to cadmium toxicity primarily through the ingestion of contaminated food or water and cigarette smoke inhalation [2]. In man or animal cadmium is mainly accumulated in kidney and liver with long biological half life. Cadmium makes a cumulative toxin, chronic exposures could still result in direct toxic effects of the residual metal. Cadmium induces oxidative stress and cell death [3]. Furthermore, cadmium acetate increases glucose-6-phosphate dehydrogenase (G-6-PD) protein level in lung of male rat [4]. However, G-6-PD activity is significantly decreased by cadmium exposure [5].

More than 400 million people worldwide are affected by G-6-PD deficiency. The highest prevalence of G-6-PD deficiency is reported in Southeast Asia, including Thailand. G-6-PD is the rate-determining step of the pentose phosphate pathway. Its most important function is to supply NADPH for protection against oxidative agents in all cells. G-6-PD deficiency causes hemolytic anemia in response to fava beans consumption, viral illnesses and certain medications such as antimalarial agents [6]. The human *G-6-PD* gene is located near the telomeric region of the X chromosome (Xq28). The gene consists of 12 introns and 13 exons, spanning nearly 20 kb in total; it encodes 515 amino acids, and a GC-rich (more than 70%) promoter region. G-6-PD deficiency is an X-linked, hereditary genetic defect caused by mutations in the *G-6-PD* gene, resulting in protein variants with different levels of enzyme activity [7]. *G-6-PD* mRNA is elevated in Kupffer and endothelial cells by TNF-alpha [8] and in HepG2 cells by oxidative stress [9]. The human *G-6-PD* gene is arranged head-to-head with the NF-kappaB essential modulator (*NEMO*) gene [10].

NEMO controls the activation of the transcription factor NF-kappaB. NEMO is required for IkappaB kinase (IKK) function in most situations [11]. Although NEMO is the only subunit absolutely essential for activation of the IKK complex by diverse stimuli, such as TNF-alpha and IL-1, very little is known about its mechanism of action [12]. It is involved sequential small ubiquitin-like modifier and ubiquitin modification occurs in both of cytoplasm and nucleus. Up-regulation of NEMO in antiestrogen-resistant breast cancer cells (MCF-7/LCC9) enhances the kinase activity of IKK. The formation of the IKK complex is required for the activation of NF-kappaB in response to external stimuli such as tumor necrosis factor-alpha (TNF-alpha) [13].

Beclin is the tumor suppressor protein that functions in the lysosomal degradation pathway of autophagy (program cell death type I) [14]. The structure of beclin, as well as its essential role in autophagosome formation, is evolutionarily conserved throughout all eukaryotic cells [15]. Autophagy is defined as a process in which proteins and organelles are degraded by lysosomal proteases. Autophagy may prevent a normal cell to become a malignant cell by degrading damaged organelles and thereby reduce cellular stress [16]. Cadmium is a minimal alteration of beclin-1 expression level in transformed human urothelial cells [17].

Chatkul Techakitiroj is with Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (corresponding author to provide phone: 66 2 5907160 ; e-mail: tverakit@ gmail.com).

Wisit Tangkeangsirisin is now with the Department of Biopharmacy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand (e-mail: twisit@gmail.com.

Palarp Sinhaseni is with the Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (e-mail: spalarp@chula.ac.th).

Cadmium is initially distributed to the liver and accumulates at high levels. Cadmium decreases NADPH when defense mechanisms are overloaded. Cells death is related to the liver becomes one of the primary site of injury in human hepatocyte [18]. However, mechanisms are not yet clearly understood. *G-6-PD* is induced expression by TNF-alpha and oxidative stress [8, 19]. *G-6-PD* and *NEMO* mRNA expression may be driven and regulated by the same set of transcription factors since their promoter align in a head-to-head direction. Therefore, the oxidative stress induced by cadmium may effect to both *G-6-PD* and *NEMO* mRNA expression on hepatocyte. Thus, we investigate the effect of cadmium to *G-6-PD* gene expression, as a key enzyme to produce NADPH, and the effect of cadmium on *NEMO* gene expression related to *G-6-PD* gene in HepG2 cells.

II. MATERIAL AND METHODS

Cell line maintenance

The human hepatocellular cancer cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). Minimal essential medium (MEM) was obtained from Invitrogen (Carlsbad, CA). HepG2 cells were maintained in MEM supplemented with 5% FBS, 1% Non-Essential Amino Acid and 0.5% L-glutamine. The medium was renewed every 3 days, and the cells were subcultured once a week.

Cadmium treatments

HepG2 cells were seeded onto a 6-well plates ($1x10^6$ cells per well) and used for the cadmium exposure experiments. After being cultured overnight, HepG2 were exposed with 0.5, 1.0, 5.0 10.0 µM for 12 or 24 hours cadmium chloride. Cadmium chloride hemi (pentahydrate) ($CdCl_2 \cdot 2.5H_2O$) were obtained from Sigma (St.Luis, MO).

Total RNA isolation

All RNA samples from attached HepG2 were purified by the Trizol^{TM} reagent from Invitrogen (Carlsbad, CA). RNA pellet was dissolved in RNase-free DNase-free DEPC-treated water. RNA concentration was determined by UV-spectrophotometry and stored at -80°C.

Reverse transcription-PCR

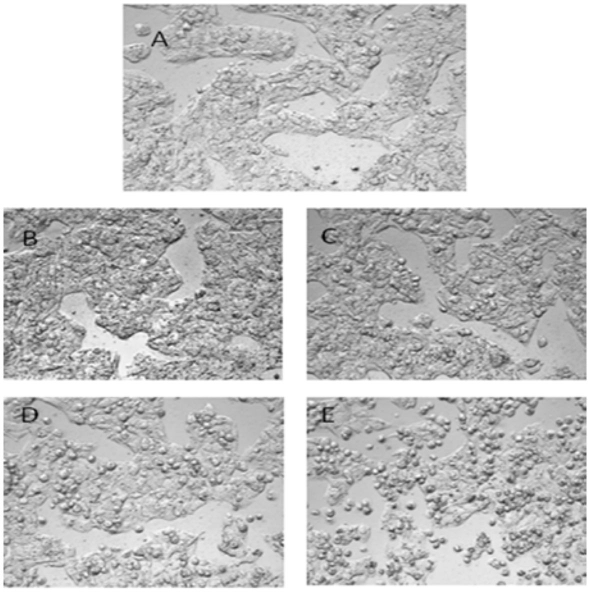
Five micrograms of total RNA was reversely transcribed by random hexamer with Super-scrip^{TM} II reverse transcriptase from Invitrogen (Carlsbad, CA). The enzymes were added and the reactions were carried on at 42°C for 50 minutes, followed by a 15-minutes heat inactivation step at 70°C. The DNA was stored at -20°C. Polymerase chain reactions were performed by the protocol following the manufacturer's recommendation (Invitrogen^{TM}). Briefly, each reaction was performed in the 0.5-ml thin wall tubes (Corning, NY). Tubes were incubated in a thermal cycler (Biometra T-Gradient. Germany). The reactions were performed 25-28 cycles. Denature 94°C for 20 s, annealing 63°C for 20 s, extension 72°C for 20 s. The reactions were carried out for an additional 10 minutes at 72°C and maintained at 4°C. PCR products were resolved in 1.5% agarose in 1 x TAE buffer and detected by ethidium bromide staining. Intensity of bands were determined by densitometric analysis and normalized to GAPDH bands (as an internal control). The specific primer pairs used were: GAPDH: forward primer, 5'- TGA AGG TCG GAG TCA ACG GAT TTG GT -3' and reverse primer, 5'- CAT GTG GGC CAT GAG GTC CAC CAC -3' ; G-6-PD: forward primer, 5'- GAT GCC TTC CAT CAG TCG GA -3' and reverse primer, 5'- GCT CAC TCT GTT TGC GGA TG -3'; NEMO: forward primer, 5'- ACG TAC TGG GCG AAG AGT CTC C-3' and reverse primer, 5'- GAC GTC ACC TGG GCT TTC AC-3'; Beclin: forward primer, 5'- CTG GCA CAG TGG ACA GTT TGG C-3' and reverse primer, 5'- CTG CAC ACA GTC CAG GAA AGC C-3'.

III. RESULTS

Effect of cadmium on cell morphology of HepG2 cells for 24 hours

The HepG2 cells morphology was cultivated in the presence of cadmium. We found that HepG2 cells morphology are rounded, indicated cell death. Increasing of cadmium concentration results in higher toxicicity and cell death (Fig. 1). This result indicated that cadmium induced HepG2 cells death.

Fig. 1 Effect of cadmium of HepG2 cells morphology for 24 hours. HepG2 cell morphology measured by light microscopic (magnitude 200X). (A) Control; (B) Cadmium 0.5 µM; (C) Cadmium 1.0 µM; (D) Cadmium 5.0 µM; (E) Cadmium 10.0 µM

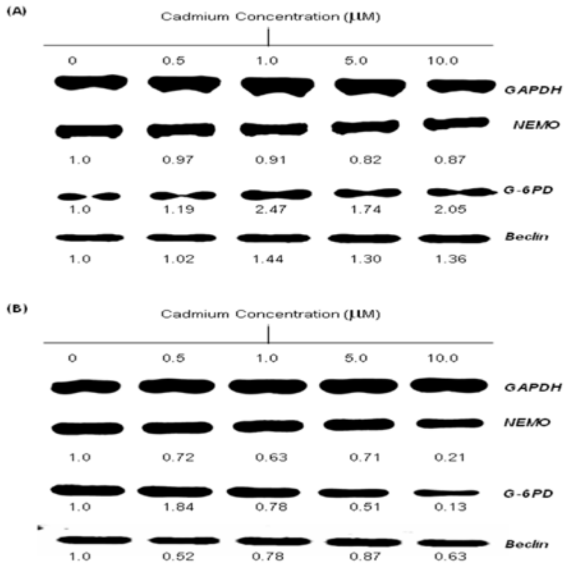


Effect of cadmium on G-6-PD, NEMO and Beclin mRNA expression in HepG2 cells

Total mRNA was isolated and *G-6-PD*, *NEMO* and *Beclin* mRNA expression were determined by reverse transcriptase

PCR. The *G-6-PD*, *NEMO* and *Beclin* mRNA expression level were investigated in HepG2 cells-treated with cadmium. At 12 hour, *G-6-PD*, *NEMO* and *Beclin* mRNA expression in HepG2 cells cultivated in the presence of cadmium are not different from the control group (Fig. 2A). This result indicated that *G-6-PD*, *NEMO* and *Beclin* mRNA expression were not reduced by cadmium for 12 hour. 24 hour, both of *G-6-PD* and *NEMO* mRNA expression in HepG2 cells cultivated in the presence of cadmium decreased. There is no change in *Beclin* mRNA expression (Fig. 2B). These results indicated that cadmium induced HepG2 cell death related to *G-6-PD* and *NEMO* mRNAs expression in dose- and time-dependent manner. However, HepG2 cell death may not related with autophagy pathway.

Fig. 2 Effect of cadmium on *NEMO*, *G-6-PD* and *Beclin* mRNA expression in HepG2 cells. (A) 12 hour; (B) 24 hour



IV. DISCUSSION

Cadmium decrease Glucose-6-phosphate dehydrogenase mRNA expression

G-6-PD is an enzyme that catalyses in the pentose phosphate pathway and essential in regenerating the reduced form of glutathione, which can reduce the oxidative stress [7]. It has been reported that oxidative stress can induce *G-6-PD* mRNA expression on HepG2 cells [9]. *In vivo* study, cadmium induces oxidative stress in rat kidney tissue by decreased level both of G-6-PD protein and reduced from of glutathione protein. These effects cause kidney tubular cell death [20]. Moreover, *in vitro* studies, cadmium induces oxidative stress in a time- and concentration-dependent manner. It has been also reported that cadmium increased *G-6-PD* mRNA expression in silver sea bream hepatocyte and primary rat hepatocyte [21, 22]. On the other hand, we found that cadmium decreased *G-6-PD* mRNA expression in dose-dependent manner. Previous reports are opposed to result with our result because cell types are difference. We also propose that cadmium has hormetic effect, a biphasic dose–response phenomenon, on cell proliferation [23]. Cadmium induced oxidative stress to cells which leads to the reduction of glutathione (GSH) level. GSH depletion induced G-6-PD expression. On the contrary, *G-6-PD* mRNA expression may decrease in response to extremely high cadmium levels and long duration because the cells fail to defense mechanism [21]. Our finding showed the significant role of cadmium on *G-6-PD* gene expression which may disturb GSH/GSSG cycle by alternating the antioxidant defense ($NADP^+$/NADPH) in human hepatoma cell cause of cell death.

Cadmium decrease NEMO mRNA expression but not affect to NEMO protein expression

The human *G-6-PD* gene maps to Xq28 and is arranged head-to-head with the *NEMO* gene [24]. Expression control of both genes may be shared by the same set of transcription factors and inducers. The loss of NEMO protein mediate to hepatic cell damage [25]. The NF-kappaB–mediated anti-apoptotic response is inhibited by the lack of NEMO protein in hepatocytes [26]. NEMO-mediated NF-kappaB activation in mice hepatocytes has an essential physiological function to prevent the spontaneous development of steatohepatitis and hepatocellular carcinoma [27]. We found cadmium decreased *NEMO* mRNA expression but not affected to NEMO protein expression (data not shown). The post-translational modification of NEMO is also necessary for NF-kappaB activation in response to certain genotoxic agents [28]. NEMO ubiquitination is necessary for NF-kappaB activation [29]. NF-kappaB activation by genotoxic stress provides an attractive paradigm for nuclear-to-cytoplasmic signaling pathways [11]. Cadmium induced genotoxicity and oxidative stress [30]. However, cadmium is weakly genotoxic and mutagenic in mammalian cells. Indirect effects of cadmium increases reactive oxygen species (ROS) generation and DNA damage [31]. We found that NEMO protein expression was not changed by cadmium (data not shown), their result lead to less genotoxic and carcinogenic effect. Moreover, we found that cadmium induced cell death, represent by increase cleavage-PARP (data not shown). Our results suggest that cadmium induce HepG2 cell death may via oxidative stress pathway more than genotoxicity.

Cadmium induces HepG2 cell death not related with autophagy pathway

Cadmium affects adversely a number of organs in human such as kidneys, liver, and pancreas. Cadmium induced hepatic cell death depend on dose [19, 32]. The effect of cadmium on the viability of HepG2 cell has been investigated previously, Lawel and Ellis [33] indicated that the human hepatoma cell line (HepG2) is the most sensitive to cadmium toxicity when compared with a human astrocytoma cell line (1321N1) and human embryonic kidney cell line (HEK293). Moreover, cadmium depleted of ATP production in HepG2 cells in a dose-dependent manner. The purpose of this study is to investigate the effect of cadmium induced HepG2 cell death and it may be not related with autophagy. The data obtained from the present work indicated that human hepatoma cells

(HepG2) is more sensitive to cadmium than monkey kidney (LLC-MK2) cells (data not shown) as mentioned in the earlier study [33]. HepG2 cell death may be not related with autophagy pathway (*Beclin* mRNA expression is not changed) but apoptotic and/or necrotic pathway. Since cadmium induced poly(ADP-ribose) polymerase (PARP) cleavage in dose dependent manner (manuscript in preparation).

V.CONCLUSION

The studies presented here demonstrate the role of cadmium-induced HepG2 cells death. Cadmium decreases *NEMO* and *G-6-PD* mRNA expression in dose dependent manner for human hepatoma cell. These results indicated that cadmium induced cell death related to alteration of intracellular redox state. Furthermore, this is the first report on the relationship and effect of cadmium on *NEMO* and *G6PD* genes expression in the HepG2 cells.

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