The Role of Oxidative Stress in Citreoviridin-Induced DNA Damage in Human Liver-Derived HepG2 Cells

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ABSTRACT: We hypothesize that citreoviridin (CIT) induces DNA damage in human liver-derived HepG2 cells through an oxidative stress mechanism and that N-acetyl-L-cysteine (NAC) protects against CIT-induced DNA damage in HepG2 cells. CIT-induced DNA damage in HepG2 cells was evaluated by alkaline single-cell gel electrophoresis assay. To elucidate the genotoxicity mechanisms, the level of oxidative DNA damage was tested by immunoperoxidase staining for 8-hydroxydeoxyguanosine (8-OHdG); the intracellular generation of reactive oxygen species (ROS) and reduced glutathione (GSH) were examined; mitochondrial membrane potential and lysosomal membranes’ permeability were detected; furthermore, protective effects of NAC on CIT-induced ROS formation and CIT-induced DNA damage were evaluated in HepG2 cells. A significant dose-dependent increment in DNA migration was observed at tested concentrations (2.50–10.00 μM) of CIT. The levels of ROS, 8-OHdG formation were increased by CIT, and significant depletion of GSH in HepG2 cells was induced by CIT. Destabilization of lysosome and mitochondria was also observed in cells treated with CIT. In addition, NAC significantly decreased CIT-induced ROS formation and CIT-induced DNA damage in HepG2 cells. The data indicate that CIT induces DNA damage in HepG2 cells, most likely through oxidative stress mechanisms; that NAC protects against DNA damage induced by CIT in HepG2 cells; and that depolarization of mitochondria and lysosomal protease leakage may play a role in CIT-induced DNA damage in HepG2 cells. © 2014 The Authors. Published by Wiley Periodicals Inc. Environ Toxicol 30: 530–537, 2015.

Keywords: CIT; HepG2 cells; single-cell gel electrophoresis assay; 8-OHdG; ROS; GSH

INTRODUCTION

Citreoviridin (CIT) is a toxic secondary metabolite produced mainly by Penicillium citreoviride. It has an unusual structure that consists of a lactone ring conjugated to a furan ring, and has a molecular weight of 402.5. CIT has been mostly identified primarily in moldy rice and corn in parts of Asia and South America (Rosa et al., 2010; Almeida et al., 2012). CIT has also been isolated from uranium mines and miners’ throats (Sram et al., 1993a, b). It has been shown that the
contamination levels of CIT in grains were between 4.9 and 33.2 μg/kg in the target areas of China (Hou et al., 2013). However, the safety limits of CIT in food products have never been established by regulatory agency in China as well as other countries.

The acute toxicity, cardiototoxicity, and neurotoxicity of CIT have been studied since the 1970s. It has been found that CIT causes vomiting, convulsions, ascending paralysis, respiratory arrest, neurological symptoms, and depressed sensory responses in animals (Ueno, 1972; Ueno and Ueno, 1972; Datta and Ghosh, 1981). In mice, oral and intraperitoneal LD50s of CIT are 20 mg/kg and 7.5 mg/kg, respectively (Ueno and Ueno, 1972). In humans, CIT can cause acute cardiac beriberi and atherosclerosis; and initiate Keshan disease probably through oxidative stress mechanisms (Sun, 2010; Almeida et al., 2012; Hou et al., 2013). A study indicated that the toxic effects of CIT may result from respiratory and cardiovascular failure, which led to central nervous system depression due to systemic hypoxia (Nishie et al., 1988).

CIT functions as an uncompetitive inhibitor of ATP hydrolysis and a noncompetitive inhibitor of ATP synthesis through the inhibition of the beta-subunits of membrane-bound F1-ATPase (Sayood et al., 1989). A recent study showed that CIT inhibited ectopic ATP synthesis, which ultimately led to the inhibition of lung cancer cell growth (Chang et al., 2012).

Reactive oxygen species (ROS), a byproduct of the normal metabolism of oxygen produced by the cells, have multifaceted roles in cell signaling, immune response, inflammation, aging, and apoptosis in both normal and pathologic cells (Devasagayam et al., 2004; Valko et al., 2007; Kozina et al., 2012; Padgett et al., 2013). Endogenous sources of ROS include the following: NADPH oxidases; the mitochondrial respiratory chain; the flavoenzyme ERO1 in the endoplasmic reticulum; xanthine oxidase; lipoxigenases; cyclooxygenases; cytochrome P450s; a flavin-dependent demethylases; oxidases for polyamines and amino acids; and nicotinic oxide synthases (Nathan and Cunningham-Bussel, 2013). The intracellular ROS levels are regulated by a complicated web of enzymes that produce (oxygenases) or scavenge (glutathione peroxidase) ROS (Celardo et al., 2011). An imbalance between ROS generation and the cellular antioxidant defense system leads to oxidative stress, which is thought to be involved in the development of numerous human diseases (Halliwell, 2007; Valko et al., 2007; Mohsenzadegan and Mirshafiey, 2012; Ramond et al., 2013). ROS serve as a source to trigger DNA damage and mitochondrial dysfunction (Xu et al., 2011; Qu et al., 2013). In addition, ROS induce lysosomal protease leakage, which plays a role in apoptosis and DNA damage (Ogawa et al., 2004).

Liver is one of the primary organs responsible for detoxification and the target for some mycotoxins. For example, aflatoxin B1 is considered the most toxic aflatoxin and has been directly implicated in the etiology of human hepatocellular carcinoma. CIT mainly deposits in liver, kidney, and heart after injection in animals. Compared with its neurotoxicity and cardiototoxicity, hepatotoxicity of CIT has been less investigated and remains largely unknown. In this study, the metabolically competent human hepatoma cells (HepG2) were used to examine the induction of DNA damage and the underlying mechanisms after CIT treatment. HepG2 cells are frequently used in vitro as an experimental model to detect mycotoxicity and gene expression. These cells are similar to hepatocytes and possess most of inducible enzymes necessary for xenobiotic metabolism. It has been shown that HepG2 cells are suitable for genotoxicity testing (Knasmuller et al., 2004).

MATERIALS AND METHODS

Reagents
CIT (C23H32O6; CAS No.25425-12-1; purity ≥97%) was purchased from Santa Cruz Biotechnology. Dimethyl sulfoxide (DMSO), cytochalasin B, RNAase A, Ethidium Bromide (EB), O-phthalaldehyde (OPT), acridine orange (AO), rhodamine 123, 2,7-dichlorofluorescein diacetate (DCFH-DA), and N-acetyl-l-cysteine (NAC) were purchased from Sigma (St. Louis, MO). Monoclonal 8-OHdG antibody was purchased from JaI CA (Fukuori, Japan). The ultrasensitive streptavidin–peroxidase kit was purchased from Maixin-Bio (Fujian, China). Cell culture flasks and dishes were purchased from Falcon (Becton Dickinson, San Jose, CA). All tissue culture reagents, that is, Minimum essential Eagle’s medium (MEM), fetal bovine serum, antibiotics (penicillin and streptomycin), and trypsin–EDTA solution were supplied by Invitrogen (Carlsbad, CA).

Cell Culture
The HepG2 cell line (American Type Culture Collection [ATCC] HB-8065) was obtained from Peking Union Medical College (Peking, China) and cultivated in MEM containing 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 mg/mL) at 37°C in a 5% CO2-humidified incubator.

Preparation of Chemical
CIT powder was dissolved in DMSO, and stored at -20°C as stock solutions at the concentration of 10 mM. The fresh dilutions of CIT at indicated concentrations were made in medium immediately before each experiment. The final concentration of DMSO in the culture medium was 0.1% (v/v). Thus, 0.1% DMSO was used as the control for comparison.

Single-Cell Gel Electrophoresis Assay
To detect cellular DNA damage, a single-cell gel electrophoresis (SCGE) assay was performed according to Zhang’s protocol (Zhang et al., 2011) with slight modification.
Aliquots of HepG2 cells (1 × 10^6 cells) were suspended in 2 mL medium and then incubated with CIT (0.00, 1.25, 2.50, 5.00, and 10.00 μM) at 37°C for 1 h. To avoid artifacts resulted from necrotic and apoptotic cells, Hoechst 33342 (8 mg/mL) and trypan blue (50 mg/mL) were used to detect the apoptotic cells and cell viability. Only cell suspensions with no apoptotic cells and cell viabilities >90% were further treated for the determination of DNA migration. The comet images were taken by fluorescent microscopy (Olympus BX-51, Omachi, Japan) with an excitation filter of 549 nm and barrier filter of 590 nm. Image analysis was performed using Comet Assay Software Project casp-1.2.2 (University of Wroclaw, Poland). Three independent experiments were performed by following the procedures described by Yarborough (1996). The images were recorded by a microscope (Olympus BX-51, Omachi, Japan). For each experimental point, three cultures were treated in parallel and from each culture, the intensity of the nuclear staining of 50 randomly chosen cells was subsequently quantified using a multiparameter image analysis program (Image-pro Plus 4.5.1). The staining data represented the average absorbance multiplied by 1000.

Measurement of Intracellular ROS

The intracellular level of ROS was measured using DCFH-DA method (Sohn et al., 2005) with slight modification. Briefly, HepG2 cells were suspended in 2 mL medium and then incubated with CIT (0.00, 1.25, 2.50, 5.00, and 10.00 μM) at 37°C for 1 h. Cells were washed twice with cold PBS, resuspended in PBS at 5 × 10^5 cells/mL, and incubated with DCFH-DA at a final concentration of 5 μM at 37°C for 40 min in the dark. To detect the effect of NAC in ROS formation, cells were pre-treated with or without NAC (0.00 and 2.50 mM) at 37°C for 1 h. After the pre-treatment, the cells were incubated with 5.00 μM CIT for 1 h at 37°C. The fluorescence intensity of the cell suspensions was detected using a fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan). Excitation and emission wavelengths were 485 and 550 nm, respectively.

Measurement of Intracellular Glutathione

Reduced Glutathione (GSH) was determined using a modified method of Hisin and Hilf (1976). HepG2 cells were treated with CIT (0.00, 1.25, 2.50, 5.00, and 10.00 μM) at 37°C for 1 h. After incubation, the medium was discarded and the cells were washed with PBS twice. Then 0.4 mL of 5% trichloroacetic acid (TCA) was added. After 30 min of incubation at 4°C to extract GSH, 50 μL of the extract was added to tubes containing 1.00 mg/mL OPT (50 μL) in 800 μL of 50 mM phosphate/5 μM EDTA buffer (pH 8.0). The tubes were incubated at 37°C in the dark for 15 min. Fluorescence was detected by fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan). The concentration of GSH was measured from a GSH standard. Excitation and emission wavelengths were 350 and 420 nm, respectively.

Mitochondrial Membrane Potential (Δψm) Assay

HepG2 cells were treated with CIT (0.00, 1.25, 2.50, 5.00, and 10.00 μM) for 30 min, and cells were then harvested and washed with PBS twice. The cell pellet was suspended in 2 mL of fresh incubating medium containing rhodamine 123 (1.00 μM) and was incubated in a thermostatic at 37°C for 10 min with gentle shaking. Hepatocytes were then separated by centrifugation, and the amount of rhodamine 123 remaining in the incubation medium was measured using a fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan). Excitation and emission wavelengths were 490 and 520 nm, respectively. Results were expressed as the fluorescence retained within the cells.

Lysosomal Membrane Stability Assay

Lysosomal membrane stability was measured with a modified method (Pourrahmad et al., 2001). After the treatment with CIT (0.00, 1.25, 2.50, 5.00, and 10.00 μM) for 1 h, the cells were harvested and washed twice with PBS and then incubated with AO at a final concentration of 5 μM at 37°C in the dark for 15 min. The cells were washed twice to remove the fluorescent dye from the media. The fluorescence intensity from cell suspensions was measured by a fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan). Excitation and emission wavelengths were 495 and 530 nm, respectively.

Statistical Analysis

Results are presented as mean ± S.D. Statistical analyses were performed with one-way ANOVA. The level of significance was set at P ≤ 0.05 and P ≤ 0.01 for all statistical analysis. Three independent experiments have
RESULTS

DNA Damage Induced by CIT

CIT increased the DNA migration in a dose-dependent manner at all tested concentrations. When the cells were treated with CIT at 2.50–10.00 μM concentrations, DNA migration was significantly increased compared with the control group (Table I). These results indicate that CIT can induce obvious DNA damage even at a low concentration.

Effect of CIT on 8-OHdG Formation

The immunocytochemical staining for 8-OHdG in HepG2 cells is shown in Figure 1. The nuclei of cells treated with CIT (1.25–10.00 μM) indicated strong positive staining for 8-OHdG, compared with the control group. This result suggests that CIT induces a significant oxidative DNA damage in HepG2 cells.

TABLE I. DNA strand breaks induced by CIT in HepG2 cells evaluated by SCGE assay

| CIT (μM) | Tail Length (μm) | Tail DNA (%) | Tail Moment (μm) |
|----------|------------------|--------------|------------------|
| 0.00     | 4.47 ± 1.50      | 2.51 ± 1.54  | 0.13 ± 0.12      |
| 1.25     | 4.32 ± 1.70      | 2.88 ± 2.03  | 0.17 ± 0.14      |
| 2.50     | 19.13 ± 8.11**   | 13.71 ± 6.74** | 2.96 ± 2.64*   |
| 5.00     | 44.67 ± 16.60*** | 34.46 ± 12.19** | 21.05 ± 11.16** |
| 10.00    | 76.47 ± 20.71*** | 55.62 ± 14.19** | 49.52 ± 17.21** |

The cells were treated with different concentrations of CIT (0.00–10.00 μM) for 1 h. Results are the mean ± S.D. of three independent experiments (*P ≤ 0.05 vs. control; **P ≤ 0.01 vs. control).

Increase in Intracellular ROS and Depletion of Intracellular GSH by CIT

The generation of ROS in HepG2 cells was determined by changes in the DCF fluorescence intensity [Fig. 2(A)]. The DCF fluorescence intensity significantly increased when the cells were treated with 2.50–10.00 μM CIT.

In addition, Figure 2(B) depicts a significant decrease of intracellular GSH which was observed in the cells treated with 2.50–10.00 μM CIT.

Effect of CIT on Δψm Collapse

Measurement of Δψm is critical for an integrated appraisal of mitochondrial function. Figure 3 suggests that CIT decreased Rhodamine 123 fluorescence intensity in HepG2 cells in a dose-dependent manner, compared with the control group. However, only at the dose of 5.00–10.00 μM, CIT induced significant decline in the Δψm in HepG2 cells.

![Fig. 1. 8-OHdG formation induced by CIT in HepG2 cells. Each bar represents mean ± S.D. of three independent experiments (**P ≤ 0.01 vs. control).](image1)

![Fig. 2. The role of oxidative stress in CIT-induced DNA damage. A: ROS formation measured by DCFH-DA in HepG2 cells incubated with or without CIT for 1h. B: Depletion of GSH induced by CIT in HepG2 cells. Each bar represents mean ± S.D. of three independent experiments (*P ≤ 0.05 vs. control; **P ≤ 0.01 vs. control).](image2)
These data indicate that exposure to CIT results in a depolarization of mitochondria and Δψm collapse.

**Effect of CIT on Lysosomal Membrane Stability**

To explore the mechanisms of CIT-induced DNA damage, the effect of CIT on the lysosomal membrane stability in HepG2 cells was evaluated. Figure 4 shows that AO fluorescence intensity increased significantly in all test groups, compared with the control group ($P < 0.01$ vs. control).

**The Protective Effect of NAC on DNA Damage and Intracellular ROS Induced by CIT**

To investigate the protective effects of NAC on CIT-induced DNA damage, DNA migration and intracellular ROS were evaluated after the treatment with 5.00 μM CIT in the presence or absence of 2.50 mM NAC. The results showed that NAC significantly decreased the DNA migration and ROS generation induced by CIT (Table II and Fig. 5).

**DISCUSSION**

In this study, an SCGE assay was conducted to identify the CIT-induced DNA damage in HepG2 cells. By detecting 8-OHdG formation, intercellular ROS and GSH levels, we tested the effects of oxidative stress on CIT-induced DNA damage. Depolarization of mitochondria and lysosomal protease leakage was also evaluated in HepG2 cells treated with CIT. We also demonstrate that NAC protected against the DNA damage induced by CIT.

It has been shown that CIT has cardiotoxic and neurotoxic effects, but very little information is available on its genotoxicity. SCGE assay, also known as comet assay, has been widely used to examine DNA damage (Lorenzo et al., 2013). The SCGE assay revealed that CIT (2.50–10.00 μM) caused a significant increase of DNA migration in HepG2 cells. Each bar represents mean ± S.D. of three independent experiments (*$P \leq 0.05$ vs. control; **$P \leq 0.01$ vs. control).
cells. This result indicates that CIT exerts genotoxicity in HepG2 cells even at a very low concentration.

8-OHdG is one of the most specific markers for oxidative DNA damage (Takeuchi et al., 1994). In this study, 8-OHdG formation was detected using immunocytochemistry staining to examine oxidative DNA damage in HepG2 cells. The results depicted that CIT at all the tested concentrations (1.25–10.00 μM) increased 8-OHdG formation in HepG2 cells. It is inferred that CIT induces a significant oxidative DNA damage in HepG2 cells.

The mechanisms through which CIT-induced DNA damage in HepG2 cells were also investigated in this study. Oxidative stress occurs when the production of ROS exceed their catabolism, and holds a major role in cell and organismal biology (Jiang et al., 2011). ROS include superoxide, hydrogen peroxide, singlet oxygen, ozone, hypohalous acids, and organic peroxides (Nathan and Ding, 2010). Many of those may have a very complex set of multiple, unrelated and opposite effects on cell death, inflammation, cancer, and aging. ROS are known as mediators of DNA damage through modulating the activities of key enzymes of DNA damage response (Caputo et al., 2012). ROS interact with other biological molecules and disrupt the normal synthesis and repair of DNA (Kryston et al., 2011). GSH functions as a major endogenous antioxidant produced by the cells, involved directly in the neutralization of free radicals and ROS. GSH protects most tissues and cell lines against injury from oxidants and reactive electrophiles (Shan et al., 1990). Our data showed that CIT (2.50–10.00 μM) significantly increased the levels of ROS and decreased the levels of GSH in HepG2 cells. These results suggest that oxidative stress plays an important role in the pathogenesis of CIT’s genotoxicity.

The mitochondrial electron transport chain is recognized as the major source of ROS generation. CIT acts as a potent inhibitor of mitochondrial ATPase that provides energy for the electron transport chain through catalyzing the decomposition of ATP into ADP. Inhibition of electron transport will lead to increased electron leakage and more ROS production (Musatov and Robinson, 2012). Although the mechanisms responsible for the ROS generation by the electron transport chain are still not well known, ΔΨm-dependent mechanisms appear to play an important role in at least part of production of ROS (Votyakova and Reynolds, 2001). In this study, the state of the ΔΨm after application of CIT was examined by measuring the relative differences in fluorescence of the rhodamine 123 between control and CIT-treated HepG2 cells. After exposure to CIT for 30 min, a dose-dependent decline in the ΔΨm was observed. These results indicate that mitochondrial depolarization may act as a prerequisite for CIT-induced DNA damage by mediating the generation of ROS in HepG2 cells.

It has been shown that lysosomal membranes’ permeability can be provoked by overproduction of ROS after mitochondrial dysfunction, leading to lysosomal protease leakage (Huai et al., 2013), and that lysosomal rupture may be partly dependent on mitochondrial disruption (Paquet et al., 2005). Lysosome contains numerous acid hydrolase enzymes that remove cellular debris. And lysosomal protease leakage has been linked to apoptosis and oxidative stress (Ogawa et al., 2004). Our results depicted that CIT at all the tested concentrations (1.25–10.00 μM) increased lysosomal membranes’ permeability, indicating that lysosomal protease leakage may participate in CIT-induced DNA damage in HepG2 cells.

NAC prevents oxidative stress and diseases as a scavenger directly reacting with oxidative metabolites, or as a precursor of cysteine for GSH synthesis indirectly clearing ROS (Fishbane et al., 2004; Wu et al., 2004). In this study, we observed that the presence of NAC blocked ROS production and DNA damage induced by CIT in HepG2 cells. These results further verify that oxidative stress plays a role in CIT-induced DNA damage, and provide an insight that NAC may act as an agent to reduce the genotoxicity of CIT.

In conclusion, our results demonstrate that CIT induces DNA damage in HepG2 cells, probably through oxidative stress mechanisms; that NAC protects against DNA damage induced by CIT in HepG2 cells; and that depolarization of mitochondria and lysosomal protease leakage may play a role in CIT-induced DNA damage in HepG2 cells.

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