Synergistic hepatotoxicity by cadmium and chlorpyrifos: Disordered hepatic lipid homeostasis

WEI HE[1,2], WENLI GUO[2], YI QIAN[2], SHUPING ZHANG[2], DIFENG REN[1] and SIJIN LIU[2]

[1]Beijing Key Laboratory of Forestry Food Processing and Safety, College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083; [2]State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, P.R. China

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Abstract. Due to its extensive application, chlorpyrifos (CPF) has contaminated a diverse range of environmental substrates, fruits and vegetables. A number of studies have suggested that CPF may incur adverse effects on human health, including neurotoxicity, hepatotoxicity and endocrine disruption. Additionally, cadmium (Cd) is one of the most prevalent environmental heavy metals, as a result of considerable use in a wide spectrum of industrial fields. Exposure to Cd can cause several lesions in various organs, including the liver, kidneys and lungs. CPF and Cd often co-exist in the environment, food and crops, however, their joint exposure and potential synergistic toxicity are largely neglected and unrecognized. Our previous study characterized an interaction between CPF and Cd, which may occur via bonding between Cd^{2+} and the nitrogen atom in the pyridine ring of CPF, or the chelation between one Cd^{2+} and two CPF molecules. Our previous study also identified increased hepatotoxicity induced by CPF and Cd together compared with the individual compounds. In the present study, the effects of the concomitant exposure of CPF and Cd on lipid metabolism in hepatocytes was investigated. The results demonstrated an accumulation of lipids in hepatocytes, induced by the CPF and Cd complex, which was fundamentally distinct from its parental chemicals. Notably, the molecular mechanism by which the CPF-Cd complex significantly induced hepatic lipogenesis was revealed, elevating the concentrations of sterol regulatory element-binding protein-1 and fatty acid synthase. These findings pave the way for future studies in recognizing synergistic biological effects between pollutants.

Correspondence to: Professor Difeng Ren, Beijing Key Laboratory of Forestry Food Processing and Safety, College of Biological Sciences and Biotechnology, Beijing Forestry University, 35 Qinghua East Road, Haidian, Beijing 100083, P.R. China
E-mail: rendifeng@bjfu.edu.cn

Professor Sijin Liu, State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Road, Haidian, Beijing 100085, P.R. China
E-mail: sjliu@rcees.ac.cn

Abbreviations: CPF, chlorpyrifos; Cd, cadmium; OPPs, organophosphorus pesticides; FASN, fatty acid synthase; SREBP, sterol regulatory element-binding protein; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; ACC, acetyl-coenzyme A carboxylase; CoA, coenzyme A

Key words: chlorpyrifos, cadmium, synergistic toxicity, lipid metabolism

Introduction

Organophosphorus pesticides have been widely used in agricultural environments, since the ban of organochlorine insecticides, to protect crops against a range of pests due to their broad spectrum of insecticidal activity, effectiveness and their non-persistence in the environment (1). Despite the benefits of pesticides, residues may remain in crops, animal feed and environmental substrates, leading to contamination and potential human exposure through the food chain (2-4). Chlorpyrifos (CPF) is a major type of organophosphorus pesticide and is currently used in the production of >40 crops and fruits, including peaches, citrus fruits, almonds and grapes (1). CPF and its metabolites have been detected in farm animals, including cattle and sheep (1,5), and CPF residues or metabolites have also been detected in food and human urine (6). In addition, CPF has been found in the umbilical cord blood from female individuals living in urban environments (7). CPF has been confirmed as a neurotoxin by inhibiting acetylcholinesterase in the central nervous system (8). This can damage the nervous system, resulting in dizziness, nausea, confusion and, at a high concentration, respiratory paralysis and mortality. Additionally, a previous study demonstrated that CPF incurs developmental toxicity in rats at concentrations of 25 mg/kg/day, which was a maternally toxic dose (9). Similar to the majority of environmental pollutants, CPF elicits significant cytotoxicity through generating oxidative stress (10-12).

Cadmium (Cd) is one of the most prevalent environmental heavy metals, and human exposure to Cd is associated with a number of toxicities, including hepatotoxicity, DNA damage and cell apoptosis (13-15). Notably, CPF and Cd often coexist in the same environmental media and food chains, causing simultaneous exposure to organisms (16,17) and resulting in

common toxicities, including carcinogenicity and hepatotoxicity (18-20). Our previous study characterized an interaction between Cd and CPF, and this interaction likely occurs due to bonding between Cd and nitrogen atoms in the pyridine ring of CPF, or the chelation between one Cd^{2+} and two CPF molecules (21). The joint hepatotoxicity of Cd and CPF to HepG2 cells was also demonstrated, with the Cd-CPF complex increasing the level of apoptosis by compared with its parental components (21).

Hepatic lipid metabolism is important in governing the whole body energy metabolism, as the liver is the major site for the storage and release of glucose and lipids (22,23). Lipid accumulation within liver has been suggested to cause obesity, insulin resistance and type II diabetes (22-24), and also predisposes individuals to nutritional stresses. Increasing evidence suggests that fatty acid synthase (FASN) is a critical regulator of hepatic lipid homeostasis, including fat and cholesterol synthesis (25,26). FASN encodes one of the key enzymes involved in fatty acid synthesis and is required for the *de novo* synthesis of fatty acids (27). Upregulated expression levels of FASN have been reported in various types of human cancer, and has been suggested to contribute to poor prognosis and recurrence of these types of cancer (28). Sterol regulatory element-binding protein (SREBP) is the predominant transcriptional activator of FASN (29,30), and previous studies have revealed that the increased expression of SREBP-1 is significantly associated with the extent of fatty liver in mouse models of diabetes mellitus (31-33). The present study aimed to investigate the synergistic effect of Cd and CPF on fat metabolism and fat accumulation in hepatocytes.

Materials and methods

Chemicals and reagents. CPF was purchased from Shuangma Fine Chemical Co., Ltd. (Nantong, China) and was dissolved in dimethyl sulfoxide (DMSO) (Solarbio Science & Technology Co., Ltd., Beijing, China). The final concentration of DMSO was <0.1% in the culture medium. $CdCl_2$ was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sterile water was used to dissolve the $CdCl_2$, and the stock solution was filtered through a 0.45 mm membrane (Solarbio Science & Technology Co., Ltd.).

Cell culture. The HepG2 human hepatic carcinoma cell line was purchased from Shanghai Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The HepG2 cells were cultured at a concentration of $5.0x10^3$ cells/well in RPMI-1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT. USA) and 100 U/ml penicillin-streptomycin (HyClone) at 37˚C in an atmosphere of 5% CO_2. The cell culture medium was changed every day and the cells were passed every other day.

Cytotoxicity assessment. The cytotoxicity was initially screened using an MTT assay (Invitrogen Life Technologies, Carlsbad, CA, USA). Briefly, the cells were inoculated and cultured overnight at 37˚C in 96-well plates (Corning, Inc., New York, NY, USA) at a density of $8.0x10^3$ cells/well in RPMI-1640 medium supplemented with penicillin, streptomycin (100 mM) and 1% FBS. Following culture, the HepG2 cells were treated with various concentrations of CPF (10, 50, 100, 500, 1,000, 1,500, 2,000 or 2,500 μM) or Cd (5, 10, 20, 40, 60, 80 or 100 μM) for a further 24 h at 37˚C, and 20 μl MTT (5 mg/ml) was then added to each well. Following an additional 4 h incubation at 37˚C, 100 μl DMSO was added to each well, followed by absorption assessment at 490 nm on a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Co., Ltd., Waltham, MA, USA). The synergistic toxicity was further determined using an Alamar Blue assay (Invitrogen Life Technologies) and a bromodeoxyuridine (BrdU) assay (Roche Diagnostics, Mannheim, Germany). Similar to the MTT assay, following treatment with 10 μM CPF or Cd, 10 μl Alamar Blue reagent (Invitrogen Life Technologies) was added to each well and incubated for 2 h at 37˚C, prior to the plates being read on a microplate reader with an emission wavelength of 590 nm and an excitation wavelength of 540 nm. The BrdU assay was performed according to the manufacturer's instructions, as described previously (34).

High glucose exposure. The cells were exposed to high glucose, as described previously (35). Briefly, the HepG2 cells were seeded into 6-well plates in RPMI-1640 medium supplemented with 10% FBS. The cells were grown to 70% confluence, prior to being maintained in serum-free RPMI-1640 medium, containing high concentrations of glucose (30 mmol/l) overnight. The cells were subsequently treated with Cd and CPF, as described above.

Determination of total cholesterol and triglyceride levels. The intracellular concentrations of total cholesterol and triglycerides were assayed in the HepG2 cell lysates, according to the manufacturer's instructions of the Triglycerides Assay and Total Cholesterol Assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The cell pellet was collected and 0.3 ml Triton-X 100 (1-2%) was added. It was lysed for 30 min prior to the start of the assays according to the manufacturer's instructions. The mixture was incubated at 37˚C for 5 min, followed by reading with the microplate reader at 590 nm.

Western blotting. Following the treatment with Cd and CPF, described above, the HepG2 cells were collected and washed twice with phosphate-buffered saline (Solarbio Science & Technology Co., Ltd.). The harvested cells were lysed in radioimmunoprecipitation lysis buffer supplemented with protease inhibitor cocktail (Roche Diagnostics) on ice for 30 min. The cells were subsequently centrifuged at 12,000 x g for 10 min. The supernatants were collected and were subjected to 10% SDS-PAGE (gel, Beijing ComWin Biotech Co., Ltd., Beijing, China; SDS buffer, Solarbio Science & Technology Co., Ltd.) and were transferred onto nitrocellulose membranes (Solarbio Science & Technology Co., Ltd.). The membranes were incubated with primary antibodies against SREBP-1 (1:200; bs-1402R), FASN (1:200; bs-1498R-PE) and β-actin (1:1,000; bs-0061R) (Bioss, Beijing, China) in 5% milk overnight at 4˚C. They were then washed 3 times in Tris-buffered saline with Tween-20 (Solarbio Science & Technology Co., Ltd.) for 5 min, and then incubated with anti-rabbit-horseradish peroxidase secondary antibodies (1:8,000; ComWin Biotech Co., Ltd.) for 1 h at 37˚C in 5% milk, then washed again in a similar manner. The target proteins were colored by a chemical

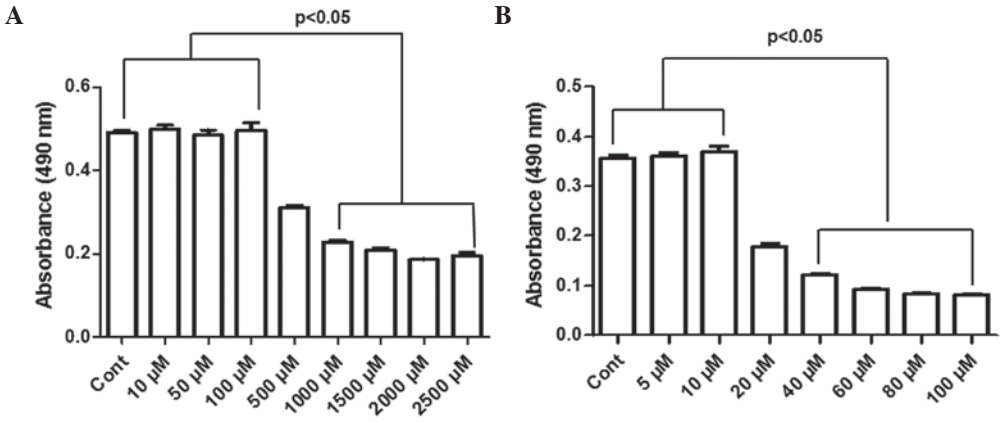

Figure 1. Cytotoxicity assay of HepG2 cells treated with various concentrations of CPF or Cd^{2+}. The cytotoxicity was assessed by measuring absorbance following an an MTT assay of the HepG2 cells 24 h after treatment with 10-2,500 μM (A) CPF and (B) Cd^{2+} (n=5). CFP, chlorpyrifos; Cd, cadmium; Cont, untreated control.

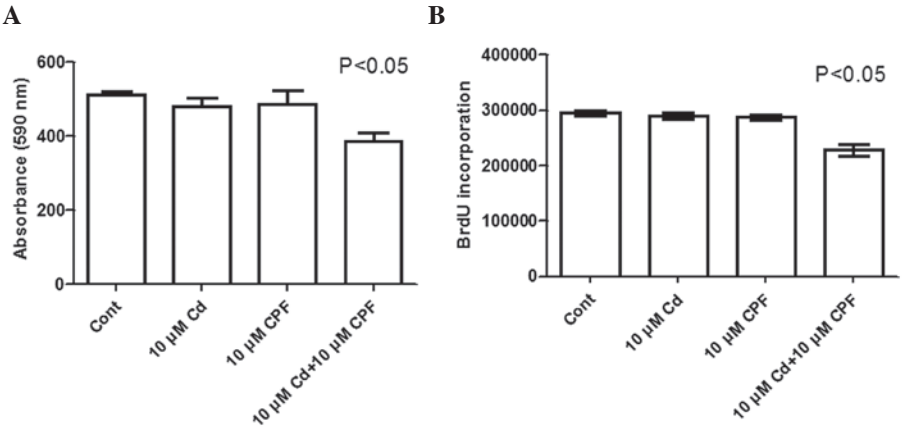

Figure 2. Cell viability following concomitant exposure of CPF and Cd^{2+} in HepG2 cells. (A) Alamar Blue and (B) BrdU assays were performed on HepG2 cells treated with CPF and Cd^{2+} either alone (10 μM each) or concomitantly for 24 h (n=4-5). CPF, chlorpyrifos; Cd, cadmium; BrdU, bromodeoxyuridine; Cont, untreated control.

method. The intensity of the protein bands were assessed using Image J software, version 1.48 (NIH, Bethesda, MD, USA).

Statistical analysis. Using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) Student's two-tailed t-test was performed to analyze the experimental data between two groups, and one-way analysis of variance was used to analyze the mean differences between groups relative to the control. The data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

The toxicity of combinations of environmental pollutants is important when determining the health risks of each individually. Cd and CPF have been demonstrated to have common targets and elicit similar phenotypes, including hepatotoxicity (11,36-38). Our previous study revealed a novel interaction between Cd ions and CPF, and described the cytotoxicity induced by the Cd-CPF complex (21). The present study hypothesized that the synergistic cytotoxicity between CPF and Cd^{2+} was likely to cause metabolic disorders in hepatocytes. Initially, a series of experiments were performed to confirm the synergistic toxicity, as previously discussed (21). As shown in Fig. 1A, the MTT assay revealed that CPF induced significant toxicity to the HepG2 cells following 24 h treatment with 0.5, 1, 1.5, 2 and 2.5 mM, in a dose-dependent manner, compared with the control (P<0.05). The half maximal inhibitory concentration (IC_{50}) was ~0.5 mM in the HepG2 cells (Fig. 1A). No cytotoxicity was observed in the HepG2 cells at CPF concentrations ≤100 μM (P>0.05; Fig. 1A). In addition, the cytotoxicity of Cd was determined in HepG2 cells treated with 5, 10, 20, 40, 60, 80 and 100 μM $CdCl_2$. Following treatment for 24 h, no toxicity was observed ≤10 μM (P>0.05; Fig. 1B), however, $CdCl_2$ induced significant cell death at Cd concentrations >10 μM (P<0.05). The IC_{50} value for Cd^{2+} was ~20 μM (Fig. 1B).

Previous studies have suggested that the formation of a complex between chemicals alters their transport across the cell membrane and increases intracellular localization, resulting in enforced cytotoxicity, which may not occur with individual chemicals (39-41). A classical interaction of CPF was identified with methyl mercury, as the formation of this complex significantly increased the bioaccumulation of methyl mercury, coupled with increased toxicity (42). Our previous study demonstrated

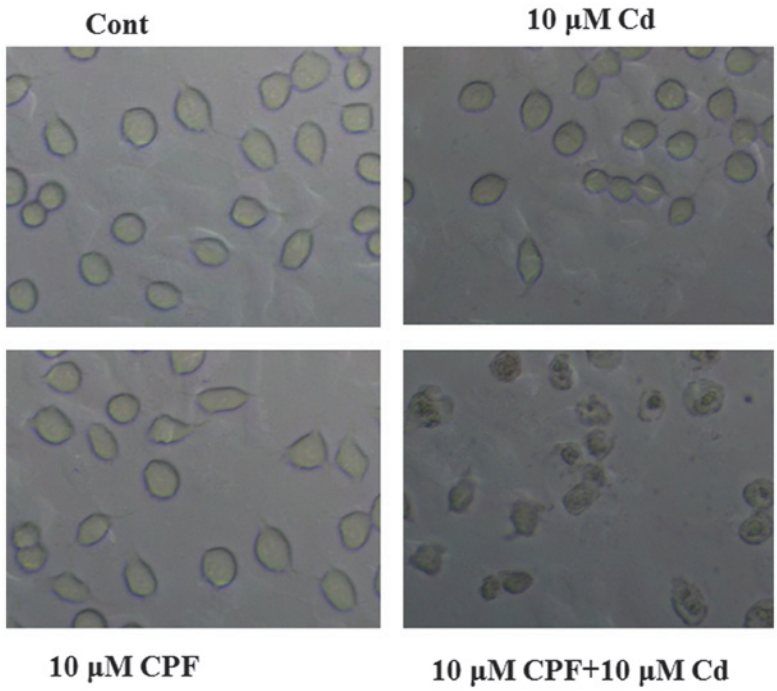

Figure 3. Morphological changes of the HepG2 cells following concomitant exposure of CPF and Cd^{2+}. The phase-contrast images demonstrated morphological changes of the HepG2 cells following treatment with CPF and Cd^{2+} either alone or concomitantly (magnification, x200). CPF, chlorpyrifos; Cd, cadmium; Cont, untreated control.

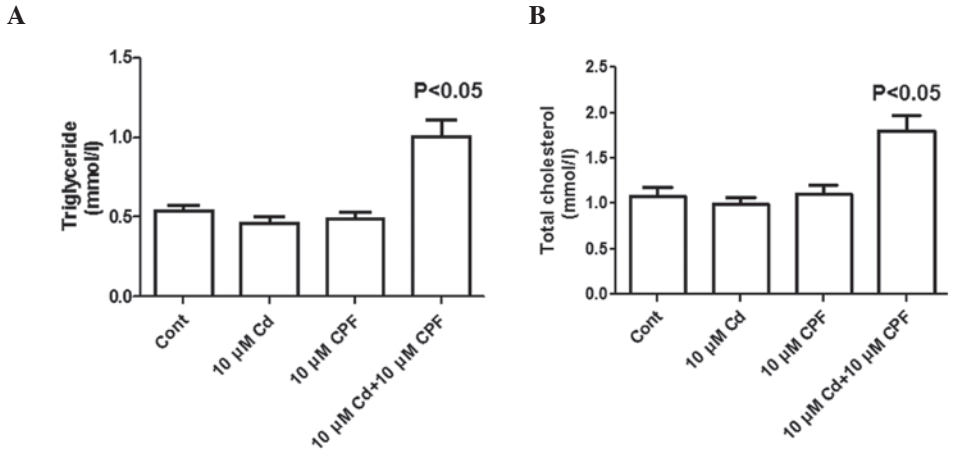

Figure 4. Lipid alterations due to the synergistic toxicity of CPF and Cd^{2+}. The levels of intracellular (A) triglycerides and (B) total cholesterol in the HepG2 cells treated for 24 h with CPF and Cd^{2+} (10 μM each) either alone or concomitantly (n=4-5). CPF, chlorpyrifos; Cd, cadmium; Cont, untreated control.

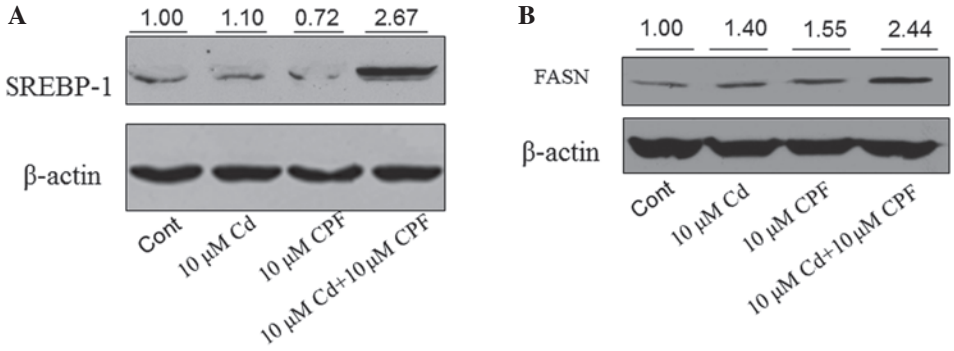

Figure 5. Western blot analysis of the proteins responsible for lipid metabolism in HepG2 cells treated with CPF and Cd^{2+}. The cells were exposed to 10 μM CPF and/or Cd^{2+} for 24 h and were subsequently collected and subjected to western blotting to determine the expression levels of (A) SREBP-1 and (B) FASN. β-actin was used as a loading control. CPF, chlorpyrifos; Cd, cadmium; SERBP, sterol regulatory element-binding protein; FASN, fatty acid synthase; Cont, untreated control.

that the combined toxicity of Cd and CPF was attributable to the Cd-CPF complex-facilitated intracellular transport (21). In the present study the combined toxicity of CPF and Cd was further confirmed using Alamar Blue and BrdU assays, and by selecting non-toxic concentrations (10 μM each) of CPF and Cd. As shown in Fig. 2A, cell viability was significantly reduced by 21% upon concomitant exposure of Cd^{2+} and CPF ($P<0.05$) compared with the control or following individual treatment with either Cd^{2+} or CPF, as demonstrated using an Alamar Blue assay. Similarly, in the BrdU incorporation assay, the concomitant exposure of Cd^{2+} and CPF markedly inhibited cell proliferation by 23%, relative to the control or following individual treatment with Cd^{2+} or CPF ($P<0.05$; Fig. 2B). In addition, a marked morphological change, of smaller and rounder cells, was observed in the cells simultaneously treated with CPF and Cd compared with the cells in the control and individual treatment groups (Fig. 3). Consistent with the cytotoxicity results, as discussed above, these morphological changes were indicative of cell death (Fig. 3). Taken together, these results revealed a significant synergistic cytotoxic effect of Cd^{2+} and CPF on the HepG2 cells, which was distinct from the effects of the individual chemicals.

To investigate the potential disturbance to lipid metabolism by the synergistic exposure of CPF and Cd, the present study investigated the lipid concentrations in HepG2 cells post-treatment. To improve characterization of lipid metabolism, this was performed using a cell model treated with high glucose, as previously described (35). As shown in Fig. 4A, the concentration of triglycerides increased ~2-fold in cells exposed to simultaneous treatment of CPF and Cd compared with the untreated cells or single compound-treated cells. Similar to the changes in triglyceride levels, the concomitant exposure to CPF and Cd^{2+} (10 μM each) significantly increased the total cholesterol concentration by 80% ($P<0.05$) compared with the untreated cells or those treated with individual components ($P<0.05$; Fig. 4B). These results collectively suggested that the synergistic effect of CPF and Cd markedly altered hepatic lipid metabolism, associated with cholesterol and triglyceride accumulation, in the hepatocytes.

The present study subsequently aimed to investigate the molecular mechanism underlying CPF/Cd-mediated disorders in lipid metabolism. SREBP is a critical regulator of hepatic lipid metabolism, including glucose transport, gluconeogenesis and lipolysis (43). The SREBP family consists of SREBP-1a, SREBP-1c and SREBP-2 (44), and these members are essential for regulating the expression of lipogenic enzymes, including acetyl-coenzyme A (CoA) carboxylase and FASN (45,46). SREBP-1 is an important transcription factor, which stimulates lipogenic enzymes involved in liver fatty-acid synthesis, whereas SREBP-2 is relatively specific to the regulation of genes responsible for cholesterol synthesis and uptake, including low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl CoA reductase (44). The lipid accumulation, induced by exposure to CPF/Cd, may reside in the dysfunction of SREBP-1. The concentration of SREBP-1 was assessed in HepG2 cells following various treatments. Western blot analysis revealed that the expression of SREBP-1 was markedly increased by >2-fold in the cells following combined treatment with CPF and Cd compared with the untreated cells or those treated with CPF or Cd alone (Fig. 5A). The expression of FASN was also markedly induced following treatment with CPF/Cd (>2-fold) compared with the untreated cells or those treated with the individual chemicals (Fig. 5B). These results demonstrated that the concomitant exposure of CPF and Cd induced hepatic fat accumulation through an increase in hepatic lipogenesis by increasing the expression levels of SREBP-1 and FASN.

Organophosphorus pesticides and heavy metals are amongst the most serious environmental pollutants. CPF, as a broad class of organophosphorus pesticides, is widely used throughout the world for agricultural and non-agricultural purposes (1-4,13). Cd is a toxic metal, commonly found in industrial workplaces and diverse environmental substrates, which contaminates food chains, including crops and fruits (47). Cd is toxic to various cell types, even at low concentrations, and has a long biological half-life in humans (10-30 years) (13,21). However, CPF and Cd are often detected in identical environmental substrates and food chains, and they elicit similar damage to organisms, including hepatotoxicity (16,17). Our previous study demonstrated the synergistic effect of CPF and Cd on reducing the viability of HepG2 cells (21). The present study demonstrated a novel HepG2 cell phenotype, caused by the CPF/Cd complex, with significant cholesterol and triglyceride accumulation in the hepatocytes. The molecular mechanism underlying hepatic lipogenesis elicited by joint exposure of CPF+Cd was found to occur through inducing the expression levels of SREBP-1 and FASN. Future investigations are required to determine the detailed mechanisms responsible for CPF/Cd-mediated action in lipogenesis.

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