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Toxicological biomarkers of 2,3,4,7,8-pentachlorodibenzofuran in proteins secreted by HepG2 cells

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ABSTRACT

Using a proteomic approach, a study was conducted for determination of the effects of 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PCDF) on proteins secreted by HepG2 cells. Briefly, HepG2 cells were exposed to various concentrations of 2,3,4,7,8-PCDF for 24 or 48 h. MTT and comet assays were then conducted for determination of cytotoxicity and genotoxicity, respectively. Results of an MTT assay showed that 1 nM of 2,3,4,7,8-PCDF was the maximum concentration that did not cause cell death. In addition, a dose- and time dependent increase of DNA damage was observed in HepG2 cells exposed to 2,3,4,7,8-PCDF. Therefore, two different concentrations of 2,3,4,7,8-PCDF, 1 and 5 nM, were selected for further analysis of proteomic biomarkers using two different pI ranges (4–7 and 6–9) and large two dimensional gel electrophoresis. Results showed identification of 32 proteins (29 up- and 3 down-regulated) by nano-LC-ESI-MS/MS and nano-ESI on a Q-TOF2 MS. Among these, the identities of pyridoxine-5'-phosphate oxidase, UDP-glucose 6-dehydrogenase, plasminogen activator inhibitor I precursor, plasminogen activator inhibitor-3, proteasome activator complex subunit 1, isoform 1 of 14-3-3 protein sigma, peptidyl-prolyl cis-trans isomerase A, 14-3-3 protein gamma, protein DJ-1, and nucleoside diphosphate kinase A were confirmed by western blot analysis. The differential expression of protein DJ-1, proteasome activator complex subunit 1 and plasminogen activator inhibitor-3 was further validated in plasma proteins from rats exposed to 2,3,4,7,8-PCDF. These proteins could be used as potential toxicological biomarkers of 2,3,4,7,8-PCDF.

1. Introduction

Dioxins are generally formed as by-products of chlorine-containing manufacturing processes or from incineration. Exposure to dioxins in humans occurs primarily through foods, and, as a result of their environmental stability and lipophilicity, these compounds persist in biological systems for a long period of time [1,2]. In addition, dioxins have cytotoxic, hematotoxic, immunotoxic, and genotoxic properties, and findings from long term toxicological studies have demonstrated their carcinogenicity in mice and rats [3].

Dioxin-like compounds can be divided into three groups; polychlorinated dibenzo-p-dioxins, 2,3,4,7,8-pentachlorodibenzofuran (PCDFs), and polychlorinated biphenyls [2,4]. Among these, PCDFs are found in high concentrations in fly ash from municipal incinerators and industrial heating facilities [5,6]. In addition, PCDFs have been focused on as environmental toxins because of the "Yusho" accident, which occurred as a result of contamination of rice brain oil by PCDFs [7,8]. 2,3,4,7,8-PCDF, which is the most abundant molecule in PCDFs [9], together with 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin was found to account for about 60% of the toxic equivalency quantity value which is based on congener-specific toxic equivalency factors relative to the most toxic dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin, in a human study concerning dioxin intake and concentration [10]. In

Abbreviations: 2,3,4,7,8-PCDF, 2,3,4,7,8-pentachlorodibenzofuran; MTT, 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; LC-ESI-MS/MS, liquid chromatography electrospray ionization-tandem mass spectrometry; TOF2 MS, time-of-flight 2 mass spectrometry; PCDFs, polychlorinated dibenzofurans; NMA, normal point melting agarose; LMA, low point melting agarose; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2-DE, two dimensional gel electrophoresis; PNPO, pyridoxine-5′-phosphate oxidase; UDP-GlcDH, UDP-glucose 6-dehydrogenase; PAI-1, plasminogen activator inhibitor I precursor; PAI-3, plasminogen activator inhibitor-3 precursor; PA28α, proteasome activator complex subunit 1; 14-3-3 σ, isoform 1 of 14-3-3 protein sigma; PPIase A, peptidyl-prolyl cis-trans isomerase A; 14-3-3 υ, 14-3-3 protein gamma; DJ-1, protein DJ-1; NDK A, nucleoside diphosphate kinase A.

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addition, the concentrations of atmospheric polychlorodibenzo-*p*-dioxin (PCDD)/PCDFs have been widely measured globally [11,12]. With respect to TEQ congener profiles, the TEQ value of PCDFs was higher than that of PCDDs. Especially, congener 2,3,4,7,8-PCDF was the single dominant contributor to the TEQPCDD/Fs accounting for an average contributuion of 53% to the toxic equivalents of PCDD/Fs. This result is also consistent with the world average ambient air [12]. Therefore, studies of changes in protein secretion in *in vitro* and *in vivo* systems by 2,3,4,7,8-PCDF could provide valuable information regarding 2,3,4,7,8-PCDF-induced toxicity.

Proteomic analysis is a method employed for analyzing of differential gene expression at the protein level and identification of biomarkers by comparison of patterns of proteomes under different conditions after exposure to toxicological compounds [13-17]. Specifically, proteomic studies conducted to evaluate the proteins secreted from cells and tissues have been used to search for biomarkers because they are good targets for therapeutic intervention as well as a tool for diagnosis and prognosis of diseases such as cancers [18].

In the present study, we evaluated the toxicological biomarkers of 2,3,4,7,8-PCDF in proteins secreted by HepG2 human hepatoma cells, which display cellular morphology similar to that of liver parenchymal cells and which may play a role in the synthesis of major plasma proteins, receptors for insulin, transferrin, epidermal growth factor, low density lipoprotein and asialoglycoprotein [19,20]. Moreover, HepG2 cells retained the activities of various phase I and phase II enzymes which play a crucial role in the activation/detoxification of genotoxic procarcinogens [21]. Although CYP2E1 is expressed at a low level in HepG2 cells compared with HepaRG and human primary hepatocytes, expression of CYP1A2 and CYP3A4 are comparable between both hepatoma cell lines [22,23]. For these reasons, HepG2 is still the most frequently used liver-based in vitro model for toxicogenomics and high-throughput toxicity screening studies [22]. Recently, a number of studies showed that dioxins induced cytochrome P450 in HepG2 cells [24–29]. Specifically, two studies reported that CYP1A1 protein was significantly up-regulated by 1 nM of 2,3,7,8-tetrachlorodibenzo-p-dioxin [25,27].

For proteomic analysis, two concentrations of 2,3,4,7,8-PCDF that did not cause cytotoxicity, but induced genotoxicity in HepG2 cells were selected (1 and 5 nM based on cytoxicity and genotoxicity assays) and toxicological biomarkers were determined using two different pI ranges (4–7 and 6–9) and large size two dimensional gel electrophoresis. Identified biomarkers were confirmed by western blot analysis. In addition, the 2,3,4,7,8-PCDF biomarkers found in HepG2 cells were evaluated in plasma obtained from rats exposed to 2,3,4,7,8-PCDF to validate their usefulness as toxicological biomarkers *in vivo*.

2. Materials and methods

2.1. Chemicals

MTT assay chemicals, urea, thiourea, CHAPS, DTT, acrylamide, NN'-methylene-bisacrylamide, iodoacetamide, and sodium thiosulfate were purchased from Sigma Chemical (St Louis, MO). HPLC grade solvents including acetonitrile, acetic acid, and methanol were purchased from Merck (Merck Co. Damstadt, Germany). 2,3,4,7,8-PCDF was purchased from Cerilliant CIL, Inc (Austin, TX, USA).

2.2. HepG2 cell culture

HepG2 cells obtained from the American Type Culture Collection (ATCC-HB 8065) were maintained in DMEM containing 10% fetal bovine serum (Gibco BRL, Grand Island, USA), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C under 5% CO_2. MTT and Comet assays were performed using HepG2 cells cultured in DMEM without fetal bovine serum for a period of either 24 or 48 h with 1, 2.5, 5, 10, 25 or 50 nM of 2,3,4,7,8-PCDF. Final concentration of DMSO in the media did not exceed 0.2%.

2.3. MTT assay

Following incubation of HepG2 cells (1×10^4) with different concentrations of 2,3,4,7,8-PCDF in 96 well plates, the MTT assay (Sigma Chemical) was performed for determination of cell viability. Briefly, different concentrations of 2,3,4,7,8-PCDF were applied to HepG2 cells. After 24 or 48 h, 20 μl of 5 mg/ml MTT in PBS was added to each well, followed by incubation of the sample for an additional 3 h at 37 °C. After removal of the media, formazan crystals in cells were dissolved in 100 μl of lysis buffer (10% w/v of SDS in 0.01 N HCl). Absorbance was then read at 570 nm using an ELISA reader (Molecular Devices Co., Sunnyvale, CA, USA). Comparison of optical densities of cells exposed to different concentrations of toxicants with those of controls was performed for determination of the percentage of cell viability.

2.4. Comet assay

NMA and LMA were dissolved in PBS (Gibco, BRL) by heating in a microwave. 100 μl of 1% NMA was then added to frosted slides precoated with 50 μl 1% NMA to ensure firm attachment, and the slides were allowed to solidify with cover slips in the refrigerator for 5 min. Once the gel had solidified, the cover slips were removed, followed by addition of 50 μl of lymphocytes mixed with 50 μl of 1% LMA. Following addition of cover slips to the layer, the slides were placed in the refrigerator for an additional 5 min and allowed to solidify. Following removal of cover slips, and addition of 100 μl of 0.5% LMA to the third layer, slides with cover slips were refrigerated again for 5 min. The slides were then submerged in the lysing buffer solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris–HCl, pH 10; 1% Triton X-100 and 10% DMSO, pH 10 were added fresh) for a period of 1 h and were then placed in unwinding buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 20 min. The same solution was used in performance of electrophoresis for 20 min at 25 V and 300 mA (0.8 V/cm). After electrophoresis, for neutralization, slides were washed three times with neutralization buffer (400 mM Tris–HCl, pH 7.4) for 5 min each, followed by staining with 50 μl of 10 μg/ml ethidium bromide. A Komet 4.0 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm was used for examination of slides. For each treatment group, two slides were prepared and each 50 randomly chosen cells (total 100 cells) were scored manually. The Komet 4.0 image analysis system was used for automatic calcuration of the parameter, Olive tailmoment [=(Tail.mean-Head.mean)*Tail%DNA/100], which was used for global comet description.

2.5. Animal experiment

Specific pathogen free male, Sprague–Dawley rats used in the study were obtained from the Samtaco Animal Breeding Company (Osan, Korea), and housed under standard laboratory conditions (Tm; 24 ± 2 °C, humidity; 50 ± 10% and 12-hour day/night cycles). Following 1–2 weeks period of acclimatization to the facility, and the animals were then observed for abnormal behavior. They were provided *ad libitum* access to a diet of Samtaco Animal chow (PMI Nutritional Int. LLC, MO) and drinking water. Animals were 6–8 weeks old on the first day of exposure. 2,3,4,7,8-PCDF was administered by gavage at a single oral dose of 25 or 50 ug/kg body weight in corn oil. The control group received corn oil only. Seven rats were exposed (a total: 21 rats) and all animals were sacrificed after 4 weeks as described previously [30]. Cardiac