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The roles of ER stress and P450 2E1 in CCl_4-induced steatosis

Geum-Hwa Lee[a], Bidur Bhandary[a], Eun-Mi Lee[b], Jin-Kyu Park[b], Kyu-Shik Jeong[b], In-Ki Kim[c], Hyung-Ryong Kim[d,*], Han-Jung Chae[a,**]

[a] Department of Pharmacology and Institute of Cardiovascular Research, School of Medicine, Chonbuk National University, Jeonju, Chonbuk, Republic of Korea
[b] College of Veterinary Medicine, Kyungpook National University, Daegu, Republic of Korea
[c] ASAN Institute for Life Science, Seoul, Republic of Korea
[d] Department of Dental Pharmacology and Wonkwang Dental Research Institute, School of Dentistry, Wonkwang University, Iksan, Chonbuk, Republic of Korea

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ABSTRACT

The role of ER stress on hepatic steatosis was investigated in a rat model. We injected CCl_4 into rats and found that CCl_4 could induce hepatic lipid accumulation, confirmed by Oil Red O staining and by measurement of triglyceride and cholesterol. The expression of ApoB, an apolipoprotein, was decreased in plasma and increased in the liver of CCl_4-treated animals. The ER stress response was also significantly increased by CCl_4. P450 2E1 expression and activity were increased through interactions of P450 2E1 with NADPH-dependent P450 reductase (NPR) under CCl_4-treated conditions. In HepG2 cells, intracellular lipid accumulation and its signaling were comparable to *in vivo* results. In order to elucidate the effect of the ER stress response itself, tunicamycin, an N-acetyl-glycosylation inhibitor, was injected into rats, followed by Oil Red O staining, lipid/triglyceride/cholesterol accumulation analysis, and examination of ApoB expression. Additionally, the ER stress response and upregulation of P450 2E1 were also confirmed in the tunicamycin-treated rats. All of the responses were similar to those seen with CCl_4. The P450 2E1 inhibitor diallyl sulphide (DAS), N-acetylcysteine (NAC), and reduced glutathione (GSH) antioxidants also regulated processes, including ApoB expression and lipid accumulation in CCl_4-treated animals. In the presence of tunicamycin, DAS or NAC/GSH regulated all of the pathological phenomena with the exception of the ER stress response. In summary, CCl_4 induces liver steatosis, a process involving ER stress-induced P450 2E1 activation and ROS production.

1. Introduction

Hepatic accumulation of triglycerides (steatosis) is a major complication of obesity, insulin resistance, and alcoholic/nonalcoholic fatty liver disease (Browning and Horton, 2004). If left untreated, benign steatosis can advance to steatohepatitis, fibrosis, and cirrhosis.

Carbon tetrachloride (CCl_4) is a colorless liquid that is found in low levels in ambient air. After oral administration, CCl_4 concentrates in the liver. A distinctive feature of CCl_4 toxicity is rapid accumulation of triglyceride in the liver (Pan et al., 2007; Paz Gavilan et al., 2006; Weber et al., 2003). Triglyceride secretion requires the assembly and secretion of apolipoproteins by the liver, which is an essential function of the endoplasmic reticulum (ER). When the ER is not functional, secretion of apolipoproteins such as apolipoprotein B (ApoB) is inhibited, leading to hepatic lipotoxicity (Pan et al., 2007; Hussain et al., 2003). Recently, CCl_4 was shown to cause rough endoplasmic reticula to swell in hepatocytes, leading to expression of GRP78 and XBP-1, a phenomenon of ER stress (Marumoto et al., 2008). Three ER-resident transmembrane proteins, endoribonuclease IRE1, PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) have been identified as the proximal sensors of ER stress. The ER has evolved highly specific signaling pathways to protect cells against ER stress, which are collectively known as the unfolded protein response (UPR) (Zhang and Kaufman, 2004). Activation of UPR causes upregulation of the genes that encode ER chaperone proteins such as GRP78, which increases protein folding activity and prevents protein aggregation (Paz Gavilan et al., 2006). It has also been reported that the accumulation of misfolded proteins within the lumen of the ER can lead to prolonged UPR activation, which in turn causes pathological phenomena such as hepatic lipid accumulation (Sozio et al., 2010; Bowes et al., 2009). Severe or prolonged ER stress can lead to accumulation of reactive oxygen species (ROS) in cells which in turns induces pathological conditions directly or indirectly

Abbreviations: ROS, reactive oxygen species; ER, endoplasmic reticulum; UPR, unfolded protein response; GRP78, glucose response protein 78; CHOP, C/EBP homologous protein; eIF-2α, eukaryotic initiation factor; NADPH, nicotinamide adenine dinucleotide phosphate.

* Corresponding author. Tel.: +82 63 850 6640; fax: +82 63 854 0285.
** Corresponding author. Tel.: +82 63 270 3092; fax: +82 63 275 2855.
E-mail addresses: hrkimdp@wonkwang.ac.kr (H.-R. Kim), hjchae@chonbuk.ac.kr, hjchae@jbnu.ac.kr (H.-J. Chae).

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(Hayashi et al., 2005). However, there is no clear explanation as to how ROS is involved in alteration of lipid metabolism induced by ER stress.

Recently, the relationship between cytochrome P450 2E1 and ROS has been studied with regards to ER stress (Kim et al., 2009; Wong et al., 1998). Bioactivation of the CCl_4 molecule to the trichloromethyl free radical CCl_3 is performed by cytochrome P450 isozymes (P450s) (Slater, 1984; Recknagel et al., 1989). The free radicals then attack polyunsaturated fatty acid portions of membrane lipids to propagate a chain reaction leading to lipid peroxidation, which alters membrane structure and disrupts protein synthesis (Recknagel et al., 1989). Of the multiple forms of P450s present in the liver ER, P450 2E1 has been implicated as a key metabolizing enzyme for CCl_4 bioactivation and CCl_4-mediated pathological alteration. For activation of P450, electron transfer between NADPH-P450 reductase (NPR) and P450 is required. During the process of electron transfer, a small percentage of electrons leak from the transfer system. This 'electron leakage' also contributes significantly to ROS production during redox cycling between NPR and P450, especially P450 2E1. In addition to the bioactivation of CCl_4, electron leakage due to P450 2E1 activation may be important for CCl_4-induced hepatic alterations such as steatosis. Our hypothesis is that CCl_4 induces ER stress that is associated with P450 2E1 activity and the subsequent accumulation of hepatic lipids.

In this study, we show that CCl_4-induced steatosis is related to the ER stress response and P450 2E1 activity through both *in vitro* and *in vivo* approaches in a rat model.

2. Materials and Methods

2.1. Materials

Goat anti-human ApoA1 and ApoB were purchased from Biodesign (Saco, Maine, USA). Stock solutions of reagents were prepared in dimethyl sulphoxide (Me_2SO). For *in vivo* experiments, CCl_4 (99.9% CCl_4) was mixed with olive oil. The final concentrations of Me_2SO did not exceed 0.25%.

2.2. Animals

Rats were housed in an air-conditioned room at 22 °C with a 12 h lighting schedule and were fed with rodent chow. Male rats (10–11 week-old) were used in this study. Food was removed at the night before the experiments. Each experimental rat (approximately 300 g body weight) was intraperitoneally injected with 1 mg/kg CCl_4 (150 μl). For convenience, 6.6 μl CCl_4 was mixed with 3 ml of olive oil for 20 rats and then 150 μl was injected into each rat. Control rats received an equal volume of olive oil (150 μl). Blood was collected from the heart, and serum lipid levels were determined from plasma using commercial kits (Thermo Trace). Cholesterol and triglycerides were measured after precipitation of ApoB with phosphotungstate-magnesium chloride reagent.

2.3. Histological analysis

Liver samples fixed in 10% formalin were embedded in paraffin and cut into 2 μm sections for hematoxylin–eosin staining. Liver fibrosis was assessed in paraffin sections by Sirius red staining. Briefly, liver sections were incubated for 10 min in 0.5% thiosemicarbazide, stained in 0.1% Sirius red F3B in saturated picric acid for 1 h, and then washed with an acetic acid solution (0.5%). Sections were visualized under a Nikon Eclipse E600 microscope (Kawasaki, Kanagawa, Japan) at a magnification of 40×, and the relative fibrosis area (expressed as % of positive Sirius red staining) was quantified by histomorphometry using a computerized image analysis system (AnalySIS, Soft Imaging System, Munster, Germany). A minimum of four independent fields were quantified and the results were expressed as percentage of area occupied by fibrous tissue. Hepatic steatosis was assessed by Oil Red O staining. Briefly, liver cryosections were fixed for 10 min in 60% isopropanol followed by staining with 0.3% Oil Red O in 60% isopropanol for 30 min and then washed with 60% isopropanol. Sections were counterstained with Gill's hematoxylin, washed with acetic acid solution (4%), and then mounted with aqueous solution. Once stained, sections were quantified by histomorphometry.

2.4. Cells

HepG2 liver cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine or 10% horse serum (Invitrogen).

2.5. Metabolic labeling and immunoprecipitation

Cells were pre-incubated in methionine-free DMEM for 30 min and pulse-labeled with [^{35}S]methionine (100–200 μCi/ml). For pulse-chase experiments, cells were incubated with [^{35}S]methionine (200 μCi/ml) for 2 h in methionine-deficient media (Sivaram et al., 1996; White et al., 1993). Cells were washed and chased in DMEM containing 100 μM methionine in the presence and absence of various agents including CCl_4 for varying durations. For immunoprecipitation, cell lysates were prepared in 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, 0.015% phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 1 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, and 1% SDS. Cell lysates were incubated at 70 °C for 15 min to ensure complete cell lysis and diluted with lysis buffer to achieve an SDS concentration of 0.1% (w/v). Media were collected and centrifuged at 13,000 rpm for 10 min to remove cell debris. Supernatants (0.9 ml) were combined with 0.1 ml of 10× lysis buffer containing 1% SDS. Cell lysates and media were incubated with antibodies (1:100 dilution) for 2 h and then with 20 μl of protein A/G-Sepharose (10% solution) for an additional 2 h. Immunocomplexes were collected by centrifugation, washed three times with 10 mM Tris, pH 7.5, containing 0.1 M NaCl and 1% Triton X-100, eluted in 50 μl of sample buffer, separated on SDS–polyacrylamide gel, and exposed to a phosphorimaging screen for metabolic labeling of samples.

2.6. Western blot analysis

Proteins were separated under non-reducing conditions, transferred to nitrocellulose membranes, blocked for 2 h in 20 mM Tris, pH 7.5, 137 mM NaCl, containing 0.1% Tween 20 and 5% nonfat dry milk at room temperature. Blots were washed three times and incubated overnight at 4 °C in the same buffer containing 0.5% dry milk and a primary antibody (1:1000 dilution). They were then washed and incubated with mouse horseradish peroxidase-conjugated secondary antibody (1:4000) in 1.0% skim milk for 1 h at room temperature. Immune reactivity was detected by chemiluminescence.

2.7. Microsomal membrane fractionation and lipid peroxidation assay

The microsomal fraction was obtained as previously described (Yang et al., 2004). Briefly, liver tissues and cells were resuspended in buffer A (250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1× protease inhibitor complex (Roche Diagnostics, Mannheim, Germany) on ice for 30 min. The lysates were then homogenized and centrifuged at 750 × *g* for 10 min at 4 °C. The supernatant from the