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Aconitum carmichaelii protects against acetaminophen-induced hepatotoxicity via B-cell lymphoma-2 protein-mediated inhibition of mitochondrial dysfunction

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ABSTRACT

We previously reported the clinical profile of processed *Aconitum carmichaelii* (AC, Aconibal®), which included inhibition of cytochrome P450 (CYP) 2E1 activity in healthy male adults. CYP2E1 is recognized as the enzyme that initiates the cascade of events leading to acetaminophen (APAP)-induced toxicity. However, no studies have characterized its role in APAP-induced hepatic injury. Here, we investigated the protective effects of AC on APAP-induced hepatotoxicity via mitochondrial dysfunction. AC (5–500 μg/mL) significantly inhibited APAP-induced reduction of glutathione. In addition, AC decreased mitochondrial membrane potential ($\Delta\psi m$) and B-cell lymphoma 2 (Bcl-2)-associated X protein levels (% change 46.63) in mitochondria. Moreover, it increased Bcl-2 (% change 55.39) and cytochrome C levels (% change 38.33) in mitochondria, measured using immunofluorescence or a commercial kit. Furthermore, cell membrane integrity was preserved and nuclear fragmentation inhibited by AC. These results demonstrate that AC protects hepatocytes against APAP-induced toxicity by inhibiting mitochondrial dysfunction.

1. Introduction

Acetaminophen (N-acetyl-p-aminophenol, APAP, Tylenol®) is a commonly used analgesic and antipyretic agent that is considered safe at therapeutic ranges (Graham and Scott, 2005). However, with overdosage it has a high toxicity and the potential to cause death in healthy adults (McGill et al., 2014). The key event in APAP toxicity is its transformation into the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) mediated by the cytochrome P450 (CYP) microsomal enzyme superfamily, which mainly include CYP450 2E1 and 1A2 (Bunchorntavakul and Reddy, 2013; McGill et al., 2014). Normally, NAPQI is rapidly detoxified by conjugation with glutathione (GSH), thereby forming a nontoxic APAP-GSH conjugate (Bunchorntavakul and Reddy, 2013). However, with overdoses NAPQI saturates the conjugation detoxification process and may ultimately deplete GSH, induce mitochondrial dysfunction, ATP depletion, and oxidative stress by covalently binding to cellular proteins (Bunchorntavakul and Reddy, 2013; Jaeschke et al., 2012). This hepatic oxidative damage induces the formation of mitochondrial peroxynitrite, which in turn triggers mitochondrial DNA damage, nitration of mitochondrial proteins, consequently leading to the opening of membrane permeability transition pores (Jaeschke et al., 2012). Collapse of the mitochondrial membrane potential ($\Delta\psi m$) eventually decreases B-cell lymphoma 2 (Bcl-2) protein levels, which inhibits cell death by preventing depolarization and increasing Bcl-2-associated X (Bax) protein levels; this promotes cell death by inducing depolarization and cytochrome C (Cyt-C) release (Badmann et al., 2011; McGill et al., 2011). Therefore, in this study we investigated the regulation of mitochondrial dysfunction as a possible therapeutic target in APAP toxicity with a focus on the changes associated with APAP-induced cell death.

Recently, the HepaRG cell line has been considered an important tool for biomedical research because several studies have demonstrated a higher expression and activity of xenobiotic metabolizing enzymes in these cells than there is in primary human hepatocytes and HepG2 cells (Andersson et al., 2012). This new human liver cell line was derived from a female patient with hepatocarcinoma, and the bipotent progenitor HepaRG cells from this line

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differentiate into hepatocytes and biliary epithelial cells that maintain liver-specific functions (Andersson et al., 2012).

Aconitum carmichaelii (AC), one of the most common species of the genus *Aconitum*, is widely cultivated in East Asia and eastern Russia (Zhou et al., 2015). Processed AC is extensively used in traditional Eastern medicine and is known as Kyeong-Po Buja, Fuzi, Bushi, Chinese aconite, monkshood or Chinese wolfsbane (Zhou et al., 2015). It has been used to treat various symptoms such as acute myocardial infarction, pain, renal failure, low blood pressure, and heart failure (Nyirimigabo et al., 2015; Zhou et al., 2015). Moreover, Aconibal®, a commercial processed AC, has been used to treat the signs and symptoms of spondylosis deformans and rheumatic pain in Korea (Oh et al., 2009; Oh et al., 2004). Previous clinical studies have shown that processed AC moderately inhibited the activity of CYP2E1, which is an important CYP 450 isoform that mediates the generation of oxidative damage and toxic effects of a variety of xenobiotics (Oh et al., 2009; Oh et al., 2004; Kim et al., 2004). Processed AC, which has demonstrated inhibitory effects against CYP2E1, may be a useful antidote to APAP overdose-induced hepatotoxicity. Therefore, this present study investigated the protective effects of processed AC on APAP-induced hepatotoxicity and determined possible underlying mechanisms or therapeutic target by assessing its effects on mitochondrial dysfunction signaling pathway, such as GSH levels, $\Delta\psi m$, Bcl-2, Bax, and Cyt-C.

2. Materials and methods

2.1. Cell line, chemicals, and reagents

HepaRG cells were obtained from Biopredic International (St Gregoire, Rennes, France) and stored in liquid nitrogen. William's E medium, L-glutamine, penicillin/streptomycin, Gluta-max supplement, HepaRG Tox medium supplement, and trypsin-EDTA were acquired from Life Technologies® (Carlsbad, CA, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), and APAP were purchased from Sigma–Aldrich (St. Louis, MO, USA). The CytoScan™ lactate dehydrogenase (LDH)-cytotoxicity assay kit was purchased from Geno Technology® (St. Louis, MO, USA). The mitochondrial/cytosolic fraction kit was purchased from BioVision (Mountain View, CA, USA). All other reagents used were of guaranteed or analytical grade.

2.2. Sample preparation

Excluding the excipient, the processed AC powder was kindly supplied by HanPoong Pharmaceutical Co., Ltd. (Seoul, Korea), and a voucher specimen (KIOM-ACO) was deposited at the herbarium of the Korea Institute of Oriental Medicine (Daejeon, Korea). Furthermore, the benzoyl alkaloid (553 mg/g) of the processed AC (Aconibal®) was previously analyzed and characterized by our research group.

2.3. Cell culture

Differentiated HepaRG cells were cultured according to the supplier's instructions. Cells were maintained in William's E medium containing HepaRG thaw, plate general purpose medium supplement; HepaRG tox medium supplement; GlutaMAX supplement; 100 units/mL penicillin; and 100 μg/mL streptomycin under conditions of 95% air and 5% CO_2 at 37 °C. The hepatocyte-like cells were incubated in a differentiation medium, to induce them into more granular cells closely resembling primary hepatocyte (1% DMSO added to the full medium). The medium was renewed every other day for over 2 weeks. After the final medium renewal, it was switched to a DMSO-free medium for 1 day, and then the cells were ready for use in the experiments.

2.4. Measurement of GSH levels

Total GSH levels were determined using a total GSH quantification kit according to the instruction manual (Dojindo Molecular Technologies, Japan). Briefly, the treated cells were lysed in 10 mM hydrochloric acid solution by freezing and thawing. Then, they were treated with 5% 5-sulfosalicyclic acid, centrifuged at 8000 × *g* for 10 min at 4 °C, and the supernatant was assessed for GSH levels. A co-enzyme working solution, buffer solution, and enzyme working solution were added to the wells containing the supernatant and incubated at 37 °C for 5 min. Then, the GSH standard, sample, and substrate working solutions were added with a 10-min incubation for each. The absorbance was measured using a spectrophotometer at a wavelength of 405 nm, and the GSH concentrations were determined using a standard calibration curve.

2.5. Measurement of mitochondrial morphology

Cells were seeded on coverslips in 24-well plates and treated simultaneously with AC and APAP for 24 h. The cells were then fixed with 4% paraformaldehyde at 25 °C for 30 min, rinsed with PBS, incubated with MitoTracker dye (dilution 1:1000) at 25 °C for 15 min, washed with PBS, and mounted. Representative images were acquired using a fluorescence microscope (Olympus Microscope System BX51, Olympus, Tokyo, Japan). Data were presented as the percentages of control values. To validate the intra-experimental variability, three independent experiments were conducted in triplicate.

2.6. Measurement of $\Delta\psi$m activity

Cells were seeded on coverslips in 24-well plates and treated simultaneously with AC at concentrations of 5, 50, and 500 μg/mL and 25 mM APAP for 24 h. The cells were then fixed with 4% paraformaldehyde at 25 °C for 30 min, rinsed with PBS, incubated with diluted 5,5′,6,6′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) at 37 °C for 30 min, washed with PBS, and then mounted on slides. The fluorescence intensity was determined at 485 and 535 nm excitation and emission wavelengths, respectively using a fluorescence microplate reader (SpectraMax Gemini EM, Molecular Device, Sunnyvale, CA, USA). Representative images were acquired using a fluorescence microscope (Olympus Microscope System BX51).

2.7. Measurement of Bax, Bcl-2, and Cyt-C proteins levels

For the detection of Bax, Bcl-2, and Cyt-C levels, proteins samples were separated into the mitochondrial and cytosolic fractions using a fractionation kit according to the manufacturer's instructions. Briefly, the cells were resuspended in cytosol extraction buffer mix containing DTT and protease inhibitors, then, they were incubated on ice for 10 min and after that, they were homogenized using a dounce tissue grinder. The homogenates were centrifuged at 700 × *g* for 10 min and the supernatant was used as the cytosolic fraction. The saved pallet was resuspended in mitochondria extraction buffer mix and used as the mitochondrial fraction. Then, Bax, Bcl-2, and Cyt-C protein levels were determined using human Bax, PathScan, total Bcl-2 sandwich, and human Cyt-C Quantikine ELISA kits, according to the R&D System instruction manuals. In addition, the fractionation lysates were separated by 15% SDS-PAGE, and were then transferred to a membrane. The membranes were incubated with 5% skim milk in TBST for 1 h. Then they were incubated with rabbit Bax, and Bcl-2 (1:1000 dilutions), mouse anti-Cyt-C