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Organic cation transporter 1 mediates the uptake of monocrotaline and plays an important role in its hepatotoxicity

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ABSTRACT

Monocrotaline (MCT) is a kind of toxic retronecine-type pyrrolizidine alkaloids (PAs) from plants of Crotalaria, which can be bio-activated by cytochrome P450 (CYP) enzymes in liver and then induce hepatotoxicity. Since CYPs are localized in the endoplasmic reticulum, the influx of MCT to the liver is the key step for its hepatotoxicity. The objective of the present study was to investigate the role of organic cation transporter 1 (OCT1), a transporter mainly expressed in liver, in the uptake of MCT and in hepatotoxicity induced by MCT. The results revealed that MCT markedly inhibited the uptake of 1-methyl-4-phenylpyridinium (MPP^+), an OCT1 substrate, in Madin–Darby canine kidney (MDCK) cells stably expressing human OCT1 (MDCK-hOCT1) with the IC_{50} of 5.52 ± 0.56 μM. The uptake of MCT was significantly higher in MDCK-hOCT1 cells than in MDCK-mock cells, and MCT uptake in MDCK-hOCT1 cells followed Michaelis–Menten kinetics with the K_m and V_{max} values of 25.0 ± 6.7 μM and 266 ± 64 pmol/mg protein/min, respectively. Moreover, the OCT1 inhibitors, such as quinidine, *d*-tetrahydropalmatine (*d*-THP), obviously inhibited the uptake of MCT in MDCK-hOCT1 cells and isolated rat primary hepatocytes, and attenuated the viability reduction and LDH release of the primary cultured rat hepatocytes caused by MCT. In conclusion, OCT1 mediates the hepatic uptake of MCT and may play an important role in MCT induced-hepatotoxicity.

1. Introduction

Monocrotaline (MCT, Fig. 1), a retronecine-type pyrrolizidine alkaloids (PAs), is present abundantly in Crotalaria genus and has hepatotoxic and pneumotoxic effects on animals (Copple et al., 2002; DeLeve et al., 2003; McLean, 1970; Nobre et al., 2004; Yan and Huxtable, 1995a), such as liver sinusoidal and central venular endothelial cell injury, hepatic sinusoidal obstruction syndrome or even hepatocytes apoptosis (Copple et al., 2004; Nakamura et al., 2012). PAs are common natural compounds distributed in more than 6000 kinds of plants, and 1,2-dehydro pyrrolizidine ester alkaloids are poisonous to humans and livestock (Boppre, 2011).

It has been reported that the toxicity of MCT mainly depends on its bioactivation to reactive pyrrolic metabolites by cytochrome P450 (CYP) enzymes, among which CYP3A are the major isozymes for the metabolic activation (Fu et al., 2004; Yan and Huxtable, 1995b). Dehydromonocrotaline (DHM), the major metabolite, is chemically and biologically reactive and tends to bind with DNA and proteins (Barreto et al., 2008; Lamé et al., 2005; Wang et al., 2005), which was responsible for the toxicities. It was demonstrated that DHM inhibits the activity of respiratory chain complex I NADH oxidase, which interferes with mitochondrial energy metabolism (Mingatto et al., 2007). DHM was also found to induce membrane permeability transition and cause the release of cytochrome *c* from mitochondria in isolated rat liver mitochondria (Santos et al., 2009).

For the severe hepatotoxicity of MCT, metabolism catalyzed by liver CYPs is a key step. However, in hepatocytes, CYPs are localized in the endoplasmic reticulum, therefore the influx of MCT is a necessary step for its hepatotoxicity. It has been confirmed that polyspecific organic cation transporters (OCTs) of the SLC22 family, including three organic cation transporters (OCT1–OCT3), play an important role in the transport of organic cations,

Abbreviations: PAs, pyrrolizidine alkaloids; MCT, monocrotaline; OCT1, organic cation transporter 1; MDCK, Madin–Darby canine kidney; MPP+, 1-methyl-4-phenylpyridinium; *d*-THP, *d*-tetrahydropalmatine; DHM, dehydromonocrotaline; CYP, cytochrome P450; ASP^+, 4-(4-(dimethylamino)styryl)-N-methylpyridinium; TEA, tetraethylammonium; SDS, sodium dodecyl sulfonate; DMSO, dimethyl sulfoxide; HBSS, Hank's balanced salt solution; MES, 2-(N-Morpholino)ethanesulfonic acid.

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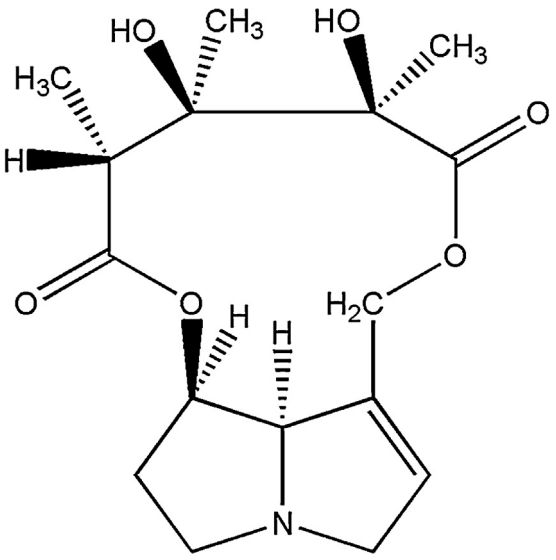

Fig. 1. Chemical structure of monocrotaline (MCT).

including weak bases and some non-charged compounds (Koepsell and Endou, 2004; Nies et al., 2011). OCT1 is highly expressed in rat and human liver (Koepsell et al., 2003; Nies et al., 2009), and mainly localized in the sinusoidal (basolateral) membrane of hepatocytes (Meyer-Wentrup et al., 1998; Nies et al., 2008). Since MCT is a weak base and can be partly ionized to organic cation at physiological pH, we deduced that OCT1 might mediate the influx of MCT to hepatocytes.

With this in mind, the aim of this study was to investigate the role of OCT1 in hepatotoxicity induced by MCT using Madin–Darby canine kidney cells stably expressing human OCT1 (MDCK-hOCT1) and primary cultured rat hepatocytes. The results will give us more information about the mechanism of the toxicity induced by MCT and provide a promising way to prevent the toxicity.

2. Materials and methods

2.1. Materials

G418, penicillin, streptomycin, fetal bovine serum (FBS), trypsin, Dulbecco's modified Eagle medium (DMEM) and RPMI 1640 medium were purchased from GIBCO (Invitrogen Life Technologies, USA), 1-methyl-4-phenylpyridinium iodide (MPP^+) and 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) were obtained from Sigma–Aldrich (St. Louis, MO, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Tetraethylammonium bromide (TEA) was purchased from Baoman Co., Ltd. (Shanghai, China). Quinidine sulfate was purchased from Nanjing DeBioChem Co., Ltd. (Nanjing, China). MCT was provided by Aladdin Co., Ltd. (Shanghai, China). *d*-Tetrahydropalmatine (*d*-THP) was purchased from Huatuo Co., Ltd. (Shanghai, China). Acetonitrile was obtained from Tedia (Cincinnati, OH, USA). Lactate dehydrogenase activity assay kit was obtained from Jiancheng Co., Ltd. (Nanjing, China). All other chemicals were purchased from commercial sources and were of analytical grade.

2.2. Animals

Male Sprague Dawley (SD) rats, 200–220 g, were provided by Experimental Animal Center of Zhejiang Academy of Medical Sciences. The animals were maintained in cages at controlled temperature and humidity with 12:12 h light–dark cycles and exposed to a free access to food and water. Animals were fasted but free access to water for 12 h before experiment. The experimental procedures were performed according to an approved animal use protocol of Zhejiang University.

2.3. Cell culture, stable transfection and isolation of rat primary hepatocytes

MDCK cells stably transfected with plasmid pcDNA3.1 (+) containing the human OCT1 cDNA sequence (GenBank accession number NM_003057; MDCK-hOCT1) were constructed as reported previously (Shu et al., 2003). MDCK cells were also transfected with empty vector pcDNA3.1 (+) as mock cells. The activity of hOCT1 in the stably transfected cells was validated by model substrates, such as ASP^+ and MPP^+ with or without OCT1 inhibitor/substrate TEA (Ahlin et al., 2008; Gründemann et al., 2003). Cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 700 μg/mL G418 (for transfected cells), at 37 °C in a 5% CO_2 and 95% air humidified incubator. The cells were sub-cultured after being 90% confluent.

Hepatocytes were isolated from male SD rats using a modified two-step collagenase perfusion method (Lu et al., 2011). The viability of the isolated hepatocytes was validated by Trypan blue uptake, and the cells with more than 85% viability were applied to the experiment. The hepatocytes were suspended in RPMI 1640 medium supplemented with heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and then seeded in poly-D-lysine-coated 12-well or 96-well plates (Costar Corning Inc., NY, USA) at a destiny of 3×10^5/well or 1×10^4/well, respectively. The cells were cultured at 37 °C with a 5% CO_2 and 95% air humidified atmosphere for 4 h to be attached to the plate.

2.4. Cellular uptake assays

MDCK-hOCT1 and mock cells were seeded in 24-well plates coated with poly-D-lysine (Costar Corning Inc., NY, USA) at a destiny of 2×10^5/well. On day 3 after seeding, the uptake studies were performed as the method reported with minor modifications (Saadatmand et al., 2012). Briefly, the cells were pre-incubated with Hank's balanced salt solution (HBSS, with 5 mM HEPES adjusted to pH 6.4, 7.4, 8.4, or with 5 mM MES adjusted to pH 5.4) for 10 min at 37 °C, and then 200 μL of HBSS (pH 7.4, except in pH-dependent experiment) containing MPP^+ (1–50 μM) or MCT (1–100 μM) in the absence or presence of different inhibitors was added to initiate the uptake, the incubation was performed at 37 °C for the designated period of time. At the end of the incubation, cells were washed thrice with ice-cold buffer after removing the incubation buffer and lysed with 100 μL 0.1% sodium dodecyl sulfonate (SDS). All experiments were performed in triplicate. The uptake of MPP^+ in the cells were quantified with LC–MS/MS and normalized to the total protein content in the lysates using a bicinchoninic acid protein assay kit with bovine serum albumin as a standard.

The primary cultured rat hepatocytes in 12-wells plate were also used to determine the uptake of MCT. At 4 h after seeding, the uptake studies were performed as described above except that the incubation buffer was replaced with 500 μL of HBSS (pH 7.4) containing 1 μM MCT with or without inhibitors.

The inhibition of MPP^+ uptake mediated by hOCT1 was checked for quinidine (0.1–100 μM), *d*-THP (0.3–100 μM, an OCT1 inhibitor demonstrated by our lab, unpublished data) and MCT (0.3–100 μM), TEA (3 mM), ASP^+ (100 μM), quinidine (100 μM) and *d*-THP (100 μM) were applied to confirm whether the uptake of MCT was reduced by the OCT1 inhibitors.

2.5. LC–MS/MS determination of MPP^+ and MCT

The concentrations of MPP^+ and MCT in the uptake samples were determined by LC–MS/MS according to the reported methods (Lin et al., 1998; Zhang et al., 2008) with some modification. 160 μL of acetonitrile (with internal standard) was added into 80 μL of the cell lysates to precipitate the proteins, the mixture was vortexed for 2 min and centrifuged at 16,000 × *g* for 15 min, and the supernatant was analyzed by LC–MS/MS directly for MPP^+. With regards to MCT, the supernatant was evaporated to dryness under vacuum, and the residue was re-suspended in 100 μL water, after being vortex-mixed and centrifuged at 16,000 × *g* for 15 min, the supernatant was analyzed with LC–MS/MS. The LC–MS/MS was performed on a Waters Acquity UPLC/TQD system (Waters, Milford, MA), which consisted of an ultra performance liquid chromatography with a triple quadrupole mass spectrometer (MS/MS). An Agilent Extend C_{18} column (3.5 μm, 2.1 mm × 50 mm) was applied for separation, and the mobile phase consisted of solvent A: 0.1% formic acid in water–acetonitrile (H_2O:ACN = 95:5, v/v) and B: 0.1% formic acid in acetonitrile. The gradient mobile phase was pumped with the flow rate of 0.2 mL/min. For MPP^+ assay, the gradient started at 0% B for 0.5 min, followed by a linear increase of solvent B to 80% from 0.5 min to 2.5 min, maintained for 0.5 min, then returned to the initial conditions at 3.1 min and maintained for 1 min to equilibrate the column. For MCT, the gradient was as follows: at 0–2.5 min, 0% B was linear increased to 20% B; at 2.51–3 min 90% B, and at 3.1–4 min, 0% B. The mass spectrometer with a electrospray interface was operated in positive ion mode with the following settings: ion source temperature, 140 °C; desolvation temperature, 350 °C; capillary voltage, 3.8 kV; cone voltage, 50 V; collision energy, 35 eV; desolvation gas (nitrogen), 850 L h^{-1}; multiple reaction monitoring (MRM) scan mode monitored the ion pair of MPP^+ at m/z 170 > 128, or MCT at m/z 326.2 > 120. The measurements were linear ($r^2 > 0.999$) in the range of 20–500 nM for MPP^+ and 10–500 nM for MCT.

2.6. Evaluation of hepatotoxicity

The cytotoxic effect of MCT on the primary cultured rat hepatocytes was assessed by MTT assay (Hansen et al., 1989) and by measuring the lactate dehydrogenase (LDH) activity in the cell culture medium. After the rat primary hepatocytes seeded in the 96-well plate were attached to the plates, they were incubated with 50–200 μM MCT or 0.25% DMSO (as control) for 48 h at 37 °C. For study of the effects of OCT1 inhibitors on the MCT toxicity, cells were incubated with 50–200 μM MCT under the presence of *d*-THP (40 μM) or quinidine (20 μM). At the end of the incubation,