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Necroptosis in 3-chloro-1, 2-propanediol (3-MCPD)-dipalmitate-induced acute kidney injury *in vivo* and its repression by miR-223-3p

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ABSTRACT

Fatty acid esters of 3-chloro-1, 2-propanediol (3-MCPD) are a group of processing-induced food contaminants with nephrotoxicity. This study investigated whether and how necroptosis played a role in the nephrotoxic effect of 3-MCPD-dipalmitate (2.5 g/kg BW) in C57 BL/6 mice. The results showed that the principal components in necroptosis pathway including receptor-interacting protein 1 (RIPK1), RIPK3, and mixed lineage kinase domain-like protein (MLKL) were up-regulated in 3-MCPD-dipalmitate-induced acute kidney injury (AKI). Deletion of RIPK3 or MLKL, and inhibition of RIPK1 suppressed AKI. The up-regulation of inflammatory cytokines in the kidney of 3-MCPD-dipalmitate treated mice were attenuated in RIPK3- or MLKL- deficient mice, suggesting a positive feedback loop involving necroptosis and inflammation. The microRNA analysis revealed that 38 known miRNAs and 40 novel miRNAs were differentially expressed (DE) in the kidney treated with 3-MCPD-dipalmitate. Of these miRNAs, miR-223-3p was significantly up-regulated during 3-MCPD-dipalmitate-induced AKI. In cultured mouse proximal tubular cells, a miR-223-3p mimic suppressed RIPK3 expression, which was blocked by miR-223-3p inhibitor. The luciferase reporter assay confirmed that miR-223-3p was able to inhibit RIPK3 expression by targeting the 3′ un-translated region of RIPK3. These results suggest that necroptosis contributes to 3-MCPD-dipalmitate-induced acute kidney injury, and that may be attenuated by miR-223-3p.

1. Introduction

Fatty acid esters of 3-chloro-1, 2-propanediol (3-MCPD) in thermally-processed foods have been known for their organ toxicities and became one of the important food safety concerns (Arisseto et al., 2014; Stadler, 2014; Liu et al., 2017). Accumulating evidence suggested that kidney is a primary target organ for 3-MCPD esters (Barocelli et al., 2011; Liu et al., 2012; Onami et al., 2014). A few studies have investigated the molecular mechanisms behind the kidney injury induced by 3-MCPD esters. In 2016, Liu and colleagues demonstrated that 3-MPCD-1-palmitate increased the level of cleaved caspase 3 via JNK/p53 pathway at a dose of 1.0 g/kg BW in rats or 1.5 g/kg BW in mice (Liu et al., 2016), indicating their induction of apoptosis pathway(s). Recently, necroptosis was also associated with kidney injury (Linkermann et al., 2013; Xu et al., 2015). The inhibition of the core components of necroptotic pathway including receptor interacting protein kinase 1 (RIPK1) and RIPK3, or mixed lineage kinase domain-like protein (MLKL) by gene knockout or a chemical inhibitor was found to diminish cisplatin-induced proximal tubule damage in mice (Xu et al., 2015). This was consistent with Linkermann's research, in which RIPK1/RIPK3-mediated necroptosis was found to play an important role in cisplatin-induced acute kidney injury (AKI) in mice (Linkermann et al., 2013). Moreover, Linkermann and colleagues demonstrated that necroptosis occurred in ischemia-reperfusion injury (IRI) in the kidney, and the mice deficient in RIPK3 were protected from IRI (Linkermann et al., 2013). However, whether and how necroptosis might play a role in 3-MCPD esters-induced kidney injury remains unclear.

MicroRNAs (miRNAs) are critical regulators of gene expression by binding to the 3′ un-translated region (3′UTR) of target mRNAs (Hao et al., 2017). Previous studies have found that the differently expressed

Abbreviations: 3-MCPD, 3-chloro-1,2-propanediol; 3′UTR, 3′ un-translated region; AKI, acute kidney injury; BMD_{10}, Benchmark doses; $BMDL_{10}$, BMD Lower Confidence Limit; BW, body weight; CXCl, chemokine (C-X-C motif) ligand; DE, differentially expressed; DR6, death receptor 6; GSK3β, glycogen synthase kinase 3β; H&E, hematoxylin and eosin; HT, homozygotes; IGF-1R, insulin-like growth factor-1 receptor; IHC, immunohistochemistry; IL, Interleukin; IRI, ischemia-reperfusion injury; KO, knockout; miRNA, microRNA; MLKL, mixed lineage kinase domain-like protein; MUT, mutant; Nec-1, necrostatin-1; NOAEL, no observed adverse effect level; PTC, proximal tubular cell; RIPK, receptor-interacting protein; SD, standard deviation; SOCS1, suppressor of cytokine signaling 1; TEM, transmission electron microscopy; TNF, tumor necrosis factor; WT, wild type

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(DE)-miRNAs play possible protective roles in kidney injury. Wei and colleagues demonstrated that miR-489 was induced by hypoxia-inducible factor-1 during ischemic acute kidney injury (AKI) protecting kidney tubular cells against apoptosis (Wei et al., 2016). Moreover, miR-17-5p significantly attenuated renal IRI in mice by targeting death receptor 6 (DR6) (Hao et al., 2017). On the other hand, some miRNAs were found to mediate kidney injury. For example, miR-150 mediated inflammation, interstitial fibrosis and cell apoptosis in AKI through suppressing insulin-like growth factor-1 receptor (IGF-1R) and suppressor of cytokine signaling 1 (SOCS1) pathways (Zhou et al., 2013; Ranganathan et al., 2015). The miR-135a and miR-135b regulated the expression of glycogen synthase kinase 3β (GSK3β), and mediated podocyte injury and the disorder of podocyte cytoskeleton in kidney disease (Yang et al., 2015). Accordingly, studies on the role of DE-miRNAs in AKI may suggest potential therapeutic strategies for AKI-related kidney disease. However, little is known whether and how DE-miRNAs may play a role in 3-MCPD ester-induced AKI.

The present study was to investigate the role of necroptosis in 3-MCPD esters-induced AKI, and the potential relationship between miRNAs and necroptosis. 3-MCPD-dipalmitate was chosen as a 3-MCPD ester probe compound as about 85% of the total 3-MCPD esters in foods is in diester form (Seefelder et al., 2008). The treatment dose was selected according to our previous study (Liu et al., 2012) demonstrating that the LD_{50} value of 3-MCPD-dipalmitate was greater than 5 g/kg BW in Swiss mice. A dose range-finding preliminary study was performed to assess the dose response in mice using the doses of 1.25, 1.66 and 2.5 g/kg BW, showing that animals developed kidney toxicity after a single dose of 2.5 g/kg BW without death. The present study may advance our understanding of the molecular mechanisms involved in the 3-MCPD esters-induced kidney injury and serves as a scientific foundation for their safety investigations.

2. Materials and methods

2.1. Antibodies and reagents

Phosphorylated- (p-) MLKL (Ser345) (ab196436), RIPK1 (ab72139), RIPK3 (ab62344) antibodies were purchased from the Abcam (Cambridge, MA, USA). RIPK1 (#3493), RIPK3 (#15828), MLKL (#28640) and β-actin (#4970) antibodies were purchased from the Cell Signaling Technology (Beverly, MA, USA). The SignalStain® Boost IHC Detection Reagents (HRP, anti-Mouse #8125; HRP, anti-Rabbit #8114) and DAB substrate kit (#8059) were also purchased from the Cell Signaling Technology (Beverly, MA, USA). RIPK1 inhibitor Necrostatin-1 (Nec-1) was purchased from Selleck Chemicals China (Shanghai, China). 3-MCPD-dipalmitate was synthesized in the laboratory following a published protocol (Liu et al., 2017) with a purity greater than 98%.

2.2. Animals and treatment

Male C57 BL/6 mice (8–10 weeks) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). Animals were acclimatized for one week before assigned into experimental groups. The homozygotes RIPK3 knockout ($RIPK3^{+/-}$, RIPK HT) mice and homozygotes MLKL knockout ($MLKL^{+/-}$, MLKL HT) mice heterozygous mice were provided as a gift by Professor Jiahuai Han in the Xiamen University (Xiamen, China). The RIPK3 knockout ($RIPK3^{-/-}$, RIPK KO) and MLKL knockout ($MLKL^{-/-}$, MLKL KO) mice were generated by mating between the heterozygous mice. Genotypes were confirmed by tail-snip PCR (Fig. S1) using the primers list at Table S1. Animals were housed in a specific pathogen-free (SPF) facility and maintained under the controlled conditions with a temperature of 24 ± 1 °C, relative humidity of 50–70%, and a 12-h light/12-h dark cycle. All procedures requiring the use of animals were conducted in accordance with the Animal Care and Welfare Committee of the Laboratory Animal Center of Shanghai Jiao Tong University (Project permission number: A2015024).

2.3. Experimental design

2.3.1. Experiment 1- the effect of RIPK1 inhibitor on the kidney toxicity induced by 3-MCPD-dipalmitate

C57 BL/6 mice were given a single dose of 2.5 g/kg BW 3-MCPD-dipalmitate and the vehicle control animal received same volume of olive oil (n = 6/group). And all the mice were received 250 μl PBS with DMSO or 1.65 mg/kg Nec-1 30 min before 3-MCPD-dipalmitate treatment (Xu et al., 2015). Mice were anaesthetized with CO_2 (Mice CO_2 inhalation to effect. Flow rate must displace no more than 30% of the chamber volume/min) and decapitated to collect the tissues 24 h after 3-MCPD-dipalmitate treatment.

2.3.2. Experiment 2- the role of RIPK3 and MLKL in the regulation of kidney toxicity induced by 3-MCPD-dipalmitate

Twelve RIPK3 wild type (RIPK3 WT) mice, 12 RIPK3 KO mice, 12 MLKL WT mice and 12 MLKL KO mice were used to test the role of RIPK3 and MLKL in the kidney toxicity induced by 3-MCPD-dipalmitate. Mice were randomly assigned to two sub-groups (olive oil or 3-MCPD-dipalmitate-treatment, n = 6/group), respectively. Then mice were given a single dose of 2.5 g/kg BW 3-MCPD-dipalmitate, and the vehicle control animal received same volume of olive oil. Mice were anaesthetized with CO_2 (Mice CO_2 inhalation to effect. Flow rate must displace no more than 30% of the chamber volume/min) and decapitated to collect the tissues 24 h after 3-MCPD-dipalmitate treatment.

2.4. Clinical chemistry

Animals were anaesthetized with CO_2 (Mice CO_2 inhalation to effect. Flow rate must displace no more than 30% of the chamber volume/min) and the blood samples were collected from retro-orbital sinus. The serum was obtained by centrifugation at 3500 rpm for 15 min at 4 °C. Serum urea nitrogen and creatinine levels were measured by KingMed Diagnostics, Inc. (Shanghai, China).

2.5. RNA isolation and qRT-PCR

Total RNA was isolated from kidney cortex using the TRIzol reagent (Thermo Fisher Scientific China, Shanghai, China), followed by the reverse transcription cDNA using the IScript Advanced cDNA Synthesis kit (Hercules, CA, USA). Real-time PCR was carried out on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using AB Power SYBR Green PCR Master Mix. The following amplification parameters were used for PCR: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min. Details of primers were listed in Table S1. Fold changes were quantified using $2^{-\Delta\Delta Ct}$ values.

2.6. Western blot analysis

For western blot analysis, total protein lysate was extracted from kidney or cell samples. Subsequently, equal amounts (20–80 μg) of protein samples were separated on 12% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After incubating for 1 h in 5% nonfat milk, the membranes were incubated with primary antibodies against RIPK1 (1:1000 dilution), RIPK3 (1:1000 dilution), MLKL (1:1000 dilution), p-MLKL (1:1000 dilution) and β-actin (1:1000 dilution) overnight at 4 °C, followed by incubating with a HRP conjugated secondary polyclonal antibody at ambient temperature for 1.5 h. Blots were developed using a Bio-Rad Clarity Western ECL (Hercules, CA, USA). The signal intensities of the bands were estimated using a ChemiDoc™ XRS+ System (Hercules, CA, USA) and quantified using an Image Lab™ Software (Hercules, CA, USA).