Functional modulation of liver mitochondria in lipopolysaccharide/drug co-treated rat liver injury model
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(Received June 24, 2019; Accepted September 18, 2019)

ABSTRACT — Drug-induced liver injury is not readily detectable using conventional animal studies during pre-clinical drug development. To address this problem, other researchers have proposed the use of co-administration of lipopolysaccharide (LPS), an endotoxin produced by gram-negative bacteria, and a drug. Using this approach, liver injury that is otherwise not detected following drug administration alone can be successfully identified. Previous studies have demonstrated that such injury is suppressed by heparin; therefore, the mechanism may involve coagulation-dependent ischemia. However, it has not been established how LPS-induced ischemia might sensitize hepatocytes to a potentially hepatotoxic drug. In the present study, we aimed to determine the effect of LPS-induced ischemia on liver mitochondrial function and downstream toxicologic responses. Consistent with previous findings, plasma alanine transaminase (ALT) activity was higher in rats co-administered with LPS (1 mg/kg) and diclofenac (100 mg/kg), but reduced by heparin. Liver mRNA expression of Hmox1, encoding heme oxygenase-1, an oxidative stress indicator, was three times higher at 2 hr after LPS administration. Furthermore, respiratory activity via mitochondrial complex II, lipid peroxidation in mitochondria, and the susceptibility to mitochondrial permeability transition pore opening in response to diclofenac administration were significantly increased by LPS administration. The increase in plasma ALT activity and the sensitization to mitochondrial permeability transition pore opening were reduced by the co-administration of heparin. In conclusion, LPS-induced transient ischemia disrupts respiratory chain complex activities, enhances reactive oxygen species production, especially in mitochondria, and sensitizes mitochondria to permeability transition pore opening when testing a potentially hepatotoxic drug in vivo.

Key words: Drug-induced liver injury, Lipopolysaccharide, Diclofenac, Mitochondria, Mitochondrial permeability transition

INTRODUCTION

Drug-induced liver injury (DILI) is a serious adverse reaction that can lead to restrictions of the clinical use or withdrawal from the market of the drug (Dykens and Will, 2007). Because post-marketing withdrawal is associated with a huge financial loss for the manufacturer, it is important to predict the risk of DILI as early as possible during the pre-clinical drug testing phase (Giri et al., 2010). Moreover, an appropriate therapeutic agent should be selected for each patient on the basis of his/her DILI susceptibility. However, the risk of DILI is frequently overlooked in pre-clinical animal studies, and even in clinical studies, because the number of patients in which the candidate drug can be tested is limited and relatively healthy patients are typically recruited.

It is thought that multiple factors are involved in the development of DILI, but animal experiments have suggested that inflammation is the most important of these factors (Roth et al., 1997; Deng et al., 2009). Rats with mild hepatic inflammation induced by lipopolysaccharide (LPS) co-administration become more susceptible to liver injury (Roth et al., 2003) by some drugs, including diclofenac (Ramm and Mally, 2013; Kishida et al., 2012), amiodarone (Lu et al., 2013; Zou et al., 2009), and ranitidine (Luyendyk et al., 2003). By contrast, the suscep-
tibility of the liver to injury by drugs with a lower clinical DILI risk, such as famotidine, is not affected by LPS co-administration (Luyendyk et al., 2003). Although the results obtained using this experimental animal model and in the human clinical setting have broadly been consistent with respect to the DILI risk associated with particular drugs, the model differs from humans in several respects. For example, the injury occurs within 24 hr of drug administration and liver histology is characterized by an infiltration of neutrophils in this animal model (Ramachandran and Jaeschke, 2019), whereas clinical DILI is generally characterized by delayed onset (Wang et al., 2018) and mononuclear leukocyte infiltration. However, even though the model does not perfectly mimic DILI in humans, it is considered useful for the screening of candidate drugs for potential liver toxicity and/or exploring the pathologic mechanism of any liver injury induced.

LPS is an endotoxin produced by gram-negative bacteria. It binds to toll-like receptor (TLR) 4 on liver-resident macrophages (Kupffer cells) (Su et al., 2000) and platelets (Feng et al., 2018), resulting in the release of inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, interferon (IFN)-γ, and IL-6 (Akira et al., 2001). These inflammatory cytokines bind to specific receptors on hepatocytes, in which complex cellular signals related to cell death or survival are initiated (Schwabe and Brenner, 2006). In addition, LPS activates blood coagulation, which can induce ischemia. This may play a key role in the induction of liver injury by LPS/drug co-administration because heparin, an inhibitor of blood coagulation, ameliorates liver injury in this model (Luyendyk et al., 2005; Zou et al., 2009; Lu et al., 2013; Shaw et al., 2009). Interestingly, the ischemia induced by LPS is transient and the activation of the blood coagulation cascade soon ceases, permitting reperfusion (Shaw et al., 2009). In the LPS/drug co-administration liver injury model, the mechanism principally involves an LPS-stimulated inflammatory response mediated by Kupffer cells, cyclooxygenase-2 derived eicosanoids, neutrophils, and the coagulation cascade (Roth et al., 1997; Deng et al., 2009). However, the role of transient ischemia or hypoxia in the increase in susceptibility of hepatocytes to hepatotoxicity induced by particular drugs has yet to be fully established.

Nearly half of the drugs with a Food and Drug Administration black box warning for hepatotoxicity induce mitochondrial dysfunction (Dykens and Will, 2007; Porceddu et al., 2012). Similarly, the co-administration of LPS and drugs such as diclofenac, amiodarone, and ranitidine is known to induce mitochondrial permeability transition (MPT) pore opening, a key indicator of mitochondrial dysfunction. MPT pore is composed of c-rings of ATP synthase and regulated by the ATP synthasome, which includes ATP synthase, adenine nucleotide translocator, and possibly other proteins (Biasutto et al., 2016). When these pores are opened, multiple mitochondrial proteins, including activators of the intrinsic pathway of apoptosis, are released into the cytosol, where they can induce cell death (Higuchi et al., 2001; Chen et al., 2019). We have previously shown that the sensitivity of mitochondria to MPT pore opening is higher in liver mitochondria isolated from type 2 diabetic rats, in which mitochondrial oxidative stress is present (Segawa et al., 2018). Considering that mitochondrial oxidative stress affects MPT pore opening and LPS-induced ischemia or hypoxia causes oxidative stress, we hypothesized that mitochondrial function may be disturbed in the liver of rats administered LPS. To test this hypothesis, we determined the functional changes and MPT pore opening sensitivity of liver mitochondria in a model of LPS/diclofenac co-administration-induced liver injury.

MATERIALS AND METHODS

Chemicals
LPS from *Escherichia coli* O127:B8 (lot number: 103V4051, 064M4021V, or 037M4067V) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The endotoxin content was > 500,000 endotoxin units (EU)/mg. Diclofenac sodium and heparin sodium were from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan).

Animals
Male Wistar rats (150-200 g, 7-8 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed in an air-conditioned room (25°C) under a 12 hr light-dark cycle for 1 week before use. Food (MF diet, Oriental Yeast Co., Tokyo, Japan) and water were provided *ad libitum*. The animals were treated in accordance with guidelines issued by the National Institutes of Health (Bethesda, MD, USA) and all procedures were approved by the Animal Care Committee of Chiba University.

Animal treatment and plasma ALT activity measurement
The dose of each reagent and the time course used have been previously described (Zou et al., 2009). To assess the effect of LPS and diclofenac on plasma alanine transaminase (ALT) activity, rats were administered LPS (1 mg/kg) or saline i.p. and simultaneously admin-
Lipoplysaccharide increases liver mitochondrial permeability

istered heparin (3,000 units/kg) or saline s.c. Two hours later, the rats were administered diclofenac (100 mg/kg) or vehicle (10% dimethyl sulfoxide [DMSO] in saline) i.p. Twelve hours after diclofenac administration, blood samples were taken and plasma ALT activity was measured using a previously reported method (Reitman and Frankel, 1957), with some modifications as described (Takemura et al., 2019). Briefly, rat blood samples were centrifuged at 15,000 × g for 30 sec to obtain plasma. Fifty microliters of ALT substrate in PBS (pH 7.4) containing 30 mL of the same buffer. The homogenates of residual blood, and then cut into small cubes with scissors and homogenized five times in a Potter homogenizer. The livers were infused with ice-cold buffer composed of 250 mM sucrose, 10 mM HEPES-KOH (pH 7.4), and 0.3 mM EGTA to remove mitochondrial fractions were prepared as described previously (Okuda et al., 1951). The livers were infused with 225 mM sucrose, 10 mM KCl, 5 mM MgCl₂, 5 mM KH₂PO₄, 0.5 mM EGTA, and 20 mM Tris-HCl (pH 7.4). Mitochondrial respiratory activity was evaluated as previously described (Chance and Williams, 1955), with modifications. Four millimolar glutamate, 0.8 mM malate (complex I substrate), and 4 mM succinate (complex II substrate) were used as respiratory substrates. Freshly-prepared mitochondrial suspensions were diluted to 1 mg/mL protein using respiration buffer composed of 225 mM sucrose, 10 mM KCl, 5 mM MgCl₂, 5 mM KH₂PO₄, 0.5 mM EGTA, and 20 mM Tris-HCl (pH 7.4). Respiratory substrates were then added to each suspension, which was incubated for 2 min at 30°C. Then, the mixture was incubated at room temperature for 20 min. Finally, 500 µL of 0.4 M NaOH was added, and the mixture was incubated at room temperature for 5 min, and the absorbance at 492 nm was measured using a Multiskan JX (Labsystems, Inc., Vienna, VA, USA). A calibration curve was constructed using 1.5 mM sodium pyruvate and 0.5% chloroform in PBS (pH 7.4), and the Karmen value for each sample was calculated.

Isolation of liver mitochondria

Rats were administered LPS (1 mg/kg) or saline i.p. and simultaneously administered heparin (3,000 units/kg) or saline s.c. Two hours later, the rats were decapitated and their livers were isolated. Liver mitochondria were centrifuged at 7,500 × g for 5 min, the ethanol was removed, and the pellets were dried. The RNA pellets were dissolved in diethylpyrocarbonate-treated water and the RNA concentrations were measured using a BioSpec-nano (Shimadzu Corporation, Kyoto, Japan). Reverse transcription (RT) was performed using ReverseTra Ace® qPCR RT Master Mix (Toyobo, Tokyo, Japan) and an MJ Mini-i™ Gradient Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). qPCR was performed using Thunderbird® SYBR® qPCR Mix (Toyobo) and a LightCycler® Nano (Roche Diagnostics, Rotkreuz, Switzerland). The reaction mixture and protocol have been described previously (Aoto et al., 2018). The primer sequences were as follows: β-actin forward, 5'-TTCAACACCCCAAGCATGTACG-3’; and reverse, 5'-GTGGTGGTGGAAGCGTGTAGCC-3’; inducible nitric oxide synthase (Nos2) forward, 5'-GCTGAAATCCCTCCAGAAT-3’, and reverse, 5'-CTCTTGTCCTTTGACCCAGCATGTACG-3’; chemokine (C-X-C motif) ligand 10 (Ccl10) forward, 5'-GATCGACTTCATTCCAGAAGTT-3’, and reverse, 5'-GATTCCCCATTCAAACCTTTC-3’; and heme oxygenase 1 (Hmox1) for- ward, 5'-GATCGACTTCATTCCAGAAGTT-3’, and reverse, 5'-GATTCCCCATTCAAACCTTTC-3’. Measurement of mitochondrial respiratory activity using oxygen consumption rate

Mitochondrial respiratory activity was evaluated as previously described (Chance and Williams, 1955), with modifications. Four millimolar glutamate, 0.8 mM malate (complex I substrate), and 4 mM succinate (complex II substrate) were used as respiratory substrates. Freshly-prepared mitochondrial suspensions were diluted to 1 mg/mL protein using respiration buffer composed of 225 mM sucrose, 10 mM KCl, 5 mM MgCl₂, 5 mM KH₂PO₄, 0.5 mM EGTA, and 20 mM Tris-HCl (pH 7.4). Respiratory substrates were then added to each suspension, which was incubated for 2 min at 30°C. Then,
200 µM ADP was added to the suspension to start the reactions. The oxygen consumption rate was measured using an oxygen sensor (GU-AM; Iijima Electronics Corporation, Aichi, Japan) and a dissolved oxygen meter (B-505; Iijima Electronics Corporation).

**Evaluation of lipid peroxidation using a TBARS assay**

A thiobarbituric acid-reactive substance (TBARS) assay was performed using a previously described method (Díaz et al., 2014), with modifications. Liver or mitochondrial suspensions were centrifuged at 15,000 × g and 4°C for 15 min, and the supernatant was removed. The pellet was washed three times using MilliQ water and then resuspended in 700 µL of the same. The wash and centrifugation processes were then repeated. 1,1,3,3-tetraethoxypropane was used as a standard, and the following procedures were performed using this and the samples. The samples were transferred to shading spitz, to which 0.5 mL of 15% (w/v) trichloroacetic acid and 2 mL of TBA reagent containing 20 mM 2-thiobarbituric acid and 3.86% perchloric acid were added, and the mixtures were then incubated in a boiling water bath for 15 min. After cooling on ice, the mixtures were centrifuged at 1,600 × g for 15 min and the absorbance spectrum of the supernatants was measured between 400 and 600 nm using a UV-2550 spectrophotometer (Shimadzu Corporation). The color of the standard was pink (the spectrum peak was at 532 nm), while the color of the samples was yellow, likely because of other endogenous substance(s) with an overlapping absorption spectrum (the spectrum peaks were ~440 nm). To obtain the net absorption corresponding to TBARS, a straight line connecting the absorption at 480 nm and 570 nm was regarded as the background and subtracted from the magnitudes of the absorption peaks at 532 nm. The protein concentrations of the samples were determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

**Cardiolipin extraction and analysis**

Mitochondrial cardiolipin was extracted using a previously described method (Martens et al., 2015), with modifications. Two hundred microliters of mitochondrial suspension (50 mg/mL) were added to 4.2 mL of chloroform/methanol (2/1, v/v) containing 0.05% butylated hydroxytoluene. The lipid and aqueous phases were separated by the addition of 800 µL of 0.01 M HCl, vigorous shaking, and centrifugation at 1,600 × g for 10 min. After centrifugation, the lipid (lower) phase was collected and dried under a nitrogen atmosphere. The thin film of cardiolipin was suspended in 0.8 mL n-hexane/2-propanol/methanol/H₂O (5/15/2/3, v/v/v/v), and then filtered through Millex®-LH 0.45 µm filters (Merck Millipore, Tokyo, Japan). Cardiolipin was analyzed by high-performance liquid chromatography (HPLC-UV) (at 206 nm for the reduced form and 235 nm for the oxidized form of cardiolipin).

**Mitochondrial swelling assay**

Mitochondrial swelling was evaluated using a previously described method (Masubuchi et al., 2002), with modifications. Mitochondria (0.5 mg/mL) were incubated in a reaction buffer composed of 125 mM sucrose, 65 mM KCl, 5 mM succinate, 10 mM HEPES-KOH, and 2.5 µM rotenone. The absorbance at 540 nm was then monitored for 150 sec or 1,500 sec using a UV Probe (Shimadzu Corporation), after adding 100 µM diclofenac and/or 5 µM cyclosporin A (CsA).

**Statistical analysis**

Statistically significant differences between conditions were identified using one-way analysis of variance with Tukey’s Multiple Comparison Test for the rat LPS-administration experiments. A p-value < 0.05 was considered to represent statistical significance. Statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software; San Diego, CA, USA).

**RESULTS**

**Plasma ALT activity is increased by LPS/diclofenac co-administration and reduced by heparin**

Administration of 100 mg/kg diclofenac i.p. alone did not affect plasma ALT activity. However, in rats pre-administered with 1 mg/kg LPS (2 hr before diclofenac administration), 100 mg/kg diclofenac caused plasma ALT activity to increase versus rats administered LPS or diclofenac alone (Fig. 1). However, plasma ALT activity was reduced by the co-administration of heparin (Fig. 1). These results are consistent with the previously established concept that liver injury in this rodent model is coagulation-dependent.

**Effect of LPS on the expression of cytokine-induced genes**

The expression of genes downstream of cytokine receptors that are known to be induced by LPS was measured using RNA extracted from the livers of rats euthanized 2 hr after LPS administration (Fig. 2). Nos2 is a downstream gene induced by both TNF-α and IL-1β (Kwon and George, 1999; Geller et al., 1995), while Cxcl10...
and Hmox1 are downstream of IFN-γ and IL-6 signaling, respectively (Luster and Ravetch, 1987; Tron et al., 2006). As expected, the expression of all these genes was significantly increased by LPS treatment alone, and only the induction of Hmox1 by LPS was significantly suppressed by heparin.

Effect of LPS on mitochondrial respiratory chain complex activities

It has been reported that hypoxia/reperfusion promotes reactive oxygen species (ROS) production by the mitochondrial respiratory chain (Vanden Hoek et al., 1998; Chouchani et al., 2014), implying that respiratory chain complex activities may be affected by LPS administration. Therefore, respiratory chain complex I to IV activities were measured in mitochondria prepared from control, LPS-administered, and LPS/heparin co-administered rat liver. However, these activities were not affected by LPS (data not shown). In addition, the mitochondrial respiratory activity was assessed by monitoring oxygen consumption using two kinds of respiratory substrates. Respiration via complex I was measured in the presence of glutamate and malate, while that via complex II was measured in the presence of succinate (Fig. 3). There were no significant differences in oxygen consumption via complex I among the three groups, but oxygen consumption via complex II was significantly higher in LPS-administered rats than in the control group, and this effect was abrogated by the co-administration of heparin.

Effect of LPS on mitochondrial redox reactions

According to Chouchani et al. (Chouchani et al., 2014), liver mitochondria may suffer from oxidative stress on the basis of an effect of LPS on complex II. Therefore, oxidative stress in the liver and liver mitochondria were evaluated by measuring the concentrations of lipid per-oxidation end-products. Although no differences between groups were observed in liver homogenates, LPS administration caused a significant increase in these end-products in liver mitochondria versus saline-administered controls (Fig. 4). In addition, the concentration of cardiolipin, a phosphatide that is abundant in mitochondria and known to be involved in several mitochondrial functions, including MPT pore opening (Nakagawa, 2011), was measured. We found that the ratio of the oxidized to the reduced forms of cardiolipin was significantly higher in the LPS-administered group (Fig. 5), and this increase was not abrogated in the LPS/heparin co-administered group.

LPS sensitizes mitochondria to MPT pore opening by diclofenac

Some drugs induce MPT, which manifests as mitochondrial swelling. Subsequently, cytochrome c is released from mitochondria into the cytosol, where it participates in the irreversible activation of the apoptotic cascade. Mitochondrial swelling can be evaluated by monitoring the absorbance of a preparation of isolated mitochondria at 540 nm. Mitochondria from the LPS-administered group exhibited more swelling than the other groups in response to diclofenac. This swelling was inhibited by CsA, which binds to cyclophilin D, a regulator of MPT pore opening (Fig. 6A, B). Moreover, diclofenac-induced swelling was not observed in LPS/heparin co-administered rat liver mitochondria (Fig. 6C, D). Although the onset times of swelling in the two data sets (Fig. 6A vs. Fig. 6B) differed depending on the experimental day, the mitochondria of the LPS groups responded to diclofenac and showed more pronounced swelling than the other groups. These findings imply that the sensitivity to MPT pore opening is increased by LPS in a coagulation-dependent manner, and this might have a significant role in LPS/diclofenac-induced hepatocyte death.

DISCUSSION

LPS has a number of effects in vivo, including activation of the innate immune response, induction of...
Fig. 2. The effect of LPS and heparin on the expression of genes downstream of cytokine receptors. Liver mRNA expression of A) Nos2, B) Cxcl10, and C) Hmox1. Rats were administered LPS (1 mg/kg) or saline i.p. and simultaneously administered heparin (3,000 units/kg) or saline s.c. Two hours later, the rats were decapitated and their livers were isolated for RNA preparation and qPCR analysis. Data are shown as means ± S.E. (n = 3, *p < 0.05).

Fig. 3. The effects of LPS and heparin on mitochondrial respiratory activity. Four millimolar glutamate and 0.8 mM malate (complex I substrate, A) and 4 mM succinate (complex II substrate, B) were used as respiratory substrates. Rats were administered LPS (1 mg/kg) or saline i.p., and simultaneously administered heparin (3,000 units/kg) or saline s.c. Two hours later, the rats were decapitated and their livers were isolated for respiratory activity testing. Data are shown as means ± S.E. (n = 3 (via complex I), n = 4 (via complex II), *p < 0.05).
inflammatory cytokines, and the temporary induction of ischemia by activation of the blood coagulation cascade (Pernerstorfer et al., 1999). In the present study, the co-administration of heparin reduced the toxicity caused by the administration of a combination of LPS and diclofenac in rats, but did not suppress the induction of Nos2 or Cxcl10, which are genes downstream of inflammatory cytokine receptors. It has been reported that inflammatory cytokines, such as TNF-α, upregulate blood coagulation (Schleef et al., 1988), and taking these and the present findings together, it is likely that LPS initially induces the production of inflammatory cytokines, which cause local ischemia in the liver. The co-administration of heparin reduced the expression of Hmox1 mRNA, likely as a result of the inhibition of blood coagulation and the subsequent ischemia. Importantly, LPS/diclofenac-induced toxicity was suppressed by the co-administration of heparin. Thus, it is possible that ischemia is directly involved in the mechanism of LPS/diclofenac-induced liver injury. Diclofenac is well known to induce mitochondrial dysfunction (Boelsterli, 2003) and some of the drugs, including amiodarone and sulindac, that cause liver injury in the LPS-preconditioned rat model are also known to cause mitochondrial dysfunction (Seo et al., 2007; Spaniol et al., 2001). Therefore, we focused on the mitochondrial function of the liver of rats pre-administered with LPS.

We first determined whether the activities of respiratory chain complexes were affected by LPS administration. Although significant changes in activity were not identified for any of the four complexes (data not shown), respiratory activity via complex II was significantly higher in LPS administrated rat liver mitochondria (Fig. 4B). Whereas we first thought that uncoupling of the respiratory chain would lead to greater free radical generation, resulting in an apparent increase in oxygen consumption, in fact the ATP synthesis rate, coupled with oxygen consumption, was not affected by the treatments (data not shown). Consistent with our initial hypothesis, it has
been reported that ischemia or the administration of LPS upregulates respiratory activity and the expression of ATP synthase (Fuller et al., 1985; Miller et al., 2006). However, although the mechanism has not yet been fully characterized, it may be explained as follows. Complex II, a succinate dehydrogenase, is known to transform succinate to fumarate under normal conditions. However, under ischemic conditions, it transforms fumarate to succinate, and the accumulated succinate is suddenly reconverted to fumarate after reperfusion (Chouchani et al., 2014), which results in the production of a lot of ROS by complex I. This scheme is referred to as reverse electron transport (RET) (Chouchani et al., 2014). Although the mechanism of the upregulation of respiratory activity via complex II that was identified in LPS-administered rat liver is unclear, such a change may lead to an increase in RET and greater ROS production (Chouchani et al., 2014; Mills and O’Neill, 2014). However, further study is required to test this hypothesis.

The rate of lipid peroxidation was higher in mitochondria but not in liver homogenate following LPS administration, and it is possible that RET is involved in such mitochondria-specific oxidative stress. Interestingly, cardiolipin, a mitochondrial phosphatide known to be involved in many mitochondrial functions, including mitochondrial respiratory chain complex activities and MPT pore opening (Nakagawa, 2011), was significantly more oxidized following LPS administration, and this tended to be ameliorated by heparin co-administration. Recently, it has been reported that the Sod2 heterozygous knockout mouse exhibits higher sensitivity to mitochondrial damage (Lee et al., 2013), which prompted us to
determine the effect of LPS on MPT pore opening.

MPT is known to be a key event in mitochondrial dysfunction and subsequent cell death, it occurs in many diseases (Norenberg and Rao, 2007; Halestrap and Pasdois, 2009; Du and Yan, 2010), and is also induced by numerous drugs, including diclofenac (Masubuchi et al., 2002). Indeed, liver mitochondria isolated from rats administered LPS are more susceptible to the induction of MPT by diclofenac. It is probable that MPT pore opening directly induces cell death via the release of cytochrome C into the cytosol. Alternatively, MPT pore opening may indirectly induce hepatocyte death in vivo. More specifically, mtDNA released from mitochondria into the cytosol may activate TLR9, resulting in the release of chemokines/cytokines from hepatocytes and the recruitment of neutrophils to liver to kill the hepatocytes (Cai et al., 2017).

In the present study, we have demonstrated that ischemia/reperfusion changes the sensitivity of mitochondria to MPT pore opening by a potentially hepatotoxic drug. We have provided preliminary evidence that CsA, an MPT pore opening inhibitor that is thought to bind to cyclophilin D, protects against liver injury induced by a combination of LPS and diclofenac. However, CsA did not suppress the increase in ALT activity induced by LPS/diclofenac co-administration in vivo (data not shown). Recently, it has been reported that CsA not only inhibits MPT pore opening, but also inhibits bile salt export pump (Stieger et al., 2000), induces endoplasmic reticulum stress (Rao et al., 2017), and induces oxidative stress (Klawitter et al., 2019; Wu et al., 2018). These pleiotropic effects of CsA make it difficult to assess the importance of MPT in the LPS/drug co-administration rodent liver injury model. To more definitively determine whether MPT opening is involved in the increase in plasma ALT activity in vivo, it would be better to use an MPT-deficient model, such as the cyclophilin D knockout mouse (Schinzel et al., 2005).

In summary, the increase in plasma ALT activity induced by LPS/diclofenac co-administration is suppressed by heparin, the sensitivity to MPT and liver injury was increased by transient ischemia, and RET induced by the transient ischemia may be responsible for the increase in mitochondrial ROS production and MPT sensitivity. A diagram summarizing our present findings is shown as Fig. 7. The relationship between MPT and liver injury remains unclear, but LPS-induced transient ischemia is involved in both the increase in MPT sensitivity and the liver injury caused by diclofenac. The present findings suggest that oxidative stress may be responsible for the increase in MPT pore opening sensitivity that may underpin the DILI induced by particular drugs. This finding may be applicable to the construction of cell-based mitochondrial toxicity assays. We have previously succeeded in identifying respiratory chain complex inhibitors and oxidative stress inducers in a hepatocyte culture system that avoids the Crabtree effect (Liu et al., 2016). However, we have not succeeded in identifying MPT-inducers, such as troglitazone, benzbromarone, and diclofenac, using this system (Liu et al., 2016). However, when other appropriate conditions, such as hypoxia-induced oxidative stress, are combined with the in vitro system, it should be possible to also identify such latent MPT-inducers. A trial...
of such a system is currently under way in our laboratory.

ACKNOWLEDGMENTS

This work was supported by Japan Society for the Promotion of Science KAKENHI grant number 26460190, 18K06744, and 19H03386.

Conflict of interest---- The authors declare that there is no conflict of interest.

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Lipopolysaccharide increases liver mitochondrial permeability