Protective effects of cilostazol on ethanol-induced damage in primary cultured hepatocytes

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Abstract Alcoholic liver disease (ALD) caused by excessive alcohol consumption is associated with oxidative stress, mitochondrial dysfunction, and hepatocellular apoptosis. Cilostazol, a licensed clinical drug used to treat intermittent claudication, has been reported to act as a protective agent in a spectrum of diseases. However, little information regarding its role in ethanol-induced hepatocellular toxicity has been reported. In the current study, we investigated the protective effects and mechanisms of cilostazol on ethanol-induced hepatocytic injury. Rat primary hepatocytes were pretreated with cilostazol prior to ethanol treatment. MTT and LDH assay indicated that ethanol-induced cell death was ameliorated by cilostazol in a dose-dependent manner. Our results display that overproduction of intracellular reactive oxygen species (ROS) and 4-hydroxy-2-nonenal (4-HNE) induced by ethanol was attenuated by pretreatment with cilostazol. Furthermore, cilostazol significantly inhibited ethanol-induced generation of ROS in mitochondria. Importantly, it was shown that cilostazol could improve mitochondrial function in primary hepatocytes by restoring the levels of ATP and mitochondrial membrane potential (MMP). Additionally, cilostazol was found to reduce apoptosis induced by ethanol using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Mechanistically, we found that cilostazol prevented mitochondrial pathway-mediated apoptotic signals by reversing the expression of Bax and Bcl2, the level of

cleaved caspase-3, and attenuating cytochrome C release. These findings suggest the possibility of novel ALD therapies using cilostazol.

Keywords Alcoholic liver disease · Ethanol · Cilostazol · Oxidative stress · Mitochondria · Apoptosis

Introduction

Alcoholic liver disease (ALD) develops through excessive and chronic alcohol consumption and is the most common lifestyle-related liver disease (Byrne and Targher 2015). Accumulating evidence indicates that alcohol intake causes hepatocyte injury and death, both via apoptotic and necroptotic pathways (Gao and Bataller 2011). Mitochondrial dysfunction has been reported to be the earliest event in the development of ALD. Chronic ethanol consumption results in decreased mitochondrial protein synthesis, impairment of the oxidative phosphorylation system, and depression in the rate of adenosine triphosphate (ATP) synthesis in hepatic mitochondria (Cunningham and Bailey 2001). Exposure to ethanol leads to increased levels of reactive oxygen species (ROS) in hepatic mitochondria. As a matter of fact, increasing evidence has shown that oxidative stress plays an essential role in ethanol-induced liver injury (Hoek et al. 2002). Overproduction of ROS initiates lipid peroxidation, glutathione depletion, and abnormal methionine metabolism in hepatocytes. Notably, increased hepatocyte apoptosis has been found to occur during exposure to ethanol (Canbay et al. 2004). So far, a myriad of factors and signaling pathways have been associated with ethanol-induced hepatocytic injury. Prevention of mitochondrial-mediated cytotoxicity and apoptosis has become an important therapeutic strategy for the treatment of ALD.

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Cilostazol, a specific inhibitor of phosphodiesterase III, is a clinical drug used to treat intermittent claudication (Dawson et al. 1998). Recent studies have shown that cilostazol exerts strong protective effects against various diseases, such as coronary artery restenosis (Choi et al. 2002) and focal cerebral ischemia (Lee et al. 2006). A recent *in vitro* study demonstrated that cilostazol prevented cardiac mitochondrial dysfunction caused by H_2O_2 -induced severe oxidative stress by ameliorating mitochondrial swelling, inhibiting mitochondrial depolarization, and reducing ROS production in cardiac mitochondria (Takagi et al. 2014). Additionally, cilostazol was reported to ameliorate lipopolysaccharide (LPS)-induced apoptosis in human umbilical vein endothelial cells through the mitochondrial pathway (Lim et al. 2009). Inhibition of the mitochondria-dependent apoptotic-signaling pathway has been considered as the main mechanism through which cilostazol exerts its protective effect against apoptotic cell death (Choi et al. 2002). Notably, cilostazol treatment results in reversal of the decrease in Bcl-2 and increases in Bax proteins and cytochrome C release induced by LPS (Kim et al. 2002). However, little information has been reported regarding the protective effects of cilostazol against ethanol-induced hepatocytic injury. Therefore, in this study, we investigated the protective effects of cilostazol against ethanol-induced hepatocytic injury.

Materials and methods

Isolation and culture of rat primary hepatocytes

Animal experiments were carried out under protocol approved by the Institutional Animal Care and Use Committee at Zhengzhou University and conformed with the guidelines for the care and use of laboratory animals. Primary hepatocytes were isolated from male Sprague–Dawley rats using a two-step collagenase perfusion method (Nagy and de Silva 1994). Seven-week-old male rats were anesthetized via intravenous injection with pentobarbital (50 mg/kg). Liver perfusion was conducted using Hank's-HEPES buffer (135 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.34 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , and 3 mg/mL HEPES) with 0.58 mM EGTA (Hank's-EGTA solution) for 3 min, followed by secondary perfusion using Hank's-HEPES buffer with 5 mM $CaCl_2$ and 1 mg/mL of collagenase (Hank's-collagenase solution) for 5 min at 37 °C. Cell viability was >90% as determined using the trypan blue dye exclusion method. Primary hepatocytes were seeded at 2.13×10^5 cells per cm^2 in 10 cm dishes (Nalge Nunc) in Ham's F-12/Dulbecco's modified Eagle's basal growth medium supplemented with 10% fetal bovine serum (Hyclone Lab Inc., Logan, UT), and 1% antibiotics. Primary cultured hepatocytes were treated with cilostazol at concentrations of 5, 10, and

25 μM for 24 h, followed by treatment with 50 mM ethanol (Bakhautdin et al. 2014) for another 24 h.

Cell viability determination

Cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) method.

Primary hepatocytes were seeded into 96-well plates at 5×10^5 cells/well and incubated for 24 h. Then, cells were treated with cilostazol at concentrations of 5, 10, and 25 μM for 24 h, followed by treatment with 50 mM ethanol for another 24 h. MTT reagent was added to each well at a final concentration of 5 mg/mL and incubated for 4 h at 37 °C in darkness. The insoluble formazan was dissolved in dimethyl sulfoxide (DMSO). Absorbance measured at 570 nm using an ELISA reader (Tecan, Salzburg, Austria) was used to calculate the viability percentage. The “relative values” presented in the data were achieved by normalizing the results to the “untreated” group, which represents the “fold of control.”

Measurement of lactate dehydrogenase release

Release of lactate dehydrogenase (LDH) into the culture medium is a marker for cell membrane damage. Primary hepatocytes were seeded into 96-well plates at 5×10^5 cells/well and incubated for 24 h. Then, cells were treated with cilostazol at concentrations of 5, 10, and 25 μM for 24 h, followed by treatment with 50 mM ethanol for another 24 h. Next, 50 μL of each sample medium was transferred into triplicate wells in a 96-well flat-bottom plate, and then 50 μL of reaction mixture was added to each sample well and mixed using a multi-channel pipette. The plate was maintained in darkness at room temperature (RT) for 30 min. The reaction was stopped using 50 μL stop solution. Absorbance was recorded at 490 nm to calculate the amount of LDH release (Sheng et al. 2009). The “relative values” presented in the data were achieved by normalizing the results to the “untreated” group, which represents the “fold of control.”

Reactive oxygen species determination

Levels of intracellular total ROS were determined using a fluorescence probe—2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Primary hepatocytes were treated with cilostazol at concentration of 5, 10, and 25 μM for 24 h, followed by treatment with 50 mM ethanol for another 24 h. Hepatocytes were washed with HBSS and loaded with 10 μM DCFH-DA (Sigma-Aldrich, USA) in a CO_2 incubator for 30 min at 37 °C in darkness. Cells were then washed three times with HBSS. Green fluorescent signals were captured by an LSM5 live configuration Variotwo VRGB microscope (Zeiss, Jena, Germany) equipped with an objective water

immersion lens (485 nm excitation wavelength/538 nm emission wavelength) at a magnification of $\times 20$. Average optical intensity of the green dye was analyzed using Image J software to index the level of intracellular ROS. The “relative values” presented in the data were achieved by normalizing the results to the “untreated” group, which represents the “fold of control.”

4-Hydroxy-2-nonenal immunofluorescence staining

Intracellular 4-hydroxy-2-nonenal (4-HNE) was determined using the immunofluorescence staining method. Hepatocytes were washed three times with PBS and fixed in 4% paraformaldehyde in PBST (PBS + Tween-20) for 10 min at RT. Then, cells were permeabilized with 0.4% Triton X-100 on ice for 15 min. Cells were then blocked with 5% BSA and 2.5% FBS in PBST for 1 h at RT and incubated with anti-4-HNE primary antibody (Cell Signaling Technology, USA) for 2 h at RT and Alexa-594-conjugated secondary antibodies (Thermo Fisher Scientific, USA) for another 1 h at RT. Staining signals were captured using an LSM5 live configuration Variotwo VRGB microscope (Zeiss, Jena, Germany) equipped with an objective water immersion lens (550 nm excitation wavelength and 600 nm emission) at a magnification of $\times 20$. Average optical intensity of the green dye was analyzed using Image J software to index the level of intracellular 4-HNE. The relative values presented in the data were achieved by normalizing the results to the untreated group, which represents the fold of control.

Assessment of mitochondrial ROS

Levels of mitochondrial ROS were evaluated using a MitoSOX Red (Invitrogen, USA) fluorescence probe in accordance with the manufacturer's instructions. Primary cultured hepatocytes were seeded onto 35 mm dishes with a glass insert designed for live cell imaging (MatTek Corporation, Ashland, USA). After 12 h, cells were treated with cilostazol at concentrations of 10 and 25 μM for 24 h, followed by treatment with ethanol for another 24 h. Cells were washed with PBS and incubated with MitoSOX Red at a concentration of 5 μM solution in phosphate buffer saline (PBS) containing 100 mM calcium and magnesium (PBS/Ca/Mg) for 10 min at 37 °C in darkness.

Fluorescent signals were captured with a Zeiss LSM510 laser scanning confocal microscope (Zeiss, Jena, Germany) using the $\times 40$ objective. Fluorescent intensity was evaluated using LSM510 Zen software, which allowed for the measurement of fluorescent intensity within a selected region of interest. All confocal image acquisition settings were constant throughout the experiment. The relative values presented in the data were achieved by normalizing the results to the untreated group, which represents the fold of control.

Determination of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured using a tetramethylrhodamine methyl ester (TMRM) fluorescence probe. Hepatocytes were washed three times with PBS and loaded with 20 nmol/L TMRM (Invitrogen, USA) for 60 min at RT. Fluorescence signals captured using a fluorescence microscope were used to calculate the average fluorescence intensity to index the level of MMP. The relative values presented in the data were achieved by normalizing the results to the untreated group, which represents the fold of control.

Intracellular adenosine triphosphate determination

Intracellular levels of ATP were measured using a Molecular Probes ATP Determination Kit containing a convenient bioluminescence assay for quantitative determination of ATP with recombinant firefly luciferase and its substrate D-luciferin (Life Technologies, USA). Primary hepatocytes were treated with cilostazol at concentrations of 5, 10, and 25 μM for 24 h, followed by treatment with 50 mM ethanol for another 24 h. Next, 100 μL of cell lysis buffer was transferred into triplicate wells in a 96-well flat-bottom plate. Then, 100 μL of luciferin/luciferase reagent was added to each sample well and mixed using a multichannel pipette to catalyze the light production from ATP and luciferin. Light output from the reaction was measured using a luminometer. Light intensity was used to index the level of intracellular ATP. The relative values presented in the data were achieved by normalizing the results to the untreated group, which represents the fold of control.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay

Cell apoptosis of primary cultured hepatocytes was evaluated via terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay using a commercially available kit (Promega, USA) in accordance with the manufacturer's instructions. Primary hepatocytes were treated with cilostazol at concentrations of 5, 10, and 25 μM for 24 h, followed by treatment with 50 mM ethanol for another 24 h. After the indicated treatment, hepatocytes were fixed with 4% formaldehyde in PBST for 20 min and incubated with 70% ethanol at -20 °C for 30 min. Then, hepatocytes were permeabilized in 0.1% Triton X-100 for 15 min on ice, followed by incubation with 3% H_2O_2 for 5 min at RT.

Afterwards, primary hepatocytes were incubated with a terminal deoxynucleotidyl transferase enzyme (Promega, USA) in darkness at 37 °C for 90 min. Nuclei of hepatocytes were counterstained by 4'-6-diamidino-2-phenylindole (DAPI). Combining DAPI staining with TUNEL staining

allows for the comparison of TUNEL-positive nuclei with surrounding normal nuclei. Signals were captured using an LSM5 live configuration Variotwo VRGB microscope (Zeiss, Jena, Germany) equipped with a $\times 20$ objective lens. Cells with TUNEL-positive nuclei were apoptotic. The number of positive stained cells is expressed as a percentage of the total number of cells counted in the 10 tissue sections. The percentage of TUNEL-positive cells was calculated from the total number of 2000 cells.

Subcellular fractionation

Fractionation of mitochondria and cytosol was carried out using a mitochondria isolation kit for cultured cells (#89874, Thermo Fisher Scientific, USA). After the indicated treatment, hepatocytes were harvested and suspended in 800 μL of mitochondria isolation reagent A. Cells were homogenized in a Dounce tissue grinder on ice and mixed with 800 μL of mitochondria isolation reagent C. Lysed cells were centrifuged at $700\times g$ for 10 min at 4°C . The supernatant was transferred to a new 2.0-mL tube and centrifuged at $12,000\times g$ for 15 min at 4°C . The supernatant was then collected for cytosol fraction. Isolated mitochondria were contained within the pellet. Mitochondrial Cox 4 was used for the negative control of the purity of cytosol fraction.

Western blot analysis

After the indicated treatment, proteins were extracted from hepatocytes using a commercial cell lysis buffer (Cell Signaling, USA) with protease inhibitor cocktail (Roche, USA). A bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, USA) was used to determine protein concentrations. An equal amount of protein (20 μg) from each sample was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Sheng et al. 2012). Membranes were then blocked with 5% non-fat milk in TBS–Tween 20 for 1 h at RT. Afterwards, membranes were probed with primary antibodies overnight at 4°C and horseradish peroxidase-conjugated secondary antibody for 2 h at RT. Blots were developed using a western chemiluminescence system (Thermo Fisher Scientific, USA) and exposed onto an x-ray film. Protein expression levels were quantified by densitometric evaluation of antibody specific bands on scanned x-ray films using Image J imaging software. The relative values presented in the data were achieved by normalizing the results to the untreated group, which represents the fold of control. The following antibodies were used in this study: Rabbit monoclonal antibody against Bax (#5023, 20KD, 1:2000, Cell Signaling Technology, USA); Rabbit monoclonal antibody against Bcl-2 (#2876,

28 KD, 1:3000, Cell Signaling Technology, USA); Rabbit monoclonal antibody against cytochrome C (#4280, 14 KD, 1:1000, Cell Signaling Technology, USA); Rabbit monoclonal antibody against COX4 (#4850, 17 KD, 1:1000, Cell Signaling Technology, USA), Rabbit monoclonal antibody against cleaved caspase-3 (#9661, 17 KD, 1:1000, Cell Signaling Technology, USA), and Rabbit monoclonal antibody against β -actin (#4967, 43 KD, 1:10,000, Cell Signaling Technology, USA).

Statistical analysis

Statistical analyses were performed using IBM SPSS version 20.0 software. All experimental data, analyzed at the 95% confidence interval, are expressed as means $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to evaluate the effects of cilostazol on cell viability. Two-way ANOVA was used to evaluate the effects of cilostazol on ethanol-induced cytotoxicity, followed by Bonferroni's post-hoc analysis. p values <0.05 were considered statistically significant.

Results

To determine the concentration of cilostazol to be used in this study, we evaluated the effects of cilostazol on cell viability in primary cultured hepatocytes. Treatment of primary cultured hepatocytes with cilostazol at concentrations of 5, 10, and 25 μM had no significant effects on cell viability. However, administration of cilostazol at a final concentration of 100 μM significantly reduced mean cell viability in primary cultured hepatocytes (Fig. 1). Therefore, we used cilostazol at concentrations of 5, 10, and 25 μM to evaluate its effects on ethanol-induced reduction of cell viability in primary cultured hepatocytes.

As shown in Fig. 2a, exposure to ethanol for 24 h led to a 38% reduction in cell viability in primary cultured

Fig. 1 Cells were treated with cilostazol at concentrations of 5, 10, 25, and 100 μM for 24 h. Cell viability was determined by MTT assay. Experiments were repeated four times. (asterisk, vs. untreated control, $n = 5$)

Fig. 2 Cilostazol improved hepatocyte injury. Primary cultured hepatocytes were treated with cilostazol at concentration of 5, 10, and 25 μM for 24 h, followed by treatment with 50 mM ethanol for another 24 h (–, without ethanol; +, with 50 mM ethanol). Experiments were

repeated four times. **a** Cell viability after 24 h treatment and **b** concentration of LDH released into the culture medium (asterisk, <0.01 vs. untreated group; number sign, <0.01 vs. ethanol-treated group, $n = 6$)

hepatocytes. Notably, pretreatment with cilostazol at concentrations of 5, 10, and 25 μM significantly increased cell viability. The results in Fig. 2b indicate that, as expected, pretreatment with cilostazol at concentrations of 5, 10, and 25 μM (24 h) ameliorated ethanol-induced LDH release.

The results in Fig. 3a show that ethanol treatment resulted in a marked increase in intracellular levels of ROS in primary cultured hepatocytes, which was significantly inhibited by pretreatment with cilostazol in a dose-dependent manner (10, 25 μM). Consistent with these results, incubation with ethanol resulted in a significant increase in intracellular levels of 4-HNE (Fig. 3b), which could be prevented by pretreatment with cilostazol (10, 25 μM).

Mitochondria are important targets of free radicals. Thus, we examined ethanol-induced mitochondrial oxidant generation in primary cultured hepatocytes. As shown in Fig. 4, results indicate that levels of mitochondrial ROS were significantly increased in ethanol-treated primary

cultured hepatocytes; however, cilostazol significantly inhibited this ethanol-induced generation of mitochondrial ROS.

We then investigated the effects of cilostazol on MMP, which is an essential parameter of mitochondrial function. Our results reveal that ethanol treatment significantly reduced MMP in primary cultured hepatocytes, which could be rescued by treatment with cilostazol (Fig. 5a). Additionally, ATP levels in primary cultured hepatocytes were determined to evaluate mitochondrial function. We found that the level of ATP was significantly reduced in response to ethanol treatment, but could be restored by pretreatment with cilostazol in a dose-dependent manner (Fig. 5b).

Then, we assessed the patterns of apoptosis. The quantitative results in Fig. 6 show that ethanol exposure greatly increased cell apoptosis, while pretreatment with cilostazol partially abolished this effect. The effects of cilostazol on ethanol-induced alternation of pro-apoptotic and anti-apoptotic

Fig. 3 Cilostazol attenuated ethanol-induced oxidative stress in primary cultured hepatocytes. Cells were treated with cilostazol at concentrations of 10 and 25 μM for 24 h, followed by treatment with 50 mM ethanol for another 24 h (–, without ethanol; +, with 50 mM ethanol). Experiments were repeated for four times. **a** Levels of intracellular ROS were measured by DCFH-DA assay; **b** levels of intracellular 4-HNE were measured by immunostaining (asterisk, vs. untreated group; number sign, vs. ethanol-treated group, $n = 6$)

Fig. 4 Effects of cilostazol on mitochondria-generated reactive oxygen species (ROS). Primary cultured hepatocytes were treated with cilostazol at concentrations of 10 and 25 μ M for 24 h, followed by treatment with 50 mM ethanol for another 24 h (–, without ethanol; +, with 50 mM ethanol). Experiments were repeated four times. **a** Representative

fluorescent images of MitoSOX Red staining in primary cultured hepatocytes; **b** quantitative analyses of MitoSOX Red fluorescence signal intensity (asterisk, vs. untreated group; number sign, vs. ethanol-treated group, $n = 6$)

pathways were also determined. The Bax/Bcl-2 family comprises important regulators of the mitochondria-dependent apoptotic pathway. It was found that ethanol treatment increased the level of Bax but reduced the level of Bcl-2, which could be restored by pretreatment with cilostazol (Fig. 7).

Furthermore, the effects of cilostazol on the release of cytochrome C and activation of caspase-3 were determined. The release of cytochrome C from mitochondria into the cytoplasm plays a crucial role in programmed cell death. Enriched mitochondrial and cytosolic fractions can be used to study the release of cytochrome C. Protein was extracted from enriched cytosol fractions. The mitochondrial protein COX4 was used as a negative cytosol purity control. Western blot results indicate that cytochrome C in the cytoplasm was increased in response to ethanol treatment as

compared to that of the normal group ($p < 0.01$). However, cilostazol treatment prevented the release of cytochrome C from mitochondria into the cytosol (Fig. 8). Additionally, the results indicate that ethanol treatment significantly increased the level of cleaved caspase-3 (activated caspase-3) (Fig. 9), which was inhibited by pretreatment with cilostazol in a dose-dependent manner.

Discussion

ALD develops through excessive and chronic alcohol consumption and is a major cause of chronic liver disease. Alcohol consumption causes hepatocytic injury and cell death. It has been reported that hepatocytic injury is one of

Fig. 5 Cilostazol improved ethanol-induced mitochondrial dysfunction in primary cultured hepatocytes. Primary cultured hepatocytes were treated with cilostazol at concentrations of 10 and 25 μ M for 24 h, followed by treatment with 50 mM ethanol for another 24 h (–, without ethanol; +, with 50 mM ethanol). Experiments were repeated four times. **a** Mitochondrial membrane potential was measured by fluorescence dye tetramethylrhodamine methyl ester (TMRM) staining. **b** Intracellular level of ATP (asterisk, vs. untreated group; number sign, vs. ethanol-treated group, $n = 5-6$)

Fig. 6 Effects of cilostazol on ethanol-induced apoptosis. Primary cultured hepatocytes were treated with cilostazol at concentration of 10 and 25 μ M for 24 h, followed by treatment with 50 mM ethanol for another 24 h (–, without ethanol; +, with 50 mM ethanol). Cell apoptosis was determined by TUNEL assay. Experiments were repeated four times. Quantitative analyses of TUNEL staining are shown (asterisk, vs. untreated group; number sign, vs. ethanol-treated group)

the major hallmarks of alcoholic liver injury (Gao and Bataller 2011). Although targeted therapies for the management of ALD are still unavailable, prevention of ethanol-induced hepatocytic injury has been considered as an essential strategy for the treatment of ALD. In the current study, primary hepatocytes were used to evaluate the direct hepatocellular protective effects of cilostazol in response to challenge with ethanol. Our results indicate that cilostazol works to protect rat primary hepatocytes against ethanol-induced cytotoxicity by ameliorating oxidative stress, increasing cell viability, optimizing

mitochondrial function, and preventing apoptosis through the mitochondrial pathway.

Mitochondria-mediated ethanol metabolism plays an essential role in regulating intracellular oxidative stress. Acute and chronic alcohol treatments increase the supply of mitochondrial NAD(P)H and promote the formation of mitochondrial ROS in the liver (Hoek et al. 2002). Among the broad spectrum of pathogenesis that constitute ALD, mitochondrial pathway-mediated hepatocellular apoptosis is considered an important early response of liver cells to hepatic injury (Stewart et al. 2001). Therefore, mitochondria have become an important target for ALD therapy. Indeed, maintaining mitochondrial homeostasis is essential to cell survival in various cell types, including hepatocytes. As a licensed clinical drug, cilostazol has been used to treat patients suffering from intermittent claudication (Sudo et al. 2000). Various physiological and pharmacological properties of cilostazol have been investigated in previous studies. Importantly, cilostazol has been reported to regulate mitochondrial homeostasis by promoting mitochondrial biogenesis. Administration of cilostazol in human umbilical vein endothelial cells increases intracellular ATP, mitochondrial DNA, cytochrome B expression, and mitochondrial mass (Luning et al. 2013). In the current study, we found that cilostazol ameliorated ethanol-induced generation of ROS and the production of 4-HNE. ROS production has been linked to damage to mitochondrial DNA, membranes and protein, and alcohol-dependent metabolic derangements in the liver (Hoek et al. 2002). Mitochondria

Fig. 7 Cilostazol reversed the effects of ethanol on the expression of Bax and Bcl-2. Primary cultured hepatocytes were treated with cilostazol at concentrations of 10 and 25 μ M for 24 h, followed by treatment with 50 mM ethanol for another 24 h (–, without ethanol; +, with 50 mM ethanol). Experiments were repeated four times. **a** Representative images of immunoblots for Bax and Bcl-2; **b** quantitative analysis of Bax protein levels; **c** quantitative analysis of Bcl-2 protein levels (asterisk, vs. untreated group; number sign, vs. ethanol-treated group, $n = 5$)

Fig. 8 Cilostazol abolished ethanol-induced release of cytochrome C. Primary cultured hepatocytes were treated with cilostazol at concentrations of 10 and 25 μ M for 24 h, followed by treatment with 50 mM ethanol for another 24 h (–, without ethanol; +, with 50 mM ethanol). Cytochrome C release was determined by western blot analysis.

Experiments were repeated four times. **a** Representative images of cytochrome C in cytosol; **b** Quantitative analysis of cytochrome C in cytosol (*asterisk*, vs. untreated group; *number sign*, vs. ethanol-treated group, $n = 5$)

are important sources of free radicals and pro-apoptotic factors. Notably, our results indicate that cilostazol inhibited the generation of mitochondrial ROS induced by ethanol, suggesting that it has a direct impact on ameliorating mitochondrial oxidative stress. A previous study showed that cilostazol and its analogs inhibit apoptotic cell death by scavenging hydroxyl radicals and intracellular ROS (Kim et al. 2002). Overproduction of intracellular free radicals leads to attacks on mitochondrial membrane phospholipids and induces the collapse of MMP, which then promotes the release of cytochrome C from mitochondria into the cytosol and triggers caspase-3 activation, thereby inducing apoptosis. In the current study, we showed that cilostazol restored the effects of ethanol on Bax/Bcl-2 expression, cytochrome C release, and

caspase-3 activation, suggesting that cilostazol protects primary hepatocytes against ethanol-induced apoptosis through the mitochondrial pathway. Consistently, cilostazol exerted a strong protective effect against apoptotic cell death in endothelial cells by destroying the mitochondrial permeability transition and preventing cytosolic cytochrome C release (Lim et al. 2009).

Taken together, the results of the present in vitro primary hepatocyte culture and cellular experiment study show that cilostazol offers significant protection against ethanol-induced hepatocytic injury by modulating the mitochondrial apoptotic pathway. The pleiotropic effects of cilostazol on ethanol-induced cytotoxicity suggest that cilostazol may have a potential role in the successful prevention and treatment of ALD.

Fig. 9 Cilostazol reversed the effects of ethanol on the expression of cleaved caspase-3. Primary cultured hepatocytes were treated with cilostazol at concentrations of 10 and 25 μ M for 24 h, followed by treatment with 50 mM ethanol for another 24 h (–, without ethanol; +, with 50 mM ethanol). Cleaved caspase-3 was determined by western blot analysis. Experiments were repeated four times. **a** Representative images of cleaved caspase-3; **b** quantitative analysis of cleaved caspase-3 (*asterisk*, vs. untreated group; *number sign*, vs. ethanol-treated group, $n = 5$)

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interests.

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