Cilostazol attenuates murine hepatic ischemia and reperfusion injury via heme oxygenase-dependent activation of mitochondrial biogenesis

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Hepatic ischemia-reperfusion (I/R) injury can arise as a consequence of circulatory shock, liver transplantation, or partial hepatectomy. I/R injury can trigger the inflammatory response and hepatocyte apoptosis, both of which may contribute to organ dysfunction (15, 29, 30). The molecular mechanisms underlying hepatocellular damage induced by I/R currently remain incompletely understood but may involve the generation of reactive oxygen species (ROS) and the activation of inflammatory pathways (18, 29). In addition, the mitochondria may play a critical role in the propagation of I/R injury. Since increases in ROS generation during I/R may arise from impaired mitochondria (7, 22), the preservation of mitochondrial function in I/R injury may represent a potentially promising therapeutic strategy.

Heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme degradation, is a stress protein that participates in cellular defense (28). HO-1 is induced by a variety of chemical and physical agents, including electrophiles and oxidants, and responds to transcriptional regulation by the NF-E2-related factor-2 (Nrf2) transcription factor (17). Upregulation of HO-1 can confer cellular protection in organ injury models including various forms of I/R injury (3, 36).

The mechanisms underlying HO-1-dependent cytoprotection remain unclear but may involve the generation of bioactive end-products from heme degradation, including biliverdin IXα, iron, and carbon monoxide (CO) (28). Increasing evidence suggests that HO-1, which can localize to mitochondria, may preserve mitochondrial function (31).

CO, a product of HO activity, has been shown to stimulate transcription of critical regulators of mitochondrial biogenesis, including the respiratory enzyme-coding (NRF1) and -2, as well as the PGC-1α-coactivator and mitochondrial transcription factor A (Tfam) (13, 26, 33). In a model of sepsis, HO-1 ameliorated mitochondrial dysfunction and promoted survival in peritonitis-challenged mice (16).

Cilostazol, i.e., 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2-(1H)-quinolinone, is a potent inhibitor of Type III phosphodiesterase (14) that is currently used for the treatment of thrombotic vascular disease because of its anti-platelet aggregation and vasodilatory properties (8, 34). Furthermore, cilostazol can exert anti-inflammatory effects in human endothelial cells by reducing ROS production and increasing βcI-2 protein expression (12, 35). Therefore, cilostazol may regulate the mitochondrial apoptotic pathway in endothelial cells. Recent studies suggest that cilostazol can upregulate HO-1 through the activation of Nrf2 (25).

In the present study, we suggest that cilostazol may be used as a therapeutic drug in hepatic I/R injury by preserving mitochondrial function. We demonstrate that cilostazol increases the expression of genes involved in mitochondrial biogenesis and mitochondrial DNA (mtDNA) replication. Furthermore, we show that cilostazol stimulates HO-1 expression through the Nrf2 axis, which in turn improves I/R injury via increasing mitochondrial biogenesis. Therefore, we suggest that cilostazol may be useful as a pharmacological cytoprotec-

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tive agent against hepatic I/R injury and other vascular disorders.

MATERIALS AND METHODS

Reagents. Cilostazol (OPC-13013), [6-[4-((1-cyclohexyl-1H-tetrazol-5-yl)butoxy)-3,4-dihydro-2(1H)-quinolinone], was donated by Otsuka Pharmaceutical (Tokushima, Japan). Zinc protoporphyrin-IX (ZnP) was from Frontier Scientific (Logan, UT). Antibodies against PGC1α, TFAM, lamin A/C, Nrf-2, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA), and antibody against HO-1 was from Assay Designs (Ann Arbor, MI). Nrf-2 siRNA were from Santa Cruz Biotechnology. All other chemicals were obtained from Sigma-Aldrich.

Cell culture. HepG2 cells were obtained from the Korean cell line bank (Seoul, Korea). HepG2 cells and primary hepatocytes were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin solution, at 37°C in humidified incubators containing 5% CO2. Primary hepatocytes were exposed to 10 μM ZnP for 1 h before cilostazol treatments.

Isolation of primary hepatocytes. Primary hepatocytes from wild-type (WT) and Nrf2 knockout (KO) mice were isolated. Livers were perfused with Ca2+- and Mg2+-free HBSS containing EGTA (2.5 mM) and then digested with a collagenase buffer containing collagenase (0.5 mg/ml, C5138, Sigma), NaCl (66.7 mM), KCl (67.7 mM), HEPES (50 mM), and CaCl2 (4.8 mM). Digested livers were dissected and then gently teased with forceps until they were in solution. The cell suspensions were filtered through a 100-μm nylon cell strainer (BD Biosciences). The cells were centrifuged for 3 min at 700 rpm and resuspended with HBSS. After the pellet suspensions were centrifuged with 25% Percoll for 5 min at 800 rpm with the brake option off. The pellets were washed with DMEM supplemented with 10% FBS, and then cells were seeded into collagen-precocoted 100-mm tissue culture plates. After 24 h, nonadherent cells were removed by aspiration, and fresh medium was added.

Animals. Animals were maintained in a specific pathogen-free facility. Animal studies were approved by the University of Ulsan Animal Care and Use Committee. The mice were maintained under specific pathogen-free conditions at 18–24°C and 40–70% humidity, with a 12-h light-dark cycle. Food and drinking water were available ad libitum. Nrf2 mice were provided by Dr. Young-Joon Surh (Seoul National University) and BALB/c HO-1 KO mice (Hmox1−/−) were provided by Dr. Mark A. Perrella (Brigham and Women’s Hospital, Boston, MA).

Mouse liver I/R injury model. A model of hepatic I/R used as previously described (10). An atrumatic clip was used to interrupt the arterial/portal venous blood supply to the cephalad liver lobes. After 90 min the clip was removed. Mice were euthanized 6 h after reperfusion for harvest of serum and liver samples. Sham controls underwent the same procedure, but without vascular occlusion. Mice in the sham or hepatic I/R group were given an intraperitoneal injection of either vehicle (10% DMSO in PBS) or cilostazol (10 mg·kg−1·day−1) for 3 days before the onset of liver I/R.

Myeloperoxidase assay. The presence of myeloperoxidase (MPO) was used as an index of neutrophil sequestration in the liver. Tissue samples for MPO analysis were frozen in liquid nitrogen immediately after removal from the animal and were thawed, homogenized, and centrifuged to remove insoluble materials. MPO activities were measured by using a mouse MPO DuoSet ELISA kit (DY3667, R&D Systems, Minneapolis, MN) according to the manufacturer’s instruction. The supernatants were analyzed for MPO levels by sandwich ELISA. The levels of MPO in organ extracts were expressed as nanograms per milligram of protein.

Hepatocellular damage assay. Hepatic injury was assessed by serum alanine transaminase (ALT) levels with use of the EnzyChrom Alanine Transaminase Assay Kit (BioAssay System, Hayward, CA).

Liver histology. To detect the pathological changes, liver tissues were fixed in 10% neutral-buffered formalin solution and then dehydrated in graded alcohol, embedded in paraffin, sectioned into 4-μm-thick sections, and stained with hematoxylin and eosin (H&E).

Mitochondrial staining. After experimental treatments, Mitotracker Red CMXROS (Invitrogen, Carlsbad, CA) was added into the normal culture medium at a final concentration of 500 nM, then subsequently washed twice in PBS and fixed with 4% paraformaldehyde in PBS at 37°C for 30 min. Cells were analyzed by using a FV1000 Confocal Laser Scanning Biological Microscope (Olympus, Japan) at an excitation wavelength 579 nm and emission wavelength of 599 nm.

Measurement of mitochondrial ROS. Mitochondrial superoxide production was examined by MitosOX Red (Invitrogen, Carlsbad, CA) staining. Cells were washed with warm PBS and stained with 2.5 μM MitosOX in PBS for 15 min at 37°C, protected from light, and washed three times. The analysis of fluorescently stained cells was analyzed by using the Super Resolution Structured Illumination (SR-SIM) Microscope (Zeiss, Germany).

Mitochondrial membrane potential assay. Assessment of mitochondrial membrane potential in the cells was assessed using TMRE (tetramethylrhodamine, Abcam, Cambridge, MA). TMRE is a reliable indicator of active mitochondria. Cells were cultured on glass coverslips. The coverslips were washed with PBS and stained with 1 μM TMRE for 20 min in PBS at 37°C. Then the coverslips were washed three times again and immediately imaged by using the FV1200 Confocal Laser Scanning Biological Microscope (Olympus, Japan).

Citrate synthase activity. Citrate synthase activity in liver tissue of mice was measured by using a Citrate Synthase Assay Kit (Sigma-Aldrich) according to the manufacturer’s instructions. To prepare tissue extracts, mouse livers were homogenized with use of the CellLytic MT Cell Lysis Reagent (Sigma-Aldrich). Protein concentration was measured with BCA protein assay reagent. Twenty microliters of extract protein mixed were with reaction mixture. A microplate reader set at 412 nm made measurements at 10-s intervals for a 1.5-min duration. Citrate synthase activity was measured by calculating the difference between baseline and oxaloacetate-treated samples according to the manufacturer’s instructions.

ATP measurements. Liver tissues ATP content was determined by means of the ATP assay kit (Colorimetric/Fluorometric) (Abcam) according to the manufacturer’s instructions.

Reverse transcriptase PCR. Total RNA was isolated from cells or mouse liver tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. In brief, total RNA (2 μg) was used to synthesize the first-strand cDNA by using oligo(DT) primers (Bioneer, Daejeon, Korea) and M-MLV reverse transcriptase (Promega, Madison, WI). The synthesized cDNA was subjected to the PCR-based amplification. The following primers were used: PGC1α 5′-GGAACCTGACGCTACCTAATCT-3′, reverse primer 5′-CACGTCCCCATCGTACCG-3′; NRF-1 5′-CCAGTTGGCCA-CACGAACTC-3′, reverse primer 5′-CTCCCTTTCCTTCCACT-GC-3′, Thiam 5′-ATGCTTATAGGGCGGAGTGG-3′, reverse primer 5′-TGGCCTATCA-3′, HO-1 5′-CGAATTCCAGAAGGGCCAG-3′, reverse primer 5′-GTCCTTGTGTCATGTTCA-3′; GAPDH 5′-GGGGCTTCTCCAGAAATCAT-3′, reverse primer 5′-GCGTCTAA-CATGGCA-3. The expression of GAPDH was measured as an internal control.

Real-time quantitative RT-PCR. Reactions were performed with SYBR Green qPCR Master Mix (2×, USB Production; Affymetrix) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA).

HO-1 activity assay. HO-1 activity was determined as method previously described by Erdmann et al. (4) with few modifications. Briefly, primary hepatocytes were washed twice with Dulbecco’s Phosphate Buffered Saline (DPBS, pH 7.4) and centrifuged at 5,000 rpm for 10 min at 4°C. The cell pellet was suspended in ice-cold DPBS (100 mM pH 7.4) and frozen at −80°C, thawed three times,
and homogenized properly on ice. After centrifugation, 400 μl of supernatants were transferred into new tube and then incubated in the dark with 200 μl of reaction mixture containing 2 mg protein of mouse liver cytosol (XenoTech, Lenexa, KS) as a source of biliverdin reductase, 10 μM hemin, 2 mM NADPH, 2 mM glucose-6-phosphate, and 0.2 unit glucose-6-phosphate dehydrogenase at 37°C for 1 h. The reaction was stopped by placing the tubes on ice with the addition of 1 ml chloroform. After centrifugation, supernatants were placed in a 96-well plate. The extracted bilirubin was then measured by the absorbance difference between 464 and 530 nm (extinction coefficient, 40 mM⁻¹·cm⁻¹). HO-1 activity was expressed as nanomolar bilirubin per milligram protein per hour (data in percent).

mtDNA analysis and mtDNA damage detection. Total DNA was extracted from cells and mice liver tissues by use of an AccuPrep Genomic DNA Extraction Kit (Bioneer). mtDNA copy number was measured by real-time quantitative PCR. The following primers for mtDNA were used: human complex II (succinate-ubiquinone oxidoreductase) forward primer 5'-GAAATGATGAGCTCACCAAG-3', reverse primer 5'-GGAATGATGAGCTCACCAAG-3'; mouse cytochrome b (Mus musculus domesticus mitochondrion) forward primer 5'-CCACTTCATCTTACACATTA-3', reverse primer 5'-ATCTGGATCAGTGTATACCTA-3'. The following primers for nuclear DNA (nDNA) were used: human β-actin forward primer 5'-TCACCCACCCTGTCGCCCTACTACA-3', reverse primer 5'-CAGCGGAAAG-CTGCATTGCAATGG-3'; mouse 18S RNA forward primer 5'-GGAGCGCTAGAAGAAGC-3', reverse primer 5'-GGTTCCGGTGGTAGATGATATTT-3'. Relative amounts of mtDNA and nDNA copy numbers were compared. mtDNA damage was measured by use of the Expand Long Template PCR system. This long PCR technique is based on the amplification of a long (8,636-bp) and a short (316-bp) mtDNA fragment. Forward primer 5'-TACTAGTCCGCGAGCGTGTCATCC-3' (nt 4964–4987), backward primer 5'-GGTTCCGGTGGTAGATGATATTT-3' (nt 13599–13579) was used to amplify the 8,636-bp mtDNA fragment and forward primer 5'-GGAGCGCTAGAAGAAGC-3', backward primer 5'-CCCCATCTTCCCTTCTTCTTCTT-3' (nt 785–762) was used to amplify the 316-bp mtDNA fragment. PCR was performed with the Expand Long Template PCR system (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions, with 50 pmol of primers. The thermocycler profile included initial denaturation at 94°C for 2 min; 30 cycles of 93°C for 10 s, 61°C for 30 s, and 68°C for 8 min, and final extension at 68°C for 7 min. PCR product (5 μl) was separated on 1.6% agarose gels stained with ethidium bromide. Photographs were taken under UV transillumination. The relative band density was analyzed by using Image J software (U.S. National Institutes of Health, Bethesda, MD).

Statistical analysis. All values are expressed as means ± SE. Statistical differences between groups were evaluated by one-way ANOVA or Student’s t-test. Data were analyzed and presented with GraphPad Prism software version 4 (GraphPad Software, San Diego, CA). Differences between groups were considered to be significant at *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

Cilostazol protects I/R-induced hepatic injury. We first sought to determine whether cilostazol could provide protection in a mouse model of hepatic I/R injury. To identify the effects of cilostazol during hepatic injury, the serum levels of ALT and TNF-α and the liver tissue MPO content were measured at 6 h after reperfusion. Preconditioning with cilostazol prior to the ischemic insult markedly reduced the serum levels of ALT, TNF-α, and liver MPO content relative to those of the vehicle-treated I/R group (Fig. 1, A-C). Furthermore, cilostazol treatment dramatically reduced histological evidence of hepaticcellular injury during I/R, as evidenced by H&E staining of tissue sections (Fig. 1D). Consistent with these findings, cilostazol also protected against hepatocellular mtDNA damage caused by I/R (Fig. 1E).

To assess mitochondrial function, we measured citrate synthase activity and ATP production. The decreases of citrate synthase and ATP production in liver tissue induced by I/R injury were reversed by cilostazol treatment (Fig. 1, F and G). These results suggest that cilostazol preconditioning can protect mice from I/R-induced hepatic injury, in part, by inhibiting hepatocellular mitochondrial dysfunction and mtDNA damage. To examine the role of HO-1 in the effects of cilostazol on mitochondrial biogenesis, we subjected HO-1 KO mice (Hmox1−/−) or WT mice to I/R-induced hepatic injury and detected the mRNA levels of mitochondrial genes such as NRF-1, PGC-1α, and TFAM (Fig. 1H) and protein levels for COX III, COX IV, and PGC-1α (Fig. 1I). Cilostazol increased mitochondrial biogenesis markers in the liver tissue of WT but not Hmox1−/− mice.

We next assessed the effect of cilostazol on mitochondrial function and the involvement of HO-1 in this process, using primary hepatocytes from WT or Hmox1−/− mice. To determine mitochondrial membrane potential, we used TMRE as a fluorescent indicator. As shown in Fig. 1J, H2O2 treatment reduced TMRE staining in WT primary hepatocytes. Cilostazol dramatically increased TMRE-positive cells in WT primary hepatocytes under control or prooxidant conditions. In contrast, cilostazol failed to increase the TMRE signal in Hmox1−/− mice. Furthermore, we measured mitochondrial ROS levels using MitoSOX Red as a mitochondrial superoxide indicator. Cilostazol decreased mitochondrial ROS levels in primary hepatocytes from WT mice during oxidant challenge. This antioxidant effect of cilostazol was abolished in hepatocytes from Hmox1−/− mice (Fig. 1K).

Cilostazol induces mitochondrial biogenesis by inducing HO-1 expression and reducing ROS production in vitro. We next assessed the potential of cilostazol to induce mitochondrial biogenesis in vitro, using isolated primary hepatocytes. Cilostazol treatment dose and time dependently increased the expression of NRF-1, PGC-1α, and TFAM mRNA in primary hepatocytes (Fig. 2, A and B). Cilostazol induced mitochondrial biogenesis in primary hepatocytes as evidenced by dose- and time-dependent increases in the expression of mitochondrial proteins COX III, COX IV, and PGC-1α (Fig. 2, C and D), total mtDNA contents (Fig. 2, E and F), and MitoTracker-positive staining (Fig. 2G).

Cilostazol can induce HO-1 expression in many cell types (11, 24, 25). Consistent with these observations, treatment of HepG2 cells with cilostazol dose and time dependently increased the expression of HO-1 mRNA and protein in HepG2 cells (Fig. 2, H and I).

We tested the hypothesis that HO-1 exerts an intermediate role in the induction of mitochondrial biogenesis by cilostazol. Pretreatment of primary hepatocytes with ZnPP, a HO-1 inhibitor, for 60 min before the addition of cilostazol, reduced cilostazol-induced NRF-1, PGC-1α, and TFAM mRNA expression (Fig. 3A). Consistent with inhibition of cilostazol-dependent mitochondrial biogenesis, ZnPP pretreatment also significantly reduced mtDNA content (Fig. 3B) and MitoTracker-positive staining in primary hepatocytes (Fig. 3C). Furthermore, to investigate the potential of cilostazol to scavenge cellular ROS, the production of ROS was
Fig. 1. Cilostazol reduces ischemia-reperfusion (I/R)-induced hepatic injury. A–G: mice were subjected to 60 min liver warm ischemia, followed by 6 h reperfusion. Hepatic injury was assessed by determining serum levels of alanine transaminase (ALT; A), TNF-α (B), and liver tissue myeloperoxidase (MPO) levels (C). Representative liver histology [hematoxylin and eosin (H&E) staining] is shown (D). Liver tissue mtDNA damage was analyzed by long PCR of a long mtDNA (8,636-bp) fragment. A short (316-bp) fragment served as the standard (E). Citrate synthase (CS) activity (F) and ATP production (G) in liver tissue were measured. H–K: hepatic I/R injury was induced in heme oxygenase (HO)-1 wild-type (WT) or knockout (KO). Mitochondrial biogenesis related genes, NRF-1, PGC-1α, and TFAM were measured by real-time RT-PCR (H) and for mitochondrial biogenesis-related protein levels, COX III, COX IV, and PGC-1α were analyzed by Western blot (I) in the liver tissue of HO-1 WT or KO. For the mitochondrial permeability transition (MPT), the TMRE-mitochondrial membrane potential assay was used (J), and the mitochondrial reactive oxygen species (ROS) levels with use of MitoSOX Red mitochondrial superoxide indicator were detected in primary hepatocytes of HO-WT or KO (K). All experiments were performed in triplicate (n = 7/group), and representative data are shown. Quantitative data are expressed as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant. Con, control; Cilo, cilostazol.
induced in primary hepatocytes by treatment with exogenous H$_2$O$_2$. Cilostazol treatment reduced H$_2$O$_2$-dependent mitochondrial ROS production in primary hepatocytes (Fig. 3D). However, cilostazol treatment failed to reduce H$_2$O$_2$-dependent ROS production in the presence of the HO-1 inhibitor ZnPP (Fig. 3D).

To confirm the requirement of HO-1 in cilostazol-induced mitochondrial biogenesis, we used HO-1 homozygous KO mice (Hmox1$^{-/-}$). Treatment with cilostazol increased NRF-1, PGC-1α, and TFAM mRNA expression and mtDNA content in the liver of WT mice. On the other hand, there were no effects of cilostazol on mitochondrial biogenesis in HO-1 KO mice (Fig. 3, E and F). These results suggest that mitochondrial biogenesis augmented by cilostazol is regulated by HO-1 activity in vitro and in vivo. In conclusion, these results support an intermediate role for HO-1 expression and activity in the stimulatory effect of cilostazol on mitochondrial biogenesis.

Cilostazol induces mitochondrial biogenesis through an Nrf-2-dependent pathway. Nrf-2 is an important transcription factor that regulates many genes involved in the cellular antioxidant response, including Hmox1. We further examined whether cilostazol activates Nrf-2 in association with HO-1 upregulation in HepG2 cells. Cilostazol treatment caused a time-dependent increase in the nuclear translocation of Nrf-2 that was apparent as early as 30 min and that lasted up to 2 h posttreatment (Fig. 3G). Furthermore, silencing the Nrf2 gene by using specific siRNA significantly decreased cilostazol-induced HO-1 expression compared with that in cells treated with control (scramble) siRNA (Fig. 3H). Consistent with results observed with ZnPP pretreatment, Nrf2 siRNA treatment downregulated the levels of NRF-1, PGC-1α, and TFAM mRNA and reduced mtDNA content (Fig. 3, I and J). These results confirm that stimulation of mitochondrial biogenesis by cilostazol was associated with an increase in Nrf-2 nuclear translocation and Nrf-2-dependent induction of HO activity.

The protective effect of cilostazol on I/R-induced liver injury in vivo is Nrf2 dependent. Next, we examined role of Nrf2 in the protective effects of cilostazol in a hepatic I/R injury model using WT and Nrf2 KO (Nrf2$^{-/-}$) mice. First, we examined the effect of cilostazol on HO-1 expression in I/R-induced liver. Whereas I/R treatment slightly increased hepatic HO-1 expression, cilostazol treatment had a greater effect on the expression of HO-1 in WT mice. In contrast, there was no effect of cilostazol on HO-1 expression in Nrf2$^{-/-}$ mice (Fig. 4A). Furthermore, to detect the effect of cilostazol on HO activity, isolated primary hepatocytes from WT or Nrf2 KO (Nrf2$^{-/-}$) were challenged with H$_2$O$_2$, as an in vitro model of
Fig. 3. Cilostazol induces mitochondrial biogenesis through the induction of HO-1 and reducing ROS production. A–D: primary hepatocytes were pretreated in the absence or presence of ZnPP (10 μM) and then treated with 10 μM cilostazol for 12 h. Expression levels of NRF-1, PGC-1α, and TFAM mRNA were measured by real-time RT-PCR (A). The relative mtDNA content was measured by real-time PCR. mtDNA content was normalized to nDNA content (B). Mitochondrial mass was assessed by using MitoTracker Red (red). Nuclei were stained with Hoechst dye (blue). Images of fluorescence were analyzed by confocal microscopy (C). Primary hepatocytes were pretreated in the absence or presence of ZnPP (10 μM), treated with 10 μM cilostazol for 6 h, and stressed by H2O2, then the mitochondrial ROS levels with use of MitoSOX Red mitochondrial superoxide indicator were detected (D). E and F: in WT and HO-1 KO mice, cilostazol (10 mg·kg⁻¹·day⁻¹) was given once daily for 3 days by intraperitoneal injection. Liver tissues were excised and analyzed for mitochondrial biogenesis in mice. Expression of PGC-1α, NRF-1, and TFAM mRNA (E) and mtDNA content (F) were measured by real-time RT-PCR. Cytosol (CE) and nuclear (NE) accumulation of Nrf-2 was confirmed by Western blotting. Lamin A/C and β-actin were used as a loading control (G) in HepG2 cells. H–J: HepG2 cells were transfected with control siRNA (Con) or Nrf2 siRNA to knockdown Nrf2 levels. Cells were treated with 10 μM cilostazol for 12 h. siRNA were subjected to Western blot and RT-PCR to confirm Nrf2 siRNA efficiency (H, top). Expression of HO-1 (H, bottom) and PGC-1α, NRF-1, and TFAM mRNA (J) was measured by RT-PCR. mtDNA content was measured by real-time RT-PCR (J). All experiments were performed in triplicate (n = 7/group), and representative data are shown. Quantitative data are expressed as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001; #P < 0.05 vs. cells treated with cilostazole alone.
Fig. 4. Cilostazol reduces I/R-induced hepatic injury in Nrf2 WT and KO mice. WT and Nrf2 KO mice, cilostazol (10 mg·kg\(^{-1}\)·day\(^{-1}\)) was given once daily for 3 days by intraperitoneal injection. Mice were subjected to 60 min liver warm ischemia, followed by 6 h reperfusion. Liver tissues were excised and analyzed for mitochondrial biogenesis in mice. Expression of HO-1 protein was detected by Western blot analysis (A) in the liver tissues and HO-1 activity was measured (B) in murine primary hepatocytes. Expression of NRF-1, PGC-1\(\alpha\), and TFAM mRNA and mtDNA content were measured by real-time RT-PCR (C, left) and the protein levels for COX III, COX IV, and PGC-1\(\alpha\) were analyzed with Western blot (C, right) in the liver tissues. Mitochondrial mass was assessed by using MitoTracker Red (red). Nuclei were stained with Hoechst dye (blue). Images of fluorescence were analyzed by confocal microscopy (D) in primary hepatocytes. Mitochondrial membrane potential was detected by confocal microscope (F) in the primary hepatocytes. For antioxidant enzyme activity in Nrf2 mice, the mRNA levels of Gclc, Nqo1, and Trx1 were measured by real-time RT-PCR (G). Hepatocellular function was evaluated by serum levels of ALT (H) and MPO (I). Representative liver histology (H&E staining) is shown (J) in the liver tissues. All experiments were performed in triplicate (\(n = 7\)/group), and representative data are shown. Quantitative data are expressed as means ± SE, *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).
I/R. I/R injury is associated with the increased production of ROS, including H_2O_2. Furthermore, H_2O_2 has been used as a model of oxidative stress in primary hepatocytes and shown to act as a hypoxia mimic when applied to various cell types at low concentration (5, 9). Cilostazol increased HO activity in control or H_2O_2-challenged WT hepatocytes but not Nrf2^{-/-} hepatocytes (Fig. 4B).

Treatment with cilostazol increased NRF-1, PGC-1α, and TFAM mRNA expression (Fig. 4C, left) and the protein levels for Cox III, Cox IV, and PGC-1α in Fig. 4C (right). MitoTracker-positive staining in H_2O_2-stressed primary hepatocytes (Fig. 4D), and mtDNA content in the liver of WT mice (Fig. 4E). To present the direct evidence of mitochondrial function, we detected the mitochondrial permeability transition using TMRE-mitochondrial membrane potential assay. As shown in Fig. 4F, treatment of cilostazol during I/R injury increased the TMRE-stained cells compared with I/R without cilostazol. Consistent with results observed with Hmox1^{-/-} mice, cilostazol had no effect on mitochondrial biogenesis in Nrf2^{-/-} mice. For antioxidant enzyme activity in Nrf2^{-/-} mice, we measured the mRNA level of Gclc, Nqo1, and Trx1. These enzyme levels were increased by cilostazol in Fig. 4G. To assess liver injury, we examined the concentrations of serum ALT (Fig. 4H) and MPO levels (Fig. 4I). As expected, cilostazol prevented liver damage caused by I/R in Nrf2 WT mice. Consistent with these findings, cilostazol also protected against hepatocellular damage caused by I/R in Nrf2 mice (Fig. 4J). In contrast to protective effects of cilostazol on hepatic I/R injury and mitochondrial biogenesis observed in WT mice, I/R-induced mitochondrial dysfunction could not be recovered by cilostazol in Nrf2^{-/-} mice. These results taken together suggest that, in the I/R injury model, cilostazol-induced mitochondrial biogenesis was mediated by an Nrf2 and HO-1-dependent pathway. Furthermore, these results suggest that I/R injury can impair hepatic mitochondrial biogenesis and mitochondrial function, which can be reversed by cilostazol treatment.

DISCUSSION

Cilostazol is a quinolinone compound that acts as a selective inhibitor of 3-type phosphodiesterase. This compound is used primarily as a vasodilator and antplatelet agent for the treatment of peripheral vascular disease, where it reduces symptoms of claudication (8, 14, 34). In this study, we show that cilostazol can effectively reduce tissue injury in a rodent model of hepatic I/R. The salutary effects of cilostazol in this model were dependent in part on the stimulation of hepatic mitochondrial biogenesis and the alleviation of mitochondrial dysfunction induced by hepatic I/R. We demonstrate that the stimulation of mitochondrial biogenesis by cilostazol in hepatocytes, as well as the hepatoprotective and antioxidative effects of this compound, were dependent on activation of the Nrf2/HO-1 axis.

Mitochondrial dysfunction may play an important role in cellular injury as well as disease pathogenesis (22). In the present study we have shown that cilostazol protected against loss of mitochondrial function in hepatocytes during hepatic I/R. Specifically, cilostazol treatment preserved citrate synthase activity, and ATP production, during hepatic I/R. Mitochondrial biogenesis, through regeneration of mitochondrial populations, may have beneficial and tissue-protective effects in a variety of disease models. For example, the stimulation of mitochondrial biogenesis has been shown to confer protection against experimental Staphylococcus aureus-induced sepsis and pneumonia (1, 6). Recent studies suggest that protective effects of certain pharmaceuticals, such as natural antioxidants, against oxidative injury, may involve induction of mitochondrial biogenesis (19, 20). For example, the protective effects of resveratrol during endotoxemia involved stimulation of mitochondrial biogenesis (13).

HO-1, an inducible stress protein, can exert cytoprotective and anti-inflammatory effects through enzyme activity-dependent generation of heme catabolites, including biliverdin-IXα, ferrous iron, and CO, which may act individually or in concert to mediate the biological effects of this protein (32). The HO-1/CO system has been implicated as a mediator of mitochondrial biogenesis in several models (21, 27, 38). Recently, we have reported that the HO-1/CO system can mediate mitochondrial biogenesis in response to resveratrol, nitric oxide stress, CO-releasing molecules, and activation of the endoplasmic reticulum stress pathway (13, 38). In the present study, we show that the stimulation of mitochondrial biogenesis by cilostazol in hepatocyte cells requires the activation of endogenous HO-1 expression and activity, as shown by inhibition of the response by genetic interference or chemical inhibition using ZnPP.

Nrf-2 is a master regulator of the antioxidant response that regulates HO-1 expression in response to antioxidants, and electrophiles, as well as other stimuli (2, 23, 37). In this study, using Nrf2^{-/-} mice, we found that Nrf-2 is required for the effects of cilostazol on mitochondrial biogenesis, and hepato-protection in vivo.

In the present study we have demonstrated that the induction of mitochondrial biogenesis in hepatocytes by cilostazol involves a signaling pathway requiring endogenous Nrf-2 activation and HO activity. Cilostazol may be exploited as a potential therapeutic to maintain mitochondrial populations and preserve mitochondrial homeostasis during I/R injury and other vascular disorders.

REFERENCES


