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Genipin protects the liver from ischemia/reperfusion injury by modulating mitochondrial quality control

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ABSTRACT

Hepatic ischemia and reperfusion (IR) injury is closely linked to oxidative mitochondrial damage. Since mitochondrial quality control (QC) plays a pivotal role in the recovery of impaired mitochondrial function, mitochondrial QC has emerged as a potential therapeutic target. Genipin, an iridoid compound from *Gardenia jasminoides*, has been showed antioxidant and anti-inflammatory properties. In this study, we investigated the hepatoprotective mechanism of genipin against IR-induced hepatic injury, particularly focusing on mitochondrial QC. Male C57BL/6 mice underwent liver ischemia for 60 min, followed by reperfusion for 6 h. Genipin (100 mg/kg, i.p.) or vehicle (10% Tween 80 in saline) was administrated to mice 1 h before ischemia. Liver and blood samples were collected 6 h after reperfusion. Hepatic IR increased hepatocellular oxidative damage and induced mitochondrial dysfunction. These phenomena were ameliorated by genipin. Hepatic IR also increased the level of mitochondrial fission, such as dynamin-related protein 1 and the level of PINK1 protein expression. In contrast, hepatic IR decreased the levels of mitochondrial biogenesis related proteins (e.g., peroxisome proliferator-activated receptor gamma coactivator 1α, nuclear respiratory factor 1, and mitochondrial transcription factor A), mitophagy related proteins (e.g., Parkin), and fusion related protein (e.g., mitofusin 2). Furthermore, hepatic IR decreased the levels of sirtuin1 protein and phosphorylation of AMP-activated protein kinase. Genipin alleviated these IR-induced changes. These data indicate that genipin protects against IR-induced hepatic injury via regulating mitochondrial QC. (225/250).

1. Introduction

Hepatic ischemia and reperfusion (IR) injury appears in many clinical situations, such as transplantation, liver surgery for trauma, and hepatic failure after shock. Significant research efforts over the past few decades have revealed that excessive reactive oxygen species (ROS) production during IR can elicit direct damage to organ, which results in a cascade of deleterious cellular responses leading to inflammation, cell death, and ultimately organ failure. However, the precise mechanisms underlying IR-induced hepatic dysfunction are still unknown.

Mitochondria are major organelles for ATP generation and cellular homeostasis. A wide array of functional alterations caused by reperfusion injury in mitochondria leads to bioenergetics failure, excessive generations of ROS, and intracellular Ca^{2+} overload and subsequently results in cell death (Lemasters et al., 2009). Emerging evidences demonstrated that mitochondrial quality control (QC) maintains optimal mitochondrial functionality by acting through multiple dynamic mechanisms such as mitochondrial biogenesis (regulating the number of mitochondria optimal cellular function), fission/fusion and mitophagy (selective mitochondrial autophagy) (de Castro et al., 2011; Rugarli and Langer, 2012). Recent studies have revealed that a critical linkage between mitochondrial QC and oxidative stress is an important in the pathogenesis of various ischemic diseases (Ikeda et al., 2015; Tompkins et al., 2006). Enhanced mitochondrial biogenesis increased mitochondrial mass and membrane potential in cultured hepatocytes after hypoxia/reoxygenation (HR) (Khader et al., 2016). Mild and transient oxidative stress induced mitophagy, thereby rescuing cell viability in H_2O_2-treated HeLa cells (Frank et al., 2012). Furthermore, pretreatment with mitochondrial division inhibitor (Mdivi-1), a well-known mitochondrial fission inhibitor, decreased oxidative stress, suppressed ROS production and attenuated cell apoptosis in a mouse model of cerebral IR (J. Wang et al., 2014). Despite this foundation of research, however, there remains only limited understanding of the integrative mitochondrial QC and its regulatory signaling during in vivo hepatic IR.

Gardenia jasminoides Ellis has been widely used for centuries in traditional Chinese medicine. *Gardenia* fruits have been used as a traditional medicine in clinical practice for the treatment of inflammation,

Abbreviations: IR, Ischemia and reperfusion; QC, quality control; ROS, reactive oxygen species; ALT, alanine aminotransferase; H&E, hematoxylin and eosin; GDH, glutamate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; PGC1α, proliferator-activated receptor-gamma coactivator 1α; NRF1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; PINK1, PTEN-induced putative kinase 1; MFN2, mitofusin 2; SIRT1, sirtuin1; p62, sequestosome1/p62; Drp1, dynamin-related protein 1.

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jaundice, edema, and hypertension (Koo et al., 2006; Wang et al., 2010). Genipin, an iridoid glucoside aglycone and a major component of *Gardenia* fruits, has diverse biological and pharmacological activities such as anti-oxidant, anti-inflammation, and anti-apoptosis (Kim et al., 2012; S. Kim et al., 2013a; Yamamoto et al., 2000). Genipin also exerts potent cytoprotective effects against ROS-induced cytotoxicity by directly scavenging superoxide radical and peroxynitrites in experimental models (Hughes et al., 2014). Moreover, J. Kim et al. (2013b) reported that genipin ameliorated oxidative stress and enhanced antioxidant capacity in hepatic IR. Recently, we demonstrated that genipin restored impaired autophagy in a mouse model of sepsis (Cho et al., 2016).

Therefore, we investigated the hepatoprotective mechanisms of genipin against a post-ischemic liver injury, with a particular focus on mitochondrial biogenesis, dynamics and mitophagy.

2. Materials and methods

2.1. Materials

ChemiLab alanine aminotransferase (ALT) assay kit was purchased from IVDLab Co. Ltd. (Uiwang, Korea). Interleukin (IL)-1β and IL-6 enzyme-linked immunosorbent (ELISA) assay kits were purchased from eBiosciences (San Diego, CA, USA). 1,1,3,3-tetraethoxypropane, 2-vinylpyridine, 2-oxoglutarate, ethylenediaminetetraacetic acid (EDTA), rotenone, sirtinol, succinate, and anti-human beta (β)-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNeasy Blood & Tissue kit was purchased from Qiagen (Valencia, CA, USA). SYBR Green detection system was purchased from Roche Applied Science (Mannheim, Germany). Anti-BCA protein assay was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA). Antibodies against peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM), mitofusin 2 (Mfn2), p62/SQSTM1 (p62) and sirtuin1 (SIRT1) were purchased from Abcam (Cambridge, MA, UK). Antibodies against dynamin-related protein 1 (Drp1), Parkin, and 5′ adenosine monophosphate-activated protein (AMPK) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against microtubule-associated protein 1 light chain 3 (LC3) was purchased from Novus Biologicals (Littleton, CO, USA). Secondary antibodies against rabbit or mouse conjugated to horseradish peroxidase (HRP) were purchased from Dako (Carpinteria, CA, USA). ECL detection system for western blotting was purchased from iNtRON Biotechnology (Seongnam, Korea). Genipin (PubChem:442424; Batch no. CTF5047) of high performance liquid chromatography grade with purity > 97% was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Animals and hepatic IR model

Male C57B/L6 mice (21–23 g) were supplied by Daehan Biolink (Eumsung, Korea). All animals were acclimatized to the laboratory conditions at Sungkyunkwan University for at least one week and were housed in cages in a temperature-controlled room with a 12 h light-dark cycle and freely received water and food. All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Institutes of Health (NIH publication No. 86-23, revised 1985) and the guidelines of the Sungkyunkwan University Animal Care Committee. Hepatic IR was performed according to Dinbar et al. (1970). Anesthetized mice were maintained at 37 °C during the anesthesia process. An atraumatic clip was placed across the portal triad, above the right lateral lobe. After 60 min of ischemia, the clamp was removed to allow reperfusion for 6 h. Sham-operated mice underwent in a similar manner without clamping. Mice were sacrificed after 6 h of reperfusion. The liver tissues were immediately treated for histological staining and the remaining parts of liver and serums were frozen in liquid nitrogen at −80 °C until biochemical analyses were performed.

2.3. Administration of genipin and experimental design

For treatment, genipin (I.P., dissolved in 10% Tween80/saline) was administered at doses of 50, 100 and 200 mg/kg 1 h before ischemia. The dosage of genipin treatment was determined based on a previously published report (Koo et al., 2006), as well as our preliminary study. Based on serum ALT activity, a genipin dose of 100 mg/kg was selected as the optimal dose for further biochemical assays. In the present study, mice were randomly assigned to 4 groups (n = 6–8 per group): (a) vehicle-treated sham (sham); (b) genipin-treated sham (genipin); (c) vehicle-treated IR (IR); and (b) genipin-treated IR (genipin + IR). To confirm the involvement of SIRT1/AMPK in mitochondrial QC modulation by genipin during hepatic IR, sirtinol (Sigma-Aldrich), a SIRT1 inhibitor, was dissolved in 5% DMSO-saline and administered (10 mg/kg, i.p.) 30 min before reperfusion. The dosage and timing of sirtinol administration were selected based on previously published studies (Kaur et al., 2015; Cho et al., 2017).

2.4. Serum ALT activities

Serum ALT activities were measured by spectrophotometric procedures via commercial kits (IVDLab Co. Ltd.).

2.5. Histological analysis

For light microscopic investigations, liver tissue was removed from a portion of the left lobe and fixed immediately in 10% neutral buffered formalin, dehydrated in alcohol series, cleared in toluene and finally embedded in paraffin. Embedded sample was cut serially into 5 μm sections, and stained with hematoxylin and eosin (H&E) and evaluated under a microscope. All samples sections were evaluated using selected fields for characterization of histological and pathological changes in a blind fashion at ×400 histological magnification (Olympus Optical Co., Tokyo, Japan).

2.6. Cytokines levels

Serum IL-1β and IL-6 levels were quantified using commercial IL-1β and IL-6 ELISA assay kits (eBiosciences), respectively, according to the manufacturer's instructions.

2.7. Lipid peroxidation and hepatic glutathione contents

The levels of thiobarbituric acid for reactive substrate in liver homogenates were analyzed at 535 nm to measure the malondialdehyde (MDA), the end-product of lipid peroxidation (Buege and Aust, 1978). The total glutathione levels were determined spectrophotometrically at 412 nm using 5,5′-dithio-(2-nitrobenzoic acid), yeast glutathione reductase, and NADPH (Tietze, 1969). The same procedure was performed to measure the oxidized glutathione (GSSG) in the presence of 2-vinylpyridine (2 μl) within 2 h in the dark (Griffith, 1980). Finally, the GSH/GSSG ratio was calculated.

2.8. Serum glutamate dehydrogenase activity

The level of serum glutamate dehydrogenase (GDH) activity was measured according to the previously published methods (Ellis and Goldberg, 1972). Briefly, serum (100 μl) was added to the mixed-reaction solution, which contains triethanolamine buffer (50 mM), NADH (0.19 mM), ADP (0.95 mM), and ammonium sulfate (50 mM) and then incubated at 37 °C for 15 min. After 15 min, 2-oxoglutarate (10 mM) was added and each sample was incubated at 37 °C for 6 min. The mixture (1 mL) was measured by spectrophotometers at 340 nm to measure GDH activity.