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Bilirubin nanoparticle preconditioning protects against hepatic ischemia-reperfusion injury

Jin Yong Kim [a], Dong Yun Lee [a], Sukmo Kang [b], Wenjun Miao [b], Hyungjun Kim [b], Yonghyun Lee [b], Sangyong Jon [a, b, *]

[a] Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea
[b] KAIST Institute for the BioCentury, Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea

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ABSTRACT

Hepatic ischemia-reperfusion injury (IRI) remains a major concern in liver transplantation and resection, despite continuing efforts to prevent it. Accumulating evidence suggests that bilirubin possesses antioxidant, anti-inflammatory and anti-apoptotic properties. However, despite obvious potential health benefits of bilirubin, its clinical applications are limited by its poor solubility. We recently developed bilirubin nanoparticles (BRNPs) consisting of polyethylene glycol (PEG)-conjugated bilirubin. Here, we sought to investigate whether BRNPs protect against IRI in the liver by preventing oxidative stress. BRNPs exerted potent antioxidant and anti-apoptotic activity in primary hepatocytes exposed to hydrogen peroxide, a precursor of reactive oxygen species (ROS). In a model of hepatic IRI in mice, BRNP preconditioning exerted profound protective effects against hepatocellular injury by reducing oxidative stress, pro-inflammatory cytokine production, and recruitment of neutrophils. They also preferentially accumulated in IRI-induced inflammatory lesions. Collectively, our findings indicate that BRNP preconditioning provides a simple and safe approach that can be easily monitored in the blood like endogenous bilirubin, and could be a promising strategy to protect against IRI in a clinical setting.

1. Introduction

Despite well-developed surgical techniques and preservation strategies [1], hepatic ischemia-reperfusion injury (IRI) is a critical major problem in patients undergoing liver transplantation and resection, resulting in hepatocellular dysfunction, rejection, and liver failure. Although the mechanisms involved in the development of IRI are not fully understood, pro-inflammatory immune responses and oxidative stress mediated by reactive oxygen species (ROS) likely damage ischemic tissue after restoration of blood supply [2,3]. Suppression of the activated immune cascade and simultaneous scavenging of ROS and radicals in ischemic tissues may therefore minimize IRI. Although many agents have been tested for their ability to suppress pro-inflammatory immune responses and/or oxidative stress in ischemic liver, none to date has been clinically successful, indicating the need for effective therapeutic agents [3,4].

Bilirubin has long been recognized as an indicator of liver dysfunction or a waste product resulting from heme metabolism. In addition, severe elevations of unconjugated bilirubin cause damage to brain and erythrocytes in neonates [5,6]. However, accumulating evidence strongly suggests that bilirubin possesses beneficial biological functions, including antioxidant, anti-inflammatory and anti-apoptotic properties [7,8]. In fact, experimental models have demonstrated that bilirubin diminishes IRI in the liver and kidney [9,10]. In addition, Gilbert syndrome, a common genetic disorder characterized by mild hyperbilirubinemia, has been linked to a lower risk of ischemic heart disease and stroke in a meta-analysis [11,12]. Indeed, a mildly elevated preoperative bilirubin concentration in liver recipients reduces IRI after transplantation [13].

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAM, cell adhesion molecule; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; IL, interleukin; IRI, ischemia-reperfusion injury; NAC, n-acetylcysteine; PEG, polyethylene glycol; ROS, reactive oxygen species.

* Corresponding author. Graduate School of Medical Science and Engineering, Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea.
E-mail address: syjon@kaist.ac.kr (S. Jon).

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Injection of bilirubin is a type of endogenous molecule based therapeutic strategy that can feasibly be applied to boost serum bilirubin. However, the poor solubility of bilirubin in water can result in severe deposition in various tissues, accounting for the limited use of exogenously administered, purified bilirubin.

We recently overcame these solubility limitations through development of polyethylene glycol (PEG)-conjugated bilirubin, which self-assembles to form nanoscale structures, termed bilirubin nanoparticles (BRNPs), with a size of approximately 100 nm in water [14,15]. In the present study, we used a well-established *in vivo* murine model of hepatic IRI to evaluate the protective effects of BRNP preconditioning. We found that BRNPs attenuated IRI-associated hepatocellular injuries, suppressed oxidative stress and inflammatory response without causing untoward side effects. We also found that, unlike native bilirubin, BRNPs were capable of targeting the site of IRI.

2. Materials and methods

2.1. Materials

Bilirubin-IX-alpha was purchased from Tokyo Chemical Industry (Tokyo, Japan). Methoxypolyethylene glycol amine (mPEG2000-NH_2) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cypate was synthesized according to previous reports [16]. All other chemicals and reagents were purchased from Sigma-Aldrich and used as received unless stated otherwise.

2.2. Synthesis of PEGylated bilirubin

PEGylated bilirubin was synthesized as previously described [14]. Briefly, bilirubin-IX-alpha (0.5 mmol) and EDC (0.6 mmol) were dissolved in dimethyl sulfoxide (DMSO). After the reaction, mPEG2000-NH_2 (0.2 mmol) and triethylamine were added with stirring under a nitrogen atmosphere. Free bilirubin was removed using methanol with subsequent centrifugation. The supernatant was evaporated, after which the reaction mixture was dissolved in chloroform for purification by silica column chromatography using chloroform:methanol (85:15). The solvents were then evaporated to yield PEGylated bilirubin.

2.3. Preparation of BRNPs

PEGylated bilirubin was dissolved in chloroform and evaporated under a stream of nitrogen gas to yield a film layer. Uniform-sized nanoparticles were formulated by suspending the film layer in phosphate-buffered saline (PBS) and sonicating for 10 min. The size of BRNPs was characterized using a Nanosizer ZS90 (Malvern Instruments, Ltd., Malvern, UK). BRNP morphology was examined by transmission electron microscopy using a Tecnai G2 F30 system (FEI Co., Hillsboro, OR, USA). Cypate-loaded BRNPs were generated by dissolving 3.4 mg PEGylated bilirubin and 85 μg cypate in chloroform and drying under a stream of nitrogen gas. The film layer was resuspended in distilled water and sonicated for 10 min.

2.4. Animals

Male wild-type C57BL/6 mice (6–8 weeks old) were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained under pathogen-free conditions in the animal facility for at least 1 week. All mice were fed a standard chow diet and water until used, and were kept under constant environmental conditions with 12 h light/dark cycles. All animal studies were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (National Academies Press. 2011). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea Advanced Institute of Science and Technology (KAIST).

2.5. Isolation of hepatocytes from liver

Mouse hepatocytes were prepared as described previously [17]. Mice were anesthetized, their abdomens were opened, and portal veins were exposed. Each liver was perfused *in situ* via the portal vein with 50 mL EGTA buffer, followed by 0.075% type IV collagenase. After gentle mechanical dissociation and further digestion in 0.009% collagenase, hepatocytes and non-parenchymal cells were isolated by Percoll gradient centrifugation (GE Healthcare Life Sciences, Seoul, Korea). The viability of isolated hepatocytes, as assessed by trypan blue staining, was greater than 90%. Cells were seeded at a density of 2.5×10^4 cells per well in 96-well plates and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37 °C. All *in vitro* experiments were performed 24 h later.

2.6. Intracellular ROS measurements

Intracellular ROS were measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), according to the manufacturer's instructions. H_2O_2-stimulated hepatocytes were treated with PBS, different concentrations of BRNPs (1–100 μM), and n-acetylcysteine (NAC) for 3 h, respectively. The media were removed and cells were loaded with 25 μM DCFDA (diluted in PBS) for 45 min at 37 °C. The hepatocytes were washed with PBS, and fluorescence intensity (excitation, 485 nm; emission, 535 nm) was measured using a fluorescence microplate reader (SpectraMax Gemini XPS; Molecular Devices, Sunnyvale, CA, USA).

2.7. Apoptosis assays

Apoptosis of primary mouse hepatocytes was measured by annexin V-FITC/PI staining according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). In brief, H_2O_2-stimulated hepatocytes were treated with PBS and BRNP for 4 h, respectively. Cells were detached using a standard trypsin/EDTA protocol and then were incubated with annexin V-FITC and PI in cold 1x binding buffer for 15 min. All samples were analyzed by flow cytometry. The fluorescence imaging of annexin V-FITC and DAPI staining was visualized using an ImageXpress Micro XL High-Content Imaging System (Molecular Devices).

2.8. Induction of hepatic IRI model in mice

Partial hepatic IRI was induced as described previously, with a few modifications [18]. Briefly, mice were anesthetized by intraperitoneal injection of a 30-mg kg^{-1} tiletamine/zolazepam solution containing 10 mg kg^{-1} of xylazine. Following a midline laparotomy, the hepatic hilum was carefully dissected and a micro-vascular clamp was applied to the first branch of the hepatic artery and portal vein supplying the left lateral and median lobes of the liver. Circulation in the caudal lobes was retained intact to prevent intestinal venous congestion. The peritoneum was closed with saline soaked sterile gauze to prevent dehydration, and the mice were placed on a heating pad. After 90 min of hepatic ischemia, the micro-vascular clamp was removed, initiating reperfusion, and the abdominal wall was closed with 6–0 nylon sutures. After 6 h of reperfusion, the mice were anesthetized, whole blood was