Ankaflavin ameliorates steatotic liver ischemia-reperfusion injury in mice

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BACKGROUND: It is well-known that steatotic liver is more susceptible to ischemia-reperfusion (I/R) injury during liver transplantation, liver resection and other liver surgeries. The increasing incidence of non-alcoholic fatty liver disease (NAFLD) decreases the availability of liver donors. Although steatotic liver is now accepted as a source of liver for transplantation, NAFLD exacerbates the liver injury after liver surgery. The present study was to investigate the protective role of ankaflavin in steatotic liver I/R injury.

METHODS: The model of fatty liver mice was induced with high fat diet in four weeks, ankaflavin or vehicle (saline) was administrated by gavage once a day for one week. The animals were subjected to partial hepatic I/R. Blood samples were collected to measure serum aminotransferases. The liver tissues were used to examine liver steatosis, apoptosis of hepatocytes, hepatic oxidative stress, Kupffer cells and inflammatory cytokines. The effects of ankaflavin on inflammatory cytokines were evaluated in isolated Kupffer cells from the steatotic liver.

RESULTS: Ankaflavin reduced liver steatosis in high fat diet mice. Compared with normal mice, I/R induced more damage to the mice with steatosis, such as hepatocyte apoptosis, inflammatory cytokines (TNF-α, IL-6 and IL-1β), serum aminotransferases and thiobarbituric acid reactive substances. Importantly, ankaflavin administration significantly attenuated these changes. In addition, ankaflavin significantly decreased the proliferation of Kupffer cells and the expression of TNF-α, IL-6 and IL-1β protein in isolated Kupffer cells stimulated by TNF-α.

CONCLUSION: Ankaflavin has protective effects against I/R injury through anti-inflammatory, anti-oxidant and anti-apoptotic mechanisms in fatty livers, these effects are at least partially mediated by inhibiting Kupffer cell functions.

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KEY WORDS: ankaflavin; ischemia-reperfusion; Kupffer cells; steatotic liver

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common disease worldwide, especially in developed countries and its incidence is rapidly increasing.[1] In the transplant community, the disparity between the number of patients on the waiting list and available donor organs remains substantial, and steatotic livers have been considered as grafts based on extended donor criteria.[2] Notably, steatotic livers are more prone to hepatic ischemia-reperfusion (I/R) injury, suggesting a higher risk of graft dysfunction and damage after transplantation.[3]

A large amount of data indicates that reactive oxygen species (ROS) play a pivotal role in hepatic I/R injury by damaging organic compounds and increasing the expression of pro-inflammatory cytokines.[4] Additionally, it is believed that Kupffer cells, the liver resident macrophages, are activated during the development of steatotic livers and further by I/R injury.[5] The toxic mediators released by Kupffer cells, including superoxide radicals, tumor necrosis factor (TNF), and cytokines, lead to hepatocyte damage.[6] Also, there is evidence that the down-regulation of Kupffer cell function can significantly attenuate hepatic I/R injury in steatotic livers.[7]

Ankaflavin is a traditional food additive that has been used in Eastern Asia, including China, for the last few centuries. Recently, some reports[8-13] have revealed its beneficial anti-obesity, anti-tumor and anti-inflammatory effects, and its ability to prevent cardiovascular

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diseases and NAFLD. Lee and co-workers[8] reported that ankaflavin prevented fatty acid accumulation in hepatocytes by inhibiting the uptake of fatty acids and lipogenesis, promoting fatty acid beta-oxidation *in vitro*, as well as reducing total cholesterol, triglyceride and free fatty acids in the plasma *in vivo*. Recently, it was shown that ankaflavin reduced the expression of cytokines, such as TNF-α, IL-6 and IL-1β.[10] Based on these previous data, we proposed that ankaflavin protects steatotic livers against I/R injury. We also tried to figure out the potential mechanisms.

Methods

Animals and experimental protocol

Four-week-old inbred C57BL/6J male mice (Model Animal Research Center of Nanjing University, Nanjing, China) were caged in a constant temperature room (25 ℃) with a 12:12-hour light-dark cycle, and free access to tap water and food. The fatty liver model was developed by feeding the mice with high fat diet (HFD; D12492, Yongli Co., Shanghai, China) from the age of 4 weeks to 8 weeks old.[14] In contrast, the control mice were fed with normal diet (ND). Firstly, three mice were fed ND, HFD and HFD+ankaflavin individually for testing the role of ankaflavin. They were killed after one night fasting. Secondly, eighteen mice were equally divided into 3 groups for I/R: the control group, the mice were fed with ND, and administered with normal saline before I/R; the HFD group, the mice were fed with HFD, and administered with normal saline before I/R; the HFD+ankaflavin group, the mice were fed with HFD, and administered with 0.624 mg/kg ankaflavin (ANPEL Scientific Instrument, Shanghai, China; Molecular formula: $C_{23}H_{30}O_5$, Molecular weight: 386.48, CAS number: 50980320) before I/R. The saline or ankaflavin was administrated by gavage once a day for one week. The ankaflavin dose in this study was based on a reference dose for a human adult with a weight of 65 kg and a height of 170 cm according to a previous study.[12] Our pilot studies showed that this dosage is optimal in the protection of I/R-induced injury in steatotic mice. The experimental protocol was approved by the Nanjing Medical University Institutional Animal Care and Use Committee (NJMU08-092).

Surgery

The animals were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal injection). The left portal vein was isolated and clamped with a micro-vessel clamp for one hour and then released for reperfusion, the incision was then sutured. The mice were killed 3 hours after reperfusion; a blood sample via cardia puncture and a fresh liver sample were collected for future analysis.

Serum aminotransferases

The serum was separated for aminotransferases (ALT and AST) analyses with a diagnosis kit (Bioassay, Wiener Laboratories, Rosario, Argentina).

Histology

The liver tissues were embedded in paraffin, cut and stained with hematoxylin and eosin (HE). The percentage of the steatosis area in the livers was evaluated by MetaMorph software.

Immunohistochemistry

Paraffin sections were stained with a rat monoclonal antibody (R&D Systems, Minneapolis, MN, USA) against F4/80, an important surface marker of macrophages, and then the activation of Kupffer cells in the livers were assessed.

Quantitative real-time PCR

Total RNA was extracted from frozen liver tissues using RNA-Bee reagent (Bio-Connect, Huissen, the Netherlands). A Super-Script First-Strand Synthesis System (Invitrogen, CA, USA) was then used for reverse transcription. Real-time PCR was performed by using the Light-Cycler System (Roche, Indianapolis, IN, USA) to determine the relative amounts of cDNA molecules as previously described.[15] The following primers were used: TNF-α (5'-TGT CTA CTG AAC TTC GGG TGA T-3' and 5'-AAC TGA TGA GAG GGA GGC CAT-3'); IL-6 (5'-CTG CAA GTG CAT CAT CGT TGT-3' and 5'-TGT CTA TAC CAC TTC ACA AGT CGG A-3'); IL-1β (5'-TGG CAG TCC TCT GTC CTT G-3' and 5'-GAT CTT TCA CAG ACA CTG CTG C-3'); GAPDH (5'-GGT CAC CAG GGC TGC CAT TTG-3' and 5'-CTG GTA CTC CAT ACA CTG GCT-3').

Thiobarbituric acid reactive substances (TBARS)

TBARS was measured to evaluate the hepatic oxidative stress using a QuantiChrom™ TBARS kit (BioAssay Systems, USA).

Isolation of mouse Kupffer cells and hepatocytes

Primary Kupffer cells were isolated from normal and steatotic livers through the portal vein perfusion. Briefly, livers were perfused with Gibco Liver Perfusion Media (Invitrogen) and Gibco Liver Digestion Media (Invitrogen) *in situ*. After excised, minced and strained through a steel mesh sieve, the hepatocytes were centrifuged, col-