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Original article

Nobiletin ameliorates ischemia–reperfusion injury by suppressing the function of Kupffer cells after liver transplantation in rats

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A B S T R A C T

This study aims to explore the protective effects of nobiletin against hepatic ischemia–reperfusion (IR) injury after liver transplantation. Kupffer cells (KCs) were activated and co-cultured with different concentration of nobiletin for 24 h in vitro, inflammatory products and activity of TLR4/NF-κB signaling pathway were detected. Sprague–Dawley rats were selected and underwent orthotopic liver transplantation. Donors were injected intravenously with nobiletin (50 mg/kg) or saline solution, once a day for 1 week before the surgery. Recipients were randomly paired and sacrificed at the indicated time points (3, 6, and 24 h after the surgery), the graft liver tissues and blood samples were collected for analysis. Hepatic function, inflammatory mediators, apoptosis of hepatocytes, histological changes, KCs and CD4+ T-lymphocyte infiltration were assessed. Results showed nobiletin dose-dependently suppressed the expression of inflammatory mediators and the activity of TLR4/NF-κB signaling pathway in activated KCs. Furthermore, nobiletin alleviated liver damage induced by IR in vivo, significantly decreased the serum levels of alanine aminotransferase, aspartate transaminase, inflammatory cytokines and alleviated the histopathology changes. Moreover, liver in the nobiletin treated group exhibited less KCs and CD4+ lymphocyte infiltration and lower hepatocyte apoptosis after operation. In addition, activity of TLR4/NF-κB signaling pathway in KCs was also suppressed, consistent with the results in vitro. Collectively, Nobiletin can ameliorate IR injury after liver transplantation and may be a promising new strategy to protect against liver IR injury.

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1. Introduction

Liver transplantation is an effective treatment for various end-stage liver diseases. Ischemia–reperfusion (IR) injury is a main challenge in transplantation because it could lead to graft non-function, may predispose to late chronic rejection, and contributes to shortage of donor organs [1]. Previous studies have demonstrated that IR injury represents a complex pathophysiological process affected by various factors. The complicated process is triggered by reactive oxygen species (ROS), macrophage/endothelial activation, enhanced expression of adhesion molecules, and cell apoptosis [2,3]. Resent study showed that Kupffer cells (KCs) are the dominant resident macrophages in liver and play a pivotal role in hepatic IR injury [4]. However, the underlying mechanisms are not fully understood. Therefore, suppressing the immune function of KCs and the following inflammatory cascades can effectively ameliorate hepatic IR injury.

Nobiletin (chemical structure is shown in Fig. 1), a polymethoxyflavone mainly present in the peel of citrus fruit, possess significant biological properties. Previous studies indicated that nobiletin exhibits a broad range of biological activities, such as anti-tumor [5], anti-inflammatory [6], antioxidant [7], and anti-diabetic activities [8]. Nobiletin can also suppress ROS production and alleviate brain and renal injuries [9]. Considering the promising anti-inflammatory effects of nobiletin, in this study, we examined its effects on KCs as well as hepatic IR injury in rat models, aiming to investigate the underling mechanisms and to find new therapeutic strategies against IR injury after liver transplantation.

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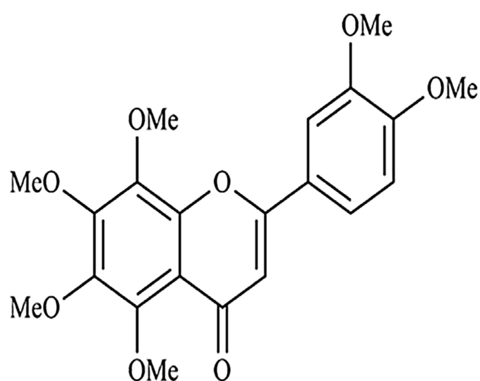

Fig. 1. Chemical structure of nobiletin.

2. Materials and methods

2.1. Materials

Nobiletin was purchased from Xian Xiaocao Botanical Development Co., Ltd. (Shanxi, China). Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide (LPS), collagenase IV, and F4/80 were provided by Abcam Trading (Shanghai) Company Ltd. (Pudong, Shanghai, China). Fetal bovine serum (FBS) was acquired from Invitrogen (Carlsbad, CA, USA). RPMI-1640 was supplied by Gibco (Gibco-BRL, Gaithersburg, MD, USA). Enzyme-linked immunosorbent assay (ELISA) kits for rat IL-1β, TNF-α, IL-6, TNF-α, and IL-10 were supplied by Abcam Trading (Shanghai) Company Ltd. (Pudong, Shanghai, China). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) kit and 3, 3′-diaminobenzidine (DAB) staining kits were provided by Roche Company (Roche, Shanghai, China). Antibodies against IRAK4, Ikkα, NF-κB p65 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against IκBα, p-IκBα, p-p65 were supplied by Cell Signaling Technology (Danvers, MA, USA); CD4, TLR4, inducible-nitric oxide synthase (iNOS), monocyte chemoattractant protein (MCP-1), Cyclooxygenase-2(COX2), chemokines CXCL2, CXCL10 and β-actin were purchased from Abcam Trading (Shanghai) Company Ltd. (Pudong, Shanghai, China).All other reagents used in this study were of commercially available analytical grade.

2.2. Isolation of KCs and culture

KCs were isolated from normal or graft liver samples 24 h after the transplantation by using the method described by Gong et al. [10]. Briefly, the liver was perfused with phosphate buffer saline containing 0.05% collagenase IV via the portal vein. The suspended cells were separated into the nonparenchymal and parenchymal cell fractions by differential centrifugation. KCs were then isolated from nonparenchymal cells by using Percoll solution. Purification was performed using selective adherence method for 2 h. KCs were collected and cultured in six-well culture plates at a density of 1×10^6 cells/well in DMEM, which was supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 U/ml streptomycin sulfate at 37 °C in the presence of 5% CO_2).

The viability of KCs was determined by trypan blue staining. In briefly, 100 μl of cells was transferred to a 1.5 ml clear tube and incubated for 3 min at room temperature (25 °C) with an equal volume of 0.4% (w/v) trypan blue solution (Beyotime, China). Cells were counted using a dual-chamber hemocytometer and a light microscope. Viable cells were unstained and nonviable cells were stained blue. These two types of cells were recorded separately, and the means of five independent cell counts were pooled for analysis. To detect the phagocytic effect, Ink (Hongxing, China) was diluted at 1:800 in the growth medium, and added to the KCs seeded in 6-well plate (1×10^5 cells/well). After incubating for 4 h at 37 °C, the cells were rinsed with PBS for three times to remove the non-phagocytosed ink, and then observed in light microscope.

KCs were activated by incubating in DMEM with LPS (100 ng/ml) and harvested at 3 h. In the nobiletin treated groups, activated KCs were added fresh medium containing different concentration of nobiletin (0, 5, 10, 20 μM) and incubated for another 24 h. KCs and culture supernatant in different groups were harvested for analysis.

2.3. Animals and experimental protocol

Male Sprague–Dawley rats (170–250 g) were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). Animals received humane care in accordance with the guidelines provided by the National Institutes of Health for the use of animals in laboratory experiments. The animal protocols used in this work were evaluated and approved by the Animal Use and Ethic Committee of 2nd Affiliated Hospital of Chongqing Medical University (Protocol 2014-16, Chongqing, China).

Rat orthotopic liver transplantation was performed using Kamada's method with slight modifications [11]. During the operation, the cold ischemic duration in compound sodium chloride solution was <60 min. The rats were randomly divided into three groups and the treatments were as follows:

(1) Sham group (n = 15), in which rats underwent laparotomy surgery without liver transplantation, and no drug was utilized;
(2) IR+ nobiletin group (with 15 donors and 15 recipients), in which the donors were injected intravenously with nobiletin (50 mg/kg) by using a previously described method [6] once a day for 1 week. Then the recipients were randomly paired and received orthotopic liver transplantation;
(3) IR group (with 15 donors and 15 recipients), in which rats were subjected to liver transplantation and donors were pretreated with isodose normal saline solution as that in the IR+ nobiletin group once a day for 1 week before the surgery.
(4) At 3, 6, and 24 h after the surgery, 5 recipients were sacrificed at each time point in each group. Blood samples were collected from inferior vena cava after the mice were anesthetic. Graft livers were removed and lavaged with normal saline solution from the portal vein to drive out the intrahepatic blood. Then liver tissues were divided into small pieces and stored for further analysis. Three biological samples were included in quantitation of each experiment.

2.4. Histopathological and immunohistochemical examination

Graft liver tissues were fixed by immersing in 10% buffered formaldehyde, embedded in paraffin, sectioned, and stained using hematoxylin and eosin. Histological findings were analyzed blindly by using modified Suzuki's criteria [12]. The sections were stained using DAB and analyzed immunohistochemically to detect the infiltration of CD4+ T lymphocytes (information of CD4 antibody are shown in Table 1). KCs were stained with FITC-F4/80, and infiltration was assessed by laser scanning confocal microscopy (LSCM).

2.5. Immunostaining of TUNEL

TUNEL was performed on paraffin sections by using commercial kits to detect hepatocyte apoptosis. The assay was conducted in

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