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Original article

Therapeutic effects of quercetin on early inflammation in hypertriglyceridemia-related acute pancreatitis and its mechanism

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ABSTRACT

Objective: To investigate the therapeutic effects of quercetin on early-stage inflammation in hypertriglyceridemia (HTG)-related acute pancreatitis (AP) both *in vivo* and *in vitro*, and its possible mechanism.

Methods: *In vivo*, rats were fed a high-fat diet to induce HTG, and AP was induced by intraperitoneal injection of cerulein (50 μg/kg × 2). Quercetin (100, 150 and 200 mg/kg) was administered by intraperitoneal injection after AP induction. *In vitro*, rat exocrine acinar cells were preincubated with palmitic acid (PA, 0.1 mmol/L, 6 h) with quercetin (5, 10, 20 and 40 μM) prior to a cholecystokinin analog CCK-8 (20 pM). Injury of the pancreas was assessed by amylase secretion and pancreatic histological evaluation. Inflammation was estimated by measuring IL-1β, IL-6, TNFα and NF-kB expression. Dynamic expression of IRE1α, sXBP1, C/EBPα and C/EBPβ was monitored by real-time PCR, immunofluorescence (IF) and western blot (WB).

Results: Quercetin intervention reduced plasma amylase level ($P < 0.001$) in a dose-dependent manner, attenuated pancreatic histopathological damage ($P < 0.05$), and reduced the mRNA and protein expression of NF-kB, IL-1β, IL-6, TNFα ($P < 0.05$) more significantly in HTG-related AP rats than in normal-lipid AP rats. Quercetin also down-regulated gene and protein expression levels of IRE1α, sXBP1, C/EBPα and C/EBPβ in a dose-dependent manner.

Conclusions: Quercetin attenuates early-stage inflammation in HTG-related AP, probably by reducing IRE1α, sXBP1, C/EBPα and C/EBPβ expression.

Introduction

Hypertriglyceridemia (HTG) is a well-established and independent risk factor for acute pancreatitis (AP). It is alleged to account for 10% of all pancreatitis episodes, and up to 50% of all gestational pancreatitis cases [1]. When serum triglyceride (TG) level exceeds 5.65 mmol/L, i.e., about three times the normal level, the risk of AP sharply increases; when TG level decreases and reaches values below 5.65 mmol/L, the risk is really low [2]. Some clinical studies have reported that HTG intensifies the course of AP and aggravates inflammatory responses [3,4]; however, the exact mechanism remains unclear. It is well known that multiple organ failure in patients with severe AP is mainly due to exaggerated inflammatory response or systemic inflammatory response syndrome (SIRS).

Endoplasmic reticulum stress (ERS) is a pathophysiological concept of AP explained at the cellular and biological level. High fat diet (HFD) itself is able to induce ERS in liver, adipose and skeletal muscle tissue [5], especially IRE1α-sXBP1 pathway, which is closely associated with lipid metabolism and inflammation [6]. Our previous study found that ERS was positively correlated with pancreatic histopathological damage in the aggravation of AP by HFD [7]. CCAAT enhancer binding proteins (C/EBPs) are a family of transcription factors including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPξ. C/EBPα and C/EBPβ are the most involved in lipid metabolism and inflammation [8,9]; however, their role in AP has

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not been reported so far. Quercetin (3,3′,4′,5,7- pentahydroxylflavone, QE), a naturally occurring plant flavonoid abundantly present in fruits, vegetables and cereals, is considered to have beneficial biological activities, including anti-inflammatory, antioxidant, antitumor, anti-atherosclerotic properties, and it has been shown to play a protective role in many diseases. In addition, QE is reported to suppress ERS by reducing IRE1α both *in vivo* and *in vitro* [10,11], and inhibit adipogenesis via the downregulation of C/EBPα and C/EBPβ in adipocytes and fibroblasts [12,13]. QE can also inhibit the transcriptional activity of C/EBPβ, and thereby suppress apolipoprotein B expression in enterocytes [14].

The present study aimed to investigate the therapeutic effects of different doses of quercetin on the early-stage inflammation of HTG-related AP both *in vivo* and *in vitro*, and the changes in expression of ERS-related molecules (IRE1α, sXBP1), C/EBPα and C/EBPβ.

Materials and methods

Experimental animals and protocols

All animal experiments were performed according to the guidelines of the Animal Care and Use Committee of Shanghai Jiaotong University. Male Sprague–Dawley (SD) rats weighing 100–110 g, normal chow and the HFD chow (20% lard and 3% cholesterol supplemented to rodent regular chow diets for two weeks) were all purchased from SLAC Laboratory Animal Co. Ltd (Shanghai, China).

In the first series of experiments, rats were fed with normal chow or HFD for 2 weeks, and then killed at 3, 6, 9 and 24 h after AP induction. The second series of experiments were designed to determine the effect of treatment with different doses of QE. Rats were randomly assigned to 10 groups (n = 6) as follows: Normal triglyceride (Nor), Nor + AP, Nor + AP + QE (100, 150 and 200 mg/kg), HTG, HTG + AP, HTG + AP + QE (100, 150 and 200 mg/kg). AP was induced by two hourly intraperitoneal injections of cerulein (Sigma), each time at a dose of 50 μg/kg body weight. QE (Sigma) was dissolved in 50% DMSO and injected intraperitoneally 30 min, 1 h, 2 h and 3 h after AP induction. The Nor, Nor + AP, HTG and HTG + AP groups were administered with the same volume of vehicle solution at the same timepoint. All rats were killed 9 h after AP induction. Blood samples were collected from abdominal aorta, a portion of each pancreas was fixed in 10% formaldehyde in phosphate-buffered saline (PBS; pH 7.4) and embedded in paraffin, and parts of pancreatic tissues were quickly frozen using liquid nitrogen and stored at −80 °C until use.

Serum and plasma biochemistry

Serum triglyceride (TG) and total cholesterol (TC) levels and plasma amylase levels were determined using the automated biochemical analyzer (Bayer Advia 1650, Germany). The percentage of amylase decrease was calculated as follows: the decreased percentage = (amylase of AP group without QE intervention − amylase of QE intervention group)/amylase of AP group without QE intervention. Plasma levels of IL-1β, IL-6 and TNFα were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols (eBioscience).

Histological examination of the pancreas

Pancreas from each rat was fixed with formaldehyde and embedded in paraffin, and then cut into 4 μm sections and stained with hematoxylin and eosin. Histopathological evaluation was performed using light microscopy by two investigators who were blind to the experimental treatment. Edema, necrosis, hemorrhage and inflammatory infiltration were evaluated according to the scoring scale reported by Schmidt et al. [15].

PAC isolation and culture

Rat pancreatic acinar cells (PAC) were isolated from rats using a collagenase digestion procedure. Pancreata from SD rats were removed, digested by collagenase at 37 °C, dispersed by pipetting, and passed through a mesh nylon cloth. For treatments, the isolated acinar cells were incubated at 37 °C in Dulbecco's modified Eagle's medium/Ham F-12 medium (DMEM-F12; Hyclone, USA) containing 10% fetal bovine serum (FBS; Hyclone, USA). PAC were preincubated with or without 0.05/0.1 mM palmitic acid (PA) for indicated timepoints (3/6/9 h) prior to 10/20 pM CCK-8 (Sigma) stimulation in 6-well culture plates. For QE stimulation group, QE was added together with PA.

Amylase and LDH activity assay

After stimulation with CCK-8, acini were separated by centrifugation and the cell-free supernatant was used to measure levels of amylase and lactate dehydrogenase (LDH) activity using the Phadebas amylase test and rat LDH ELISA kit according to the manufacturer's instructions. Net stimulated secretion in percent was calculated by subtracting the secretion in the absence of CCK-8 ("basal secretion) from the secretion noted in the presence of CCK-8 ("stimulated" secretion). The amount of amylase and LDH secreted was expressed as a percentage of the total amount of those in acinar cells.

Real-time PCR

Total RNA was isolated from pancreatic tissues and pancreatic acinar cells using Trizol (Invitrogen, USA). Reverse transcription (RT) was performed using the PrimeScript™ RT Master Mix (Takara, RR036A). Quantitative PCR was performed using the SYBR *Premix Ex Taq*™ (Takara, RR420A) following the manufacturer's protocol. The RT reaction was set at 37 °C for 15 min, and then inactivated at 85 °C for 5 s. The PCR protocol was as follows: denaturation at 95 °C 10 s, annealing at the appropriate temperature (Table 1) for 30 s, and extension at 72 °C for 30 s. Samples were run for 40 cycles. Amplification specificity was confirmed by analysis of melting curves. Relative gene expression values were calculated with $2^{-\Delta\Delta Ct}$ method [16]. Changes in mRNA expression level were calculated after normalization with β-actin expression. Primers used for RT-PCR reactions are given in Table 1.

Immunofluorescence (IF)

Paraffin-embedded sections of pancreatic tissues were subjected to IF staining as follows. The sections were dewaxed and rehydrated after deparaffinization. High-temperature antigen retrieval involved boiling the slides in citrate buffer (10 mM, pH 6.0) for 20 min. The sections were incubated with anti-IRE1α (1:200), anti-C/EBPα (1:200) and anti-C/EBPβ (1:200) primary antibodies (Santa Cruz) overnight at 4 °C. Then sections were then incubated with fluorescein-labeled secondary antibody for 1 h at room temperature. 4′6-Diamidino-2-phenylindole (DAPI) was applied to visualize the nucleus. Representative images were captured with an Olympus IX70 camera.

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