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Limonin attenuates hepatocellular injury following liver ischemia and reperfusion in rats *via* toll-like receptor dependent pathway

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ABSTRACT

Limonin has been shown to exhibit anti-inflammatory and antioxidant properties in the settings of chemically induced hepatic injury. The current study aimed to investigate the protective effects of limonin on experimentally-induced hepatic ischemia reperfusion (I/R) injury in rats. Rats were injected IP with either DMSO or limonin (100 mg/kg BW), 30 min before submission to 45 min of ischemia, followed by 1 h of reperfusion. Limonin ameliorated the deleterious effects of I/R as indicated by improvement in liver function tests, reduction of lactate dehydrogenase, reduction of oxidative stress, decrease in hepatocyte degeneration, and pyknosis. Furthermore, pretreatment of I/R rats with limonin, induced a significant down regulation in the various elements of the toll like receptor (TLR)pathway including TLR-2 and TLR-4, myeloid differentiation factor 88 (MYD88) and toll/IR-1(TIR)-domain-containing adaptor protein inducing interferon-beta (TRIF) and the downstream effectors TNF-α, TNF-α/IL-10 ratio and nuclear factor-κB (NF-κB). It also increased the anti-inflammatory cytokine IL-10 and decreased the activity of the apoptotic marker, caspase-3. These data indicate that limonin exerts antioxidant and anti-inflammatory effects in ischemic liver, thus, protecting hepatocytes against I/R injury in rats. The mechanism of these hepatoprotective effects appears to be related to the antioxidant and anti-inflammatory potential of limonin mediated by the down regulation of TLR- signaling pathway.

1. Introduction

The standard treatment of liver failure is liver transplantation. Hepatic ischemia/reperfusion injury (IRI) is commonly noticed during liver transplantation and represents the main underlying cause of post-transplantation liver damage (Peralta et al., 2013). I/R injury is caused by several mechanisms including, deleterious impact of free radicals especially reactive oxygen species and inflammation mediated *via* production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins (IL-1, IL-6) (Bilzer and Gerbes, 2000; Cheng et al., 2008; Mahmoud et al., 2012). The early phase of I/R injury (lasts for up to 2 h after reperfusion) is characterized by the activation of Kupffer cells (KC). Activated KC produce reactive oxygen species and several pro-inflammatory cytokines (Zapolska-Downar and Naruszewicz, 2009). Both reactive oxygen species and TNF-α damage hepatocytes causing lipid peroxidation of cell membranes and depletion of the protective GSH releasing liver enzymes. Therefore, the administration of exogenous free radical scavengers or anti-inflammatory agents could reduce the extent of I/R damage of the liver.

Toll like receptors (TLRs) are group of transmembrane receptors through which the innate immune system recognizes specific endogenous molecules produced by damaged tissues (DAMP) during ischemic damage (Akira and Takeda, 2004). TLRs are expressed in hepatocytes and other liver cells. They have been shown to be important for the production of the inflammatory response observed in experimental I/R injury (Chong et al., 2004).

Limonin belongs to the limonoids found in citrus fruits. Limonoids represent secondary metabolites in all citrus fruit tissues and unique highly oxygenated triterpenoid compounds. They are recognized as one of the most important components of medical foods (Ozaki et al., 1995). Limonin possesses antibacterial, antiviral, antinociceptive, anti-inflammatory, anti-carcinogenic in aflatoxin B1-induced hepatocellular carcinoma and anti-feedant effects (El-Readi et al., 2010; Langeswaran et al., 2012; Miller et al., 1992). It also can suppress HIV-1 protease activity in infected human mononuclear cells (Battinelli et al., 2003).

Limonin can induce an important detoxifying enzyme, glutathione S-transferase (Kelly et al., 2003; Lam et al., 1989). The antioxidant activity of limonin has been confirmed by the β-carotene bleaching assay (Sun et al., 2005) and by *in vitro* free radical scavenging activity (Yu et al., 2005). Limonoids exert a

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hepatoprotective effects in chloroform-induced liver damage (Germanò et al., 2005). Our previous study of the *in vivo* and *in vitro* hepatoprotective effects of limonin on D-galactosamine-induced liver injury, revealed that limonin possesses a hepatoprotective activity comparable to that of silymarin (Mahmoud et al., 2014). The current study aimed to determine the hepatoprotective effect of limonin on rats I/R injury and to explore the role of TLRs in such protective effects.

2. Materials and methods

2.1. Animals

Twenty-four adult male Wistar rats (200–220 g; Zagazig University, Zagazig, Egypt) were used in the present study. All animals were maintained under standard husbandry condition with food and water *ad libitum*. The experimental procedures were approved by the Institutional Animal Ethics Committee of the Faculty of Pharmacy, Zagazig University (approval number P2-6, 2012) and animals were handled following the International Animal Ethics Guidelines, ensuring minimum animal suffering.

2.2. Study protocol

Animals were randomly divided into three experimental groups (each containing eight animals): sham, ischemia/reperfusion (I/R) injury, limonin (100 mg/kg). Sham group received vehicle then anesthetized, the portal vein and bile duct exposed but not occluded. Rats of I/R group were anesthetized by i.p injection of ketamine (75 mg/kg) and subjected to partial liver ischemia (70%) followed by reperfusion. Ischemia was induced by occluding hepatic portal vein and bile duct with a traumatic vascular clamp. After 45 min of ischemia, the clamp was removed to start reperfusion for 1 h. Limonin dissolved in dimethyl sulfoxide (DMSO) then given i.p as single dose 30 min before ischemia. Blood was collected from the retro-orbital plexus and centrifuged (3000 × *g*, 4 °C, 20 min) for separation of serum. The obtained serum was used to analyze liver enzymes. Thereafter, animals were killed, livers were separately dissected and blood was washed off with cold saline then, livers were divided into two parts: one part was immediately flash frozen in liquid nitrogen and kept at −80 °C for measurement of tissue parameters and the other part was kept in 10% formalin for histopathological examination.

2.3. Biochemical analysis

The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT) enzyme activities and lactate dehydrogenase (LDH) level were measured using commercially available analytical kit (Biodiagnostic Co, Egypt).

2.4. Determination of oxidative stress

The generation of reactive oxygen species in response to hepatic ischemia/reperfusion injury was determined in liver tissues by the measurement of the lipid peroxidation product content, malondialdehyde (MDA), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities. SOD, MDA and GPx were measured in tissue homogenate photometrically (spectrophotometer, Jenway®, England, UK) according to Nishikimi et al. (1972) and Ohkawa et al. (1979).

2.5. Determination of proinflammatory and anti-inflammatory cytokines

The TNF-α level in liver homogenate was detected according to the previous reported method (Maskos et al., 1998), using ELISA kit (Quantikine, USA). IL-10 level in liver homogenate was detected by using ELISA kit (Bio Vendor, Germany). TNF-α/IL-10 was mathematically calculated.

2.6. Determination of TLR-2, TLR-4, MYD88, TRIF and NF-κB

Toll like receptors 2 and 4 (TLR-2 and TLR-4), their adaptor proteins, myeloid differentiation factor 88 (MYD88) and toll/IR-1 (TIR)-domain-containing adaptor protein inducing interferon-beta (TRIF) and NF-κB were determined by qRT-PCR as following:

Total RNA was extracted from liver tissue homogenate using a spin or vacuum (SV) total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The extracted RNA was reverse transcribed into cDNA using RT-PCR kit (Stratagene, USA) according to the manufacturer's instruction.

A real time-PCR reaction mixture was composed of 25 ml SYBR Green Mix (2x), 0.5 ml cDNA, 2 ml of each primer pair mix (5 pmol/ml each primer), and dH_2O to 50 ml, the primer sequences are shown in Table 1. The PCR program was used to amplify cDNA consisted of 120 s at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, followed by 10 min at 72 °C. The real time-PCR result was analyzed with the step PE Applied Biosystems (Perkin Elmer) software and the data was expressed in Cycle threshold (Ct). Target gene expression was assessed and related to reference gene (β-actin) (Cho et al., 2011).

2.7. Determination of apoptotic marker (caspase-3) activity

This assay utilizes a synthetic tetrapeptide, Asp-Glu-Val-Asp (DEVD), labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin (AFC) as substrate. DEVD-dependent protease activity is assessed by detection of the free AFC cleaved from the substrates using spectrofluorometry according to method of Gurtu et al.(1997).

2.8. Histopathological study

Liver tissues were fixed in 10% formalin and embedded in paraffin. Sections of 5–6 μm thickness were stained with hematoxylin and eosin (H and E) then examined under a light microscope for determination of histopathological changes. The histological analysis was performed by a person blinded to the treatment.

Table 1
Sequences of PCR primers.

Gene	Primer sequences
TLR2	F: 5′-GAGCATCCGAATTGCATCAC-3′
	R: 5′-TATGGCCACCAAGATCCAGA-3′
TLR4	F: 5′-TCGAATCCTGAGCAAACAGC-3′
	R: 5′-CCCGGTAAGGTCCATGCTAT-3′
MD88	F: 5′-AAGAAAGTGAGTCTCCCCTC-3′
	R: 5′-TCCCATGAAACCTCTAACAC-3′
TRIF	F: 5′-ATGGATAACCCAGGGCCTT-3′
	R: 5′-TTCTGGTCACTGCAGGGAT-3′
NF-κB	F: 5′-GTCATCAGGAAGAGGTTTGGCT-3′
	R: 5′-TGATAAGCTTAGCCCTTGCAGC-3′
β-actin	F: 5′-AGAACATCATCCCTGCATCC-3′
	R: 5′-TCCACCACCCTGTTGCTGTA-3′