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Silymarin and caffeine combination ameliorates experimentally-induced hepatic fibrosis through down-regulation of LPAR1 expression

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ABSTRACT

Aims: Lysophosphatidic acid is a lipid mediator that is supposed to be implicated in hepatic fibrosis. Silymarin and caffeine are natural compounds known for their anti-inflammatory and antioxidant effects. Our study aimed to explore the effect of silymarin, caffeine, and their combination on lysophosphatidic acid receptor 1 (LPAR1) pathway in thioacetamide (TAA)-induced hepatic fibrosis.

Main methods: Hepatic fibrosis was induced in male Sprague-Dawley rats by intraperitoneal injection of 200 mg/kg of TAA twice a week for 8 weeks. Silymarin (50 mg/kg), caffeine (50 mg/kg), and their combination (50 mg/kg silymarin + 50 mg/kg caffeine) were orally given to rats every day for 8 weeks along with TAA injection. Liver functions were measured. Histopathological examination of liver tissues was performed using hematoxylin and eosin and Masson's trichrome staining. mRNA expressions of LPAR1, transforming growth factor beta 1 (TGF-β1), connective tissue growth factor (CTGF), and alpha smooth muscle actin (α-SMA) were measured using RT-PCR. LPAR1 tissue expression was scored using immunohistochemistry.

Key findings: Silymarin, caffeine, and their combination significantly improved liver function. They caused significant decrease in fibrosis and necro-inflammatory scores. Combination of silymain and caffeine caused a significant decrease in the necro-inflammatory score than the single treatment with silymarin or caffeine. In addition, silymarin, caffeine, and their combination significantly decreased hepatic LPAR1, TGF-β1, CTGF, and α-SMA gene expressions and LPAR1 tissue expression.

Significance: Silymarin, caffeine, and their combination protect against liver fibrosis through down-regulation of LPAR1, TGF-β1, and CTGF.

1. Introduction

Chronic liver disease of different etiologies is a major world health problem. When left untreated, it leads to hepatic fibrosis. Hepatic fibrosis is described as a multicellular wound repairing process that involves excessive aggregation of extracellular matrix (ECM) inside the liver. Severe fibrosis results in disorganized tissue architecture and liver failure [1].

The major source of ECM is hepatic stellate cells (HSCs) that become activated to myofibroblasts upon liver injury. Activated myofibroblasts are mainly characterized by the expression of alpha smooth muscle actin (α-SMA) and play a crucial role in the incidence of fibrosis [2]. Transforming growth factor beta 1 (TGF-β1) is essential for the activation of myofibroblasts [3]. The pro-fibrotic effects of TGF-β are partially mediated via stimulation of its downstream mediator, connective tissue growth factor (CTGF) [4,5].

Lysophosphatidic acid (LPA) has acquired great attention after its discovery as a strong signaling molecule with effects on a wide range of tissues. Six G-protein-linked receptors for LPA have been recognized [6]. Lysophosphatidic acid receptors (LPARs) play a crucial role in physiological, developmental, and pathological processes [7]. LPAR1 is the first recognized and the best studied LPAR [8]. LPAR1 couples to three of the Gα proteins, activating downstream signaling cascades through mitogen-activated protein kinase (MAPK), phospholipase C, Rho, and Akt. Activation of LPAR1 promotes cell proliferation and migration, cytoskeletal changes, Ca^{2+} mobilization, and adenylyl cyclase inhibition [9].

Fibrosis was shown to be firmly affected by receptor-mediated LPA signaling in various organs. LPA was found to stimulate CTGF expression in both renal fibroblast cell lines [10] and pulmonary fibrosis animal model [11]. Moreover, it has been revealed that LPA stimulates proliferation of hepatocyte and HSCs [12,13]. An earlier study found increased plasma concentrations of LPA in chronic hepatitis C-induced liver fibrosis [14].

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Caffeine is an alkaloid present in a lot of beverages such as tea, cocoa, and coffee [15]. Recently, the anti-inflammatory and anti-fibrotic actions of caffeine were demonstrated in *in vivo* studies of liver fibrosis [16,17]. Moreover, increased caffeine intake was related to the decreased liver fibrosis in individuals experiencing chronic hepatitis C virus infection [18].

Silymarin is a mixture of flavonolignans extracted from milk thistle. The components of silymarin include silibinin (the main component), isosilybin A, isosilybin B, silycristin, isosilycristin, silydianin and taxifolin [19]. Silymarin is well-known for its anti-oxidant, anti-inflammatory and anti-fibrotic power and thus, it exhibits protective effects in various liver disorders such as fibrosis [20]. Although silymarin and caffeine show anti-fibrotic effects, it is still unclear whether their anti-fibrotic effects are mediated *via* LPAR1 pathway. Thus, the purpose of this study is to examine the anti-fibrotic effects of caffeine and silymarin either alone or in combination, and to explore whether their effects are mediated through LPAR1 and/or TGF-β1-CTGF pathways in hepatic fibrosis induced by thioacetamide (TAA).

2. Materials and methods

2.1. Drugs and chemicals

TAA and caffeine were obtained from Sigma Aldrich, USA. Silymarin was supplied in the form of a pharmaceutical product (Legalon tablets, MADAUS GmbH., Germany). Chemicals used in preparation of phosphate-buffered formalin were purchased from El Nasr Pharmaceutical chemicals Co., Egypt.

2.2. Animals and diet

Sixty male Sprague–Dawley rats (180–200 g) were purchased from VACSERA CO., Egypt. Rats were housed with food and tap water supplied *ad libitum* and maintained on 12 h-light/12 h-dark cycles.

The study protocols were accepted by "Research Ethics Committee" of the Faculty of Pharmacy, Mansoura University, Egypt that followed guidelines of "Principles of Laboratory Animal Care" (National Institute of Health publication No. 85-23, revised 1985).

2.3. Experimental design

Seven days after adaptation, rats were randomly classified to five groups (n = 12). Liver fibrosis was induced using freshly prepared TAA, dissolved in sterile saline and administered i.p (200 mg/kg) [21], while silymarin (50 mg/kg) [22] and caffeine (50 mg/kg) [23] were given by oral tube. The grouping and regimens of treatment are described in Table 1.

Rats were sacrificed one day after the last TAA dose and fasting overnight. Blood samples were gathered for serum preparation, while liver tissues were gathered for histopathological examination, immunohistochemistry, and RT-PCR.

Table 1
Grouping and regimens of treatment.

Group	Dose	Duration
Normal	Sterile saline, 0.2 ml	Twice per week for 8 weeks
TAA	200 mg/kg TAA	Twice per week for 8 weeks
Sily	50 mg/kg silymarin along with TAA	Silymarin daily and TAA twice per week for 8 weeks
Caff	50 mg/kg caffeine along with TAA	Caffeine daily and TAA twice per week for 8 weeks
Sily + Caff	50 mg/kg Silymarin + 50 mg/kg caffeine along with TAA.	Silymarin and caffeine daily, while TAA twice per week for 8 weeks

Table 2
Primers sequence and direction.

Gene	Direction	Sequence	Reference sequence
LPAR 1	Forward	5`- TTTCACAGCCCCAGTTCACA-3`	NM_053936.3
	Reverse	5`- GCTTGCTCACAGTGTTCCAT-3`	
TGF-β1	Forward	5`- CCGCAACAACGCAATCTATGA-3`	NM_021578.2
	Reverse	5`- GCACTGCTTCCCGAATGTCT-3`	
CTGF	Forward	5`- CGAGTCCTTCCAAAGCAGTT-3`	NM_022266.2
	Reverse	5`- ATCACACACCCACTCCTCAC-3`	
ACTA 2	Forward	5`- ACCATCGGGAATGAACGCTT-3`	NM_031004.2
	Reverse	5`- CTGTCAGCAATGCCTGGGTA-3`	
HPRT 1	Forward	5`- TGTCTTTCTCAAACCCTATTCCA-3`	NM_012583.2
	Reverse	5`- AAGGAGGATTAGTCGCAGCA-3`	

ACTA 2: actin, alpha 2, smooth muscle, aorta (gene encoding alpha smooth muscle actin protein); CTGF: connective tissue growth factor; HRPT1: Hypoxanthine-guanine phosphoribosyltransferase; LPAR 1: lysophosphatidic acid receptor 1; TGF-β1: transforming growth factor beta 1.

2.4. Biochemical analysis

The blood samples were allowed to clot, and then centrifuged at 1200 × *g* for10 min, at 4 °C, and the serum part was gathered. The serum levels of albumin and total protein were measured by the colorimetric method, following the manufacturer's protocols (Human Diagnostics, Germany). The serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by the kinetic method, following the manufacturer's protocols (Human Diagnostics, Germany). The total bilirubin serum levels were determined by the colorimetric method according to the instructions of the manufacturer (Diamond Diagnostics, Egypt).

2.5. Histopathological examination

After being fixed in 10% buffered formalin, liver tissues were embedded in paraffin, dissected into 5 µm sections, then stained with hematoxylin and eosin and Masson's trichrome stains.

2.6. Assessment of necro-inflammatory scores

Hematoxylin and eosin-stained liver sections were used for assessment of necro-inflammatory changes. They were scored according to Ishak's activity index [24]. The score is the sum of the following parameters: interface hepatitis (piecemeal necrosis) (0–4), confluent necrosis (0–6), spotty necrosis, apoptosis and focal inflammation and portal inflammation (0–4).

2.7. Quantification of fibrotic areas

Masson's trichrome- stained liver sections were used for quantitative analysis of the collagen contents. Images were captured using a digital camera placed on a BX51 Olympus optical microscope (Olympus Corporation, Japan). The NIH Image software was used for extraction and analysis of collagenous areas stained with Masson's trichrome. The intensity of fibrosis was represented as a percentage of the fibrotic (stained) area to the total tissue area by examining thirty random fields in the same slide [25].

2.8. Immunohistochemical detection of LPAR1

For immunohistochemistry, rabbit LPAR1 polyclonal primary antibody (Novus Biologicals, USA) was used in accordance with the standard protocols. Concisely, sections were dissected at 5 µm, deparaffinized, and then rehydrated. Hydrogen peroxide solution was used to