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Targeting NLRP3 inflammasome via acetylsalicylic acid: Role in suppressing hepatic dysfunction and insulin resistance induced by atorvastatin in naïve versus alcoholic liver in rats

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ABSTRACT

Background: NLRP3 inflammasome is described in many pathological conditions and is also involved in drug induced liver injury.
Aim of the work: To investigate the role of NLRP3 inflammasome in liver injury induced by chronic alcohol and/or atorvastatin ingestion.
Materials and methods: Sixty male Wistar rats were used. They were divided into 5 groups: (I) control naïve (II) Alcoholic: given ethanol 8 g/kg/day, p.o (III) Atorvastatin: given atorvastatin 10 mg/kg/day, p.o. (IV) Alcoholic + atorvastatin (V) Acetylsalicylic acid (ASA): given ASA 10 mg/kg/day, p.o together with alcohol and atorvastatin. Isolated perfused liver, biochemical and histological studies were done.
Results: Atorvastatin and alcohol induced liver inflammation with increasing the expression of NLRP3, IL-1β and caspase-8 immune-reaction. Atorvastatin and alcohol decreased the reduced form of glutathione in hepatic tissues and induced insulin resistance. ASA administration alleviated the hepatotoxic effects of alcohol and atorvastatin to a significant extent.
Conclusions: Acetylsalicylic acid alleviated the hepatotoxic effects of alcohol and atorvastatin through decreasing the production of NLRP3 inflammasome in rats' liver.

1. Introduction

Inflammasomes were described as large intracellular signaling molecules that contain nucleotide-binding oligomerization domain and leucine-rich repeat-containing receptors (NLRs). Among NLR inflammasome complexes, the NLRP3 inflammasome has been the most widely characterized that controls the maturation of the proinflammatory cytokine IL-1β [1].

Activation of NLRP3 leads to activation of pro-caspase-8 into caspase-8 which subsequently cleave pro-IL-1β into its mature form [2]. Therefore, NLRP3 inflammasome directly utilize caspase-8 as both a pro-apoptotic initiator and major IL-1β-converting protease [3].

NLRP3 inflammasome component had been found in hepatic stellate cells (HSCs) and killer cells (KCs) and promotes activation of macrophages and inflammation with production of IL-1β. This cytokine stimulates fatty acid deposition in hepatocytes and development of fatty liver [4].

The liver plays an important role in controlling blood glucose level. In liver impairment, glucose intolerance occurs frequently due to insulin resistance (IR) [5]. The impairment of insulin signaling pathway together with induction of reactive oxygen species (ROS) production and mitochondrial dysfunction lead to insulin resistance (IR) [6–8]. The role of inflammasomes in IR was suggested by Goldfine et al. [9]; they showed that mice with ablation of the NLRP3 receptor are resistant to

Abbreviations: AFL, alcoholic fatty liver; ALD, alcoholic liver disease; ALK, alkaline phosphatase; ALT, alaninetransaminase; ASA, acetylsalicylic acid; AST, aspartate transaminase; Bcl2, B cell lymphoma type 2; BSP, bromsulphthalein; COX-I, cyclooxygenase I; GSH, glutathione; GSSG, glutathione disulfide; HFD, high fat diet; HOMA-IR, homeostasis model assessment-insulin resistance; IL-1β, interleukin 1beta; KCs, Kuffer cells; MDB, Mallory-Denk bodies; NLRP3, NACHT, LRR and PYD domains-containing protein 3; OS, oxidative stress; PCR, polymerase chain reaction; rER, rough endoplasmic reticulum; ROS, reactive oxygen species; RT, PCR: reverse transcriptase polymerase chain reaction; sER, smooth endoplasmic reticulum; UDP-glucuronosyltransferase, uridine 5'-diphospho-glucuronosyltransferase

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diet-induced insulin resistance and hepatosteatosis.

Evidence supported the association between activation of the NLRP3 inflammasome and the pathogenesis of various chronic inflammatory and metabolic diseases, including alcoholic liver disease (ALD) [10]. ALD continues to be a major health concern. Prolonged ingestion of alcohol induces alcoholic fatty liver (AFL) with IR which may progress to steatohepatitis, fibrosis, and hepatocellular carcinoma [11].

Inflammasome components including NLRP3, pro-caspase-1 and mature IL-1β had been shown to be upregulated in the AFL suggesting that inflammasome activation is a component of the liver pathophysiology in ALD [12].

Statins effect on the liver is debateful. Williams et al. [13] proved that statin therapy triggers the NLRP3 inflammasome in vivo and promotes IR in metabolic tissues such as liver and pancreatic islets. They also showed that low dose aspirin can inhibit the NLRP3 inflammasome pathway and downregulate pro-IL-1β.

Therefore, the present study was undertaken to explore the role of NLRP3 inflammasome inhibition via acetylsalicylic acid (ASA) on the hepatic changes and IR induced by atorvastatin alone or in combination with chronic alcohol ingestion in rats.

2. Material and methods

2.1. Animals and grouping

All animal procedures were approved by the Institutional Animal Ethics Committee for Ain Shams University, Faculty of Medicine.

Sixty male Wistar rats (weighing 150 to 200 g) purchased from National Research Institute (Cairo, Egypt) were housed in an animal room with temperature (22 °C) and lighting (12 h (light) – 12 h (dark) cycle) control. An adaptation period of 1 week was allowed before initiation of the experimental protocol. The whole duration of the experiment was 6 weeks after acclimatization.

The rats were randomly distributed equally among 5 groups and fed on ordinary chow diet:

Group I; Control naïve group.

Group II; Alcoholic group; subjected to alcohol ingestion (8 g/kg/day) [14].

Group III; Atorvastatin group; given atorvastatin (10 mg/kg/day, p.o once/day) [15].

Group IV; Alcoholic + atorvastatin group; given alcohol + atorvastatin.

Group V; Alcoholic + atorvastatin + ASA group; given alcohol + atorvastatin + ASA (10 mg/kg/day, p.o once/day) [16].

2.2. Chemicals and drugs

Atorvastatin, ASA and Bromsulphthalein (BSP) were purchased from Sigma Chemical Company, Cairo, Egypt.

2.3. Experimental procedures

2.3.1. Induction of alcoholic liver disease

After acclimation for 6–7 days, animals were given ethanol 6 g/kg/day replacing water (solutions maximally containing 56% alcohol), and the dose was progressively increased during week 1 to a maintenance dose of 8 g/kg/day that was continued for 5 more weeks. All rats had regular standard rat chow available throughout the 6-week period [14].

2.3.2. Isolated liver perfusion

At the end of the experiment, whole liver was weighted, and liver perfusion was done in all groups according to Jäger et al [17].

After 30 min of perfusion with control medium (original Krebs Henseleit solution KHB), the water soluble BSP was added directly to KHB to give a concentration of 150 μM.

Single bile drops falling from the bile duct cannula were collected and weighed (approximately 8 mg). The concentration of BSP was determined according to the method of Seligson et al [18]. A mixture of 0.1 ml bile, 0.7 ml alkaline buffer (pH 10.6) prepared as follows (12.2 g $Na_2HPO_4.7H_2O$, 1.77 g $Na_3PO_4.12H_2O$ and 3.20 g of sodium p-toluenesulphonate, made up to 500 ml with distilled water) and 0.02 ml acid reagent (2 M NaH_2P0_4) was read spectrophotometrically at 580 nm. Bile flow was determined from the time interval between drops and from liver weight and was determined in mg/g liver/min. Canalicular excretion rate was calculated as the product of bile flow times biliary concentration.

2.3.3. Sample processing for biochemical measurements

Blood was collected, serum was separated and kept frozen at −80 °C until used in the quantitative determination of fasting glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin (total and direct). Fasting insulin was measured using rat insulin ELISA kit (RayBio, Norcross, GA, USA). Calculation of HOMA-IR was done and IR was considered when HOMA-IR ≥ 2.8 [15].

Liver samples (from left lobes) were stored at -80 °C until used in the measurement of reduced form of glutathione (GSH) according to the method of Ellman [19] and in the determination of the relative expression of IL-1β and NLRP3 by real-time PCR.

2.3.3.1. Real-time PCR. Total RNA was extracted using RNeasy mini Kit, RNA was reversed transcribed using QuantiTect Reverse Transcription Kit and real time PCR was performed for NLRP3 and IL-1β using QuantiTect SYBR Green PCR Kit. All kits were purchased from Qiagen, Hiden, Germany. β-actin was used as the housekeeping gene (Primers; Forward: 5′-AGGGAAATCGTGCGTGAC-3′, Reverse: 5′-CGCTCATTGCCGATAGTG-3′, GenBank NM_031144.3). For NLRP3, the primers were (Forward: 5′−CCAGGGCTCTGTTCATTG-3′, Reverse: 5′−CCTTGGCTTTCACTTCG-3′, GenBank NM_001191642.1) and for IL-1β, the primers were (Forward: 5′-CACCTTCTTTTCCTTCATCTTTG-3′, Reverse: 5′-GTCGTTGCTTGTCTCTCCTTGTA-3′, GenBank NM_031512.2).

In real-time PCR, the volume of the reaction mixture was 20 μl containing 500 ng cDNA and 0.5 μM of each primer. The cycling protocol was: an initial heating at 95 °C for 15 min then 40 cycles. Each cycle consisted of a denaturation step at 94 °C for 15 s, annealing step at 55 °C for 30 s and extension step at 70 °C for 30 s. Relative expression of target genes was presented as fold expression calculated using the $2^{-\Delta\Delta CT}$ equation.

2.4. Histological study

At the end of the experiment, the right lobe of liver was extracted immediately and divided into two halves for the following:

2.4.1. Light microscopic (LM) study

One half was cut into small pieces and fixed in 10% neutral buffered formalin then it was dehydrated and processed to get paraffin blocks for LM study. The paraffin blocks were cut into five micron-thick sections for H&E and Mallory's trichrome stains. Immunohistochemical staining was performed using an avidin biotin-peroxidase technique for detection of cleaved Caspase-8 (purchased from Cell Signaling Technology, USA) at a dilution of 1:100 for one hour. The reaction was developed with DAB solution for 10 min (purchased from DAKO, Denmark). Then, counterstain was done using Mayer's hematoxylin. Negative control sections were obtained by the same steps, except for the use of the primary antibody [17].

2.4.2. Transmission electron microscopic (TEM) study

The other half of liver specimen was cut into small parts (1 mm3) and fixed in 2.5% glutaraldehyde to be processed for TEM study. After