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Alpha lipoic acid exerts antioxidant effect via Nrf2/HO-1 pathway activation and suppresses hepatic stellate cells activation induced by methotrexate in rats

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ABSTRACT

Hepatic injury is a major side effect associated with methotrexate (MTX) therapy resulting from inflammatory reactions and oxidative stress induction. Therefore, liver fibrosis incidence is augmented with long-term MTX therapy. Alpha lipoic acid (ALA) is a naturally occurring compound with potent antioxidant activity. This study explored the hepatoprotective mechanisms of ALA against MTX-induced hepatic injury in rats. Hepatic injury was induced in MTX group by 20 mg/kg body weight *ip*. injection of MTX. ALA group was pretreated with ALA 60 mmol/kg body weight *ip*. for five days followed by a single dose of MTX in the sixth day. Blood samples and liver tissues were then obtained to assess several biochemical parameters as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), reduced glutathione (GSH), total antioxidant capacity (TAC) and lipid peroxidation. Nuclear factor E2-related factor 2/heme oxygenase-1 (Nrf2/HO-1) pathway was studied by determining the extent of mRNA Nrf2 expression and the level of HO-1. Hepatic stellate cells (HSCs) activation was evaluated by estimating the expression of α-smooth muscle actin (α-SMA) and hydroxyproline content. Also, tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and caspase-3 were assessed by ELISA in addition to histopathological examination of liver samples.

Results showed that ALA pretreatment improved liver function since serum ALT, AST and ALP levels were reduced. Additionally, ALA restored GSH and TAC levels when compared to MTX group and decreased lipid peroxidation. ALA exerted its antioxidant effect via Nrf2/HO-1 pathway as well as it showed anti-inflammatory and antiapoptotic effects by reducing TNF-α, iNOS, COX-2 and caspase-3 levels in liver tissue homogenate. Finally, ALA suppressed HSCs activation by decreasing α-SMA expression and hydroxyproline content in liver. It was concluded that ALA has hepatoprotective effects against MTX-induced hepatic injury mediated by Nrf2/HO-1 pathway as well as anti-inflammatory and antiapoptotic properties.

1. Introduction

Methotrexate (MTX), an antimetabolite, is used widely in cancer therapy and in the management of several autoimmune diseases such as psoriasis and rheumatoid arthritis. However, MTX therapy is usually associated with severe hepatic injury limiting its application [1]. It induces liver injury through the generation of free radicals and reactive oxygen species (ROS) leading to oxidative stress and mitochondrial injury. Cellular stress induced by MTX initiates inflammatory response evidenced by elevated levels of proinflammatory cytokine tumor necrosis factor-α (TNF-α), acute phase protein C-reactive protein (CRP), activated neutrophils and monocytes into the liver [2]. Besides it leads to oxidative DNA damage and induction of apoptotic cell death pathway [3]. Its toxicity is usually associated with elevation of liver enzymes [4]. Additionally, MTX acts as a profibrotic agent via inducing activation of resident hepatic stellate cells (HSCs) [3]. Activated HSCs result in deposition of extracellular matrix proteins (ECMs) and increased collagen production leading to hepatic cirrhosis and subsequently liver dysfunction. Also, fibrotic liver is evidenced by increased expression of α-smooth muscle actin (α-SMA), an essential marker for HSCs activation [5]. Several studies have presented the role of other molecules and mediators in the pathophysiology of liver injury and cirrhosis, such molecules as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [6,7].

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Alpha Lipoic acid (ALA) is a naturally occurring compound having anticancer and antioxidant activities. It decreases cell proliferation and induces apoptotic cell death in diverse types of tumor cells as bladder, lung, and breast cancer cell line [8]. It is also a powerful antioxidant generating glutathione (GSH) and other redox proteins thus inhibiting ROS-induced damaging effects [9,10]. Particularly, ALA is efficient in regulating the level of antioxidative enzymes as well as preventing hepatic inflammatory responses and oxidative stress [11,12]. Moreover, nuclear factor E2-related factor 2 (Nrf2) is a main regulator of the antioxidant system. Its nuclear translocation induces the cytoprotective gene heme oxygenase-1 (HO-1) for initiating cellular defense mechanisms [13,14].

This study was constructed to develop a prophylactic regimen against MTX-induced hepatic injury. Therefore, a rat model was used to study whether ALA exerts protective effects against liver injury due to MTX through its anti-inflammatory and antioxidant effects focusing on Nrf2/HO-1 pathway. Moreover, the hypothesis that ALA could hinder the activation of HSCs that may induce hepatic fibrogenesis due to MTX administration was explored.

2. Material and methods

2.1. Drugs and chemicals

All chemicals were purchased from Sigma Aldrich chemical Co. (St. Louis, MO, USA) except those mentioned elsewhere.

2.2. Animals and experimental design

The protocols of the experiment were approved by ethics committee of Modern Sciences and Arts University (MSA) Faculty of Pharmacy, Egypt (Approval letter reference: PG1/EC1/2017PD).

Wister albino male rats weighing 150:200 g, were used in this study. The rats were maintained at constant temperature (22 °C) with 12-hour dark-light cycle. A preliminary trial was done for selecting a proper dose of ALA using three doses 30, 60 and 120 mmol/kg body weight *ip*. According to liver function enzymes, the least protection against MTX hepatic injury was provided by ALA 30 mmol/kg body weight (data are not shown). Both ALA 60 and 120 mmol/kg body weight showed the most protective effects but there was no significant different between these two doses.

Three groups of animals were used in the study, seven rats for each group as follows: Group I, a control group, received normal physiological saline *ip*. Group II, a MTX-group, injected MT X 20 mg/kg body weight *ip*. single dose. Group III, an ALA + MTX treated group, were injected ALA 60 mmol/kg body weight *ip*. for five days followed by MT X 20 mg/kg body weight *ip* injection in the sixth day [15]. At day seven, rats were sacrificed by decapitation under light anesthesia. Blood samples were obtained to determine serum liver enzymes (ALT, AST, ALP). Liver organ was isolated and rinsed with ice cold saline then divided into two parts: one part for histopathological examination were fixed in 10% buffered formalin, the other part was kept frozen at −80 °C till the time of analysis.

2.3. Histopathological analysis

Fixed Liver tissues were dehydrated, embedded in paraffin, and sectioned at a thickness of 5 µm. The tissue sections were stained with hematoxylin and eosin (H&E) for histopathological analysis and examined with a light electric microscope [16]. The histopathological parameters were graded by using different symbols including (−) showing no changes, (+) showing mild degenerative changes, (++) showing moderate changes and (+++) showing severe changes.

2.4. Serum biochemical factors evaluation

The sera were used for measuring alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). These parameters were measured to evaluate hepatic injury by using a commercially supplied kit (Biodiagnostic, Cairo, Egypt).

2.5. Tissue homogenization

Liver tissue samples were weighed and divided into three parts. First part of the tissue was used to prepare 20% homogenate in phosphate buffered saline for biochemical analysis such as total antioxidant capacity (TAC), reduced glutathione (GSH) and lipid peroxides. Second part was homogenized in hypotonic lysis buffer supplemented with protease inhibitor cocktail and used for enzyme-linked immunosorbent assay (ELISA) for determining the level of TNF-alpha, COX-2, iNOS, hydroxyproline, caspase-3 and HO-1 levels. The third part was used for RNA extraction and quantifying gene expression of α-SMA and Nrf2.

2.6. Biochemical measurements

2.6.1. Liver lipid peroxidation assay

The level of malondialdehyde (MDA), which is the end product of lipid peroxidation was evaluated using a commercially supplied kit (Biodiagnostic, Cairo, Egypt). In which thiobarbituric acid reactive substances were detected spectrophotometrically at 535 nm [17].

2.6.2. Measurement of GSH level and total antioxidant capacity (TAC)

GSH content was determined by a method based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB) at 412 nm [18]. Measurement of TAC in liver tissue is indicative for its ability to counteract against oxidative stress-induced damage. The assay was done according to the manufacturer's instructions using TAC assay kit (sigma-Aldrich, USA) and TAC was estimated in Trolox equivalents at 570 nm using a microplate reader.

2.7. Enzyme-linked immunosorbent assay (ELISA)

2.7.1. Evaluation of hepatic inflammation and collagen deposition

The inflammatory mediator tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were estimated in liver homogenates by a quantitative sandwich ELISA following the manufacturer protocol (Sunlong Biotech, China). Hydroxyproline was evaluated, as a marker of increased collagen production due to HSCs activation, using sandwich ELISA kit (Shanghai Crystal Day Biotech CO., China)

2.7.2. Evaluation of apoptosis mediator and HO-1 level

Caspase-3 and HO-1 levels were measured in liver tissue homogenate according to the instructions of ELISA kit (Sunlong Biotech, China).

2.8. RNA extraction and quantification of gene expression

Total RNA was isolated from the liver tissues according to the manufacturer's instructions (Bioflux, China) and quantified at 260 nm. RNA 2 mg was used to synthesize cDNA (MyTaq Red Mix, Bioline) which was then amplified using SYBR Green master mix (Thermo scientific, USA). The primers used in amplifying the target genes are listed in Table 1. The PCR reactions were performed and the $2^{-\Delta\Delta Ct}$ method was used to analyze the amplification data (CFX96 Touch™ Real-Time PCR, BIO RAD). The results were normalized to β-actin.

2.9. Statistical analysis

All data were expressed as mean ± SEM. The difference between