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Alpha-lipoic acid protects mice against concanavalin A-induced hepatitis by modulating cytokine secretion and reducing reactive oxygen species generation

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ABSTRACT

Background: Alpha-lipoic acid (α-LA), which exits in almost all types of prokaryotic and eukaryotic cells, is a key regulator of energy metabolism in mitochondria. This study was designed to explore the protective effect of α-LA against concanavalin A (Con A)-induced hepatitis in mice and explore the potential mechanism.
Methods: Acute autoimmune hepatitis was induced by intravenous (IV) injection of Con A (15 mg/kg) in C57BL/6 mice. α-LA (100 mg/kg) was administered four days before Con A injection. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and histopathological change of the liver tissue were measured. Serum cytokine TNF-α, IL-6, IFN-γ and IL-10 were detected by ELISA. The mRNA levels of these inflammatory cytokines in the liver were detected by RT-PCR. Malondialdehyde (MDA), myeloperoxidase (MPO), superoxide dismutase (SOD) and reduced/oxidized glutathione (GSH/GSSG) in liver were determined using commercial kits. Phosphorylated NF-κB p65, IκBα and phosphorylated MAPK were measured by Western blot.
Results: Con A injection induced severe immune responses and extensive hepatocellular apoptosis within 12 h. Pretreatment of α-LA markedly reduced the serum ALT and AST activity and the increase of plasma TNF-α, IL-6, IFN-γ and IL-10. In addition, α-LA pretreatment decreased the tissue MPO activity and lipid peroxidation, but increased SOD and GSH levels. α-LA inhibited the phosphorylation of NF-κB p65, IκBα and JNK.
Conclusion: Pretreatment of α-LA markedly attenuated Con A-induced hepatitis by modulating cytokine secretion and reducing reactive oxygen species generation.

1. Introduction

Hepatitis is a severe disease that threatens human health and daily life. It can be caused by a variety of factors including alcohol, viral infections, drugs, poisons, or autoimmune disorders [1–3]. Concanavalin A (Con A)-induced hepatitis is a well-established mouse model of immune-mediated liver injury. Knowing that its pathological changes are similar to those of human hepatitis in many aspects [4], it has been used as an appropriate model for studying hepatitis. Activated T lymphocyte infiltration, apoptosis and necrosis of hepatocytes are regarded as the prominent character in autoimmune hepatitis [5–7]. Besides, reactive oxygen species (ROS), a highly reactive and diffusible free radical, is another major mediator of inflammation. Ample evidence indicates that reduction in ROS can attenuate hepatic injury [8], and antioxidant enzymes including superoxide dismutase (SOD) and glutathione (GSH) are shown to play important roles in human hepatitis [9,10]. ROS can trigger various signaling pathways and redox-sensitive signal transduction that modulates cellular mechanisms for cell proliferation, survival, death, and immune responses by inducing the production of inflammatory factors such as TNF-α, IL-6, IFN-γ and IL-10 through activation of NF-κB [11–13]. ROS accumulation inhibits mitogen-activated protein kinase (MAPK) phosphatases, resulting in *c*-Jun *N*-terminal kinase (JNK) activation, which contributes to ROS accumulation and hepatocyte death [14].

Alpha-lipoic acid (α-LA), also known as thioctic acid (TA) and 1,2 dithiolane-3-pentanoic acid, is a naturally occurring substance existing in almost all types of prokaryotic and eukaryotic cells. It is a key regulator of energy metabolism in mitochondria [15–18]. The physiologic function of α-LA has been most highlighted as a co-factor of the pyruvate dehydrogenase complex [19]. It is a potent natural antioxidant medium capable of scavenging ROS, chelating metal ions, and regenerating endogenous and exogenous antioxidants [20–22]. In addition to its well-described antioxidant activities, a-LA also exhibits a distinct regulatory effect on signal transduction processes, playing a central role in tissue damage and protection.

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The protective effect of α-LA in the liver has been reported in various liver injuries such as ischemia–reperfusion injury, alcohol-induced damage, metal intoxication, and hyperdynamic circulation in biliary cirrhosis [23–25], where α-LA suppressed apoptotic and inflammatory pathways, and restored water channels and sodium transporters, suggesting that α-LA might be a candidate for the treatment of Con A-induced autoimmune hepatitis.

The aim of the present study was to see whether administration of α-LA could protect Con A-induced hepatitis in a mouse model and explore the underlying mechanism.

2. Materials and methods

2.1. Materials

α-LA (T5625-500 mg) and Con A (C2272-2 mg) were obtained from Sigma Chemical Company (USA). All the other chemicals and reagents were of standard commercially available biochemical quality.

2.2. Model establishment and experimental design

C57BL/6 male mice (aged 6–8 weeks; 20–25 g) were obtained from the Animal Experimentation Center of the Second Military Medical University (Shanghai, China). All animals were acclimatized under controlled temperature (20 °C), humidity (60%) and 12 h light/12 h dark cycle for 1 week before initiation of the experiment. Mice were randomly divided into three groups: control group, receiving intravenous normal saline (100 μL); Con A group, receiving an injection of Con A (15 mg/kg, dissolved in 100 μL normal saline) via the tail vein [26]; and α-LA pretreatment group, receiving daily *i.p.* injection of α-LA (100 mg/kg) for four days before an injection of Con A. The effective dosage was selected on the basis of earlier investigations [27]. Blood samples and liver tissue were harvested 12 h after Con A administration.

2.3. Measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and cytokine levels

Mice were anesthetized by sevoflurane and sacrificed to collect blood samples from the heart. Plasma was separated after centrifugation at 5000 rpm for 10 min. Serum ALT and AST activities were assayed using an automatic dry biochemical analyzer (HitachiAutoAnalyzer7170, Japan). TNF-α, IL-6 a, IFN-γ (eBioscience, San Diego, CA) and IL-10 (R&D, Minneapolis, MN) levels were detected by using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

2.4. Histopathology

Liver tissues were harvested 12 h after IV administration of Con A, fixed in 4% formalin for at least 48 h and embedded in paraffin. Sections (4–5 μm) on slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E). Hepatocyte damage was scored as 0, none; 1, mild; 2, moderate; and 3, severe in terms of cell swelling and degeneration. Necrosis was analyzed with a four-point score for severity: 0, no necrosis; 1, 1–20% necrosis; 2, 20–40% necrosis; and 3, >40% necrosis.

2.5. Measurement of tissue malondialdehyde (MDA), myeloperoxidase (MPO), superoxide dismutase (SOD) level and the ratio of reduced/oxidized glutathione (GSH/GSSG)

Liver tissues (100 mg) were homogenized in saline. Homogenates were then centrifuged at 12,000 rpm for 10 min at 4 °C to obtain the supernatant. The MDA, MPO and SOD level, as well as the ratio of GSH/GSSG in liver tissue were determined by MDA detection kit, MPO detection kit, SOD detection kit and *T*-GSH/GSSG colorimetric assay kit, respectively. All of the above kits were provided by Jiancheng (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as described previously [28]. Levels of MDA, as an index of membrane lipid peroxidation (LPO), were expressed as nmol/mg tissue; MPO activity, an indicator of PMN accumulation in liver, were expressed as unit/g tissue; SOD activity were expressed as unit/mg tissue; GSH and GSSG were reported as μmol/ml of tissue and expressed as the ratio of GSH/GSSG.

2.6. RNA isolation and real-time PCR analysis

Total RNA was extracted from the liver tissue using Trizol reagent (Invitrogen). To determine the expression of target genes in the liver, SYBR Green quantitative RT-PCR was performed using a Real-time PCR Detection System (Bio-Rad Life Sciences). Primer sequences were as follows.

Mouse TNF-α (Forward, F): AAGCCTGTAGCCCACGTCGTA; and (Reverse, R): GGCACCACTAGTTGGTTGTCTTTG. Mouse IL-6 (Forward, F): ACAACCACGGCCTTCCCTACTT; and (Reverse, R): CACGATTTCCCAGAGAAC ATGTG. Mouse IFN-γ (Forward, F): CCTCAAACTTGGCAATACTCA; and (Forward, R): CTCAAGTGGCATAGATGTGGA. Mouse GAPDH (Forward, F): AGAGTGGGAGTTGCTGTTG; and (Forward, R): GCCTTCCGTGTTCCTACC.

2.7. Western blot analysis

Mice were narcotized by sevoflurane 12 h after Con A administration. The liver was harvested from each mouse, homogenized into lysis buffer (Thermo, USA), and centrifuged at 12,000 g for 10 min at 4 °C. The protein concentration was determined by Bradford protein assay kit (Thermo, USA) with bovine serum albumin as standard. Equal amounts of protein extracts separated discontinuously onto 10% polyacrylamide gels (Life Technologies, Carlsbad, CA) and transferred to nitrocellulose membranes (Life Technologies, Carlsbad, CA). After blockade of nonspecific binding sites, membranes were blocked for 2 h at room temperature in various antibodies against NF-κB p65, phosphorylated NF-κB p65, IκBα, phosphorylated IκBα, JNK, phosphorylated JNK, p38, phosphorylated p38, ERK, phosphorylated ERK (Cell Signaling Technology, Danvers, MA) with a dilution ratio of monoclonal antibody 1:2000. After primary antibody incubation, membranes were washed three times in PBST, and secondary antibody (Cell Signaling Technology, Danvers, MA). Membranes were developed by chemiluminescence using an Amersham prime ECL Plus detection system (GE Healthcare Life Sciences, Pittsburgh, PA). Signals were densitometrically assessed and normalized to the β-actin signals as reported previously by our laboratory [29].

2.8. Statistical analysis

The data are presented as mean values and standard error (SE). Statistical analyses were performed using Prism 6.0 (Graph Pad Software, USA). Statistical evaluations were compared using the standard one-way analysis of variance. $P < 0.05$ was considered to indicate a significant difference.

3. Results

3.1. α-LA pretreatment ameliorates Con A-induced hepatitis

First, we examined whether α-LA had a protective effect on Con A-induced hepatitis. Compared with normal control group, serum ALT and AST levels were increased significantly in Con A-treated mice, and α-LA pretreatment significantly attenuated the Con A-induced elevation of ALT and AST ($P < 0.01$) (Fig. 1A and 1B), suggesting that α-LA had a protective effect against Con A-induced hepatitis. Next, we injected single dose α-LA (100 mg/kg) one hour after the injection of Con A to mice to examine the therapeutic potential of α-LA and found no significant difference in ALT and AST levels compared with Con A