Study on the Relationship Between Lentinan Amelioration of Hepatic Lipid Accumulation in Arsenic-Exposed Mice and ORP8

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Date: 2025-03-05T00:00:00+00:00

Abstract

Objective To investigate the relationship between lentinan (LNT) regulation of hepatic lipid accumulation and oxysterol-binding protein-related protein 8 (ORP8) in sodium arsenite (SA)-exposed mice. Methods C57BL/6 male mice were administered SA by gavage, or SA exposure combined with LNT intervention. Oil Red O staining was used to observe histomorphological features of liver tissue, enzyme-linked immunosorbent assay was performed to detect triglyceride (TG) and total cholesterol (TC) levels; Western blotting (WB) was conducted to detect ORP8 expression and LC3-II/I ratio levels in liver tissue; molecular docking simulation was performed to analyze the interaction between LNT and ORP8. Results Compared with the control group, the SA exposure group showed hepatic lipid accumulation and elevated TG and TC levels ($P<0.05$); compared with the SA exposure group, the LNT intervention + SA group showed alleviated hepatic lipid accumulation and downregulated TG and TC levels ($P<0.05$); WB results demonstrated that ORP8 expression and LC3-II/I ratio levels were decreased in the SA exposure group compared with the control group, while LNT intervention increased ORP8 expression and LC3-II/I ratio levels ($P<0.05$). Molecular docking experiments revealed a relatively stable hydrogen bond interaction between hydrogen atoms in the LNT molecule and oxygen atoms in the amino acid residues of the ORP8 protein (average binding energy of -124.17 kcal/mol). Conclusion LNT ameliorates hepatic lipid accumulation in arsenic-exposed mice, which may be associated with LNT-ORP8 interaction.

Full Text

The Relationship Between Lentinan-Induced Improvement of Hepatic Lipid Accumulation and ORP8 Protein in Arsenic-Exposed Mice

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Objective: To investigate the relationship between lentinan (LNT) regulation of hepatic lipid accumulation and oxysterol-binding protein-related protein 8 (ORP8) in mice exposed to sodium arsenite (SA). **Methods:** C57BL/6 male mice were orally administered SA alone or in combination with LNT intervention. Oil red O staining was used to observe hepatic histomorphological characteristics, enzyme-linked immunosorbent assay (ELISA) was employed to detect triglyceride (TG) and total cholesterol (TC) levels, Western blotting (WB) was performed to measure hepatic ORP8 expression and LC3-II/I ratio, and molecular docking simulations were conducted to analyze the interaction between LNT and ORP8.

Abstract

Objective: To investigate the relationship between lentinan (LNT) regulation of hepatic lipid accumulation and oxysterol-binding protein-related protein 8 (ORP8) in mice exposed to sodium arsenite (SA). **Methods:** C57BL/6 male mice were orally administered SA or treated with SA combined with LNT intervention. Oil red O staining was used to observe the morphological characteristics of liver tissue, and enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of triglyceride (TG) and total cholesterol (TC). Immunoblotting (WB) was used to detect the expression of ORP8 and the LC3-II/I ratio in liver tissue. Molecular simulation docking experiments were used to analyze the interaction between LNT and ORP8. **Results:** Compared with the control group, the SA-exposed group showed hepatic lipid accumulation and increased TG and TC levels ($P<0.05$). Compared with the SA-exposed group, the LNT intervention+SA group showed reduced hepatic lipid accumulation and downregulated TG and TC levels ($P<0.05$). WB results showed that ORP8 expression and the LC3-II/I ratio were downregulated in the SA-exposed group compared to the control group, while LNT intervention increased ORP8 expression and the LC3-II/I ratio ($P<0.05$). Molecular docking experiments revealed stable hydrogen bonding interactions between hydrogen atoms in LNT molecules and oxygen atoms in the amino acid residues of ORP8 protein (average binding energy of -124.17 kcal/mol). **Conclusion:** LNT improves lipid accumulation in the liver of arsenic-poisoned mice, which may be associated with LNT-ORP8 interaction.

Keywords: Lentinan; Sodium arsenite; Hepatic lipid accumulation; Oxysterol-binding protein-related protein 8 (ORP8)

Funding: Guangxi Natural Science Foundation (2023JJA140042); Guangxi University Student Innovation Training Program (S202310601198).

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Sodium arsenite (SA) is a common chemical toxicant in the natural environment that can contaminate soil, drinking water, or food, causing toxic liver injury even with trace ingestion. Epidemiological investigations have revealed that environmental arsenic exposure is closely associated with the development of non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) in human populations [1-2]. Animal experiments have demonstrated that SA exposure can induce hepatic glucose intolerance, impaired insulin signaling pathways, and insulin resistance in mice [3]. Conversely, studies in insulin-resistant rat models have found increased transport of free fatty acids (FFA) and lipid deposition in the liver [4]. Abnormal lipid deposition in hepatocytes can induce insulin resistance through mechanisms including gluconeogenesis, tissue inflammatory responses, oxidative stress, and impaired insulin signaling pathways [5]. Consequently, hepatic lipid accumulation is considered a pathological mechanism underlying the development of NAFLD [6] and T2DM [7].

Lipophagy is a selective biological process that targets lipid droplets as substrates, forming autophagosomes through the binding of lipid droplets with ubiquitin proteins (Ub) and autophagosomal LC3 proteins, which then fuse with lysosomes for degradation of intracellular lipid components by lysosomal hydrolases [8]. Recently, oxysterol-binding protein-related protein 8 (ORP8) has been identified as a regulatory factor mediating lipophagy [9]. Studies have shown that macrophages with ORP8 gene silencing via shRNA technology exhibit elevated lipid levels (including free cholesterol and cholesterol esters) [10], whereas hepatic ORP8 overexpression in mice results in reduced serum and hepatic total cholesterol (TC), phospholipids, and triglyceride (TG) levels [11]. Lentinan (LNT) is a bioactive polysaccharide extracted from the edible mushroom *Lentinus edodes*. Our previous animal studies found that LNT intervention could antagonize hepatic lipid accumulation and reduce hepatic TG and NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome levels in SA-exposed mice [12-13]. However, whether ORP8 is involved in the mechanism by which LNT antagonizes lipid accumulation in SA-exposed mice remains unclear. Therefore, this study investigated the relationship between LNT regulation of hepatic lipid accumulation and ORP8 in arsenic-poisoned mice through animal experiments.

1.1 Reagents and Instruments

Reagents: SA (molecular formula $NaAsO_2$, CAS 7784-46-5) was purchased from Sigma-Aldrich. LNT (CAS 37339-90-5) was extracted by our research group using ultrasound-assisted enzymatic hydrolysis combined with thermal degradation and fermentation methods, with a polysaccharide content of 90%. ELISA kits for triglyceride (TG, DM-X6707) and total cholesterol (TC, DM-X6708) were purchased from Shanghai Duma Biotechnology Co., Ltd. Primary antibodies against LC3B/A (ab62721) and ORP8 were obtained from Abcam.

Instruments: Thermo Fisher cryostat (Cryotome FSE), 800 TS absorbance microplate reader (BioTek, USA), and infrared laser scanning imaging system (Odyssey 9120, LI-COR).

1.2 Animal Experimental Protocol

Specific-pathogen-free (SPF) grade C57BL/6 male mice were purchased from Changsha Slack Laboratory Animal Co., Ltd. (Production License No. SCXK(Xiang)2021-0002), with body weights of 26.5$±$1.0 g and ages of 9-10 weeks. Housing conditions were maintained at 20-24°C with 40-60% relative humidity and a 12-hour light-dark cycle. The experimental protocol was approved by the Guilin Medical University Animal Ethics Committee (GLMC20230712), and all procedures strictly followed the 3R principles for animal use. The experiment consisted of four groups (n=6 per group): Group I—control group (mice fed under conventional conditions with free access to water), Group II—SA exposure group (SA administered by gavage at 5.0 mg/kg body weight every other day for 8 weeks), Group III—LNT intervention+SA group (LNT administered by gavage at 50.0 mg/kg body weight 12 hours before SA exposure at the same dose as Group II, every other day for 8 weeks), and Group IV—LNT control group (treated with LNT at the same dose as Group III as an intervention control). At the end of the experiment, mice were anesthetized with 1% pentobarbital sodium and euthanized. Serum and liver tissues were collected for histomorphological, biochemical, ELISA, and Western blot analyses to assess liver function, hepatic lipid content, and expression of ORP8 and autophagy-related proteins.

1.3.1 Oil Red O Staining

Liver tissue samples were embedded in OCT compound to prepare frozen sections, which were then stained with oil red O. Lipid deposition characteristics in hepatic tissue were evaluated under microscopy. Orange-red granules or clumps in liver tissue represent fat droplets, while blue staining indicates nuclear counterstaining.

1.3.2 Hepatic Tissue Lipid Levels

Livers were isolated and rinsed with PBS buffer to remove residual blood. Hepatic tissue homogenates were prepared using a Dounce homogenizer (10% w/v)

in RPMI (Roswell Park Memorial Institute) medium containing 0.05% type II collagenase, followed by incubation at 37°C for 30 minutes. After repeated freeze-thaw cycles, samples were centrifuged at 1500×g for 10 minutes at low temperature, and supernatants were collected. Hepatic TG and TC contents were measured according to the mouse ELISA kit instructions.

1.4 Western Blotting (WB) Experiment

Hepatic tissue homogenates were lysed with RIPA buffer to extract total protein, and protein concentrations were determined. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to membranes, and blocked. Membranes were incubated with primary antibodies against ORP8 or LC3II/I, followed by enzyme-labeled secondary antibodies, with β-actin as an internal control. Target protein bands were visualized by chemiluminescence and imaging. Relative quantification was performed using integrated absorbance (IA) of bands, analyzing target protein expression (ORP8, LC3-II/I) characteristics (IA/IA_0 or IAB/IAA).

1.5 Molecular Simulation Docking Experiment

Molecular docking simulations were performed using the Yinfotek Biomedical Technology Platform (https://www.yinfotek.com/). LNT was used as the ligand and ORP8 as the receptor. Ligand mol2 files and receptor 3D PDB structure files were input into the ZDOCK online server (https://zdock.wenglab.org/), and the ZDOCK method was applied to simulate molecular docking between LNT and ORP8. Interaction prediction results between LNT and ORP8 protein were obtained after running the simulation.

1.6 Statistical Analysis

Data are expressed as mean $\pm$ standard deviation ($\mathbb{X} \pm$ SD). Statistical analysis was performed using SPSS V27.0 software. Differences were evaluated by one-way ANOVA followed by least significant difference (LSD) test, with $P \leq 0.05$ considered statistically significant.

2.1 Lentinan Reduces Hepatic Lipid Levels and Lipid Accumulation in SA-Exposed Mice

As shown in [Figure 1: see original paper], compared with Group I (control), Group II (SA exposure) exhibited elevated serum TG and TC levels ($P<0.05$). Oil red O staining of liver tissue revealed orange-red granules or clumps in some areas, with some hepatocytes showing lipid droplet vacuoles and lipid accumulation characteristics (lipids distributed as red or orange-red). In contrast, Group III (LNT intervention+SA) showed significantly reduced serum TG and TC levels compared with Group II ($P<0.05$), and frozen sections of liver tissue demonstrated alleviated lipid accumulation. These results suggest that LNT intervention improves hepatic lipid accumulation.

Figure 1-A: Characteristics of hepatic TG and TC levels after LNT intervention (n=6, X±SD; * compared with control group, P<0.05; # compared with SA exposure group, P<0.05)

Figure 1-B: Oil red O staining characteristics of mouse liver tissue ($×$40 magnification; orange-red represents fat granules, purple-blue represents nuclei)

Note: I, control group; II, SA 5.0 mg/kg body weight exposure group; III, LNT 50.0 mg/kg body weight intervention+SA group; IV, LNT intervention control group; Exposure method and duration: gavage, 8 weeks.

2.2 LNT Upregulates Hepatic ORP8 Expression and LC3-II/I Ratio

Western blot results shown in [Figure 2: see original paper] demonstrate that compared with Group I (control), Group II (SA exposure) exhibited decreased hepatic ORP8 protein expression and significantly reduced autophagy initiation protein LC3-II/I ratio (P<0.05). Compared with Group II (SA exposure), Group III (LNT intervention+SA) showed significantly increased ORP8 expression and LC3-II/I ratio (P<0.05). These results suggest that SA exposure inhibits the activity of the lipophagy regulatory protein ORP8 in mouse liver tissue, while LNT can antagonize this SA-induced suppression of lipophagy.

Figure 2: Hepatic ORP8 and LC3-II/I levels after SA exposure or LNT intervention (n=3, X±SD; * compared with control group, P<0.05; # compared with SA exposure group, P<0.05).

2.5 Molecular Docking Between LNT and ORP8 Protein

Lentinan (LNT) is a bioactive polysaccharide isolated from *Lentinus edodes* with a β-1$→$3-glucan structure as its main chain. Therefore, we used the 3D structure of this β-1$→$3-glucan backbone as the ligand to explore the interaction between LNT and ORP8 protein. Molecular docking results (shown in [Figure 3: see original paper]) revealed hydrogen bonding interactions between hydrogen atoms in the β-1$→$3-glucan backbone of LNT and oxygen atoms in the methionine (Met), lysine (Lys), asparagine (Asn), and leucine (Leu) residues of ORP8 molecules (average binding energy of -124.17 kcal/mol). These findings suggest that LNT may improve SA-induced hepatic lipid accumulation in mice through interaction with ORP8 protein, thereby mediating activation of ORP8-dependent lipophagy.

Figure 3: Molecular docking between LNT and ORP8 protein

Studies have shown that *Lentinus edodes* cultivation is highly developed in China, with annual production exceeding 12 million tons in recent years, making China the world' s leading producer and exporter of shiitake mushrooms. The bioactive polysaccharides abundant in shiitake mushrooms are known as

lentinan, with the trade name Lentinan. LNT is a bioactive polysaccharide extracted from *Lentinus edodes* with a β-1,6;1,3-glucan structure as its main chain, exhibiting a triple-helical spatial conformation and a relative molecular weight of approximately 6.5-70$×10^{4}$ [14], with strong antioxidant capacity [15] and immunomodulatory activity [16]. Previous studies have demonstrated that after absorption into the bloodstream, LNT primarily concentrates in the liver, which is the target organ for LNT metabolism [14]. LNT possesses biological activity in improving hepatic lipid accumulation in high-fat diet (HFD)-induced nonalcoholic fatty liver disease (NAFLD) model mice [17], while hepatic steatosis can promote the formation of macrophage-derived foam cells, representing an important contributing factor in the development of atherosclerosis (AS) [18]. Currently, the underlying mechanism by which LNT regulates hepatic lipid accumulation remains unclear.

In this study, we found that SA-exposed mice exhibited lipid accumulation with elevated hepatic lipid (TG, TC) levels, reduced expression of the lipophagy regulatory protein ORP8, and decreased LC3-II/I ratio, while LNT intervention demonstrated antagonistic effects on hepatic lipid levels, ORP8 protein expression, and LC3-II/I ratio in SA-exposed mice. These results suggest that LNT improves SA-induced hepatic lipid accumulation through activation of the lipophagy regulatory protein ORP8. Additionally, our ZDOCK molecular docking experiments revealed stable hydrogen bonding interactions between LNT and ORP8 protein. These findings further indicate that LNT improves hepatic lipid accumulation in SA-exposed mice through interaction with ORP8 protein, which mediates activation of cellular lipophagy, thereby ameliorating hepatic lipid accumulation.

In summary, this study demonstrates that LNT intervention can increase hepatic ORP8 levels and autophagosomal protein LC3-II/I ratio, exerting antagonistic effects on hepatic lipid content and lipid accumulation in arsenic-exposed mice. ORP8 protein may play an important role in the process of LNT regulation of hepatic lipid accumulation. However, this study lacks additional experimental evidence from ORP8 gene intervention studies on hepatic lipid accumulation. Future work should establish hepatic/hepatocyte lipid accumulation models with ORP8 gene silencing or overexpression to further explore the biological role of ORP8 in LNT regulation of hepatic lipid accumulation through targeted interventions with LNT alone or in combination with autophagy inhibitors/activators.

Author Contribution Statement: All authors declare no conflicts of interest.

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Note: Figure translations are in progress. See original paper for figures.