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β-Lapachone alleviates alcoholic fatty liver disease in rats

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ABSTRACT

Alcohol-induced liver injury is the most common liver disease in which fatty acid metabolism is altered. It is thought that altered NAD^+/NADH redox potential by alcohol in the liver causes fatty liver by inhibiting fatty acid oxidation and the activity of tricarboxylic acid cycle reactions. β-Lapachone (βL), a naturally occurring quinone, has been shown to stimulate fatty acid oxidation in an obese mouse model by activating adenosine monophosphate-activated protein kinase (AMPK). In this report, we clearly show that βL reduced alcohol-induced hepatic steatosis and induced fatty acid oxidizing capacity in ethanol-fed rats. βL treatment markedly decreased hepatic lipids while serum levels of lipids and lipoproteins were increased in rats fed ethanol-containing liquid diets with βL administration. Furthermore, inhibition of lipolysis, enhancement of lipid mobilization to mitochondria and upregulation of mitochondrial β-oxidation activity in the soleus muscle were observed in ethanol/βL-treated animals compared to the ethanol-fed rats. In addition, the activity of alcohol dehydrogenase, but not aldehyde dehydrogenase, was significantly increased in rats fed βL diets. βL-mediated modulation of NAD^+/NADH ratio led to the activation of AMPK signaling in these animals. Conclusion: Our results suggest that improvement of fatty liver by βL administration is mediated by the upregulation of apoB100 synthesis and lipid mobilization from the liver as well as the direct involvement of βL on NAD^+/NADH ratio changes, resulting in the activation of AMPK signaling and PPARα-mediated β-oxidation. Therefore, βL-mediated alteration of NAD^+/NADH redox potential may be of potential therapeutic benefit in the clinical setting.

1. Introduction

Alcoholic liver disease is a major cause of illness and death in most well-developed countries and is becoming a leading cause of disease in developing countries [1]. In the initial stages of alcoholic liver disease, fat accumulation in hepatocytes leads to the development of fatty liver, which is a reversible condition. With the continuation of alcohol consumption, fatty liver may progress to hepatitis and fibrosis, which may lead to liver cirrhosis [2]. It has been also suggested that patients with fatty liver are more susceptible to fibrotic liver diseases such as hepatitis and fibrosis [3]. Therefore, alcoholic fatty liver has long been considered benign; however, increasing evidence supports the idea that it is a pathologic condition. Blunting of the accumulation of fat within the liver during alcohol consumption may block or delay the progression of fatty liver to hepatitis and fibrosis [4]. Therefore, recovery from a fatty liver would decrease the susceptibility to, and prevent the progression of liver fibrosis or cirrhosis.

β-Lapachone (βL; also known as ARQ 501), currently in phase II clinical trials for the treatment of pancreatic cancer [5], is an o-naphthoquinone originally isolated from the bark of the lapacho tree (*Tabebuia avellanedae*) [6]. βL is a prodrug and becomes cytotoxic to cancer cells following bioreduction. The enzyme involved in the bioreduction of quinone-containing drugs is NAD(P)H:quinone oxidoreductase (NQO1) [7–9]. βL is reduced to an unstable hydroquinone that spontaneously reverts to its parent structure using two oxygen molecules [5]. As a result, reactive oxygen species are generated causing DNA damage, γ-H2AX foci formation, poly(ADP-ribose) polymerase-1 (PARP-1) hyperactivation and subsequent loss of ATP and NAD^+ [10,11]. βL induced cell death is unique because PARP-1 and p53 proteolysis occurs concomitant with calpain activation [12]. βL mediated cell

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death exhibited classic features of apoptosis, but was not dependent on typical apoptotic mediators, such as p53, Bax/Bak, or caspases [5].

Interestingly, it has recently been proposed that βL plays an important role in regulating metabolic syndrome such as obesity [13] and atherosclerosis [14] through NQO1-mediated changes of NAD^+/NADH redox potential. In the current study, we have investigated the mechanism of βL action on the improvement of alcohol-induced fatty liver in a rat model and primary hepatocytes. Our results suggested that βL prevents the onset of steatosis and plays a critical role in lipid metabolism and lipid mobilization by directly or indirectly modulating NAD^+/NADH ratio in the injured liver of rats.

2. Materials and methods

2.1. Chemical and reagents

RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin and other cell culture reagents were obtained from Invitrogen (USA). Anti-AMPK, anti-pT179 (AMPK), anti-ACC and anti-pS79 (ACC) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-PPARα, anti-PPARγ and anti-RXR antibodies were purchased from Perseus Proteomics Inc. (Tokyo, Japan). Lipid extraction kit was purchased from Wako Pure Chemical (Osaka, Japan). Alcohol dehydrogenase (ADH) assay kit was obtained from Biomedical Research Service. Oil red O and 6-methoxy-2-napthaldehyde were obtained from Sigma (St. Louis, MA).

2.2. Synthesis and formulation of compounds

β-Lapachone (βL) and other related 1,2-naphthoqunes were prepared by a two step synthetic process, resulting in a purity >99.9% as described previously [6]. Briefly, the lithium salt of 2-hydroxy-1,2-naphthoquinone was treated with several allyl-halides, such as 3-bromo-1-propene, 1-bromo-3-methyl-2-butene, 3-bromo-2-methyl-1-propene, and 1-bromo-2-butene, to give several 2-hydroxy-3-allyl-1,4-naphthoquinone derivatives. Then each 2-hydroxy-3-allyl-1,4-naphtho-quinone derivative was treated with H_2SO_4 and purified by recrystallization to give several pure 1,2-naphthoquinones, including βL.

2.3. Rat model of alcoholic fatty liver

All animal procedures were in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of the Chungnam National University School of Medicine. Four-week-old male Sprague–Dawley (SD) rats obtained from Central Lab Animal (Seoul, Korea) and housed individually in temperature- and light-controlled rooms were randomly assigned to four groups: (a) rats fed isocaloric liquid diets without ethanol for 60 days (n = 4); (b) rats fed ethanol-containing liquid diets for 30 days and then fed daily with vehicle (SLS; Sodium Lauryl Sulfate) for the next 30 days (n = 6); (c) rats pair-fed ethanol containing liquid diets for 30 days and then fed daily with SLS for the next 30 days (n = 6) and (d) rats fed ethanol-containing liquid diets for 30 days and then fed daily with 150 mg or 250 mg of βL in SLS per kilogram of body weight for the next 30 days (n = 6). The calorie distribution of liquid diet components is as follows: 17% as protein, 36% as fat, 11% as carbohydrate and 36% as either ethanol or isocaloric maltose dextrin in the isocaloric liquid diets. Body weight and food intake were measured at every 5 days. At the end of the experiment, the rats were sacrificed and blood and liver tissue were collected.

2.4. Biochemical and histopathological analyses

For measurement of hepatic lipid content, the liver was homogenized at 4 °C with a Polytron (Polytron PT-MR 2100, Kinematica, Switzerland) homogenizer in the homogenization buffer containing 50 mM Tris–HCl (pH 7.4), 0.25 M sucrose and 1 mM EDTA, and centrifuged for 10 min at 12,000 ×*g*. Lipids in the liver homogenate were extracted using chloroform/methanol (1:2 vol/vol), evaporated, and dissolved in 2-propanol. The amounts of triglyceride, total cholesterol, and phospholipids were assayed enzymatically, using kits obtained from Wako Pure Chemicals Co. (Osaka, Japan) with a spectrophotometer DU-650 reader (Beckman, Fullerton, CA) at 600 nm. For histological analysis, liver tissues were fixed with formalin, dehydrated with ethanol, embedded in paraffin, cut at a thickness of 5 μM, and stained with hematoxylin and eosin. Alternatively, hepatic lipids were stained by an Oil red O method.

2.5. Analysis of fatty acid oxidation

To determine fatty acid oxidation in the muscle, ^{14}C-palmityl-CoA-oxidation was measured in unfrozen soleus muscle using $^{14}CO_2$ and 0.2 ml of benzethonium solution as described previously [15]. Briefly, soleus muscle was mixed with the assay buffer containing 1.25 M NaCl, 0.1 M $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 0.5 M KCl, 0.2 M glucose, 1 M $NaHCO_3$, 0.25 M $CaCl_2$ and 1 mM [^{14}C]-palmityl-CoA (1 mCi/mmol; Perkin Elmer; Chicago, IL), gassed for 30 s under humidified 95/5% O_2/CO_2, covered with filter paper moistened with 0.2 ml of benzethonium solution and then incubated for 1 h at 37 °C. The reaction was stopped with 6% TCA solution and the radioactivity trapped in the filter paper was determined. Fatty acid β-oxidation activity was expressed as nmol/min/liver.

2.6. Measurement of enzymatic activity; ADH and ALDH

For the measurement of alcohol dehydrogenase (ADH) activity, unfrozen liver was homogenized by homogenizer in 0.25 M sucrose at 4 °C and cleared by centrifugation at 600 ×*g* for 10 min. The resultant supernatant was mixed with the reaction buffer containing 50 mM sodium phosphate, 300 μM formazan, 11.4 mM NAD, and 12 mM 4-methylpyrazol. The reaction was stopped by the addition of 3% acetic acid. The relative ADH activity was measured at O.D. 492 nm and normalized with total amount of protein. In the case for aldehyde dehydrogenase (ALDH) activity, 100 μg of lysates was incubated with the reaction buffer containing 11.4 mM sodium phosphate (pH 8.5), 300 μM NAD, 4-methylpyrazol (ADH inhibitor) and 6 mM 6-methoxy-2-naphthaldehyde as a substrate. The fluorescence of reaction was measured with a CytoFluor II fluorescence plate reader (excitation — 310 nm, emission — 360 nm; Applied Biosystems, Foster City, CA).

2.7. Measurement of lipids in cultured primary hepatocytes from ethanol-fed rats

Primary hepatocytes were prepared from ethanol-fed SD rats using the collagenase perfusion method as previously described [16]. Briefly, the isolated hepatocytes were placed on a culture plate coated with type I collagen at a density of 5×10^5 cells/ml and culture for 4 h in Williams' medium E supplement with 10% FBS, 1 nM dexamethasone. After serum-starvation, the cells were treated with either DMSO or 10 μM βL for 8 days, washed two times with cold PBS and then solubilized in lysis buffer containing 50 mM Tris–HCl (pH 7.4) and 0.5% SDS. Triglyceride in the cell lysates was extracted and measured. For histological detection of lipids, the cells were fixed with 10% formaldehyde for 1 h and subjected to lipid staining using Oil red O staining.

2.8. Electrophoretic mobility shift assays

Binding and electrophoresis were performed as previously described [17]. Briefly, cells were placed on ice, harvested and extracted with nuclear lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.05% NP-40 0.5 mM DTT and 0.5 mM PMSF by passing 20 times with a 1 ml Dounce Homogenizer by passing.