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RESEARCH ARTICLE

Resveratrol protects against chronic alcohol-induced liver disease in a rat model

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Abstract

Hepatic ethanol metabolism participates in the pathogenesis of alcoholic liver disease (ALD). We aimed to evaluate the protecting effects and underlying mechanisms of Resveratrol against ALD. Adult male rats were fed with liquid ethanol diet, in the presence or absence of Resveratrol for 23 weeks. It was demonstrated that Resveratrol attenuated ethanol-induced release of serum alanine and aspartate aminotransferase. Ethanol insult caused malondialdehyde elevation and the impairment of antioxidant defense system, leading to reactive oxygen species generation, which could be greatly reversed by Resveratrol treatment. Moreover, Resveratrol protected against chronic ethanol-induced upregulation in hepatic cytochrome P-4502E1 expression, whereas hepatic alcohol dehydrogenase expression was unaffected by Resveratrol. Unexpectedly, hepatic aldehyde dehydrogenase2 expression was markedly diminished in ethanol-fed rats, which was gradually improved by Resveratrol treatment. Our data demonstrate that the protecting effects of Resveratrol on ALD might be associated with changes in the regulation of alcohol-metabolizing enzymes. Our findings could provide new perspective into the pharmacological targets of Resveratrol in the treatment of ALD.

Keywords: *resveratrol*; *alcoholic liver disease*; *oxidative stress*; *alcohol dehydrogenase*; *aldehyde dehydrogenase2*; *cytochrome P4502E1*

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Chronic alcohol ingestion is believed to be associated with increased risks of many chronic and acute diseases, particularly alcoholic liver disease (ALD) (1), which could lead to hepatocellular carcinoma (2, 3). Despite of its serious consequences, to date, there has been no proven effective treatment for ALD.

ALD pathogenesis is a complex process involving different biological and molecular mechanisms, of which the consequences of alcohol metabolism are the most critical and basic pathogenesis of ALD (4, 5). Alcohol metabolism includes participation of various enzymatic systems or enzymes, such as aldehyde dehydrogenase 2 (ALDH2) pathway, alcohol dehydrogenase (ADH), and cytochrome P4502E1 (CYP2E1) system. Chronic ethanol consumption was reported to inhibit ALDH2 activity, resulting in strikingly increased tissue and plasma acetaldehyde levels (4). Moreover, as a major toxic metabolite of alcohol oxidation, acetaldehyde is one of the main culprits mediating alcohol-inflicted mutagenic and fibrogenic effects in the liver. Acetaldehyde accelerates adduct formation that leads to dysfunction of key enzymes and proteins, thereby promoting ALD (6).

Resveratrol is a polyphenol existing in various plant species (7). Resveratrol has been shown to possess a variety of diverse biochemical and physiological functions, including anti-inflammatory, antioxidant properties, vaso-protective, and antiaging effects, which has attracted extensive research attention (8–10). Growing evidence has illustrated that Resveratrol exhibits favorable hepato-protective effects against ethanol toxicity in the animal models (11, 12). It has also been found that Resveratrol could successfully suppress ALD formation (13). However, this process is mainly mediated by the antioxidant activities and upregulation of hepatic adenosine 5'-monophosphate (AMP)-activated protein kinase and sirtuin 1 to increase rates of fatty acid oxidation and

reduce lipid synthesis (14, 15). However, mechanisms underlying this progression still remain elusive. A study implied that Resveratrol suppressed ADH gene expressions, whereas increased mRNA expression of ALDH2 in human peripheral lymphocytes in vitro (16). It was revealed that Resveratrol suppressed the expression of various isoforms of CYP under pathological conditions, clearly suggesting its role in reduction of CYP-mediated reactive oxygen species (ROS) generation (17, 18). In this study, we aimed to explore the protective effects of Resveratrol on the enzymes involved in ethanol metabolism.

Materials and methods

Animals

The study was approved by Hangzhou First People's Hospital. Forty-two male Sprague–Dawley rats (150–180 g) were randomly divided into six groups with seven animals in each experimental group, and the rats were treated for 23 weeks as follows: control group received control liquid diet; ethanol groups received ethanol liquid diet: low dose of ethanol [Leth, 6.28% (1.11% vol/vol) of total calories], middle dose of ethanol [Meth, 11.48% (2.03%, vol/vol) of total calories], and high dose of ethanol [Heth, 17.19% (3.04%, vol/vol) of total calories]; Resveratrol (Shanghai Yunhao Biotech, Shanghai, China) control group (Con+Res) received control liquid diet plus 100 mg·kg body wt^{-1}·day^{-1} Resveratrol; Resveratrol plus ethanol group (Heth+Res) received Heth and 100 mg·kg body wt^{-1}·day^{-1} Resveratrol. All liquid diets included 1 kcal/ml and isocalorically substituted maltose dextrin for ethanol. The doses for ethanol and Resveratrol were based on our previous study (19, 20).

ROS determination

ROS levels were determined using dihydroethidium (DHE) following previously published method (21). Briefly, cryostat sections of liver (10 μm) were incubated with 5 mM DHE (Beyotime) at 37°C for 30–40 min. The mean fluorescence density was calculated to reflect the ROS levels.

Biochemical assays

Serum alanine aminotransferases (ALT) and aspartate (AST) were evaluated using automatic biochemistry analyzer (Mindray BS-200, Shenzhen, China) with corresponding kits. Low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), and triglyceride (TG) were measured by enzymatic colorimetric methods and performed using corresponding kits (Zhongshen Beikong Biotech, China). Levels of serum oxidized glutathione (GSSG), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) as well as hepatic SOD, Cu-ZnSOD, CAT, GSH, GSSG, and glutathione peroxidase (GPx) were determined by enzymatic colorimetric methods using commercially kits (Nanjing Jiancheng Institute). Malondialdehyde (MDA) contents in serum and liver were measured using corresponding kits (Nanjing Jiancheng Institute).

Assays for enzymatic activity of CYP2E1, ADH, and ALDH2

Liver was homogenized with ice cold isotonic saline to yield a 10% (w/v) tissue homogenate. Microsomal sample was extracted from the homogenate by high-speed centrifuge at 4°C. CYP2E1 activity was determined by isolated hepatic microsomes, according to the protocol of regent kit manufacturer, and its activity was expressed as nmol per min per mg protein. ADH catalytic activity was measured by the formation of reduced nicotinamide adenine dinucleotide (NADH) from NAD oxidation according to the introduction of regent kit manufacturer, and enzyme-specific activity was expressed as nmol per min per mg protein. The activity of ALDH2 was determined by monitoring the formation of NADH from NAD^+ at 450 nm in a spectrophotometer, which was expressed as nmol NADH per min per mg protein.

Western blot

Liver was homogenized and lysed in radioimmunoprecipitation lysis buffer. Lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were then blocked with 5% non-fat milk for 2 h, followed with incubation with corresponding primary antibodies overnight at 4°C. The membranes were further incubated with horse-radish peroxidase-conjugated secondary antibody for another 2 h. An ECL plus Western Blotting Detection System was used to detect the blots (Amersham Biosciences, Little Chalford, UK).

Statistical analysis

Data were presented as mean ± standard deviation (SD). Analysis was performed using ANOVA with SPSS 20.0 software. The comparisons were considered statistically significant at $P < 0.05$.

Results

Resveratrol prevents chronic ethanol-induced changes in body weight and energy efficiency ratio

At the end of the experiment, rats in all ethanol groups had significantly lower body weight and energy efficiency ratio compared with control group, whereas Resveratrol supplementation to the ethanol-fed rats reduced the weight gain and improved the value of energy efficiency ratio (Table 1).

Table 1. Growth parameters in rats after chronic ethanol intake with or without Resveratrol supplemented

Parameters	Groups					
	Con	Leth	Meth	Heth	Con+Res	Heth+Res
Initial body weight (g)	207 ± 14	206 ± 10	208 ± 13	203 ± 11	204 ± 9	205 ± 13
Final body weight (g)	491 ± 54	443 ± 38$^{\#}$	439 ± 33$^{\#}$	441 ± 21$^{\#}$	483 ± 35	480 ± 40*
Body weight gain (g)	289 ± 47	239 ± 35$^{\#\#}$	235 ± 38$^{\#\#}$	240 ± 16$^{\#\#}$	282 ± 33	278 ± 35*
Energy intake (kJ)	11,033 ± 33	10,502 ± 101	10,605 ± 105	10,972 ± 98	11,287 ± 23	10,613 ± 86
Energy efficiency ratio (g/kJ)	2.62 ± 0.43	2.27 ± 0.34$^{\#}$	2.21 ± 0.36$^{\#}$	2.26 ± 0.12$^{\#}$	2.49 ± 0.29	2.57 ± 0.38

Forty-two rats were randomly divided into six groups on average as follows: (1) Control liquid diet group (Con); (2) low dose of ethanol liquid diet group (Leth, 6.28% of total calories); middle dose of ethanol liquid diet (Meth, 11.48% of total calories); high dose of ethanol liquid diet (Heth, 17.19% of total calories); Con+Res (100 mg/kg/d); Heth+Res (100 mg/kg/d). The animals were euthanized after 23 weeks. All data points represent mean ± SD; $n = 7$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs Con group; $^{*}P < 0.05$ vs Heth group.

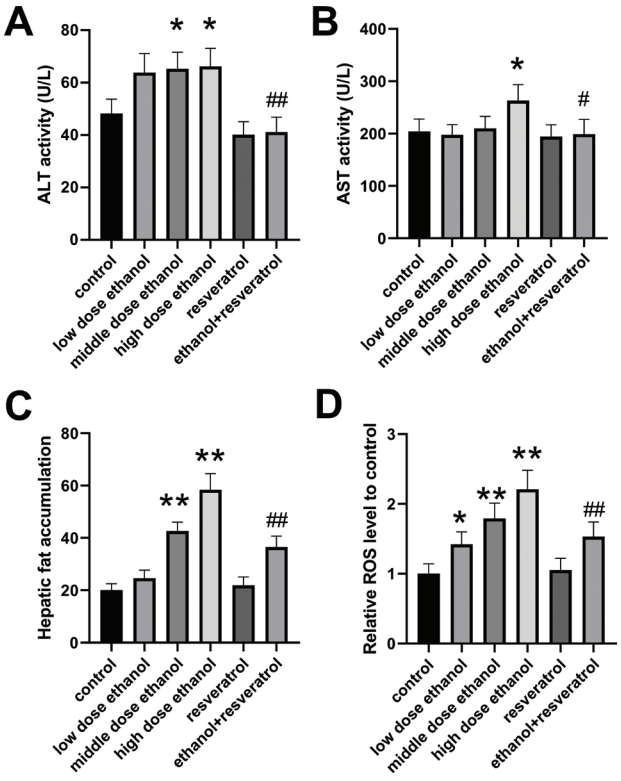

Fig. 1. Effects of dietary Resveratrol supplementation on hepatic enzymes release and ROS levels in rats. (A) Serum ALT, (B) AST level, (C) hepatic fat accumulation, and (D) relative ROS levels in the experimental groups. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs high dose ethanol group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group.

Resveratrol supplementation attenuates hepatic enzymes release in the liver

As shown in Fig. 1A and 1B, serum levels of liver damage markers, ALT and AST, were dramatically elevated in ethanol-fed groups compared with control group, which was effectively inhibited by Resveratrol supplementation. Ethanol-fed groups showed dose-dependent increase in fat accumulation compared with control group, and importantly, the increase was reversed by Resveratrol treatment (Fig. 1C). Also, relative ROS could be significantly decreased by Resveratrol treatment in the Heth group (Fig. 1D).

Resveratrol supplementation attenuates chronic ethanol-induced lipid disorder

Serum and hepatic TC, and TG levels were increased in the chronic ethanol-fed rats in a dose-dependent manner (Table 2). In addition, ethanol increased serum LDL-C and lowered HDL-C levels when compared to control rats. Interestingly, ethanol-induced abnormal serum TC, TG, HDL-C, and LDL-C levels were almost restored to normal levels upon Resveratrol supplementation to ethanol-fed rats. Disturbed hepatic TC and TG were also partially reversed by Resveratrol in spite of significant difference from control group. Of note, Resveratrol alone had no effect on lipid profile.

Resveratrol supplementation inhibits chronic ethanol-induced oxidative stress in serum and liver

Changes in the antioxidant ability and lipid peroxidation of serum and liver are listed in Table 3. Chronic ethanol exposure dose-dependently decreased levels of SOD, CAT, GSH, and GSH:GSSG for serum and liver, respectively. Resveratrol supplementation to the intoxicated rats caused moderate increase in the above parameters in comparison with the Heth group, but significant enhanced serum SOD. However, Resveratrol alone had no effect on the above parameters in comparison with the control group. Furthermore, chronic ethanol also inactivated hepatic Cn-Zn SOD and GPx, by about 27 and 29%, respectively, in the Heth group, in comparison with the control group. Resveratrol treatment alleviated Cn-Zn SOD depletion and significantly enhanced GPx in comparison with the Heth group. MDA level was used as an indicator for the extent of lipid peroxidation induced by ethanol. MDA levels were moderately increased in the serum but significantly elevated in the liver upon ethanol

Table 2. Lipid metabolism parameters in the blood serum and liver of rats after chronically ethanol intake with or without Resveratrol supplemented

Parameters	Groups					
	Con	Leth	Meth	Heth	Con+Res	Heth+Res
Serum TC (mmol/L)	1.88 ± 0.24	2.05 ± 0.32	2.16 ± 0.35	2.62 ± 0.98##	1.92 ± 0.36	2.10 ± 0.43*
Serum TG (mmol/L)	0.86 ± 0.17	0.91 ± 0.32	1.17 ± 0.48	1.36 ± 0.42##	0.82 ± 0.15	0.98 ± 0.18*
Serum HDL-C (mmol/L)	0.59 ± 0.11	0.47 ± 0.08#	0.46 ± 0.06##	0.46 ± 0.10##	0.53 ± 0.07	0.54 ± 0.12
Serum LDL-C (mmol/L)	1.24 ± 0.71	1.77 ± 0.49#	1.86 ± 0.51#	2.04 ± 0.25##	1.13 ± 0.31	1.23 ± 0.34**
Hepatic TC (μmol/g)	18.34 ± 5.28	24.39 ± 4.18	24.47 ± 5.30	27.76 ± 6.06#	18.75 ± 3.61	25.63 ± 7.92#
Hepatic TG (μmol/g)	30.00 ± 8.39	55.39 ± 9.38##	65.38 ± 8.39###	65.39 ± 6.07###	38.32 ± 9.78	53.72 ± 6.94#*

All data points represent mean ± SD; $n = 7$. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs Con group; *$P < 0.05$, **$P < 0.01$ vs Heth group.

Table 3. Antioxidant parameters and lipid peroxidation products (MDA) in serum and liver of rats after chronically ethanol intake with or without Resveratrol supplemented

Parameters	Groups					
	Con	Leth	Meth	Heth	Con+Res	Heth+Res
Serum MDA (nmol/ml)	6.94 ± 0.50	7.43 ± 0.67	7.35 ± 0.72	8.15 ± 1.33	6.68 ± 1.00	7.06 ± 0.76
Serum SOD (U/ml)	160.21 ± 27.79	136.95 ± 37.65	104.72 ± 33.82##	100.58 ± 16.22##	175.02 ± 38.90	141.94 ± 28.59*
Serum CAT (U/ml)	13.87 ± 3.11	8.67 ± 2.79	7.09 ± 2.42	5.39 ± 2.67#	14.87 ± 2.93	8.19 ± 1.63
Serum GSH (μmol/L)	28.95 ± 2.35	29.05 ± 4.66	25.93 ± 2.89	22.82 ± 2.92#	29.45 ± 4.50	25.20 ± 4.58
Serum GSSG(μmol/L)	5.78 ± 1.12	4.38 ± 1.45*	6.27 ± 1.34*	8.04 ± 1.62#	5.17 ± 1.66*	7.17 ± 1.78
Serun GSH:GSSG	5.22 ± 1.32	7.15 ± 2.13*	4.28 ± 0.85	2.99 ± 0.90#	6.58 ± 3.36	4.59 ± 2.88
Liver MDA (nmol/mg-protein)	2.93 ± 1.12	3.66 ± 1.75#	3.94 ± 1.85#	4.92 ± 1.32##	3.27 ± 1.08	3.68 ± 1.48*
Liver SOD (U/mg-protein)	115.55 ± 9.66	104.14 ± 11.30#	93.72 ± 9.84##	92.38 ± 8.11##	110.08 ± 10.66	99.61 ± 12.20
Liver Cu-ZnSOD (U/mg-protein)	55.1 ± 12.41	50.35 ± 12.28	46.12 ± 10.63	39.93 ± 14.78#	58.97 ± 8.70*	49.81 ± 11.36
Liver CAT (U/mg-protein)	23.99 ± 7.02	19.96 ± 8.99	20.22 ± 3.36	14.25 ± 4.69#	22.84 ± 6.08	19.36 ± 4.48
Liver GPx (U/mg-protein)	50.50 ± 4.80	46.20 ± 5.10	42.60 ± 3.21##	35.94 ± 3.13###	53.19 ± 5.62	43.82 ± 4.87#**
Liver GSH (μmol/g)	44.53 ± 4.38	31.36 ± 3.13##	35.55 ± 10.05#	29.92 ± 6.73##	39.55 ± 11.12	24.70 ± 10.00
Liver GSSG (μmol/g)	13.78 ± 2.28	13.80 ± 1.99	14.98 ± 2.27	15.34 ± 2.71	13.25 ± 2.97	13.18 ± 2.43
Liver GSH:GSSG	3.36 ± 0.96	2.30 ± 0.30	2.43 ± 0.80	2.04 ± 0.69#	3.42 ± 2.36	2.68 ± 0.84

All data points represent mean ± SD; $n = 7$. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs Con group; *$P < 0.05$, **$P < 0.01$ vs Heth group.

exposure and were significantly restored by Resveratrol ($P < 0.05$ for liver MDA).

Effects of Resveratrol supplementation on the catalytic activities and protein contents of hepatic ADH, ALDH2, and CYP2E1

As depicted in Fig. 2A and 2B, the expression and the activity of hepatic ADH were slightly increased after chronic ethanol ingestion in comparison with control group, and this elevation was moderately suppressed by Resveratrol ($P > 0.05$). From Fig. 2A and 2C, we found that chronic ethanol diet alone apparently decreased the hepatic expression and the activity levels of ALDH2 compared to the control group ($P < 0.05$). However, Resveratrol supplementation with ethanol ingestion attenuated chronic ethanol diet-induced down-regulation of ALDH2 ($P < 0.05$). Chronic ethanol exposure alone resulted in a dose-dependent upregulation of hepatic CYP2E1 activity level and protein content (Fig. 2A and 2D) in comparison with control group, and this increase was also reversed by Resveratrol treatment ($P < 0.01$).

Discussion

In this study, we found that ALD could be induced by alcoholic liquid diet in rats, which is consistent with previous reports (13, 22). Treatment with Resveratrol significantly mitigated chronic ethanol-induced liver injury, ROS accumulation, lipid peroxidation, and anti-oxidative system, likely through, at least in part, the regulation of alcohol-metabolizing enzymes of ADH, ALDH2, and CYP2E1.

The liver is the main organ responsible for ethanol metabolism, making it as one of the crucial targets of ethanol toxicity. The pathophysiological origin of hepatic alterations produced by ethanol starts from its metabolism. Alcohol is mainly metabolized in three ways: (1) by mitochondrial catalase, (2) by CYP2E1, and (3) by

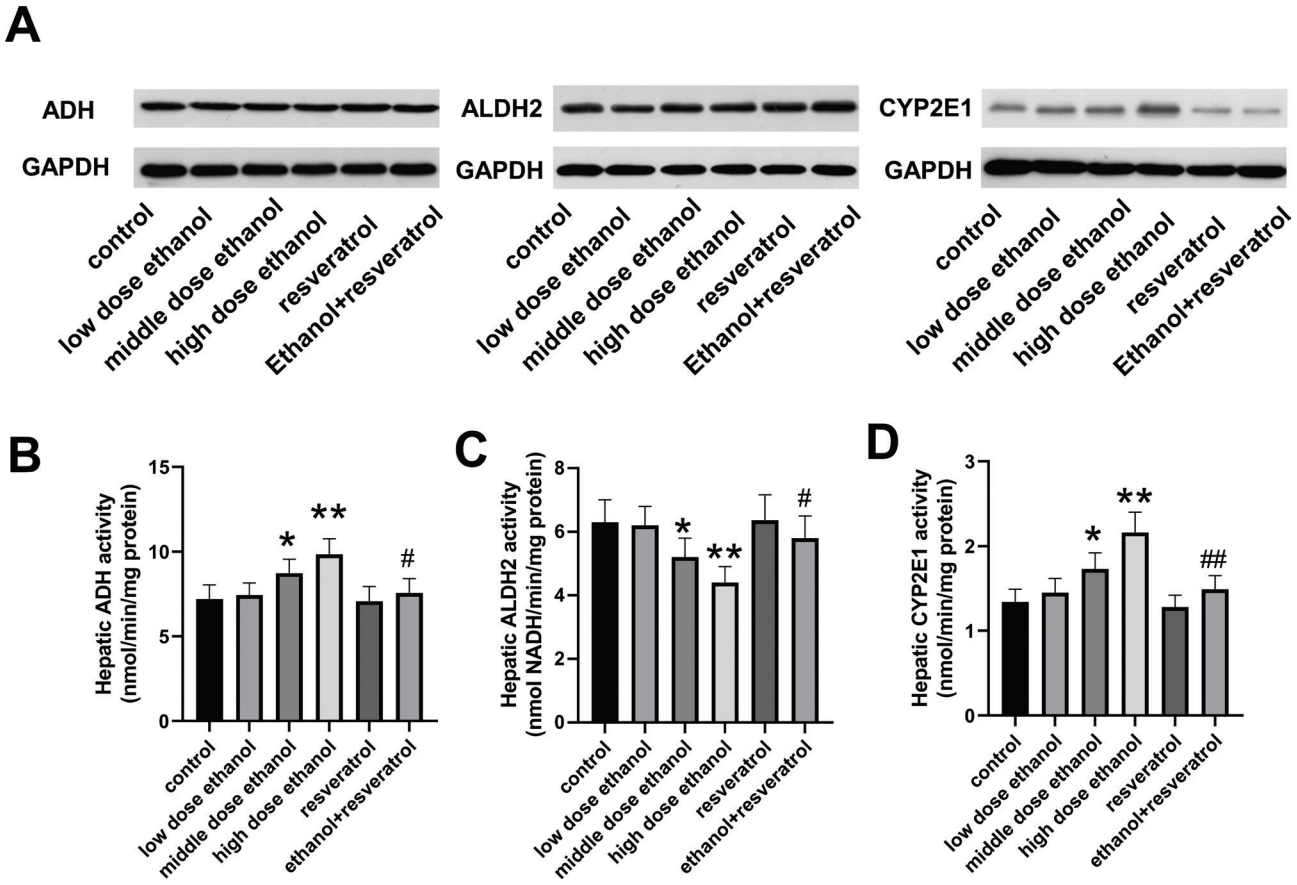

Fig. 2. Effects of ethanol and Resveratrol treatment on the protein expression and activity level of hepatic ADH, ALDH2, and CYP2E1. (A) The relative protein expression of hepatic ADH, ALDH2, and CYP2E1 was analyzed by Western blot analysis. Rat liver tissue was separated for hepatic enzymatic assay of ADH (B), ALDH2 (C), and CYP2E1 (D). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs high dose ethanol group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group.

the enzyme ADH. Among these ways, ADH is the most important way. ADH is a zinc containing metalloenzyme widely distributed in nature, localized in the soluble cytoplasm and NAD^{+}-dependent (23, 24). On the other hand, ethanol-induced CYP2E1 takes place in chronic alcohol ingestion or excessive alcohol intake. In short, alcohol is primarily metabolized by cytosolic ADH and microsomal CYP2E1 to acetaldehyde, which is further metabolized to acetate by mitochondrial ALDH2 (25).

It has been proven that the overexpression of CYP2E1 and ADH could aggravate chronic alcohol exposure-induced hepatic damage (26, 27). Our current study and other previous data (4, 28, 29) showed that ethanol upregulated CYP2E1 and ADH but downregulated ALDH2, indicating a role for acetaldehyde in alcohol ingestion-induced liver damage. Acetaldehyde was reported to inhibit hepatocyte proliferation (3, 30), generate protein adducts (31), and increase ER stress in HepG2 cells (32). Moreover, it can also regulate lipid metabolism by increasing the level of SREBP-1, which enhances hepatic lipogenesis thereby leading to the development of ALD (33). Because the hepatic acetaldehyde level was not measured in the current study, we could not infer the limitation of acetaldehyde production in liver directly. However, considering that the expression levels of ADH, CYP2E1, and ALDH2 limit acetaldehyde production, they were chosen as molecular factors involved in Resveratrol-induced improvement of hepatic acetaldehyde accumulation. Interestingly, our data revealed that Resveratrol supplementation partially ameliorated chronic ethanol-induced hepatotoxicity by inhibiting the expression of hepatic CYP2E1 and ADH as well as significantly increasing the expression of hepatic ALDH2, consistent with previous results (16–18, 34). In studies using mouse models, the overexpression of ALDH2 attenuated chronic alcohol exposure-induced hepatic damage (35), and ethanol hepatotoxicity was substantially alleviated by CYP2E1 knockout (36–38), further elucidating that ADH, CYP2E1, and ALDH2 are important in preventing hepatotoxicity.

Lipid metabolic disorders are involved in the pathogenesis of ALD. ALT and AST are well-known biomarkers

for hepatic dysfunction (39). Therefore, in order to further illustrate the hepato-protective effect of Resveratrol, we detected lipid profile, serum AST, ALT, and liver pathological sections. In our study, Resveratrol supplementation attenuated the chronic ethanol-induced lipid disorder, which is in accordance with previous study on non-alcoholic fatty liver disease (40). Furthermore, chronic ethanol ingestion led to elevated serum ALT and AST activities, suggesting a sustained liver damage. Resveratrol effectively ameliorated ethanol-derived aminotransferase leakage and liver morphological abnormity, exhibiting favorable hepato-protective effects against ethanol toxicity, which is in line with other studies employing rat models (11, 13).

Various mediators are reported to be implicated in ethanol-induced liver diseases, in which free radical damage and oxidative stress are of particular importance (41, 42). CYP2E1 was demonstrated to be a key source of ROS, which participates in alcohol-induced liver injury (29, 43). CYP2E1 knockout could suppress alcohol-induced oxidative stress and liver injury in a mouse model, whereas its overexpression aggravated the hepatic impairment, approving the causative involvement of CYP2E1 in the pathophysiology of ALD (36). In the present study, ethanol exposure increased hepatic CYP2E1 expression, resulting in numerous ROS generation, which could be reversed by Resveratrol supplementation. The eruption of ROS propagates lipid peroxidation, as seen by increased MDA level. In the current study, ethanol insult increased serum and liver MDA contents, whereas Resveratrol treatment significantly inhibited MDA production. Additionally, the impaired antioxidant defense system in the liver has long been recognized in ALD. In our experiments, ethanol exposure remarkably decreased the amount of GSH, the most abundant non-enzymatic antioxidant involved in cellular defense as well as the activities of antioxidant enzymes of SOD, CAT, and GPx. Resveratrol treatment showed a powerful antioxidant effect, as evidenced by elevated GSH concentration and increased antioxidant enzymes activities following its supplementation. Our results indicated that the ability of Resveratrol treatment to protect against ethanol-induced oxidative stress may be due to the suppression of hepatic CYP2E1 activity and the enhancement of both enzymatic and non-enzymatic defense systems.

In conclusion, naturally existing Resveratrol evidently attenuated chronic ethanol-induced ALD, which highlights its potential for the treatment of ALD.

Conflict of Interest

None.

Acknowledgments

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