Demethylenberberine, a Natural Mitochondria-Targeted Antioxidant, Inhibits Mitochondrial Dysfunction, Oxidative Stress, and Steatosis in Alcoholic Liver Disease Mouse Model

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ABSTRACT

Excessive alcohol consumption induces oxidative stress and lipid accumulation in the liver. Mitochondria have long been recognized as the key target for alcoholic liver disease (ALD). Recently, the artificial mitochondria-targeted antioxidant MitoQ has been used to treat ALD effectively in mice. Here, we introduce the natural mitochondria-targeted antioxidant demethylenberberine (DMB), which has been found in Chinese herb Cortex Phellodendri chinensis. The protective effect of DMB on ALD was evaluated with HepG2 cells and acutely/chronically ethanol-fed mice, mimicking two common patterns of drinking in human. The results showed that DMB, which is composed of a potential antioxidant structure, could penetrate the membrane of mitochondria and accumulate in mitochondria either in vitro or in vivo. Consequently, the acute drinking–caused oxidative stress and mitochondrial dysfunction were significantly ameliorated by DMB. Moreover, we also found that DMB suppressed CYP2E1, hypoxia inducible factor α, and inducible nitric oxide synthase, which contributed to oxidative stress and restored sirtuin 1/AMP-activated protein kinase/peroxisome proliferator-activated receptor-γ coactivator-1α pathway–associated fatty acid oxidation in chronic ethanol-fed mice, which in turn ameliorated lipid peroxidation and macrosteatosis in the liver. Taking these findings together, DMB could serve as a novel and potential therapy for ALD in human beings.

Introduction

Alcoholism is a major cause of morbidity and mortality worldwide. In 2004, ethanol consumption–associated chronic liver disease accounted for 3.8% of all deaths around the world (Rehm et al., 2009). The spectrum of alcoholic liver disease (ALD) ranges from simple steatosis to severe alcoholic hepatitis, fibrosis, cirrhosis, and ultimate hepatocellular carcinoma (Lieber, 2004). Accumulating evidence indicated that oxidative stress plays a critical role in ethanol-induced liver injury (Hoek et al., 2002). Metabolizing ethanol in the liver is always accompanied with abundant reactive oxygen species (ROS) production, which initiates lipid peroxidation, glutathione depletion, abnormal methanione metabolism, and malnutrition. CYP2E1 and the mitochondrial respiratory chain have been considered as two major contributors of ROS production associated with ethanol-dependent oxidative stress (Hoek et al., 2002; Lu and Cederbaum, 2008). Usually CYP2E1 has been recognized to produce abundant ROS, such as hydrogen peroxide and the superoxide anion radical in the membrane of the endoplasmic reticulum. However, in recent time, increasing attention has been paid to CYP2E1 within mitochondria, as it has been well documented that mitochondrial CYP2E1 augments local and cellular oxidative stress and acts as a critical pathogenic role in alcoholic liver diseases (Bansal et al., 2010; Knockaert et al., 2011a,b). Increased mitochondria-located CYP2E1 causes ROS overproduction and glutathione (GSH) depletion and dysfunction in mitochondria. Therefore, we can regard the specific CYP2E1 within mitochondria as a novel and potential target for preventing ALD.

Mitochondria-mediated ethanol metabolism acts as another important role in oxidative stress (Hoek et al., 2002). Increased supply of mitochondrial NAD(P)H and reducing pressure resulting from acute and chronic alcohol treatments significantly promote mitochondrial ROS formation in liver.
cells (Kurose et al., 1997; Bailey et al., 2001; Higuchi et al., 2001). In addition, CYP2E1-induced increase in the proportion of $O_2$ uptake for oxidizing ethanol competes with mitochondrial electron transport in the utilization of $O_2$ (Ingelman-Sundberg et al., 1994), which consequently develops localized and transient hypoxia in the tissue, particularly in the pericentral region of the liver acinus (Arteel et al., 1997; Cunningham and Ivester, 1999). Such conditions of hypoxia and reoxygenation further strengthen ROS formation through the mitochondrial respiratory chain. On the other hand, ethanol-medicated activation of Kupffer cells generates reactive nitrosative stress through the induction of inducible nitric oxide synthase (iNOS), which exacerbates oxidative stress and mitochondrial dysfunction in the liver (McKim et al., 2003; Venkatraman et al., 2004). Therefore, these findings suggested mitochondria as an important target of therapies for ALD. Currently, Chacko et al. (2011) have treated ALD in mice with the mitochondria-targeted antioxidant MitoQ, an artificial conjugate of ubiquinone with triphenylphosphonium cation, which targets the ubiquinone to mitochondria (McKim et al., 2003). However, several studies have indicated that cellular toxicity is associated with triphenylphosphonium cation.

Demethylenberberine (DMB), introduced in this study, is a novel cationic antioxidant, which comes from the Chinese herb Cortex Phellodendri chinensis, which has a long history of traditional Chinese medicine use. Theoretically, DMB could be guided into the mitochondrion by the high negative potential inside the mitochondrion. Recent studies revealed that Cortex Phellodendri chinensis has many pharmacological activities, such as antimicrobial, anti-inflammatory, antidiarrhea, and anticancer (Li et al., 2006; Shuanglai et al., 2011), but the particular biologic properties of DMB are still elusive. Our study proposes DMB as a potential agent for treating ALD due to its special structure, which contains both cationic and catechol groups (Fig. 1A). A series of in vitro and in vivo

![Fig. 1.](image-url)

**Fig. 1.** DMB was determined to be a mitochondria-targeted antioxidant. (A) Structure of DMB. (B) T-AOC of DMB. (C) Representative images of ROS fluorescent staining with DCFH-DA. Original magnification, 200×. Flow cytometric histograms are shown in the right panel. (D) HPLC analysis of DMB in mitochondria isolated from HepG2 cells. (E) Flow cytometric histograms of MMP indicated by Rhodamine 123. M1, low Rhodamine 123 fluorescence intensity. Bar graph represents the quantified fluorescence intensity. Values represent means ± S.D. (n = 3). ###P < 0.001 versus control; ***P < 0.001 versus H$_2$O$_2$ (1 mM) or EtOH group.
experiments were conducted to evaluate the biologic activity of DMB in this work.

Materials and Methods

Demethyleneberberine hydrochloride was synthesized in our laboratory and slightly modified from Zu et al. (2006). The purity of DMB was more than 98% when analyzed by high-performance liquid chromatography (HPLC; parameters not shown). The structure was identified by mass spectrometry, 1H-NMR, and other methods (Supplemental Fig. 1). Total antioxidant capacity (T-AOC), alanine aminotransferase (ALT), thiobarbituric acid reactive substances (TBARS), and GSH test kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rhodamine 123 and dihydrodichlorofluorescein diacetate (DCFH-DA) were products of Sigma-Aldrich (St. Louis, MO). Other chemicals were of analytical grade.

T-AOC Test. An antioxidant reduces Fe3+ to Fe2+, which coordinates with phenanthroline to form a stable and colored complex reaching the maximum absorbance at 520 nm. To determine the antioxidant property of DMB, a T-AOC test was conducted according to the protocol of the commercial kit.

Determining the Mitochondria-Targeted Potential of DMB In Vitro and In Vivo with HPLC. HepG2 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (Gibco/Life Technologies, Grand Island, NY) in a humidified incubator, supplemented with 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 mg/ml). When cells reached 80% confluence in the flasks, and DMB was added with a final concentration of 50 μM for 30 minutes. Then, cells were washed with phosphate-buffered saline (PBS) three times and then collected. This was followed by mitochondria and cytoplasm isolation with the method of differential centrifugation (Frezza et al., 2007). The isolated mitochondria were washed with an isolating solvent three times to avoid cytoplasmic contamination. DMB distributed in mitochondria and cytoplasm was identified by HPLC (parameters not shown). Adult male ICR mice (8 weeks, 24–26 g) from the College of Veterinary Medicine, Yangzhou University (Yangzhou, China), were housed in temperature- and humidity-controlled rooms. The mice were maintained at a 12-hour light/dark cycle and had free access to rodent chow and tap water. All procedures were approved by the Institutional Animal Care and Use Committee at China Pharmaceutical University and adhered to the Jiangsu Provincial Guidelines for the use of experimental animals. Mice were administrated with DMB (10 mg/kg) intravenously for one time, and every individual liver was collected after 15 minutes. The mitochondria and cytoplasm from the livers were isolated by differential centrifugation as described above. DMB distributed in the mitochondria and cytoplasm was identified by HPLC (parameters not shown).

ROS Determination. HepG2 cells were cultured in six-well plates under the same condition described above. With suitable confouency, cells were incubated with 50 μM DMB for 2 hours. After being washed with PBS buffer three times, H2O2 was then used to stimulate cells for another 15 minutes at a final concentration of 1 mM. Cells were incubated with 5 μM DCFH-DA at 37°C for 30 min in the dark and then washed with PBS three times to remove excess dye (Takanashi et al., 1997; Yeligar et al., 2012). Fluorescence microscope and flow cytometry were used to image and quantify the fluorescence intensity to reflect the ROS production.

Mitochondrial Membrane Potential Test with Cell Culture. Mitochondrial membrane potential (MMP) was evaluated in HepG2 cells cultured with a medium containing 0.5% ethanol in the presence or absence of 50 μM DMB for 1 week. As an indicator of MMP, Rhodamine 123 was used to evaluate the protective effect of DMB on the ethanol-induced MMP decrease (Yan et al., 2007) by the detection of the fluorescence intensity with fluorescence microscope and flow cytometry.

Acute Alcoholic Liver Injury Model. The acute alcohol exposure model has been proposed as a predictive/screening tool for therapies of the commercial kit.

Chronic Alcoholic Liver Injury Model. Adult male ICR mice (8 weeks, 24–26 g) were divided into three groups: 1) control; 2) Lieber-DeCarli liquid diet, and 3) LD+DMB (40 mg/kg per day i.p.); n = 10, which were pair-fed an isocaloric Lieber-DeCarli liquid diet (Trophic, Nantong, China) containing 0 or 36% ethanol by caloric content for 5 weeks to produce chronic alcoholic liver disease (Nanj et al., 1989). The dosage of DMB was based on preliminary range-finding studies. At sacrifice, animals were anesthetized with sodium pento-barbital (80 mg/kg i.p.) 6 hours after ethanol administration. Blood was collected with cardiac puncture just prior to sacrifice. Liver tissues were harvested at the time of sacrifice.

Serum ALT Assay. Serum was separated by centrifugation of 800g at 4°C for 10 minutes after 40 minutes of standing at room temperature. Serum ALT activity was measured using commercially diagnostic kits.

Histopathological Analysis. After being sacrificed, small pieces of individual liver from the same location were harvested, washed with ice-cold saline, and fixed in 4% neutral buffered formalin solution. Sections of 5-mm thickness were cut, deparaffinized, hydrated, and stained with hematoxylin and eosin.

Western Blot Analysis. Mice liver tissue or isolated mitochondria were homogenized and sonicated in ice-cold lysis buffer (radioimmunoprecipitation assay buffer) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Lysates were centrifuged at 10,000g and 4°C, and supernatant was collected, of which the protein concentration was determined using the bicinechonic acid method. Equal amounts of protein (100 μg) were loaded on 10% polyacrylamide gel (29:1 acrylamide-bisacrylamide) separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membrane was blocked for 1 hour at room temperature in Tris-buffered saline with Tween 20 (TBST) buffer containing 5% BSA, and then incubated with the primary antibody in TBST with 5% BSA overnight at 4°C. A primary antibody at an appropriate dilution ratio was used to detect CYP2E1 (AmbioBio, San Francisco, CA), iNOS, and glyceraldehyde 3-phosphate dehydrogenase (Biorod, Dublin, OH). With three times washing of TBST, the membrane was incubated with the secondary antibody (Biorod) in TBST with 5% BSA and exposed with ECL (Millipore) after another three times of washing of TBST.

Real-Time Reverse-Transcription Polymerase Chain Reaction Assay. RNA was purified and reverse transcribed with the Moloney murine leukemia virus reverse transcription and oligo-dT primers. The forward and reverse primers were designed using Oligo 6.0 and listed in Table 1. Real-time quantitative polymerase chain reaction was performed in the presence of SYBR Green Master (ROX) (Roche, Basel, Switzerland) on Step One Plus System (Applied Biosystems, Foster City, CA). Relative abundance of mRNA was normalized to that of cyclophilin mRNA.

Immunohistochemistry. Liver sections (10 μm) were mounted on glass slides. Sections were deparaffinized and incubated in 3% H2O2 for 10 minutes to quench endogenous peroxidase activity. After blocking with normal goat serum for 20 minutes, the sections were stained with polyclonal rabbit antibody raised against CYP2E1 (1:100) (Bioworld) and hypoxia inducible factor-1α (1:20) (BBI, Markham, ON, Canada) at 4°C overnight, respectively, followed by washing with PBS and incubated with goat anti-rabbit antibody at 37°C for 30 minutes. The antibody binding sites were visualized by incubation with diaminobenzidine at room temperature for 10 minutes.

Electron Microscopy. Liver tissue (1 mm3) from the same location of the liver was removed and prefixed for 2 hours at 4°C in
HepG2 cells as compared with the group treated with H2O2 changes were presented by the decrease of absorbance at 520 nm. Protein was added to the solution. Mitochondrial osmotic volume, which is reflective of viable intact cells (Fig. 1E), whereas cells displayed strong Rhodamine 123 fluorescence intensity, the effect of DMB on mitochondria by testing MMP in much higher than that in cytoplasm. Then, we evaluated concentration of DMB in mitochondria is suggested to be could target mitochondria. As a result, DMB was detectable performed an HPLC experiment to address whether DMB on mitochondria by testing MMP in Fig. 1C.

Evaluation of Mitochondrial Chemistry. Mitochondria were isolated by differential centrifugation of the liver homogenate (Frezza et al., 2007). The total protein of isolated mitochondria was measured by the bicinchoninic acid method. The mitochondrial peroxidative product (TBARS), antioxidant defense [glutathione peroxidase (GPx)/GSH], and mitochondrial aspartate aminotransferase (mAST) activity were determined by the commercial kits according to the manufacturer’s instructions.

Mitochondrial Swelling Test. The assays were performed in 1 ml of the reaction media (250 mM sucrose, 3 mM sodium succinate, 5 mM KH2PO4, and 0.3 mM CaCl2) (Galindo et al., 2003). Changes of absorbance at 520 nm in 10 minutes were recorded after 0.5 mg of protein was added to the isolated solution. Mitochondrial osmotic volume changes were presented by the decrease of absorbance at 520 nm. Damaged mitochondria reflected much less sensitivity to the Ca2+ loading, which was demonstrated by a blunted decrease of 520-nm absorbance.

Statistical Analysis. All analyses were performed with Statistical Package for Social Sciences, version 16.0 (SPSS, Chicago, IL). Data were expressed as mean ± S.D. Statistical significance was determined by one-way analysis of covariance. In all statistical comparisons, a P < 0.05 was used to indicate a statistically significant difference.

Results

DMB Was Determined To Be a Mitochondria-Targeted Antioxidant. In the first place, the antioxidant capability of DMB was detected by T-AOC and ROS tests. As shown in the result of the T-AOC test, DMB exhibited an antioxidant capacity in a concentration-dependent manner (Fig. 1B). In the ROS experiment, the probe DCFH-DA only detects intracellular ROS, reflecting the antioxidant activity of DMB in cells. As a result, DMB neutralized H2O2-originated ROS by 55% in a concentration-dependent manner (Fig. 1B). In the ROS experiment, the probe DCFH-DA only detects intracellular ROS, reflecting the antioxidant activity of DMB in cells. As a result, DMB neutralized H2O2-originated ROS by 55% in HepG2 cells alone (Fig. 1C).

In the second place, given the cationic structure of DMB, we performed an HPLC experiment to address whether DMB could target mitochondria. As a result, DMB was detectable significantly in both mitochondria and cytoplasm isolated from HepG2 cells (Fig. 1D). Due to that, the volume of mitochondria is smaller than that of cytoplasm, and the concentration of DMB in mitochondria is suggested to be much higher than that in cytoplasm. Then, we evaluated the effect of DMB on mitochondria by testing MMP in HepG2 cells exposed to 0.5% ethanol for 1 week. Control cells displayed strong Rhodamine 123 fluorescence intensity, which is reflective of viable intact cells (Fig. 1E), whereas a small amount of cells was located in the low Rhodamine 123 fluorescence (M1) population, which is reflective of damaged cells (Fig. 1E). Ethanol stimulation caused fluorescence migration to the M1 population (Fig. 1E), whereas, inspiring, coinoculation in the presence of 50 μM DMB significantly reversed such migration, protecting HepG2 cells from the decline in MMP produced by ethanol (Fig. 1E).

DMB Protected Liver Mitochondria from Oxidative Stress in Acute Ethanol–Treated Mice. Based on the above findings, the biologic activity of DMB in vivo was investigated with binge-drinking mice. As shown by H&E staining, binge ethanol caused microsteatosis, swelling, and even apoptosis in liver cells. DMB treatment (40 mg/kg) remarkably attenuated these histopathological damages (Fig. 2A). Elevated serum ALT by binge drinking was also blunted in mice after DMB treatment (Fig. 2B).

Mitochondria have long been recognized as the major targets of ethanol-induced oxidative stress (Hoek et al., 2002). Our results showed that binge drinking significantly reduced mitochondrial GSH and GPx activity by 33% and further elevated TBARS formation in mitochondria by 250% (Fig. 2C). DMB treatment completely blocked the decline of GSH, GPx activity, and TBARS formation in mitochondria (Fig. 2C). Blunted decrease in 520-nm absorbance indicated the swelling of mitochondria, suggesting that the mitochondrial membrane was disturbed by ethanol-induced ROS. DMB treatment alleviated ethanol-mediated mitochondrial swelling, which was monitored by an increased rate of the decline in 520-nm absorbance (Δ520 nm) (Fig. 2Da). In addition, binge ethanol treatment in mice decreased mAST activity by 60%, which suggested that ethanol ruptured the mitochondrial membrane, resulting in mAST leaking out (Fig. 2Db). DMB treatment significantly recovered mAST activity by 50% (Fig. 2Db).

Electron microscopy images showed that DMB treatment profoundly ameliorated the ultrastructural damages of mitochondria. Under the condition of binge ethanol, the membrane and cristae of mitochondria were ruptured and several mitochondria were even transformed to megamitochondria (Fig. 2E).

The endoplasmic reticulum became swollen and thin, and lots of lipid droplets were observed in the binge ethanol-treated group (Fig. 2E). All of these abnormal ultrastructural changes were significantly improved by DMB treatment. A rich endoplasmic reticulum and normal mitochondria but few lipid droplets were found in hepaticocytes from DMB-treated mice (Fig. 2E). Therefore, the results supported that DMB could prevent alcoholic liver injury in vivo, at least partially, by protecting mitochondria from ethanol-induced oxidative stress.

DMB Alleviated Chronic Alcoholic Liver Injury. The chronic alcoholic liver injury model was set up to mimic the initial pathogenesis of ALD in human beings due to long-term drinking (Lieber and DeCarli, 1989; Brandon-Warner.

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<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>CPT1a</td>
<td>5'-GCTGCAATATTCAAGGGAAT-3'</td>
<td>5'-TGAGACCACTAAGGGAAG-3'</td>
</tr>
<tr>
<td>ACO</td>
<td>5'-TCCGACTTCAATCAGGAGA-3'</td>
<td>5'-CTGGGCTTAGTCCGACATTA-3'</td>
</tr>
<tr>
<td>MCAD</td>
<td>5'-TGCGAGCGTTCCTGACACAG-3'</td>
<td>5'-CAGCCGACCTTCCCACCTGAT-3'</td>
</tr>
<tr>
<td>SIRT1</td>
<td>5'-GGGGTTCTGGTCTCCCTGTT-3'</td>
<td>5'-GAATGCTTTGGCTCGTTT-3'</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>5'-TGCCATGGTAAAGCCGAG-3'</td>
<td>5'-TTGGGCTCATTTGGTACG-3'</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>5'-CCXATGTCTCATCAAGGAGTTCTCAT-3'</td>
<td>5'-CTTGCACTCAGGCAAGATCTTT-3'</td>
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Mice were pair-fed with either ethanol or control liquid diets for 5 weeks in the presence or absence of DMB (40 mg/kg per day i.p.). The liver from the chronic ethanol-treated group looks rough, swollen, and xanthic (Fig. 3A). The ratio of liver to body weight and serum ALT activity increased by about 1.2- and 2.3-fold, respectively, by chronic ethanol exposure as compared with pair-fed controls, both of which were attenuated by DMB treatment (Fig. 3, B and C). In accordance, H&E staining revealed that chronic drinking in mice caused significant microsteatosis/macrosteatosis and hepatocellular ballooning (Fig. 3D). The severe pathologic changes were substantially alleviated by DMB treatment, which revealed the protective effect of DMB on chronic alcoholic liver disease (Fig. 3D).

**DMB Suppressed CYP2E1, HIF-1α, and iNOS, which Contributed to Ethanol-Dependent Oxidative Stress.** That DMB treatment (40 mg/kg per day) ameliorated oxidative stress in response to chronic alcohol consumption was reflected by decreasing hepatic TBARS formation by 25% in chronic ethanol feeding mice (Fig. 4A). The induction of CYP2E1 has been well documented to be a central pathway that contributes to ethanol-mediated oxidative stress (Hoek et al., 2002; Lieber, 2004; Lu and Cederbaum, 2008). Elevated CYP2E1 by ethanol generates excessive reactive oxygen species, such as the hydrogen peroxide and superoxide anion radical. Our results showed that DMB treatment dramatically reduced the total CYP2E1 protein expression (Fig. 4Ba) and blocked the distribution of CYP2E1 around the veins (Fig. 4Bb). In the past decades, CYP2E1 has been recognized to generate ROS in the membrane of the endoplasmic reticulum for a long time, but currently, increasing attention has been paid to CYP2E1 within mitochondria. Many studies reported that mitochondrial CYP2E1 augmented local and cellular oxidative stress and acted as a critical pathogenic role in alcoholic liver diseases (Bansal et al., 2010; Knockaert et al., 2011a,b). A recent study even indicated that mitochondrial CYP2E1 made a greater contribution to the pathogenesis of ALD than microsomal CYP2E1 (Knockaert et al., 2011a,b). We investigated the isoforms of CYP2E1 located in mitochondria (mtCYP2E1), phosphorylated CYP2E1 (P-mt CYP2E1), and truncated mtCYP2E1. Western blot showed that both forms of mtCYP2E1 were reduced by DMB treatment in mice

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**Fig. 2.** DMB protects liver mitochondria from binge ethanol-induced oxidative damage in mice. (A) Liver H&E staining. Original magnification, 400×. Arrow 1, microsteatosis; arrow 2, apoptosis. (B) Serum ALT levels. (C) Mitochondrial levels of GSH, GPx activity, and TBARS. (D) (a) Mitochondrial swelling. (b) Mitochondrial AST activity. (E) Mitochondrial electron microscope (EM, 1700×). LD, lipid droplet; M, mitochondria; N, nucleus. Values represent means ± S.D. (n = 3–8). **P < 0.01 and ***P < 0.001 versus control; *P < 0.05; **P < 0.01 versus ethanol.
fed with chronic ethanol (Fig. 4C). Consequently, CYP2E1-associated hypoxia was alleviated by DMB, which was reflected by reduced HIF-1α induction around the veins (Fig. 4D). In addition, iNOS induced by chronic alcohol consumption in mice was also suppressed after DMB treatment (Fig. 4E).

**DMB Prevented Alcoholic Fatty Liver by Restoring Sirtuin 1/AMP-Activated Protein Kinase/Peroxisome Proliferator-Activated Receptor-γ Coactivator-1α Pathway–Associated Fatty Acid Oxidation.** The onset and progression of ALD is usually characterized by steatosis (Li et al., 2006). In accordance, the triglyceride content in liver from ethanol-treated mice was significantly increased by 200% compared with control, whereas DMB treatment reduced the triglyceride by about 50% (Fig. 5A). Chronic alcohol consumption resulted in extensive accumulation of Oil Red O–stained lipids around the central vein and periportal region, which was apparently blunted by DMB treatment (Fig. 5B). In the liver, AMP-activated protein kinase (AMPK) could increase fatty acid oxidation via the activation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which coactivates the expression of enzymes involved in fatty acid oxidation, such as carnitine palmitoyltransferase 1α, acyl-CoA oxidase, and mitochondrial medium-chain acyl-CoA dehydrogenase (Ajmo et al., 2008). Recent studies demonstrated that sirtuin 1 (SIRT1) acted upstream of AMPK and could also directly activate PGC-1α to promote fatty acid utilization (Banks et al., 2008; Lan et al., 2008; Amat et al., 2009). Our study revealed that DMB substantially reversed the inhibitory effects of chronic ethanol feeding on hepatic SIRT1, phosphorylated AMPK, and PGC-1α expression at the protein or mRNA level in mice (Fig. 5C). We further determined whether the activated SIRT1/AMPK/PGC1-α pathway resulted in the altered expression of genes associated with fatty acid oxidation by real-time polymerase chain reaction. Results showed that the carnitine palmitoyltransferase 1α, acyl-CoA oxidase, and mitochondrial medium-chain acyl-CoA dehydrogenase mRNA expression of the DMB-treated group was 2-, 9-, and 2-fold higher than those of the model group, respectively (Fig. 5D).

**Discussion**

The present study characterized the diverse hepatoprotective effects of DMB on ALD. We identified a novel and unique mechanism involving the attenuation of hepatic oxidative stress and steatosis, partially through mitochondria-targeted antioxidation, downregulation of CYP2E1, and activation of the SIRT1/AMPK/PGC1-α pathway–associated fatty acid oxidation. Such an action differs from that of the well-known antioxidants S-adenosyl methionine and tocopherol, which restore glutathione content (Altavilla et al., 2005; Tilg and Day, 2007).

Alcohol-induced liver injury enhances the susceptibility of the liver to develop steatohepatitis, fibrosis, cirrhosis, and carcinoma (Lieber, 2004). Since mitochondria are both sources and targets for reactive oxygen and nitrogen species, it is reasonable to conclude that they play a central role in the pathophysiology of ethanol-mediated hepatotoxicity.
Recent studies have closely linked the production of ROS to mitochondrial DNA, membrane and protein damage, and alcohol-dependent metabolic derangements in the liver. On the basis of these findings, we thought a mitochondria-targeting antioxidant could potentially attenuate pathologic changes in response to alcoholism. DMB, which was introduced in this study, has long been identified as the ingredient of Cortex Phellodendri chinensis as well as a major metabolite of berberine (BBR), another traditional Chinese medicine, in vivo (Zuo et al., 2006). However, this is the first time that DMB was recognized as a natural mitochondria-targeted antioxidant for treating ALD in our study. First, the mitochondria-targeting potential and antioxidant activity of DMB were demonstrated by a series of in vitro experiments. Second, we further confirmed that DMB potentially targeted liver mitochondria in vivo and significantly protected liver mitochondria from acute ethanol-induced oxidative damages in mice. Third, DMB was determined to treat chronic alcoholic liver injury that resembled the initial pathogenesis of ALD in human beings due to long-term drinking, which suggested that DMB could serve as a potential therapy for human ALD.

The detrimental consequences of alcohol drinking on the liver, especially of heavy and/or long-term consumption, have been well established. Heavy and chronic ethanol consumption caused ALD in approximately 20% of individuals. Ethanol-induced robust ROS are predominantly produced by CYP2E1 and the mitochondrial respiratory chain in the liver (Hoek et al., 2002). Based on the etiopathogenesis of alcoholic liver injury, it is reasonable to regard DMB as an effective therapy for ALD, which combats mitochondrial ROS formation as well as CYP2E1-dependent oxidative stress. An interesting result in this study was that DMB treatment significantly reduced two isoforms of mitochondrial CYP2E1: a highly phosphorylated 52-kDa form and a shortened 40-kDa NH2-terminal truncated form (Fig. 4, B and C) (Knockaert et al., 2011a,b). Recent studies indicated that mitochondrial CYP2E1 could be induced by ethanol exposure and contributed to the pathogenesis of ALD, and the localization of CYP2E1 within mitochondria resulted in mitochondrial ROS overproduction, GSH depletion, and a membrane potential decrease (Bansal et al., 2010; Knockaert et al., 2011a,b). cAMP-dependent phosphorylation of CYP2E1 by protein kinase A has been thought to result in increasing the association of the protein with cytoplasmic...
Hsp70 and Hsp90 chaperones and favoring its binding to mitochondrial translocase outer membrane transporters (Robin et al., 2002; Anandatheerthavarada et al., 2009). Although DMB can target mitochondria in hepatocytes, as shown in the HPLC charts, amounts of DMB still reside in the cytoplasm. We propose that DMB might decrease mitochondrial CYP2E1 by interfering with the phosphorylation of CYP2E1 induced by protein kinase A in the cytoplasm. On the other hand, it is also possible that the DMB inside mitochondria disturbed the mitochondria location and further truncation of CYP2E1, which needs further investigation.

Accumulation of lipids as microvesicles and macrovesicles and the distinctive localization of lipid vesicles determined the characteristic tissue pathology resulting from chronic alcohol consumption. DMB-mediated inhibition of both microsteatosis and macrosteatosis suggested the direct or indirect interference with the pathways related to lipid metabolism in the liver. Both SIRT1 and AMPK have been implicated in the development of alcoholic fatty liver (You et al., 2004; García-Villafranca et al., 2008; Lieber et al., 2008). The present study indicated that DMB exerts its protective action against ethanol-induced liver steatosis by turning on the hepatic SIRT1/AMPK signaling system (Fig. 5C). SIRT1 and AMPK actions converge through PGC-1α to enhance fatty acid oxidation, suggesting that SIRT1/AMPK/PGC-1α stimulation may serve as a key mechanism for alleviating alcoholic fatty liver. Consequently, the mRNA levels of several genes associated with fatty acid oxidation and lipid transport were restored by DMB (Fig. 5D). These data indicated that DMB ameliorated hepatosteatosis in response to chronic alcohol consumption by activating SIRT1/AMPK/PGC-1α pathway–associated fatty acid oxidation. However, the specific mechanism by which DMB regulates this pathway needs to be confirmed by more evidence. Interestingly, DMB has also been identified as a major metabolite of BBR in vivo (Zuo et al., 2006). BBR is a well recognized AMPK agonist that attenuates lipid accumulation in the liver (Turner et al., 2008; Liu et al., 2013). Even though BBR has been studied for many years, it remains unclear how BBR activates AMPK, but we believe that DMB shares a structure-activity relationship with BBR in part.

In summary, this study identified the natural cationic antioxidant DMB as a potential agent to treat ALD in mice and uncovered novel mechanisms of DMB on ameliorating ethanol-dependent oxidative stress, which was distinct from other antioxidants. These findings raise the possibility that DMB might be beneficial in liver disease associated with increased oxidative stress, including alcoholic/nonalcoholic steatohepatitis and chronic hepatitis C. Further studies are needed to confirm the effects of DMB against these diseases.
Demethylenobervine Prevents Alcoholic Liver Disease

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Conducted experiments: P. Zhang, Qiang, M. Zhang, Ma Zhao, Zhou.
Wrote or contributed to the writing of the manuscript: P. Zhang, Y. Zhang.

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Demethylenberberine, a natural mitochondria-targeted antioxidant, inhibits mitochondrial dysfunction, oxidative stress and steatosis in alcoholic liver disease mouse model

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Supplementary Data
1. Identification of structure of DMB by MS and \(^1\)H-NMR

**Fig. 1 (A):** MS spectrum. HRESI-MS showed the [M-H] \(^+\) signal at \(m/z\) 324.2 and [MH] \(^+\) signal at \(m/z\) 325.2 which are consistent with the theoretical molecular weight of DMB.

**Fig. 1 (B):** \(^1\)H-NMR spectrum. The 2, 3-phenolic hydroxyl signals at \(\delta\) 9.4101(s, 1H) and \(\delta\) 10.2117(s, 1H) are clearly shown in the \(^1\)H-NMR.