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# 2-Methoxyestradiol protects against ischemia/reperfusion injury in alcoholic fatty liver by enhancing sirtuin 1-mediated autophagy

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ABSTRACT

Alcoholic fatty liver (AFL) is susceptible to ischemia/reperfusion (I/R) injury, responding with inflammation and extensive hepatocellular damage. Autophagy maintains cellular homeostasis and regulates inflammation and lipid metabolism. 2-Methoxyestradiol (2-ME2), an endogenous metabolite of estradiol, exhibits antioxidant and anti-inflammatory properties. This study examined the cytoprotective mechanisms of 2-ME2 on hepatic I/R in AFL, focusing on autophagy signaling. C57BL/6 mice were fed an ethanol diet (ED) to induce AFL, or a control diet (CD) for 6 weeks, and then subjected to 60 min of ischemia and 5 h of reperfusion. 2-ME2 (15 mg/kg, i.p.) was administered 12 h before ischemia and 10 min before reperfusion, and sirtinol (sirtuin 1 (SIRT1) inhibitor, 10 mg/kg, i.p.) was administered 30 min before reperfusion. After reperfusion, ED animals showed higher serum aminotransferase activities and proinflammatory cytokine levels, and more severe histological changes compared with CD animals. These alterations were attenuated by 2-ME2. In the ED I/R group, autophagy and mitophagy were significantly impaired, as indicated by decreased hepatic levels of microtubule-associated protein 1 light chain 3 II and parkin protein expression, and increased p62 protein expression, which were attenuated by 2-ME2. The hepatic levels of Atg12-5 complex, Atg3, Atg7, lysosomal-associated membrane protein 2 and Rab7 protein expression significantly decreased in ED I/R animals, which were attenuated by 2-ME2. In the ED I/R group, the level of SIRT1 protein expression and its catalytic activity significantly decreased, which were attenuated by 2-ME2. Sirtinol reversed the stimulatory effect of 2-ME2 on autophagy. Our findings suggest that 2-ME2 ameliorates I/R-induced hepatocellular damage in AFL through activating SIRT1-mediated autophagy signaling.


## 1. Introduction

Alcoholic steatosis sensitizes the liver to secondary stress insult, which may result in further hyperinflammation and scarring of the liver. Hepatic ischemia and reperfusion (I/R) injury remains an important complication of liver surgery or transplantation. The number of patients in need of a liver transplantation has increased, and the acceptance of fatty liver transplantation has grown due to increasingly low numbers of available donor organs [1]. The susceptibility of fatty livers to I/R injury has been attributed to fat accumulation in hepatocytes, oxidative stress, and inflammatory responses; however, the exact mechanisms are not fully understood [2].

Autophagy is an intracellular catabolic recycling process for maintaining cellular homeostasis in response to multiple stressors and dynamic processes. An unbalanced autophagic response has been implicated in the development of various pathological processes such as inflammation, hypoxia and oxidative stress [3]. In mice fed a high-fat diet and exposed to I/R, pharmacological mitigation of autophagy ameliorated hepatocellular damage and improved mitochondrial function [4]. In contrast, Zhao et al. [5] reported that an autophagy inducer, rapamycin, attenuated hepatocellular damage and hyperinflammation in steatotic hepatocytes during I/R. Our recent study showed that chronic alcohol intake impaired autophagic flux with lipid accumulation in hepatocytes, which contributed to liver injury [6].

Sirtuin 1 (SIRT1), a $NAD^+$-dependent class III protein deacetylase, plays a pivotal role in the regulation of hepatic lipid metabo-

*Abbreviations:* AFL, alcoholic fatty liver; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; CD, control diet; CQ, chloroquine; DMSO, dimethyl sulfoxide; ED, ethanol diet; H&E, hematoxylin and eosin; IL, interleukin; I/R, ischemia and reperfusion; LAMP, lysosomal-associated membrane protein; LC3, microtubule-associated protein 1 light chain 3; 2-ME2, 2-methoxyestradiol; PBS, phosphate-buffered saline; PINK1, PTEN-induced putative kinase 1; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SIRT1, sirtuin 1; TBS/T, 0.1% Tween 20 in 1 × Tris-buffered saline; TEM, transmission electron microscopy.

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lism and systemic inflammatory status. Acute and chronic ethanol intoxication disrupts hepatic SIRT1 signaling, which leads to excessive fat accumulation and inflammatory responses [7]. Recent studies indicate that SIRT1 is an important regulator of autophagy. In various *in vitro* systems, resveratrol, a SIRT1 inducer, activated autophagy signaling [8,9]. During hepatic I/R, pharmacologic stimulation of SIRT1 enhanced autophagy and restored mitochondrial mass and membrane potential [10].

2-Methoxyestradiol (2-ME2) is an endogenous mammalian metabolite of estradiol, that has low binding affinity for estrogen receptors with low toxicity [11]. 2-ME2 has been found to possess antiproliferative, antimetastatic and antiangiogenic activities, for which it attracted considerable interest as a potent anti-cancer agent [12]. Recently, 2-ME2 has been shown to exert anti-inflammatory activities in inflammatory diseases. 2-ME2 ameliorated multiple organ damage by reducing proinflammatory cytokines and nitric oxide, which improved mortality in septic mice [13]. In rodent models of cerebral and renal ischemic injuries, 2-ME2 reduced organ damage through suppression of inflammatory response and apoptotic cell death [14,15]. However, there is limited information available on the effect of 2-ME2 on liver inflammation in *in vivo* animal studies.

Therefore, we investigated the protective mechanisms of 2-ME2 on hepatocellular damage following I/R in alcoholic fatty liver (AFL) in a mouse model, in particular focusing on the autophagy signaling. Our results suggest that 2-ME2 may be a novel therapeutic strategy to counteract I/R injury in AFL.

## 2. Materials and methods

### 2.1. Animals

All experiments were approved by the Animal Care Committee of Sungkyunkwan University School of Pharmacy (SUSP15-37) and performed in accordance with the guidelines of the National Institutes of Health (NIH publication No. 86-23, revised 1985). Male C57BL/6 mice (19–21 g, Orient Bio, Inc., Seongnam, Korea) were acclimatized to the laboratory conditions at Sungkyunkwan University for 1 week. Mice were kept in a temperature- and humidity-controlled room (25 ± 1 °C and 55 ± 5%, respectively) with a 12 h light-dark cycle.

### 2.2. Induction of alcoholic fatty liver

Mice were fed a liquid diet because animals given ethanol have a tendency to reduce their solid food consumption. Diets were based on the Lieber-DeCarli formation (Dyets Inc., Bethlehem, PA, USA), and mice were fed an ethanol-containing Lieber-DeCarli diet or a caloric equivalent Lieber-DeCarli diet for 6 weeks. The ethanol diet (ED) consisted of 35% total energy from fat, 18% from protein, 11% from carbohydrates, and 36% from ethanol. In the control diet (CD), the ethanol was replaced isocalorically with maltose-dextrin. Over the first 2 weeks, mice were progressively introduced to the ED. This included liquid diet acclimation using the CD for 4 days, followed by the ED with 0.75% ethanol (w/v) for 3 days, 1.50% ethanol (w/v) for 4 days, 3.75% ethanol (w/v) for 3 days, and 5.00% ethanol (w/v) for 4 weeks.

### 2.3. Hepatic ischemia and reperfusion

After the Lieber-Decarli diet feeding for 6 weeks, mice were fasted for 18 h before the experiments but allowed free access to tap water. Following intraperitoneal administration of ketamine (55 mg/kg) and xylazine (7 mg/kg), body temperature was maintained at 37 °C using heating pads. After a midline laparotomy, partial hepatic ischemia was induced by occluding the blood supply to the left and median lobes of the liver with a micro-serrefine clip (Fine Science Tools, Inc., Vancouver, BC, Canada). After 60 min of ischemia, the clamp was removed to allow reperfusion. Sham-operated animals underwent the same procedure but without vessel occlusion. At 5 h after reperfusion, all mice were euthanized by administration of overdosed ketamine/xylazine. Blood and liver tissues were collected and stored at −75 °C until biochemical analyses were performed.

### 2.4. Experimental groups

2-ME2 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.5% dimethyl sulfoxide (DMSO) in saline and administered (15 mg/kg, i.p) 12 h before ischemia and 10 min before reperfusion. Chloroquine (CQ, Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS) and injected (60 mg/kg, i.p.) 1 h prior to ischemia. The dosage and timing of 2-ME2 and CQ administration were determined based on previous studies and preliminary investigations in our laboratory [14,16]. In preliminary study, to select an optimal dose of 2-ME2, we administered at doses of 7.5, 15 and 30 mg/kg of 2-ME2 to mice underwent I/R, measured the hepatic injury using serum alanine aminotransferase (ALT) activities (I/R: 9234.3 ± 701.9 U/L; 7.5 mg/kg + I/R: 7892.0 ± 794.3 U/L; 15 mg/kg + I/R: 5843.1 ± 498.6 U/L; 30 mg/kg + I/R: 6132.5 ± 534.2 U/L), and selected 15 mg/kg of 2-ME2 as optimal dose of hepatoprotection against I/R injury (data not shown). In the vehicle-treated CD, ED, CD + I/R, or ED + I/R groups, 0.5% DMSO-saline or PBS was administered in the same volume and route as the respective drug treatment. Animals were divided randomly into eight groups ($n = 8$ per each group) as follows: (a) vehicle-treated CD group (CD sham), (b) vehicle-treated ED group (ED sham), (c) vehicle-treated CD group operated hepatic I/R (CD I/R), (d) vehicle-treated ED group operated hepatic I/R (ED I/R), (e) 2-ME2-treated CD group operated hepatic I/R (CD I/R + 2-ME2), (f) 2-ME2-treated ED group operated hepatic I/R (ED I/R + 2-ME2), (g) CQ-treated CD group operated hepatic I/R (CD I/R + CQ), and (h) CQ-treated ED group operated hepatic I/R (ED I/R + CQ). As there were no differences in any of the parameters among vehicle-, 2-ME2- and CQ-treated sham animals in the CD and ED groups, the results of these groups were pooled and referred to as CD sham and ED sham, respectively. To confirm the involvement of SIRT1 in autophagy modulation by 2-ME2 during AFL exposed to I/R, sirtinol (Abcam, Cambridge, MA, USA), a SIRT1 inhibitor, was dissolved in 2% DMSO-saline and administered (10 mg/kg, i.p.) 30 min before reperfusion. The dosage and timing of sirtinol administration were selected based on previously published papers [17,18]. Randomization was conducted by an individual other than the operator. The animals with similar degrees of body weight were selected randomly from the pool of all cages eligible for inclusion in the study and divided into groups.

### 2.5. Serum aminotransferase activities

Serum ALT and aspartate aminotransferase (AST) activities were assayed at 37 °C by monitoring the decrease in absorbance at 340 nm for 1 min caused by the disappearance of NADH, using ChemiLab ALT and AST assay kits (IVDLab Co., Ltd., Uiwang, Korea), respectively, and a Hitachi 7600 automatic analyzer (Hitachi, Ltd., Tokyo, Japan).

### 2.6. Serum cytokine levels

Serum interleukin (IL)-6 and IL-1β levels were quantified using commercial mouse ELISA kits (eBioscience, Inc., San Diego, CA, USA) according to the manufacturer's instructions.