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Restoration of autophagy by puerarin in ethanol-treated hepatocytes via the activation of AMP-activated protein kinase

Byung-Kyu Noh [a], Jung Kyu Lee [a], Hee-jin Jun [a], Ji Hae Lee [a], Yaoyao Jia [a], Minh-Hien Hoang [a], Jung-Wan Kim [b], Kwan-Hwa Park [c], Sung-Joon Lee [a,*]

[a] Division of Food Bioscience and Technology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Republic of Korea
[b] Department of Biology, University of Incheon, Incheon 402-749, Republic of Korea
[c] Department of Foodservice Management and Nutrition, Sangmyuung University, Seoul 110-743, Republic of Korea

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ABSTRACT

We investigated the effects of puerarin, the major isoflavone in Kudzu roots, on the regulation of autophagy in ethanol-treated hepatocytes. Incubation in ethanol (100 mM) for 24 h reduced cell viability by 20% and increased the cellular concentrations of cholesterol and triglycerides by 40% and 20%, respectively. Puerarin stimulation significantly recovered cell viability and reduced cellular lipid accumulation to a level comparable to that in untreated control cells. Ethanol incubation reduced autophagy significantly as assessed by microtubule-associated protein1 light chain 3 (LC3) expression using immunohistochemistry and immunoblot analysis. The reduced expression of LC3 was restored by puerarin in a dose-dependent manner in ethanol-treated cells. The effect of puerarin on mammalian targets of rapamycin (mTOR), a key regulator of autophagy, was examined in ethanol-treated hepatocytes. Immunoblotting revealed that puerarin significantly induced the phosphorylation of 5′AMP-activated protein kinase (AMPK), thereby suppressing the mTOR target proteins S6 ribosomal protein and 4E-binding protein 1. These data suggest that puerarin restored the viability of cells and reduced lipid accumulation in ethanol-treated hepatocytes by activating autophagy via AMPK/mTOR-mediated signaling.

1. Introduction

Alcoholic liver disease is a major global health problem characterized by the accumulation of excess fat in the liver, which can cause liver steatosis or steatohepatitis [1]. Excessive alcohol intake interferes with lipid oxidation by forcing the delivery of lipids to the liver [2], resulting in the accumulation of lipid droplets in hepatocytes. This causes steatosis, alcoholic hepatitis, and alcohol-related cirrhosis [1]. Excessive alcohol consumption also induces mitochondrial damage, oxidative stress, and cell death in hepatocytes. In addition, excessive ethanol consumption often causes liver enlargement and protein accumulation by reducing the catabolism of long-lived proteins in the liver by suppressing hepatic protein synthesis [3]. These metabolic changes are associated with impaired autophagy in the liver.

Autophagy, the self-degradation of subcellular organelles [4], facilitates the degradation of cellular components by the lysosomal machinery [5]. Autophagy is a tightly regulated process that is essential for multiple cellular functions, including cellular protein homeostasis, which balances cell synthesis, degradation, and subsequent recycling [6]. Autophagy is the process by which a starving cell reallocates nutrients from unnecessary processes to more essential processes. In the liver, autophagy is important for the balance of energy and nutrients required for cellular function, as well as the removal of misfolded proteins and maintenance of pathophysical conditions. Therefore, any disturbance in autophagy in the liver could have a major impact on liver physiology and disease [7].

Mammalian target of rapamycin (mTOR) is a protein kinase complex that regulates important cellular processes; its activity is known to inhibit autophagy [8]. The main upstream regulator of mTOR in mammalian cells is 5′AMP-activated protein kinase (AMPK). AMPK is a heterotrimeric protein activated by an elevated cellular AMP-to-ATP ratio and a significant regulator of various metabolic and signal transduction pathways, including autophagy. Activated AMPK downregulates energy-requiring pathways and stimulates catabolic pathways. Therefore, the activation of AMPK induces autophagy [9], as shown in the livers of animals that have consumed ethanol [10,11]. AMPK activation suppresses mTOR activity, thereby releasing the inhibition of autophagy. Well-defined downstream targets of mTOR include 4E-binding protein 1 (4E-BP1) and S6 ribosomal protein (S6), which control protein

* Corresponding author. Fax: +82 2 3290 3494.
E-mail addresses: ropang@korea.ac.kr (B.-K. Noh), tiel83@naver.com (J.K. Lee), ijinijoa@naver.com (H.-j. Jun), jerryl@hanmail.net (J.H. Lee), janciey227@naver.com (Y. Jia), hoangminhhien@yahoo.com (M.-H. Hoang), truestar7@hanmail.net (J.-W. Kim), biakim530@gmail.com (K.-H. Park), junelee@korea.ac.kr (S.-J. Lee).

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translation. The phosphorylation of these proteins controls mTOR activity. Chronic ethanol consumption inhibits cellular autophagy due to reduced AMPK activity, which subsequently activates mTOR in the liver [12]. Therefore, pharmacologic and nutritional intervention using AMPK agonists such as AICAR and metformin *in vivo* may recover autophagy in ethanol-treated hepatocytes [13,14].

Puerarin is a major isoflavone found in a number of plants and herbs, including *Pueraria lobata*, or Kudzu roots. Puerarin is effective for treating multiple metabolic symptoms, including hyperlipidemia [15], it can ameliorate ethanol-derived cytotoxicity [16] of liver enzyme levels in the plasma of liver-damaged animals, and it can inhibit oxidative stress caused by acute alcoholism [17]. Puerarin also exhibits hypolipidemic effects and activates AMPK phosphorylation in hepatocytes [18]. Given these known activities, we investigated whether puerarin is able to ameliorate alcohol-induced hepatic lipid accumulation by recovering autophagy through AMPK/mTOR signaling in hepatocytes treated with high doses of ethanol.

2. Materials and methods

2.1. Materials

The rat hepatoma cell line H4IIE was purchased from the Korean Cell Line Bank (Seoul, Korea). All cell culture reagents and supplies were obtained from HyClone (Logan, UT, USA). Puerarin and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were acquired from Sigma Aldrich (St. Louis, MO, USA). Rapamycin was purchased from LC Laboratories (Woburn, MA, USA). Primary antibodies against AMPK, phospho-AMPK, mTOR, S6, phospho-S6, 4E-BP1, and phospho-4E-BP1 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Primary anti-microtubule-associated protein1 LC3 (LC3) antibodies were purchased from Novus Biological Inc. (Littleton, CO, USA). Primary anti-α-tubulin and all secondary antibodies (anti-rabbit and -mouse) were acquired from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies labeled with Alexa Fluor® 633 were purchased from Invitrogen (Carlsbad, CA, USA). Power Opti-ECL Western blotting detection reagent was purchased from Amersham-Pharmacia Korea (Seoul, Korea).

2.2. Cell culture, treatments, and viability testing

Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in Eagle's minimum essential medium with Earle's balanced salts (MEM/EBSS) supplemented with 20% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (HyClone). The cells were incubated in 6-well plates at 5×10^5 cells/well. The cells were preincubated in serum-containing medium then incubated with 100 mM ethanol alone or ethanol plus puerarin (30, 60, 120, 180, or 240 μM) or 100 mM rapamycin in serum-free medium. Cell viability was measured by MTT assay according to a previously reported method [16]. Briefly, subconfluent cells were incubated with MTT solution at 37 °C for 4 h before the addition of DMSO. After 5 min at room temperature, the optical density of the samples was measured at 570 nm using a Bio-Rad model 680 microplate reader (Hercules, CA, USA).

2.3. Lipid extraction and cellular lipid measurement

The cellular lipid level was measured as described previously [19]. Briefly, the cells were washed three times with 2 mL of ice-cold PBS, then soluble cell proteins were dissolved in 1 mL of 0.1 N NaOH and measured using Bradford reagent (Bio-Rad) with bovine serum albumin (BSA; Sigma Aldrich) as the standard. To determine the intracellular triglyceride (TG) and cholesterol levels, the cells were washed three times with cold PBS then treated with 1 mL of hexane/isopropanol (2:1) for 30 min at room temperature. The samples were then transferred to test tubes, the organic solvent was removed under nitrogen, and the lipids were resuspended in 95% ethanol for TG and cholesterol measurement. The cellular TG level was quantified via an enzymatic method using a Cobas C111 automatic analyzer (Roche, Basel, Switzerland). The cholesterol level was measured with an Amplex Red Cholesterol Assay Kit (Invitrogen, Foster City, CA, USA) according to the manufacturer's instructions.

2.4. Immunoblotting

Cells were treated with ethanol (100 mM) alone or ethanol plus rapamycin (100 nM) or puerarin (60 or 120 μM) for 24 h then lysed in ice-cold lysis buffer containing Tris–HCl (pH 7.4, 10 mM), 0.1 M EDTA, 10 mM NaCl, 0.5% Triton X-100, and protease inhibitor cocktail (Roche). The lysate was centrifuged at 17,000*g* for 10 min at 4 °C then the supernatant was collected for analysis. The protein concentration was determined with a Bio-Rad protein kit using BSA as the standard. The samples were boiled with equal amounts of total protein in loading buffer for 5 min then separated by SDS–PAGE and blotted onto nitrocellulose membranes (0.45 μM; Schleicher & Schuell BioScience, Keene, NH, USA). Nonspecific protein binding was blocked by incubation in Tris-buffered saline (TBS; pH 7.4) containing 0.1% Tween 20 and 5% non-fat dry milk. The following antibodies were diluted 1:500 in 5% skim milk: anti-ribosomal S6 protein (Cell Signaling Technology, 2217), anti-4E-BP1 (Cell Signaling Technology, 9452), anti-AMPKα (Cell Signaling Technology, 2532), anti-phospho-S6 (Ser235/236) (Cell Signaling Technology, 2211), anti-phospho-4E-BP1 (Thr37/46) (Cell Signaling Technology, 9459), anti-phospho-AMPKα (Thr172) (Cell Signaling Technology, 2535), and anti-α-tubulin (Santa Cruz, sc-5286). Anti-LC3 (Novus Biological, NB100-2220) antibodies were diluted 1:1000. The membranes were incubated with the primary antibodies overnight at 4 °C then rinsed three times with TBS containing 0.05% Tween-20 (TBST) and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz) diluted 1:1000 in TBST. The proteins were visualized using a Chem-iDoc XRS Imaging System (Bio-Rad) with ECL (Bio-Rad) solution.

2.5. LC3 immunohistochemistry

Cultured hepatocytes were treated with ethanol alone (100 mM) or with ethanol plus rapamycin (100 nM) or puerarin (60 or 120 μM) for 24 h then fixed with 4% paraformaldehyde for 20 min. The samples were permeabilized with 0.1% Triton-X in 1X PBS. After incubation with 1% BSA in PBS for 30 min, the cells were immunostained with anti-LC3 antibodies (Novus Biologicals) overnight then incubated with anti-rabbit IgG tagged with Alexa Fluor® 633 (Invitrogen) for 1 h. The coverslips were embedded in anti-fade mounting medium after staining with DAPI. Image analysis was performed with a Zeiss LSM510 META confocal microscope (63 × 1.4 NA) and Zeiss LSM510 v.3.2 software (Carl Zeiss, Jena, Germany). For the quantification of LC3-labeled autophagy, confocal images were captured with LSM510 v.3.2 software then analyzed with ImageJ software. At least six images per group were analyzed. A total of 100 cells were counted for the quantification of LC3-positive autophagy.

2.6. Statistical analysis

All data are expressed as the mean ± standard error (SE). Two-group comparisons were performed using Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

Alcoholic liver damage is a major global cause of morbidity and mortality; however, treatment options are limited. The development of fatty liver due to the accumulation of cytosolic lipid droplets, which is characteristic of early alcohol-induced liver damage, can progress to more serious symptoms such as steatohepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma [20]. Moreover, increased oxidative stress resulting from ethanol metabolism in the liver can damage mitochondria and induce cell death, leading to elevated serum ALT and AST levels. Thus, scientists and clinicians have recently focused their attention on the development of effective treatments for alcohol-induced liver damage.

Previous research has shown that lipid accumulation in alcoholic fatty liver is partly due to reduced hepatic autophagy, which regulates lipid and mitochondrial homoeostasis. In addition, the mTOR inhibitor rapamycin reduces alcohol-induced liver pathogenesis by promoting autophagy [21]. Specifically, rapamycin treatment in ethanol-fed mice significantly decreased the number of ethanol-treated lipid droplets and the total hepatic TG level [22]. In addition, puerarin can ameliorate the symptoms of alcoholic liver damage [17]. Therefore, we investigated whether puerarin ameliorates hepatic lipid accumulation and upregulates autophagy.

First, we examined the effect of puerarin on cell viability in ethanol-treated hepatocytes. Rat hepatoma H4IIE cells were incubated with ethanol (100 mM) or were stimulated with either puerarin (30–240 μM) or rapamycin (100 nM), a selective inhibitor of mTOR and key regulator of autophagy [23], in the presence of ethanol for 24 h. As expected, ethanol significantly reduced cell viability by 20% in H4IIE cells compared with untreated control cells. Interestingly, however, cell viability was recovered dose-dependently with rapamycin or puerarin in ethanol-treated cells up to 180 μM (Fig. 1A). Two concentrations of puerarin (60 and 120 μM) were used in our subsequent experiments. These concentrations were shown to be non-toxic and biologically functional in several *in vitro* experiments [24]. We subsequently investigated the cellular cholesterol and TG levels in hepatocytes. Ethanol significantly increased the cellular levels of cholesterol and TGs by 40% and 20%, respectively, compared with untreated control cells. Conversely, stimulation with puerarin (120 μM) significantly reduced the cellular lipid concentration (Fig. 1B). Our positive control, rapamycin, also reduced cellular cholesterol and TG levels. These results suggest that puerarin stimulation recovered cell viability and inhibited lipid accumulation in ethanol-treated hepatocytes.

We next quantified the distribution of autophagy, which is altered in ethanol-incubated cells, in puerarin-stimulated hepatocytes by immunohistochemistry using antibodies against LC3, an autophagosome-specific protein (Fig. 2A) [25]. Ethanol incubation significantly reduced the expression of LC3 by 50%, whereas puerarin and rapamycin treatment significantly recovered the level of LC3 expression in a dose-dependent manner up to the levels in untreated control cells (Fig. 2B). In addition, we examined the expression of the LC3-I and -II subtypes by Western blotting (Fig. 2C). During autophagy, LC3-I, the cytoplasmic form of LC3, is processed and translocated to autophagosomes, where LC3-II, the autophagosomic form of LC3, is generated by site-specific proteolysis near the

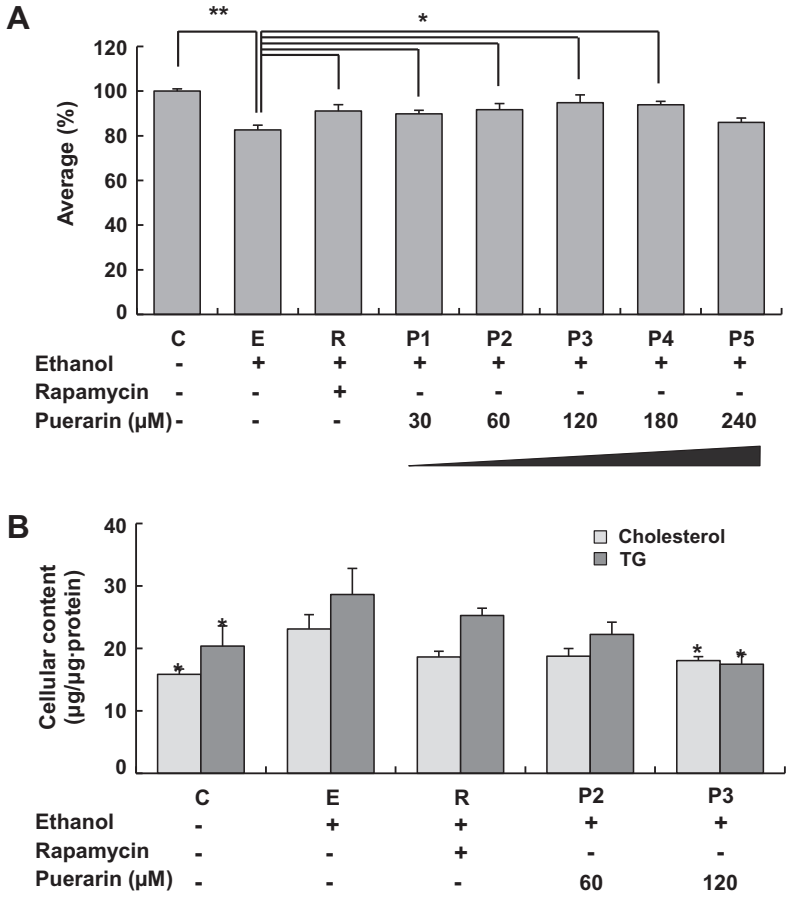

Fig. 1. Puerarin improves the viability of H4IIE cells and reduces lipid accumulation. (A) Cell viability. (B) Intracellular triglyceride levels. C, cells cultured in serum-free culture medium. E, cells incubated with ethanol (100 mM) alone for 24 h. R, cells stimulated with rapamycin (100 nM) for 24 h in the presence of ethanol. P1–P5, cells stimulated with puerarin (30, 60, 120, 180, or 240 μM) for 24 h in the presence of ethanol. Error bars, SE; $^{*}P < 0.05$; $^{**}P < 0.01$ compared with group E.

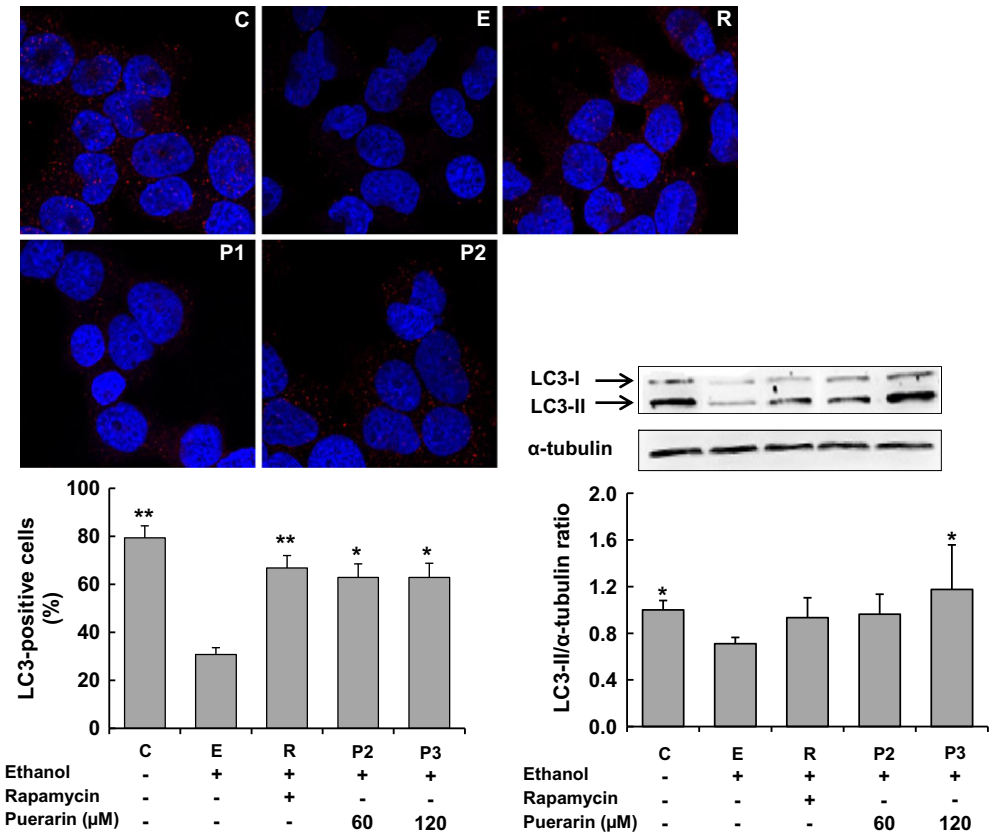

Fig. 2. Puerarin restores autophagy in ethanol-treated H4IIE cells. (A) Expression of LC3, an autophagy biomarker, in H4IIE hepatocytes. Cells were incubated with anti-LC3 antibodies, then with anti-rabbit IgG tagged with Alexa Fluor® 633 and analyzed by confocal microscopy. Confocal microscopic images show Alexa Fluor® 633-positive autophagic vesicles in the hepatocytes. (B) The percentage of LC3-positive cells. (C) Immunoblot analysis of LC3-I and -II. (D) Densitometric analysis of LC3-II. LC3-II expression was normalized to that of α-tubulin. C, cells cultured in serum-free culture medium. E, cells incubated with ethanol (100 mM) alone for 24 h. R, cells stimulated with rapamycin (100 nM) for 24 h in the presence of ethanol. P2 and P3, cells incubated with puerarin (30 and 60 μM). Error bars, SE; $^{*}P < 0.05$; $^{**}P < 0.01$ compared with group E.

C-terminus. Thus, LC3-II is often used as a specific biomarker of cellular autophagy. LC3-II expression was significantly decreased in H4IIE cells with ethanol treatment, whereas it was recovered to the level in untreated control cells when treated with rapamycin or puerarin (Fig. 2D). These observations suggest that the induction of LC3 by puerarin seen by immunohistochemistry was mainly due to an increase in LC3-II, not LC3-I. Together, these results indicate that puerarin restored autophagy in ethanol-treated hepatocytes.

We further investigated the mechanism of activation of autophagy by puerarin in ethanol-treated hepatocytes. The regulation of mTOR signaling is key in the induction of autophagy, and the inhibitory function of mTOR in autophagy is well-established [26]. Autophagy is promoted by the activation of AMPK, a major energy sensor that regulates cellular metabolism and energy homeostasis [27]. AMPK phosphorylates TSC2 to activate GTPase activity toward Rheb, and GTP-bound inactive Rheb subsequently inhibits mTOR activity, leading to the dephosphorylation of S6 and phosphorylation of 4E-BP1, two downstream substrates of mTOR. This results in the inhibition of protein translation and activation of autophagy [28].

Ethanol consumption has been reported to reduce AMPK activity in rat liver [29], and puerarin activates AMPK. Therefore, we hypothesized that puerarin treatment may increase mTOR-mediated autophagy by stimulating AMPK activity. Ethanol treatment reduced AMPK phosphorylation in H4IIE cells; however, stimulation with puerarin (120 μM) induced AMPK phosphorylation in ethanol-treated hepatocytes by nearly 2-fold compared with cells treated with ethanol alone (Fig. 3A and B).

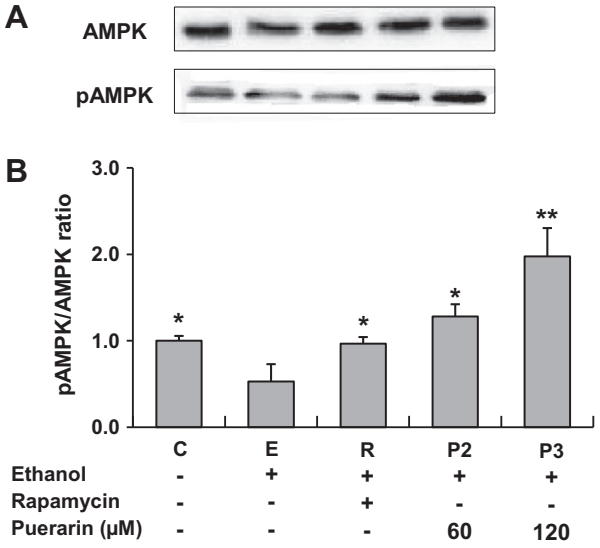

Fig. 3. Puerarin increases autophagy by stimulating AMPK phosphorylation. (A) Expression of AMPK and phospho-AMPK in ethanol-treated cells stimulated with puerarin. (B) Densitometric analysis of (A). C, cells cultured in serum-free medium. E, cells incubated with ethanol (100 mM) alone for 24 h. R, cells stimulated with rapamycin (100 nM) for 24 h in the presence of ethanol. P2 and P3, cells incubated with puerarin (30 or 60 μM). Error bars, SE; $^{*}P < 0.05$; $^{**}P < 0.01$ compared with group E.

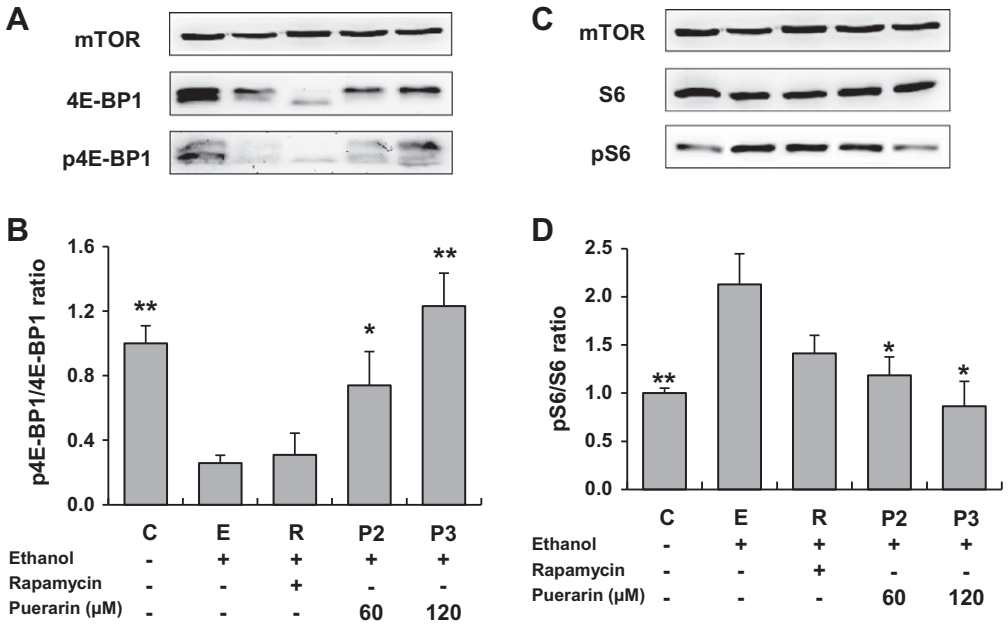

Fig. 4. Puerarin suppresses mTOR activity. (A) Expression of 4E-BP1 and phospho-4E-BP1 in ethanol-treated H4IIE cells stimulated by puerarin. (B) Densitometric analysis. (C) Expression of S6 and phospho-S6 in ethanol-treated H4IIE cells stimulated by puerarin. (D) Densitometric analysis. C, cells cultured in serum-free culture medium. E, cells incubated with ethanol (100 mM) alone for 24 h. R, cells stimulated with rapamycin (100 nM) for 24 h in the presence of ethanol. P2 and P3, cells incubated with puerarin (30 or 60 μM). Error bars, SE; $^{*}P < 0.05$; $^{**}P < 0.01$ compared with group E.

We examined whether the induction of AMPK by puerarin directly regulates the kinase activity of mTOR. Stimulation with rapamycin or puerarin in ethanol-treated hepatocytes caused significantly reduced S6 phosphorylation (Fig. 4A and B) and increased 4E-BP1 phosphorylation (Fig. 4C and D) compared with cells incubated with ethanol alone. This suggests an inhibitory effect of puerarin on mTOR that results in the induction of autophagy. Together, these results imply that puerarin increased autophagy in ethanol-incubated hepatocytes by stimulating the phosphorylation of AMPK, which suppresses mTOR phosphorylation.

Our results suggest that puerarin restores autophagy via AMPK activation, and thus plays a protective role in ethanol-induced hepatotoxicity. Alcoholic liver disease is a major cause of death throughout the world; however, there are few effective treatments available. Autophagy regulates lipid homeostasis and plays a hepatoprotective role in ethanol-treated hepatocytes. Thus, the modulation of autophagy is a viable candidate treatment for alcoholic liver disease. Our results show that puerarin may be an important agent in alcoholic liver damage, thus confirming *in vivo* observations of the role of puerarin in this condition in animal models.

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