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Activation of autophagy by globular adiponectin attenuates ethanol-induced apoptosis in HepG2 cells: Involvement of AMPK/FoxO3A axis

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ABSTRACT

Hepatocellular apoptosis is an important pathological entity of alcoholic liver disease. Previously, we have shown that globular adiponectin (gAcrp) protects liver cells from ethanol-induced apoptosis by modulating an array of signaling pathways. In the present study, we investigated the role of autophagy induction by gAcrp in the suppression of ethanol-induced apoptosis and its potential mechanism(s) in liver cells. Here, we demonstrated that gAcrp significantly restores ethanol-induced suppression of autophagy-related genes, including Beclin-1 and microtubule-associated protein light chain (LC3B) both in primary rat hepatocytes and human hepatoma cell line (HepG2). Globular adiponectin also restored autophagosome formation suppressed by ethanol treatment in HepG2. Furthermore, inhibition of gAcrp-induced autophagic process by knock-down of LC3B prevented protection from ethanol-induced apoptosis. In particular, the autophagic process induced by gAcrp was involved in the suppression of ethanol-induced activation of caspase-8 and expression of Bax. Moreover, knock-down of AMPK by small interfering RNA (siRNA) blocked gAcrp-induced expression of genes related to autophagy, which in turn prevented protection from ethanol-induced apoptosis, suggesting that AMPK plays an important role in the induction of autophagy and protection of liver cells by gAcrp. Finally, we also showed that gAcrp treatment induces translocation of the forkhead box O member protein, FoxO3A, into the nucleus, which may play a role in the induction of autophagy-related genes. Taken together, our data demonstrated that gAcrp protects liver cells from ethanol-induced apoptosis via induction of autophagy. Further, the AMPK-FoxO3A axis plays a cardinal role in gAcrp-induced autophagy and subsequent inhibition of ethanol-induced apoptosis.

1. Introduction

Alcoholic liver disease (ALD) developed by excessive and chronic alcohol consumption is a major cause of chronic liver disease and accompanied by various pathogeneses, including steatosis, inflammation, fibrosis and cirrhosis [1]. Ethanol treatment is also well known to induce excessive and sustained hepatocellular apoptosis. Recent studies have highlighted that alcohol- and/or its metabolite-induced generation of reactive oxygen species (ROS) along with inflammatory mediators plays a crucial role in the activation and release of pro-apoptotic molecules, leading to massive cellular organelle damage in the liver [2,3]. This is considered an important pathological event in the progression of human alcoholic liver disease and emerging evidence suggests that therapeutic modulation of dysregulated apoptosis may constitute a critical remedial approach for the treatment of ALD. Apoptosis of liver cells is regulated by both the extrinsic pathway integrated by caspase-8 and the intrinsic pathway regulated by Bcl-2 family proteins, such as Bcl-2 and Bax. Both of these pathways ultimately activate caspase-3, the final executioner of apoptotic cell death [4], and are implicated in ethanol-induced apoptosis in hepatic cells.

Autophagy, a different type of self-destructive process from apoptosis, is the major cellular pathway for the degradation of damaged proteins and cytoplasmic organelles. The canonical process of autophagy involves the formation of double-membraned autophagosomes, which envelop potential toxic substrates and finally fuse with lysosomes for degradation [5,6]. It is an evolutionarily conserved process in eukaryotes and acts as a crucial process in the development and differentiation of cells and the maintenance of cytoplasmic organelle turnover [5]. Even if autophagy was observed in dying cells (i.e., autophagic cell death) and has been classified as type 2 programmed cell death (apoptosis is type 1) [7], emerging evidence has shown that the autophagic process provides an efficient cellular defense mechanism and plays a cytoprotective role in various stressful conditions, such as starvation, endoplasmic reticulum stress and pathogen infection [8,9]. Furthermore, dysregulated modulation of autophagy accompanies various pathophysiologic conditions, including cancer [10].

Growing evidence also highlights that the cytoprotective function of autophagy is mediated at least in part by negative modulation of apoptosis [8,11]. For example, activation of the autophagic process leads to negative modulation of Bax [12,13]. Also, Beclin-1 mediated

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autophagy is known to inhibit caspase-8 activity, thereby preventing apoptosis [14]. Recent studies have reported that chronic ethanol consumption causes down-regulation of autophagy-related proteins, including beclin-1 and micro-tubule associated protein light chain (LC3-II) [15], and inhibits autophagy of liver cells through inhibition of AMPK activity [16]. Moreover, activation of autophagy reduces ethanol-induced hepatotoxicity in mice [17]. In addition, the degradation and removal of damaged and unnecessary constituents in the hepatocellular environment through autophagy have been shown to limit ethanol-induced hepatotoxicity and promote survival along with proper cellular functioning [18], suggesting that dysregulated autophagy is involved in the pathogenesis of alcohol-induced liver disease.

Adiponectin predominantly secreted from adipose tissue plays a key role in the regulation of various biological responses, including lipid and glucose metabolism [19] and also possesses potent anti-inflammatory, anti-diabetic, anti-atherogenic and anti-apoptotic properties [20–22]. In addition, adiponectin has been shown to reduce liver damage from chronic alcohol consumption [23]. As mentioned earlier, apoptosis of liver cells is a critical feature of ALD and it has recently been shown that globular adiponectin (gAcrp) prevents ethanol-induced apoptosis of liver cells [24], suggesting that down-regulation of apoptotic signaling is responsible for the protective effect of adiponectin on ethanol-induced liver injury. While a growing body of evidence suggests that autophagy negatively regulates the apoptotic process in various cellular conditions [8] and adiponectin possesses potent anti-apoptotic properties, the effect of adiponectin on autophagic process and its role in the suppression of ethanol-induced apoptosis have not been explored yet.

AMP-activated protein kinase (AMPK), a conserved energy sensor in eukaryotic cells, is well recognized as a key mediator of various biological responses induced by adiponectin [25]. In addition to its established role in metabolism, the AMPK signaling pathway has been intensively studied in recent years because it coordinates metabolism with both apoptosis and autophagy [26]. Ethanol consumption has been linked to reduced intracellular AMPK activity in the liver [27]. Furthermore, decreased AMPK activity by ethanol has been shown to inhibit autophagy in liver cells, which ultimately promotes hepatocyte cell death [18,28,29]. With regards to mediation of various biological responses by AMPK signaling, AMPK regulates the activities of a number of transcription factors, including the peroxisome proliferator-activated receptor (PPAR) gamma, PPAR-alpha, cAMP response element-binding (CREB), and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [30]. Among these, the forkhead box O (FoxO) family of transcription factors, particularly FoxO3A, has been shown to induce expression of genes related to autophagy [30–32].

Thus, to better understand the mechanisms underlying suppression of apoptosis by adiponectin in ethanol-treated liver cells, herein we investigated the role of autophagy in the suppression of apoptosis by globular adiponectin (gAcrp). In the present study, we have demonstrated for the first time that gAcrp activates the autophagy process in a human liver cell line and this is implicated in the suppression of ethanol-induced apoptosis. Furthermore, we have identified that the AMPK-FoxO3A signaling pathway plays a critical role in gAcrp-induced expression of genes related to autophagy.

2. Material and methods

2.1. Materials

All the cell culture reagents used in the present study were procured from Hyclone laboratories (South Logan, Utah, USA). Recombinant human globular adiponectin (gAcrp) was obtained from Peprotech Inc. (Rocky Hill, NJ, USA). Absolute ethanol was purchased from Merck chemicals (Whitehouse Station, NJ, USA). Caspase-3 and caspase-8 activity assay kits were purchased from Promega Corporation (Madison, WI, USA). Rapamycin and antibodies against Beclin-1, LC 3B and β-actin were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Goat anti-rabbit secondary antibody was obtained from Pierce biotechnology (Rockford, IL, USA). 3-Methyladenine (3-MA), a class III phosphoinositol 3-kinase (PI3K) inhibitor, and used as a selective inhibitor of autophagy, was obtained from Tocris Bioscience (Bristol, UK). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) unless mentioned elsewhere.

2.2. Isolation and culture of rat primary hepatocytes

All the animal experiments were performed in accordance with the guidelines for Animal Care and Use Committee at Yeungnam University. Male Sprague–Dawley rats, weighing 225–250 g, were used for the isolation of hepatocytes, via a two-step collagenase perfusion method [33].Cell viability for isolated hepatocytes was assessed by trypan blue exclusion, which was found to be above 90%. Isolated rat hepatocytes were then suspended in William's medium E (Sigma) and incubated in collagen-coated 6-well culture plates. After overnight incubation, the cells were treated with gAcrp in the absence or presence of ethanol for the indicated time points as mentioned in the figure legends.

2.3. Cell cultures

HepG2 cells, a human hepatoma cell line purchased from American Type Culture Collection (Rockville, MD, USA), were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin along with 0.1% amphotericin at 37 °C in an incubator with 5% CO2.

2.4. MTS assay

For the determination of cell viability, HepG2 cells were seeded in 96 well plates and transfected with siRNA targeting LC3B for 48 h, followed by ethanol treatment in the absence or presence of gAcrp. Finally, cell viability was monitored after addition of MTS solution via a Versamax microplate reader (Sunnyvale, CA,USA) by measuring absorbance at 490 nm as described previously [24].

2.5. Caspase-3 and caspase-8 activity assay

Caspase-3 and caspase-8 activities were determined using Caspase-Glo 3/7 and Caspase-Glo 8 assay kits (Promega Corporation, Madison, USA) respectively as described previously [24]. In brief, primary rat hepatocytes and HepG2 cells were seeded at a density of 1×10^5 cells per well in 2 ml culture media as described above in 6-well plates and 35 mm dishes respectively. The cells were incubated overnight followed by treatment with the indicated concentrations of globular adiponectin for 24 h followed by treatment with ethanol (50 mM) for additional 24 h. Finally, 100 μl of cell lysates was used in duplicate for the measurement of luminescence from the cleavage of luminogenic substrate Ac-DEVD-pNA and LETD sequence for caspase-3 and caspase-8 activities respectively with a micro-plate reader (Flurostar Optima, BMG Labtech, Ortenberg, Germany).

2.6. RNA isolation, reverse transcription (RT) and quantitative PCR (qPCR)

For the measurement of mRNA levels of genes of interest, total RNAs were isolated from the cultured cell with Qiagen lysis solution (Qiagen, Maryland, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed for the synthesis of cDNA. Real time-PCR amplification was then performed with a Roche LightCycler 2.0 (Mannheim, Germany) using the absolute QPCR SYBR green capillary mix AB gene system (Thermoscientific, UK) at 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 45 s. The primer sequences used

for amplification of target human genes are listed in Table 1. The amount of target mRNA was determined as described previously [24] via comparative threshold (Ct) method after normalizing target mRNA Ct values to those for glyceraldehyde-3-phosphate dehydrogenase GAPDH (ΔCt) which was taken as a housekeeping gene.

2.7. Transient transfection with small interfering RNA (siRNA)

HepG2 cells were initially seeded in a six well plate at a density of 2×10^5 cells per well in 2 ml culture media. After overnight incubation, cells were transfected with corresponding siRNA of target gene or scrambled control siRNA in 1 ml growth medium with Hiperfect transfection reagent (Qiagen) according to the manufacturer's instructions. The transfection efficiency was assessed by qRT-PCR after 24–48 h of transfection, followed by gAcrp treatment for the indicated time points. The siRNA duplexes were chemically synthesized by Bioneer (Daejeon, South Korea) and used for the study (Table 2).

2.8. Preparation of cellular extracts and Western blot analysis

Primary hepatocytes and HepG2 cells were seeded in 6-well plates and 60 mm dishes respectively at the density of 1×10^6 cells per well. After overnight incubation, cells were treated with ethanol in the absence or presence of gAcrp for the indicated time points. Total proteins were then isolated after lysing cells in RIPA buffer containing halt protease inhibitor cocktail (Thermoscientific, Rockford, USA) as described previously [24]. For the preparation of nuclear and cytosolic fractions, the cells were washed in ice-cold PBS twice, and then lysed using subcellular fractionation buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and halt protease inhibitor cocktail). The cellular lysates were then passed through 25G needle 10–15 times for homogenization followed by centrifugation at 720 *g* for 5 min at 4 °C to remove the nuclear pellet. The remaining supernatants were centrifuged at 10,000 *g* for 15 min. The cellular debris was then removed and the obtained supernatants were taken as cytosolic fractions. For the preparation of nuclear fraction, nuclear pellets were lysed in RIPA buffer for 1 h followed by centrifugation at 10,000 *g* for 15 min and the obtained supernatants were taken as nuclear fraction. All the procedures mentioned above were carried out on ice. Cytosolic and nuclear fractions were stored at −70 °C until further use. For immunoblot analysis, 30–60 μg of solubilized proteins was then loaded and resolved by 10–15% SDS-PAGE. The proteins were then transferred to PVDF membranes, blocked, incubated with the designated primary antibodies, washed and incubated with the secondary HRP-labeled antibody. Chemiluminescent images of the blots were finally captured using a Fujifilm LAS-4000 mini (Fujifilm, Tokyo, Japan). The membranes were then stripped and reprobed with β-actin or lamin b1 antibody as the loading control.

2.9. Confocal microscopic analysis

For confocal microscopic analysis, HepG2 cells were transfected with enhanced green fluorescent protein (eGFP)-LC3 expression plasmid using Fugene HD transfection reagent (Promega, Madison, USA) in cover glass bottom dishes according to the manufacturer's instructions.

Table 1
Sequences of human primers used in quantitative RT-PCR.

Target gene	Primer	Nucleotide sequence
GAPDH	F	5′-ACCACAGTCCATGCCATCAC-3′
	R	5′-TCCACCACCCTGTTGCTGTA-3′
Beclin-1	F	5′-CTTACCACAGCCCAGGCGAAAC-3′
	R	5′-GCCAGAGCATGGAGCAGCAA-3′
LC3B	F	5′-ACCATGCCGTCGGAGAAG-3′
	R	5′-GGTTGGATGCTGCTCTCGAA -3′

Table 2
Sequences of small interfering RNA used in transfection.

Target gene	Primer	Nucleotide sequence
LC3B	F	5′-GACUGUCUCGUUUAGACUG-3′
	R	5′-CAGUCUAAACGAGACAGUC-3′
AMPK alpha-1	F	5′-CUGAGUUGCAUAUACUGUA-3′
	R	5′-UACAGUAUAUGCAACUCAG-3′
FoxO3A	F	5′-GACGAUGAUGCGCCUCUCU-3′
	R	5′-AGAGAGGCGCAUCAUCGUC-3′
Scrambled control	F	5′-CCUACGCCACCAAUUUCGU-3′
	R	5′-ACGAAAUUGGUGGCGUAGG-3′

After 36 h of transfection, cells were treated with gAcrp in the absence or presence of ethanol for the indicated time. Cells were fixed with 4% paraformaldehyde solution and finally, the confocal images were captured using an A1 Confocal Laser Microscope System (Nikon Corp., Tokyo, Japan). For quantification of autophagic puncta, confocal images obtained from triplicate experiments were used and expressed as percentage of cells with GFP-LC3 dots obtained from at least 100 cells with Image Inside software version 2.32.

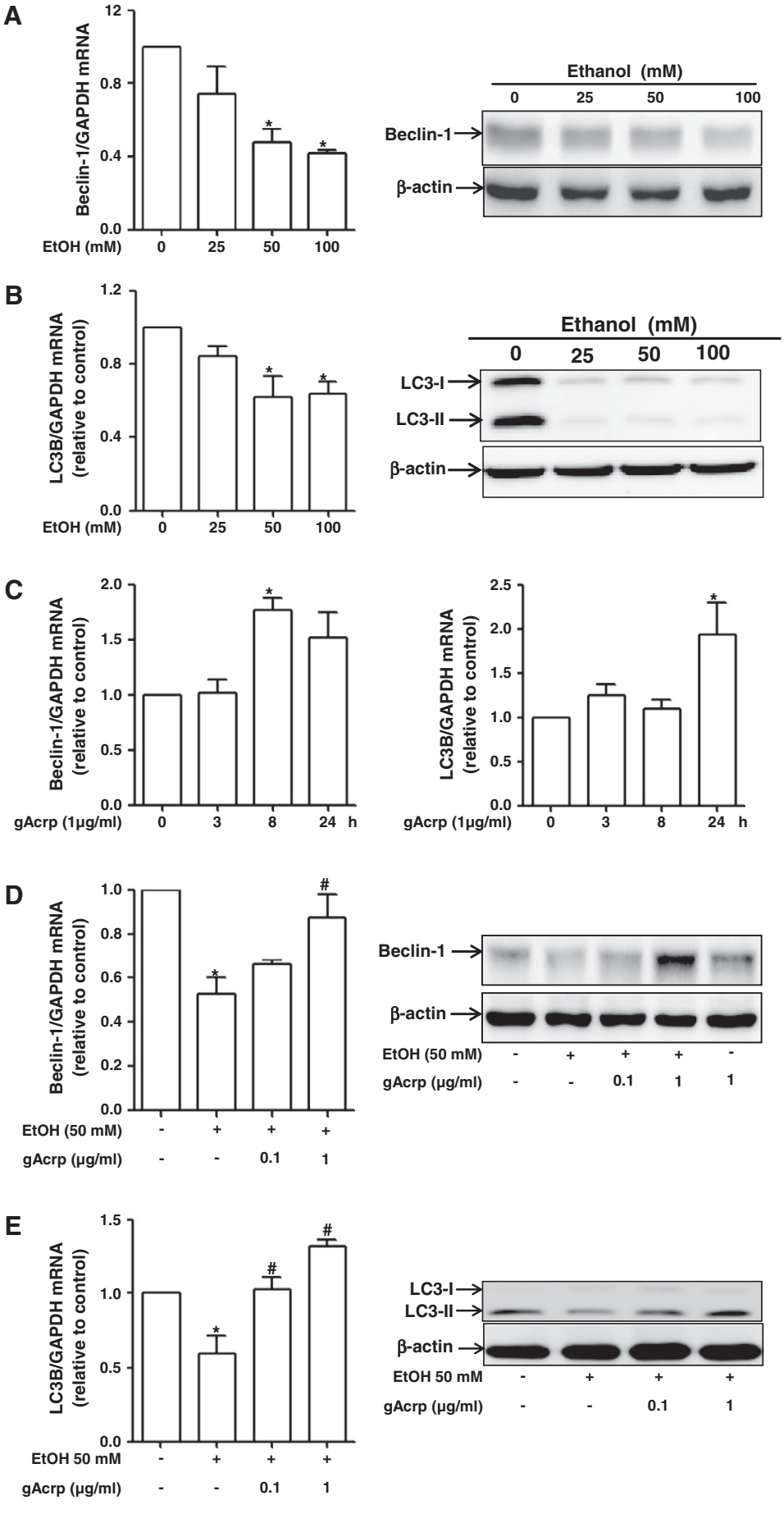
2.10. Statistical analysis

Values are presented as mean ± SEM of at least three experiments. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests using GraphPad prism software version 5.01 (California, USA). Differences between groups were considered to be significant at $p < 0.05$.

3. Results

3.1. Globular adiponectin restores ethanol-suppressed expressions of autophagy-related proteins in HepG2 and primary hepatocytes isolated from rat

Ethanol treatment has been shown to decrease expression of autophagy-related proteins in the liver. We hypothesize that adiponectin restores suppression of autophagy-related proteins by ethanol in liver cells. To examine this, we first confirmed inhibitory effects of ethanol on the expression of proteins related to autophagy in human hepatoma cell line (HepG2 cells). In these experiments, we measured the expression of beclin-1, which is considered essential for the formation of autophagosomes (required for the initial stage of autophagy) [34], and microtubule-associated protein light chain 3B (LC3B), which is regarded as a major constituent of autophagosomes (essential for relatively late stage of autophagic process) [5]. As shown in Fig. 1A, ethanol treatment decreased the expression of beclin-1 both at the mRNA and protein levels in a dose-dependent manner. It also decreased LC3B mRNA and LC3 II protein expression levels (Fig. 1B) in a dose-dependent manner. Next, to investigate the effects of gAcrp on the expression of autophagy-related proteins, cells were treated with gAcrp for the indicated times. Interestingly, gAcrp treatment significantly increased mRNA expressions of beclin-1 and LC3B (Fig. 1C) in a time-dependent manner. Finally, the effects of gAcrp on restoration of ethanol-suppressed autophagy-related proteins were examined. As indicated in Fig. 1D, gAcrp dose-dependently restored ethanol-inhibited beclin-1 mRNA and protein expression. In addition, LC3B mRNA and LC3 II protein levels were also returned to the normal level by gAcrp treatment in HepG2 cells (Fig. 1E). Similar results were also observed in primary hepatocytes isolated from rat. As shown in Fig. 1, ethanol treatment reduced expression of beclin-1 (Fig. 1F) and LC3 (Fig. 1G) in rat hepatocytes, which was restored by pretreatment with gAcrp. All these data indicate that gAcrp restores expression of autophagy-related genes suppressed by ethanol in liver cells.


A
Beclin-1/GAPDH mRNA
12
0.8
0.4
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EtOH (mM) 0 25 50 100
Ethanol (mM)
0 25 50 100
Beclin-1
β-actin
B
LC3B/GAPDH mRNA (relative to control)
1.2
0.8
0.4
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EtOH (mM) 0 25 50 100
Ethanol (mM)
0 25 50 100
LC3-I
LC3-II
β-actin
C
Beclin-1/GAPDH mRNA (relative to control)
2.0
1.5
1.0
0.5
0.0
gAcrp (1μg/ml) 0 3 8 24 h
LC3B/GAPDH mRNA (relative to control)
2.5
2.0
1.5
1.0
0.5
0.0
gAcrp (1μg/ml) 0 3 8 24 h
D
Beclin-1/GAPDH mRNA (relative to control)
1.0
0.8
0.6
0.4
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EtOH (50 mM) - + + +
gAcrp (μg/ml) - - 0.1 1
Beclin-1
β-actin
EtOH (50 mM) - + + + -
gAcrp (μg/ml) - - 0.1 1 1
E
LC3B/GAPDH mRNA (relative to control)
1.5
1.0
0.5
0.0
EtOH 50 mM - + + +
gAcrp (μg/ml) - - 0.1 1
LC3-I
LC3-II
β-actin
EtOH 50 mM - + + +
gAcrp (μg/ml) - - 0.1 1

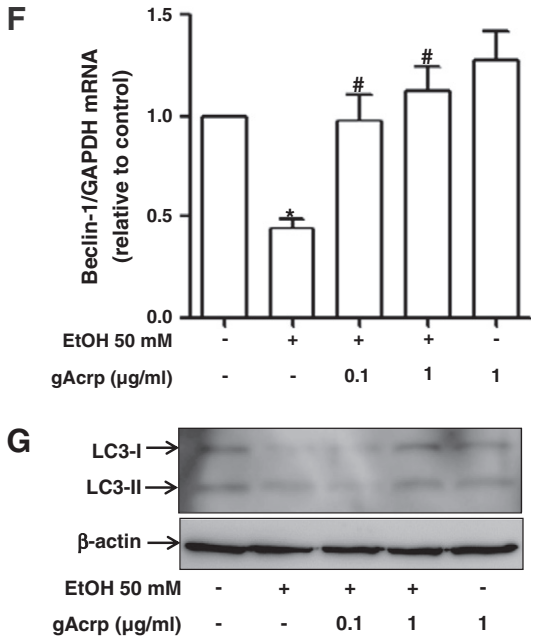


Fig. 1. Effects of globular adiponectin on the expression of autophagy-related proteins suppressed by ethanol. (A) Effect of ethanol on beclin-1 expression. Cells were incubated with the indicated concentration of ethanol for 24 h. (left panel) Beclin-1 mRNA expression level was assessed by qRT-PCR as described in the Material and methods section and the level was normalized to that of GAPDH mRNA. Values represent fold increase in comparison to cells not treated with ethanol and are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$ compared to the cells not treated with ethanol. (right panel) Beclin-1 protein expression level was determined by Western blot analysis. β-actin was used as an internal control. Images are representative of three independent experiments that showed similar results. (B) Effect of ethanol on LC3 expression. Cells were incubated with indicated concentration of ethanol for 24 h. (left panel) LC3B mRNA expression level was measured by qRT-PCR as described previously and normalized to GAPDH mRNA. Values represent fold increase compared to cells not treated with ethanol and are expressed as mean ± SEM (n = 4). $^{*}P < 0.05$ compared to cells not treated with ethanol. (right panel) LC3 II protein expression level was determined by Western blot analysis. β-actin was used as an internal control. Images are representative of three independent experiments that showed similar results. (C) Cells were incubated with gAcrp (1 μg/ml) for the indicated time periods. (left panel) Beclin-1 mRNA expression was analyzed by qRT-PCR as described previously and the level was normalized to GAPDH mRNA. Values represent fold increase relative to control and are expressed as mean ± SEM, n = 4. $^{*}P < 0.05$ compared with control group. (right panel) Cells were incubated with gAcrp (1 μg/ml) for the indicated time periods. LC3B mRNA expression was analyzed by qRT-PCR as described previously and normalized to GAPDH mRNA. Values represent fold increase relative to control and are expressed as mean ± SEM, n = 3. $^{*}P < 0.05$ compared with control group. (D) Effect of gAcrp on the expression of beclin-1 suppressed by ethanol. Cells were preincubated with the indicated concentrations of gAcrp for 24 h followed by stimulation with ethanol for additional 24 h. (left panel) Beclin-1 mRNA expression was analyzed by qRT-PCR as described previously and normalized to GAPDH mRNA. Values are presented as mean ± SEM (n = 4) $^{*}P < 0.05$ compared to cells not treated with ethanol, $^{\#}P < 0.05$ compared to ethanol-treated group. (right panel) Total cell lysates were prepared as described in the Material and methods section and used for the measurement of beclin-1 protein level by Western blot analysis. Representative image from three independent experiments is shown along with β-actin for internal loading control. (E) Effect of gAcrp on expression of Beclin-1 and LC3B suppressed by ethanol. HepG2 cells were pretreated with the indicated concentrations of gAcrp for 24 h followed by incubation with ethanol for additional 24 h. (left panel) LC3B mRNA expression was analyzed by qRT-PCR as described previously and normalized to GAPDH mRNA. Values are presented as mean ± SEM (n = 3) $^{*}P < 0.05$ compared to cells not treated with ethanol, $^{\#}P < 0.05$ compared to ethanol-treated group. (right panel) Total cell lysates were prepared as described previously and used for the measurement of LC3II protein level by Western blot analysis. Representative image from three independent experiments is shown along with β-actin for internal loading control. (F) Effect of gAcrp on expression of Beclin-1 suppressed by ethanol in primary rat hepatocytes. Hepatocytes were isolated from rat and treated with the indicated concentrations of gAcrp for 24 h followed by incubation with ethanol for additional 24 h. Beclin-1 mRNA expression was analyzed by qRT-PCR as described previously. Values are presented as mean ± SEM (n = 3) $^{*}P < 0.05$ compared to cells not treated with ethanol, $^{\#}P<0.05$ compared to ethanol-treated group. EtOH: Ethanol, gAcrp: globular adiponectin. (G) Effect of gAcrp on protein expression of LC3 suppressed by ethanol in primary rat hepatocytes. Hepatocytes were isolated from rat and treated with gAcrp for 24 h followed by incubation with ethanol for additional 24 h. LC3 protein expression levels were analyzed by Western blot analysis as described previously.

3.2. Globular adiponectin-induced autophagosome formation correlates to the suppression of ethanol-induced apoptosis

We next confirmed gAcrp-induced expression of LC3 II protein inside cells by transfection of eGFP-LC3 plasmid under confocal microscopy. As depicted in Fig. 2A, ethanol treatment significantly decreased LC3 II expression (LC3 dots) and pretreatment with gAcrp restored LC3 II expression (punctuated pattern), indicating the effect of gAcrp on autophagosome formation in ethanol-treated HepG2 cells. Next, we assessed whether gAcrp induces autophagic flux in these conditions. For this, cells were pretreated with Bafilomycin A1, a specific autophagosome-lysosome inhibitor, followed by ethanol treatment in the absence or presence of gAcrp. Interestingly, as depicted in Fig. 2B, pretreatment with Bafilomycin A1 further increased LC3 II protein levels, suggesting that gAcrp induces autophagic flux in HepG2 cells. Our lab data also revealed that gAcrp decreased expression of p62 (SQSTM1/sequestosome 1), which is a marker of autophagic flux [35] (data not shown),strongly suggesting that gAcrp not only increases LC3 II protein levels but also activates autophagic flux in HepG2 cells. Recent studies have shown a potential linkage between autophagy and apoptosis in various experimental conditions [8,11]. To elucidate whether gAcrp-induced autophagy is linked to ethanol-induced apoptosis, we investigated the effect of gAcrp on ethanol-induced caspase-3 activation. As shown in Fig. 2C, ethanol-induced caspase-3 activation was returned to the normal level by gAcrp in HepG2 cells and primary hepatocytes (Fig. 2D), which was consistent with previous observations and indicated a correlation between induction of autophagy and suppression of apoptosis by gAcrp.

3.3. Globular adiponectin-induced autophagy contributes to the suppression of ethanol-induced apoptosis in HepG2 cells

To further investigate the role of autophagy induction by gAcrp in the suppression of ethanol-induced apoptosis, we first measured the effect of rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), which is known to induce autophagy on ethanol-induced caspase-3 activation. As shown in Fig. 3A, pretreatment of cells with rapamycin significantly inhibited ethanol-induced activation of caspase-3, indicating

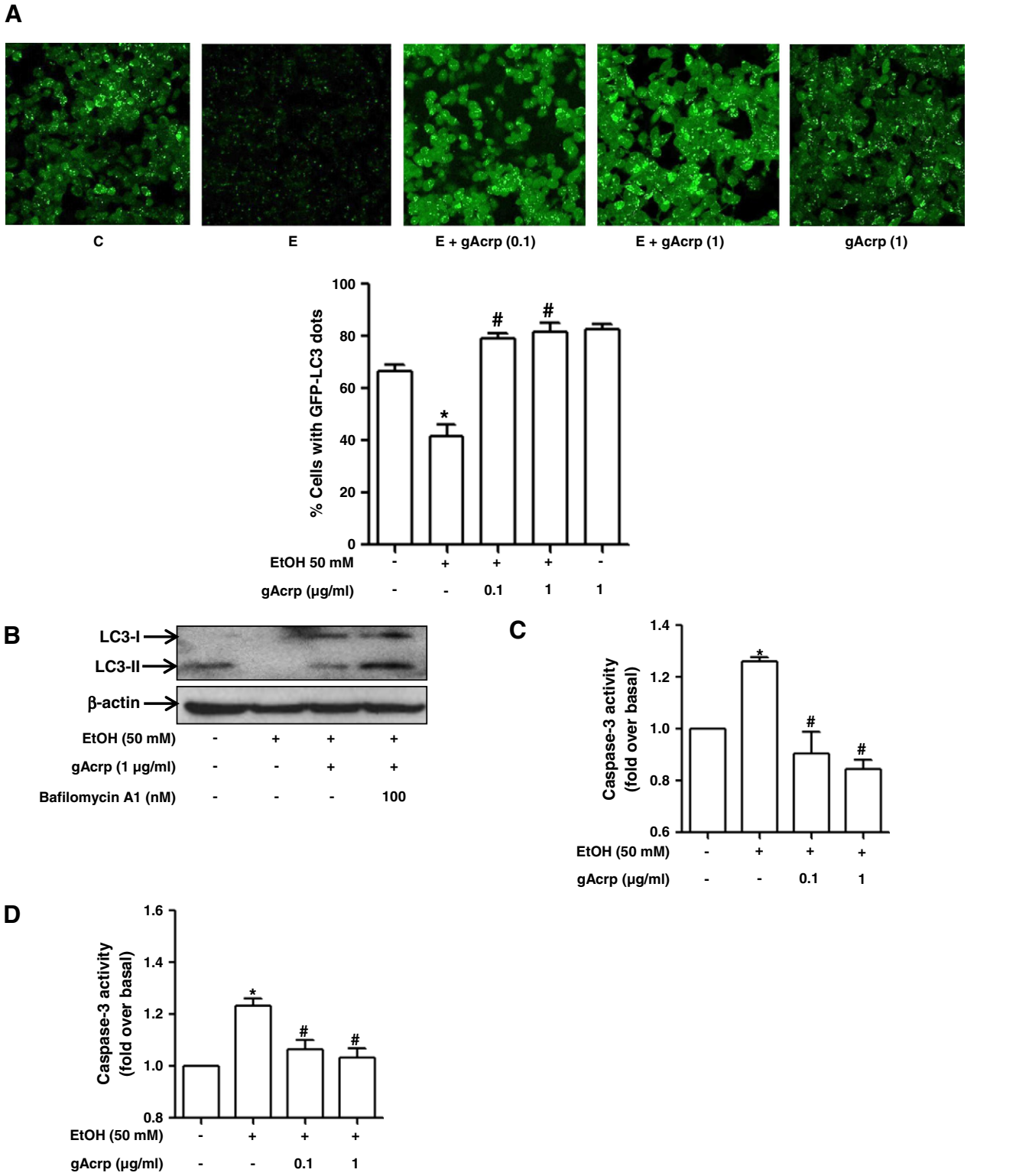


Fig. 2. Effects of ethanol and gAcrp on autophagosome formation and its inverse relationship with apoptosis. (A) Cells were transfected with eGFP-LC3 expression plasmid for 36 h. Cells were then pretreated with gAcrp for 24 h followed by stimulation with ethanol for additional 24 h. GFP-LC3 was viewed with A1 Confocal Laser Microscope System as described in the Material and methods section. Representative images from three independent experiments are shown along with quantitation of LC3 dots on the lower panel. Values are expressed as percentage of cells with GFP-LC3 dots obtained from at least 100 cells. C: Control, E: ethanol, gAcrp: Globular adiponectin. $^{*}P < 0.05$ compared with cells not treated with ethanol; $^{\#}P < 0.05$ compared with cells treated with ethanol. (B) Cells were pretreated with Bafilomycin A1, a specific autophagosome-lysosome inhibitor, followed by gAcrp treatment in the absence or presence of ethanol. LC3 II protein expression level was determined by Western blot analysis. Representative image from three independent experiments is shown along with β-actin for internal loading control. (C) HepG2 cells were incubated with the indicated concentrations of gAcrp for 24 h followed by stimulation with ethanol for additional 24 h. Caspase-3 activity was determined as described in the Material and methods section. Values shown are the results of three independent experiments and are expressed as mean ± SEM. $^{*}P < 0.05$ compared with cells not treated with ethanol; $^{\#}P < 0.05$ compared with cells treated with ethanol. (D) Hepatocytes were isolated from rat and incubated with the indicated concentrations of gAcrp for 24 h followed by stimulation with ethanol for additional 24 h. Caspase-3 activity was determined as described previously. Values shown are the results of three independent experiments and are expressed as mean ± SEM. $^{*}P < 0.05$ compared with cells not treated with ethanol; $^{\#}P < 0.05$ compared with cells treated with ethanol.

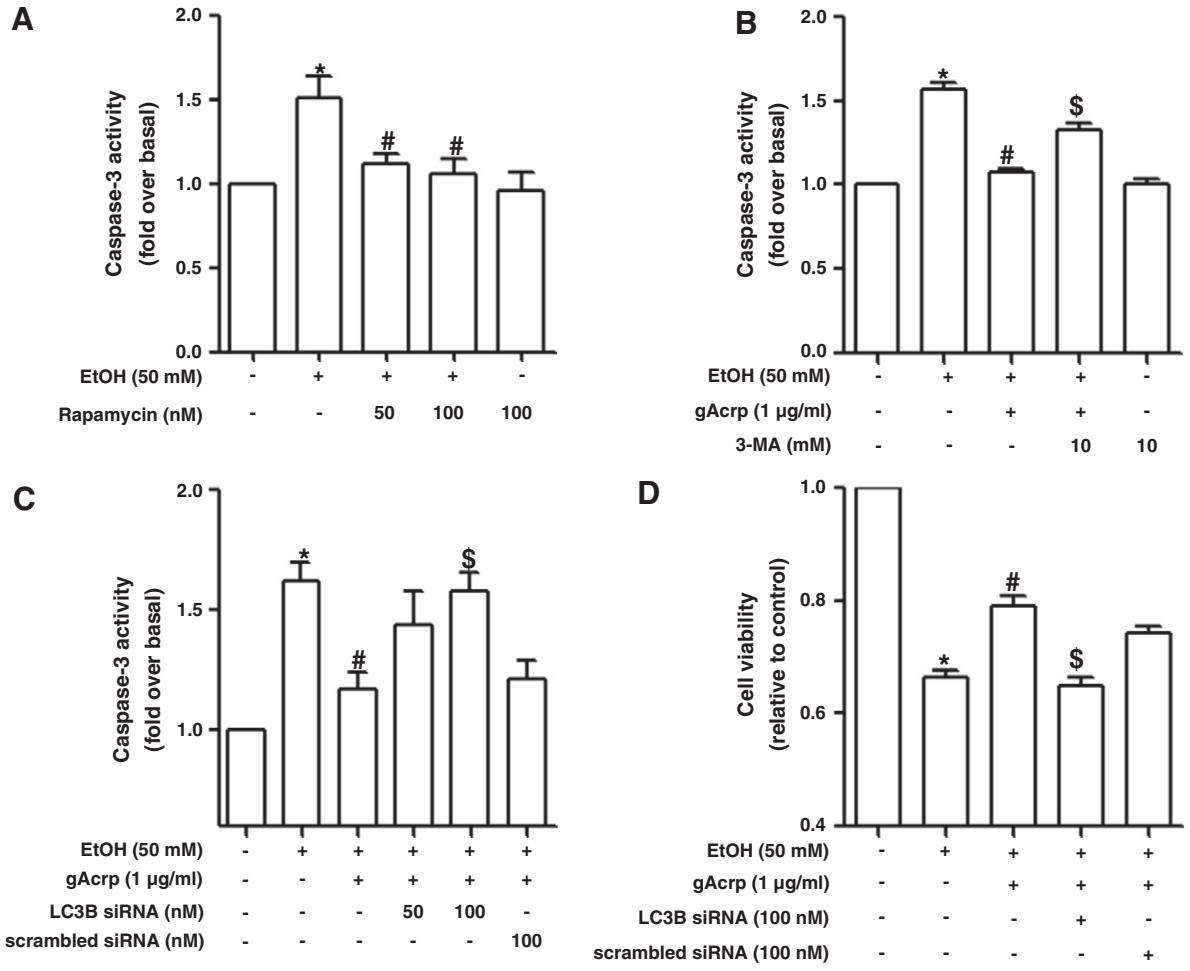


Fig. 3. Role of induction of autophagy in the prevention of ethanol-induced apoptosis by globular adiponectin in HepG2 cells. (A) Cells were pretreated with the indicated concentration of rapamycin for 2 h followed by incubation with ethanol for 24 h. Caspase-3 activity was determined as described in the Material and methods section. Values represent fold increase compared to control and are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$ compared with cells not treated with ethanol; $^{\#}P < 0.05$ compared with cells treated with ethanol. (B) Cells were pretreated with the indicated concentration of 3-MA used as an inhibitor of autophagy in the absence or presence of gAcrp for 24 h followed by further stimulation with ethanol for 24 h. Caspase-3 activity was determined as described previously and values represent fold increase compared to control of three independent experiments and are expressed as mean ± SEM. $^{*}P<0.05$ compared to cells not treated with ethanol; $^{\#}P<0.05$ compared with ethanol-treated group; $^{\$}P < 0.05$ compared with cells treated with gAcrp and ethanol. (C) Cells were transfected with siRNA targeting LC3B or scrambled control siRNA. After 24 h incubation, cells were pretreated with gAcrp followed by stimulation with the indicated concentration of ethanol for 24 h. Caspase-3 activity was then measured as described previously. (D) Cells were transfected with siRNA targeting LC3B or scrambled control siRNA. After 48 h incubation, cells were pretreated with gAcrp followed by stimulation with the indicated concentration of ethanol for 24 h. Cell viability was measured by MTS assay as described in the Material and methods section. Values shown are results of three independent experiments and are expressed as mean ± SEM. $^{*}P < 0.05$ compared to cells not treated with ethanol; $^{\#}P < 0.05$ compared with cells treated with ethanol; $^{\$}P < 0.05$ compared with cells treated with gAcrp but not transfected with siRNA of LC3B. EtOH: Ethanol, gAcrp: globular adiponectin.

a possibility that up-regulation of autophagy may suppress ethanol-induced apoptosis in HepG2 cells. Next, to further determine whether autophagy plays a role in the suppression of ethanol-induced apoptosis by gAcrp, cells were pretreated with 3-methyl adenine (3-MA), a class III phosphoinositol 3-kinase (PI3K) inhibitor widely used as an inhibitor of autophagy, and caspase-3 activity was measured. Interestingly, pretreatment of cells with 3-MA restored caspase-3 activity suppressed by gAcrp in ethanol-treated cells (Fig. 3B), providing evidence that gAcrp-induced autophagy may play a role in the suppression of ethanol-induced caspase-3 activity in HepG2 cells. The role of autophagy induction by gAcrp in the inhibition of ethanol-induced apoptosis was confirmed by gene silencing of LC3B, which is a specific biomarker of cellular autophagy. Importantly, knocking down the LC3B gene restored caspase-3 activity suppressed by gAcrp to the normal level (Fig. 3C). Interestingly, silencing of LC3 expression also abrogated enhancement of cell viability by gAcrp in ethanol-treated cells (Fig. 3D). All these data indicate that autophagy induction plays a critical role in the prevention of ethanol-induced apoptosis by gAcrp.

3.4. Globular adiponectin-induced autophagy causes suppression of ethanol-induced Bax expression

We next investigated mechanisms underlying prevention of ethanol-induced apoptosis by induction of autophagy. Bax, a pro-apoptotic Bcl-2 family protein, is known to play a crucial role in the induction of apoptosis in ethanol-treated hepatocytes through activation of the intrinsic pathway of apoptosis [36]. In the present study, we investigated whether gAcrp regulates the intrinsic pathway of apoptosis by modulating ethanol-induced Bax expression. For this, we first examined the role of autophagy in ethanol-induced Bax expression. As shown in Fig. 4A, ethanol treatment increased Bax expression as expected (compare 1st and 2nd column). The ethanol-induced increase in Bax protein expression was significantly reduced in the presence of rapamycin, an inhibitor of mTOR and used as an autophagy inducer, implying that ethanol may induce Bax protein expression via modulation of autophagy. Furthermore, pretreatment with gAcrp also significantly suppressed ethanol-induced Bax protein expression (Fig. 4B). To further verify the

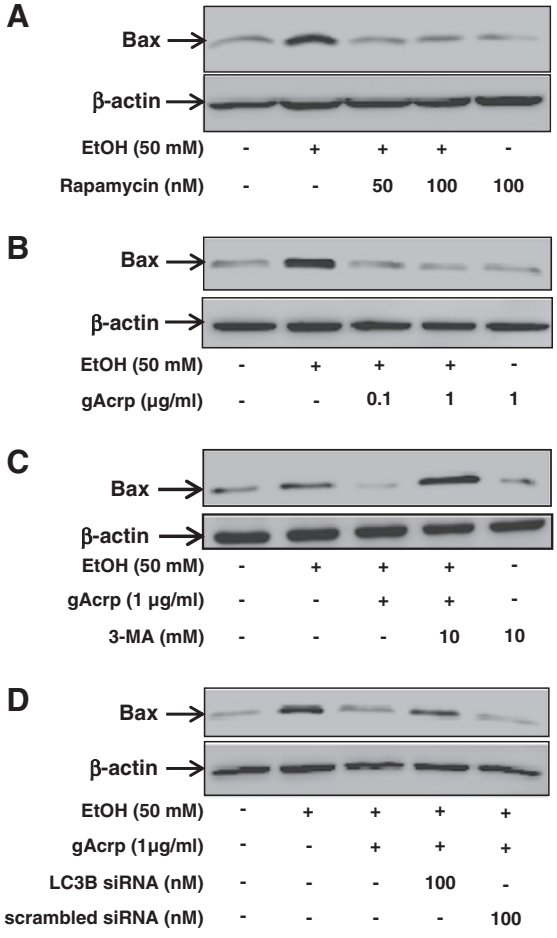


Fig. 4. Role of autophagy induction by globular adiponectin in the suppression of ethanol-induced Bax expression. (A) Cells were pretreated with indicated concentrations of rapamycin for 24 h followed by stimulation with ethanol (50 mM) for 8 h. Bax protein level was measured by Western blot analysis as described previously. Representative images from three independent experiments are shown. (B) Cells were pretreated with the indicated concentration of gAcrp for 24 h followed by incubation with ethanol (50 mM) for 8 h. Bax protein level was determined as described previously. Representative images from three independent experiments are shown. (C) Cells were pretreated with the indicated concentrations of 3-MA, an inhibitor of autophagy, and gAcrp for 24 h followed by further stimulation with ethanol for additional 8 h. Bax protein levels were determined by Western blot analysis. Images shown are representative of three independent experiments that showed similar results. (D) HepG2 cells were transfected with siRNA targeting LC3B or scrambled control siRNA. After 48 h incubation, cells were pretreated with gAcrp for 24 h and stimulated with ethanol (50 mM) for 8 h. Bax protein level was then measured as described previously. 3-MA: 3-Methyl adenine.

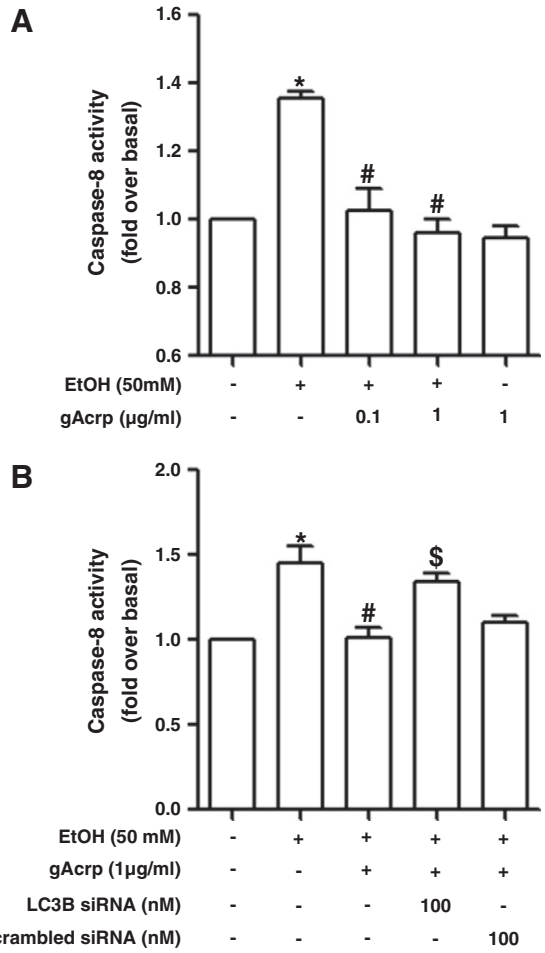


Fig. 5. Role of autophagy induction by globular adiponectin in the suppression of ethanol-induced caspase-8 activation. (A) Cells were incubated with the indicated concentration of ethanol for 24 h in the absence or presence of gAcrp and caspase-8 activity was measured as described in the Material and methods section. Values are depicted as mean ± SEM (n = 3). $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to ethanol-treated cells. (B) Cells were transfected with siRNA targeting LC3B or scrambled control siRNA for 48 h. After transfection, cells were incubated with gAcrp for 24 h followed by treatment with ethanol for additional 24 h. Caspase-8 activity was then measured as described previously. Values are shown as mean ± SEM, n = 3. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with cells treated with ethanol; $^{\$}P < 0.05$ compared with cells treated with gAcrp and ethanol but not transfected with LC3B siRNA.

role of autophagy induction by gAcrp in the suppression of ethanol-induced Bax expression, cells were treated with gAcrp in the presence or absence of 3-MA. As shown in Fig. 4C, pretreatment with 3-MA reverted suppression of Bax protein expression by gAcrp in ethanol-treated cells. Moreover, gene silencing of LC3B by siRNA restored suppressive effect of gAcrp on ethanol-induced Bax protein expression (Fig. 4D). Taken together, these data provide vital evidence that autophagy induction by gAcrp plays a central role in the suppression of ethanol-induced Bax expression.

3.5. Autophagy induction by globular adiponectin suppresses ethanol-induced caspase-8 activation

Ethanol treatment has been shown to induce caspase-8 activation, an integral component of the extrinsic pathway of apoptosis, in liver cells [37]. We also examined whether autophagy induction by gAcrp modulates caspase-8 activation in ethanol-treated HepG2 cells. As shown in Fig. 5A, ethanol treatment caused a significant increase in caspase-8 activity in HepG2 cells, and pretreatment with gAcrp inhibited ethanol-induced caspase-8 activation, implying that gAcrp inhibits ethanol-induced apoptosis in HepG2 cells through modulation of the extrinsic pathway of apoptosis. Furthermore, knocking down LC3B gene restored caspase-8 activity suppressed by gAcrp to a level similar to that in cells treated with ethanol alone (Fig. 5B). Taken together, these data suggest that gAcrp-induced autophagy plays a putative role in the suppression of ethanol-induced apoptosis via modulation of the extrinsic pathways of apoptosis in HepG2 cells, as well as intrinsic pathways (modulation of Bax).

3.6. AMPK signaling is implicated in expression of autophagy-related genes and suppression of ethanol-induced caspase-3 activation by gAcrp

To further explore the potential signaling mechanisms involved in gAcrp-induced expression of autophagy-related genes, we first examined the role of AMPK in gAcrp-induced LC3B mRNA expression, since AMPK activation mediates numerous biological responses by gAcrp [19,26]. Knocking down the AMPK gene by AMPK α-1 siRNA transfection significantly decreased both gAcrp-induced LC3B mRNA (Fig. 6A, left panel) and LC3 II protein (Fig. 6A, right panel) expression in HepG2

cells, suggesting that AMPK signaling is involved in gAcrp-induced LC3 expression. Next, we determined the role of AMPK in the restoration of LC3B expression by gAcrp in ethanol-treated cells. As depicted in Fig. 6B, AMPK gene silencing blocked the restoration of LC3B mRNA (left panel) and LC3 II protein expression (right panel) by gAcrp in ethanol-treated cells. The expression level of LC3 gene was similar to that observed in cells treated with ethanol alone, implying that AMPK signaling plays a critical role in the up-regulation of autophagy-related gene expression by gAcrp in ethanol-treated cells. Moreover, confocal microscopic analysis showed suppression of LC3 dots (punctuated pattern) by AMPK silencing in gAcrp and ethanol-treated cells (Fig. 6C). Finally, we aimed to verify the role of AMPK signaling in the suppression of ethanol-induced caspase-3 activation by gAcrp in HepG2 cells. As shown in Fig. 6D, gene silencing of AMPK blocked the protective effect of gAcrp against ethanol-induced caspase-3 activation, suggesting that AMPK signaling plays a cardinal role in the suppression of ethanol-induced apoptosis by gAcrp.

3.7. FoxO3A signaling is involved in globular adiponectin-induced expression of genes related with autophagy

FoxO family proteins translocate into the nucleus in response to stimuli and cause transcriptional activation of target genes. FoxO family transcription factors, particularly FoxO3A, are commonly associated with induction of autophagy-related genes [30,31]. Thus, to further characterize the transcription factor responsible for induction of autophagy-related genes by gAcrp, we investigated the role of FoxO3A in beclin-1 and LC3 expression. For this, we first measured the subcellular localization of FoxO3A protein on gAcrp treatment. As shown in Fig. 7A, gAcrp treatment decreased FoxO3A protein level in the cytosol, whereas nuclear FoxO3A accumulation was increased in a time-dependent manner. In addition, ethanol-treatment inhibited nuclear translocation of FoxO3A, which was restored to normal levels by gAcrp (Fig. 7B), suggesting that gAcrp causes activation of FoxO3A that might play a role in the induction of autophagy-related genes. Furthermore, transfection of FoxO3A siRNA significantly inhibited expression of autophagy-related proteins such as beclin-1 mRNA (Fig. 7C), LC3B mRNA (Fig. 7D, left panel) and LC3 II proteins (Fig. 7D, right panel) upon gAcrp treatment. In addition, gene silencing of FoxO3A almost completely negated the up-regulation of beclin-1 (Fig. 7E) and LC3B (Fig. 7F) by gAcrp in ethanol-treated cells, providing crucial evidence that FoxO3A plays a critical role in the gAcrp- induced increase in the expression of autophagy-related genes in HepG2 cells. Finally, to explore the involvement of AMPK activation in FoxO3A translocation to the nucleus, cells were transfected with AMPK α-1 siRNA and nuclear FoxO3A protein levels were assessed by Western blot analysis. Importantly, AMPK silencing significantly decreased nuclear FoxO3A protein level (Fig. 7G), suggesting that AMPK activation, at least in part, plays a crucial role in the translocation of FoxO3A, which contributes to induction of autophagy-related genes by gAcrp.

4. Discussion

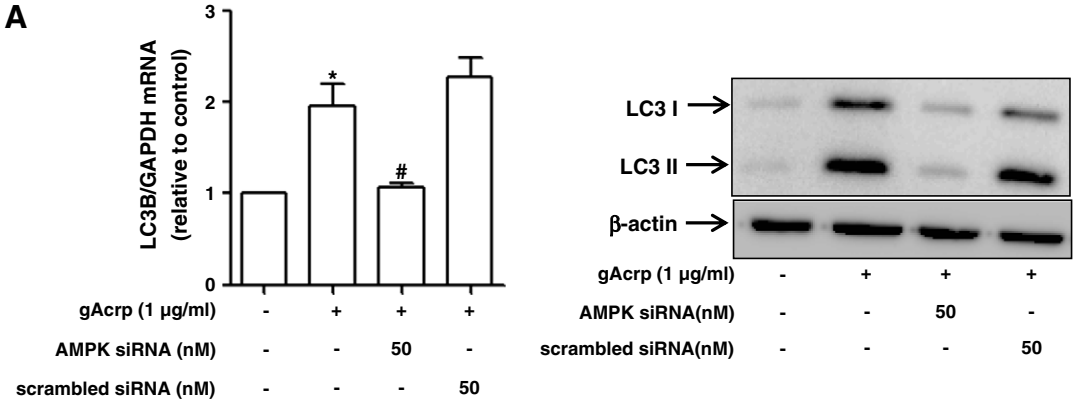
Alcoholic liver disease (ALD) caused by excessive alcohol consumption is a major cause of chronic liver disease throughout the world. Despite advancements in our understanding of the mechanisms underlying ALD, targeted therapies for the management of ALD are still unavailable. Among the broad spectrum of pathogenesis that constitutes ALD, hepatocellular apoptosis is considered an important early response of liver cells to hepatic injury [38]. Adiponectin, the most abundant adipokine secreted from adipose tissue, has been shown to prevent ethanol-induced liver injury in various experimental settings. In particular, a recent study has demonstrated that globular adiponectin (gAcrp) inhibits ethanol-induced apoptosis in hepatoma cell lines [24]. A series of recent studies have also indicated that autophagy counteracts dysregulated apoptosis under conditions of stressful insults and promotes cellular survival [8,39]. Furthermore, alcohol consumption has been linked to the inhibition of autophagic process in liver cells [15,17,18]. Therefore, in an attempt to elucidate the mechanisms underlying the protection of liver cells from ethanol-induced cell death by adiponectin, we investigated the effect of globular adiponectin on the autophagic process in a human liver cell line and further delineated the role of autophagy induction in the suppression of ethanol-induced apoptosis. In the present study, we have demonstrated for the first time that globular adiponectin protects liver cells from ethanol-induced apoptosis through activation of autophagy. Moreover, we have also presented that the AMPK-FoxO3A signaling pathway plays a central role in the gAcrp-induced expression of genes related to autophagy in liver cells and the prevention of ethanol-induced apoptosis by globular adiponectin.

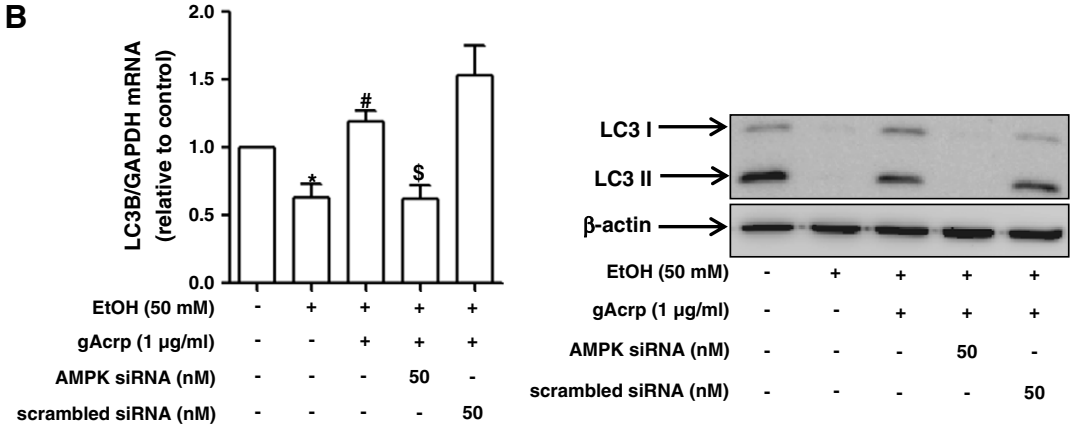
In the present study, we have demonstrated that gAcrp restores autophagy suppressed by ethanol in liver cells. However, the effects of ethanol on autophagy are controversial. Ding et al., demonstrated that ethanol activates autophagy in mice liver and cultured liver cells [17], and suggested that enhanced autophagy plays a protective role against ethanol-induced liver damage, while many other previous reports have shown that ethanol decreases autophagic process. For example, Noh et al. [16] has shown that ethanol reduces autophagy in rat hepatoma cell lines. Ethanol treatment has been also demonstrated to inhibit expression of autophagy-related proteins in immune cells, including human monocytic U937 and CD4 Jurkat cells [15], and inhibit autophagic process in MCF-7 cells and neuronal cells [40,41]. It has been shown that ethanol disrupts lysosomal machinery and inhibits fusion of autophagosome with lysosomes, a critical late step in autophagy process for the degradation of misfolded proteins [42]. Recently, a review paper has highlighted that oxidative stress and reactive oxygen species (ROS) production during ethanol treatment can also impair autophagy in liver cells. Similarly, ethanol treatment has been shown to impair intrahepatic amino-acid pool that can mediate suppression of autophagy [28]. Moreover, ethanol treatment has been well known to affect various signaling pathways, such as JNK and AMPK in the liver that has potential to impair autophagy in the liver cells [40,43]. Based on these controversial reports, the exact effects of ethanol on autophagy are not conclusive, and seem to depend on the experimental conditions, such as cell type, origin of cell, incubation time and the way of treatment. At this stage, while we cannot directly dissect the discrepancy on the different results from the previous report by Ding et al.,[17], we found potential differences between Ding's et al., and our experiments. While we performed our experiments on HepG2 cells and primary hepatocytes isolated from rats, Ding et al., used ethanol binge model in mice as well as primary hepatocytes from mice. We assume that this may be responsible for the different responses.

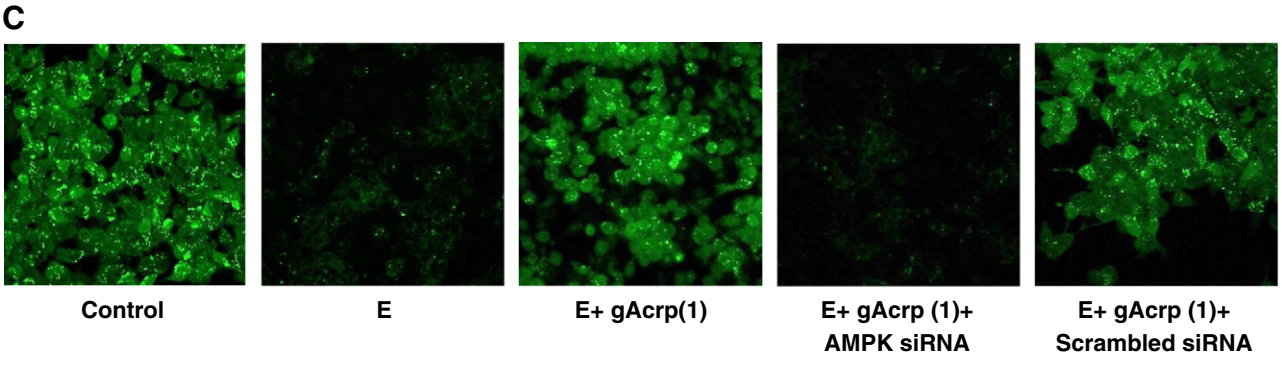
In the present study, we demonstrated that ethanol suppresses the expression of autophagy-related (Atg) genes in a human hepatoma cell line and primary hepatocytes isolated from rat and that gAcrp restores the ethanol-suppressed expression of Atg genes and proteins at both mRNA and protein levels (Fig. 1A–F). Among various Atg genes, beclin-1 and LC3 play a critical role in coordination of the cytoprotective function of autophagy while counteracting the apoptotic process [44]. Beclin-1 has been suggested to play a cardinal role in autophagosome formation through localization of autophagy-related proteins to a pre-autophagosomal structure that is dependent on interaction with class III phosphoinositide 3-kinase/Vacuolar sorting protein 34 [44,45]. LC3, an important constituent of autophagosomes, also plays an essential role in the fusion of autophagosomes with lysosomes for degradation of damaged organelles by lysosomal enzymes [8], suggesting that inducing expression of these genes is closely associated with induction of the autophagic process by gAcrp.

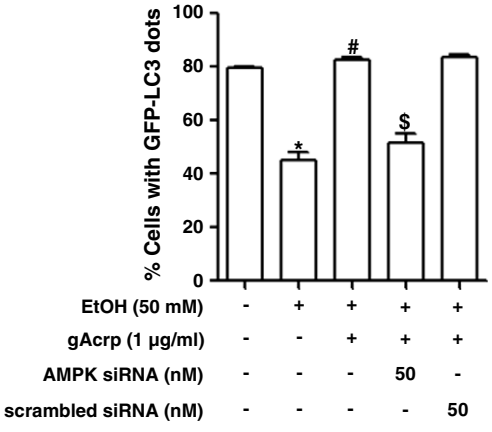
It has been reported that there are direct interactions between beclin-1 and Bcl-2 family members. Interestingly, the relationship between autophagy and apoptosis is a matter of intense debate. The important points in the interconnection between apoptosis and autophagy include autophagy-induced suppression of caspase activity

and/or caspase-mediated cleavage of autophagy-related proteins [46]. Many studies supported the notion that the apoptotic process usually leads to the suppression of other cell death pathways such as autophagy and necrosis [7], while other studies have suggested that the autophagic process prevents apoptosis and subsequently protects cells from various harmful stimuli. For example, a study by Xiao showed that









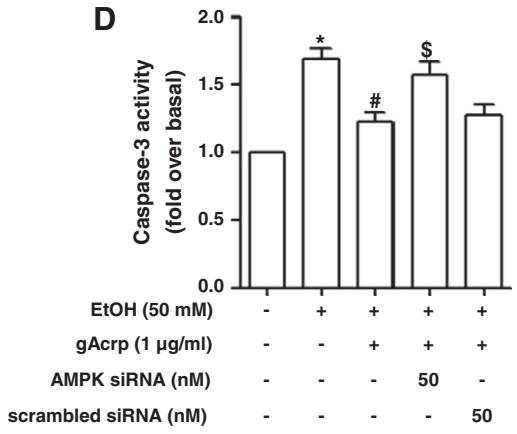


Fig. 6. Involvement of AMPK signaling in globular adiponectin-induced autophagy-related gene expression and attenuation of ethanol-induced caspase-3 activation in HepG2 cells. (A) Cells were transfected with siRNA targeting AMPK or scrambled control siRNA and treated with gAcrp (1 μg/ml) for 24 h. The level of LC3B mRNA (left panel) and LC3II protein (right panel) was assessed as described previously. In left panel, results are expressed as fold increase compared to control group and shown as mean ± SEM of three independent experiments. *P < 0.05 compared with control group; #P < 0.05 compared with cells treated with gAcrp but not transfected with AMPK siRNA. (B) After transfection with siRNA targeting AMPK or scrambled control siRNA, cells were pretreated with indicated concentration of gAcrp for 24 h and then stimulated with ethanol for additional 24 h. LC3B mRNA(left panel) and LC3II protein expression(right panel) was determined as described previously. Results are expressed as fold increase compared to control group and shown as mean ± SEM of three independent experiments. *P < 0.05 compared with control group; #P < 0.05 compared with ethanol treated group, $P < 0.05 compared with cells treated with gAcrp but not transfected with AMPK siRNA. (C) Cells were transfected with eGFP-LC3 expression plasmid for 24 h followed by transfection with siRNA targeting AMPK or scrambled control siRNA and treated with ethanol for 24 h in the absence or presence of gAcrp. GFP-LC3 was viewed with A1 Confocal Laser Microscope as described in materials and methods. Representative images from three independent experiments are shown along with quantitation of LC3 dots on the lower panel. Values are expressed as LC3 dots percentage of cells with GFP-LC3 dots obtained from at least 100 cells. C: Control, E: ethanol, gAcrp: globular adiponectin. *P < 0.05 compared with cells not treated with ethanol; #P < 0.05 compared with cells treated with ethanol. $P < 0.05 compared with cells treated with gAcrp and ethanol but not transfected with siRNA targeting AMPK. (D) Cells transfected with AMPK siRNA were pretreated with gAcrp (1 μg/ml) for 24 h and stimulated with ethanol for additional 24 h. Caspase-3 activity was then assessed as described in the Material and methods section. Data are expressed as fold change relative to control group. Values are shown as mean ± SEM, n = 5. *P < 0.05 compared with control group; #P < 0.05 compared with cells treated with ethanol; $P < 0.05 compared with cells treated with gAcrp and ethanol but not transfected with siRNA of AMPK.

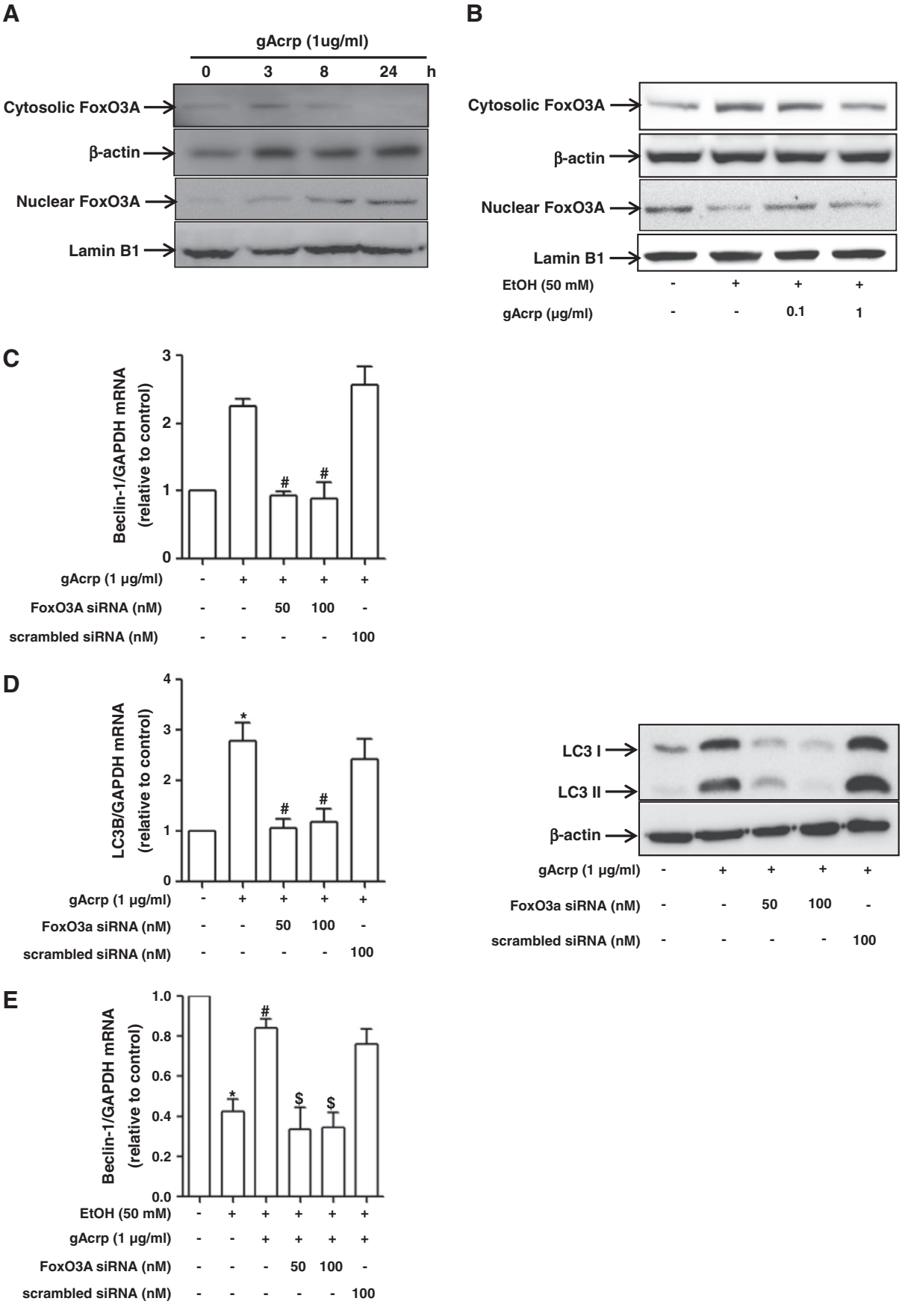
autophagy itself is not solely responsible for cell death and the pro-survival role of autophagy is optimally manifested in conditions associated with cell death that may occur simultaneously [47]. It has been reported that adiponectin protects the liver from ethanol-induced damage [23] and inhibits ethanol-induced apoptosis [24]. Even though a close (negative) relationship between autophagy and apoptosis has been previously suggested, the role of autophagy in the hepatoprotective effect of adiponectin has not been explored. The current study clearly demonstrates for the first time that gAcrp induces an increase in the expression of autophagy-related genes and that the gAcrp-induced autophagic process plays an important role in the amelioration of ethanol-induced apoptosis.

The results presented in current study are in agreement with recent studies showing that adiponectin inhibits ethanol-induced apoptosis through modulation of heme oxygenase-1 (HO-1) [24] and activation of autophagy mediated by HO-1 contributes to prevention of hepatic cell death from infection/sepsis in mice [48]. HO-1 is implicated in adiponectin-induced protective actions in various pathophysiological conditions. Data presented here and in previous studies further suggest a possible role of HO-1 in adiponectin-induced autophagy and protection of cells from various stimuli. It would be interesting to further elucidate the molecular mechanisms underlying how autophagy activation by gAcrp prevents ethanol-induced cell death in liver cells.

Dysregulated autophagy is closely associated with a number of disease conditions. In particular, autophagy increases the threshold for death stimuli in many cellular conditions [49] and the survival advantage offered by autophagy in cells undergoing apoptosis has important implications in the pathogenesis of several human diseases including alcoholic liver disease and cancer [18,46]. In fact, Habeeb et al. recently reported that adiponectin supports survival of colorectal cancer cells in glucose-deprived conditions by enhancing autophagy [50]. However, less attention has been paid regarding the role of autophagy in mediating adiponectin-induced various biological responses. In the current study, we have demonstrated a crucial role of autophagy in the protective effect of adiponectin against ethanol-induced cell death. This is the first report, to our knowledge, showing a role for the autophagic process in the protective response by adiponectin from a certain harmful stimulus. Data presented here suggest that the autophagic process would be a promising target mediating various biological functions of adiponectin and, indeed, our results have opened up a new research area for identifying the role of autophagy in diverse biological responses of adiponectin.

AMP-dependent protein kinase (AMPK) acts as a sensor of cellular energy level and its activity is regulated by various cellular stresses such as hypoxia, glucose deprivation, and oxidative stress [26]. The activation of AMPK increases the catabolic process inside the cells, which modulates numerous pathophysiological processes. It has now been firmly established that many beneficial biological responses induced by adiponectin are attributed to AMPK activation and its downstream transcriptional mediators [19,30]. A recent study by Katsiougiannis et al. revealed that the anti-apoptotic effect of adiponectin is mediated by AMPK activation in salivary gland epithelial cells [51]. Furthermore, AMPK activation is essential for the induction of autophagy in hepatocytes, Hela cells and HT-29 cells [52]. A number of studies have also shown that ethanol decreases AMPK activity in the liver [43,49]. Based on these previous reports, we hypothesized that AMPK signaling is involved in the induction of autophagy by adiponectin and plays a critical role in the prevention of ethanol-induced apoptosis. The results shown in the current study clearly demonstrate that modulation of AMPK plays a key role in the regulation of the autophagic process in HepG2 cells treated with gAcrp and ethanol. AMPK mediates various biological responses through modulation of downstream targets, particularly via regulation of various transcription factors. Chiacchirea et al. reported that AMPK activation causes nuclear translocation of Forkhead box O3A (FoxO3A), thereby inducing expression of genes involved in autophagy [53]. We also demonstrated that knocking down FoxO3A prevents gAcrp-induced expression of beclin-1 and LC3 (Fig. 7C and D), while gene silencing of AMPK prevents nuclear translocation of FoxO3A (Fig. 7G) and expression of LC3 (Fig. 6A and B), indicating that the AMPK/FoxO3A axis plays a critical role in the prevention of ethanol-induced apoptosis in HepG2 cells. Emerging evidence has demonstrated that the FoxO3A transcription factor is implicated in various biological responses, including tumor suppression, development and energy metabolism [54]. Adiponectin has been also shown to regulate many of these responses. Even if there is no report regarding the role of FoxO3A in mediating adiponectin-induced biological responses, it is highly likely that FoxO3A is the transcription factor that mediates autophagic responses by adiponectin. The data presented here suggest that FoxO3A is a promising target mediating various biological responses induced by adiponectin.

Ethanol treatment induces apoptosis of liver cell through modulation of extrinsic and intrinsic pathways of apoptosis. Previous studies have demonstrated that pro- and anti-apoptotic members of Bcl-2 family play a critical role in the intrinsic pathway of apoptosis. In particular, expression of Bax, a pro-apoptotic protein, activates apoptotic process in ethanol-treated hepatocytes and many other experimental conditions [36,55]. It translocates into mitochondria from cytosol upon sensing various cell death stimuli and initiates efflux of cytochrome c,


A
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LC3B/GAPDH mRNA (relative to control)
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FoxO3a siRNA (nM) - - 50 100 -
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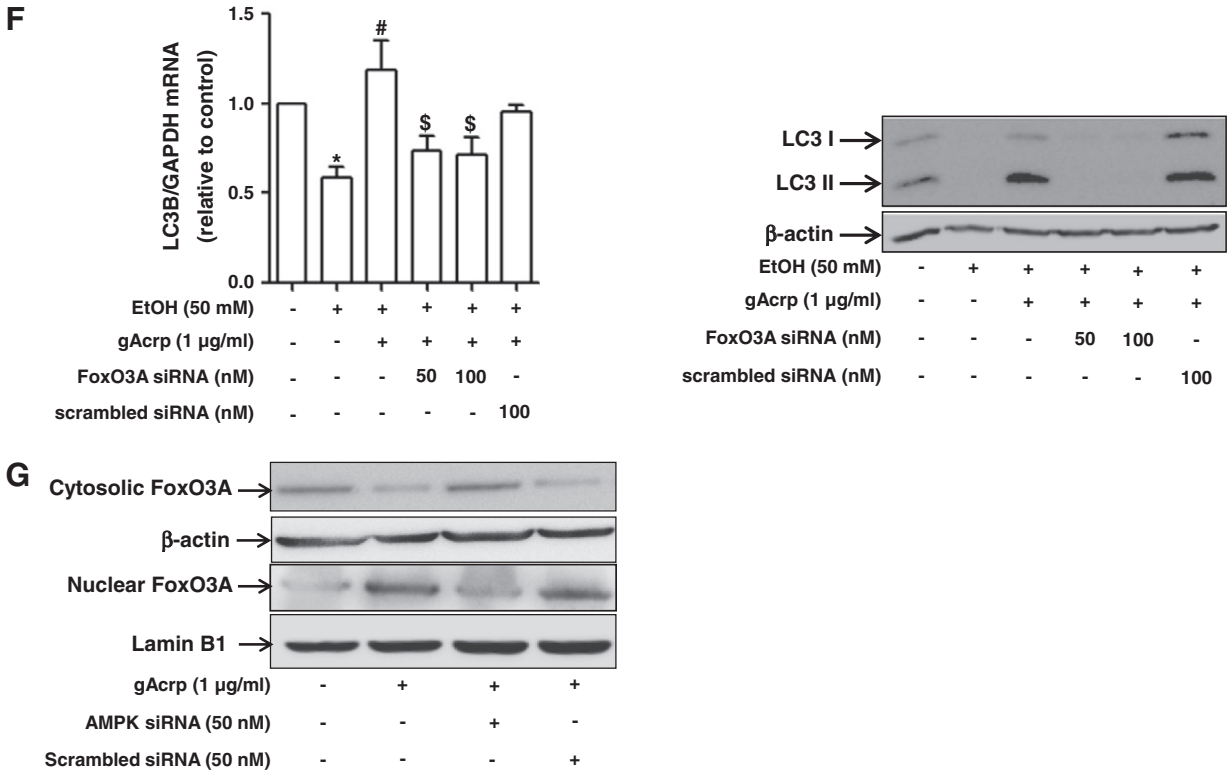

Fig. 7. Role of AMPK-FoxO3A signaling in expression of autophagy-related genes by globular adiponectin in HepG2 cells. (A) Cells were incubated with gAcrp (1 μg/ml) for the indicated time periods. Cytosolic and nuclear fractions were prepared as described in the Material and methods section and the level of FoxO3A protein in each fraction was determined by Western blot analysis. β-actin and lamin b1 was used as an internal control for cytosolic and nuclear fractions. Images are representative of three independent experiments that showed similar results. (B) Cells were pretreated with the indicated concentration of gAcrp for 24 h then treated with ethanol (50 mM) for additional 24 h. Cytosolic and nuclear FoxO3A protein levels were determined by Western blot analysis as described previously. Images shown are representative of three independent experiments that showed similar results. (C) Cells were transfected with siRNA targeting FoxO3A or scrambled control siRNA for 48 h and were treated with gAcrp (1 μg/ml) for 24 h. Beclin-1 expression level was determined by qRT-PCR as described previously. Values are expressed as mean ± SEM ($n = 5$). $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with cells treated with gAcrp but not transfected with FoxO3A siRNA. (D) Cells were transfected with siRNA targeting FoxO3A or scrambled control siRNA for 48 h and were treated with gAcrp (1 μg/ml) for 24 h. In left panel, LC3B mRNA level was determined by qRT-PCR. Values are expressed as mean ± SEM ($n = 5$). $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with cells treated with gAcrp but not transfected with FoxO3A siRNA. In right panel, LC3 II protein expression level was determined by Western blot analysis. (E) Cells were transfected with siRNA targeting FoxO3A or scrambled control siRNA for 48 h. Cells were pretreated with gAcrp (1 μg/ml) for 24 h and then treated with the indicated concentration of ethanol stimulation for additional 24 h. Beclin-1 mRNA level was determined by qRT-PCR as described previously. Values shown are the results of five independent experiments and are expressed as mean ± SEM. $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with cells treated with ethanol; $^{\$}P < 0.05$ compared with cells treated with ethanol and gAcrp but not transfected with siRNA of FoxO3A. (F) Cells were transfected with siRNA targeting FoxO3A or scrambled control siRNA for 48 h. Cells were pretreated with gAcrp (1 μg/ml) for 24 h and then treated with the indicated concentration of ethanol stimulation for additional 24 h. In left panel, LC3B mRNA level determined by qRT-PCR. Values shown are the results of five independent experiments and are expressed as mean ± SEM. $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with cells treated with ethanol; $^{\$}P < 0.05$ compared with cells treated with ethanol and gAcrp but not transfected with siRNA of FoxO3A. In right panel, LC3 II protein expression level was determined by Western blot analysis as described previously. (G) After transfection with siRNA targeting AMPK or scrambled control siRNA, cells were treated with gAcrp (1 μg/ml) for 24 h and FoxO3A protein levels were determined by Western blot analysis as described previously. Images are representative of three independent experiments that showed similar results. β-actin and lamin b1 was used as an internal control.

which in turn activates downstream caspases such as caspase-9 and caspase-3 [4,55]. Furthermore, a number of studies have implicated Bax in the balance between autophagy and apoptosis [7,56]. Previous studies have shown that over-expression of Bax inhibits autophagy by degrading beclin-1 [57], while a credible role of autophagy in the suppression of Bax expression has been shown in human glioma cells treated with arsenic trioxide [58] and in cardiac myocytes undergoing ischemia-reperfusion injury [12]. The current study reinforced these findings that autophagy may counteract apoptosis as evidenced by suppression of ethanol-induced Bax protein expression by treatment with rapamycin, an inducer of autophagy (Fig. 4B). The results from induction of autophagy and its role in the suppression of ethanol-induced Bax protein expression (Fig. 4C–E) intriguingly suggest that autophagy may have a more dominant role than apoptosis in the regulation of cell death in HepG2 cells treated with globular adiponectin. Bax is implicated in the initiation of the intrinsic pathway of apoptosis, suggesting that gAcrp efficiently rescues cell death by acting on the intrinsic pathway of apoptosis.

Ethanol treatment also causes activation of the extrinsic pathway of apoptosis. In ethanol-treated cells, the extrinsic pathway of apoptosis is initiated by binding of FasL and/or TNF-α to its receptors followed by recruitment of its death domain and ultimately activation of caspase-8 and downstream effectors, including caspase-3 [4]. The data presented in the current study also showed that gAcrp significantly suppresses ethanol-induced activation of caspase-8, an important modulator of extrinsic pathway of apoptosis (Fig. 5A), corroborating earlier findings that adiponectin regulates the extrinsic pathway of apoptosis in the suppression of ethanol-induced apoptosis [24]. Our study further showed that inhibition of autophagy negates the suppressive effect of adiponectin on ethanol-induced activation of caspase-8 (Fig. 5B), which is in agreement with a previous study showing that autophagy counter-balances the apoptotic response through inhibition of caspase-8 activity in human colon cancer cells (HCT116) that are TRAIL-resistant [14]. Therefore, it is reasonable to speculate that induction of autophagy by gAcrp might prevent ethanol-induced apoptosis by modulating ethanol-induced activation of caspase-8. Taken together, these data imply that gAcrp suppresses ethanol-induced apoptosis through modulation of the extrinsic and intrinsic pathways of apoptosis.

With regards to the role of LC3B in autophagy induction and suppression of apoptosis, it has been recently reported that LC3B interacts

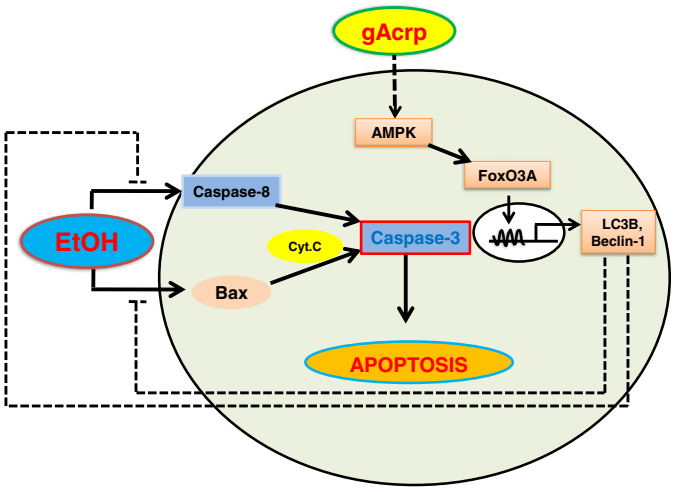

Fig. 8. Proposed model for the activation of autophagy by globular adiponectin and its protective effect against ethanol-induced apoptosis in HepG2 cells. Ethanol treatment causes aberrant apoptosis of liver cells. This is modulated by both the intrinsic pathway, which is regulated by Bcl-2 family members including Bax, and the extrinsic pathway, which is mediated by activation of caspase-8. Activation of both pathways ultimately leads to activation of caspase-3, the executioner of apoptosis. Globular adiponectin induces nuclear translocation of FoxO3A in an AMPK-dependent manner. AMPK/FoxO3A signaling plays a key role in gAcrp-induced expression of genes involved in the autophagic process, such as Beclin-1 and LC3B. Importantly, gAcrp-induced autophagy plays an important role in the prevention of ethanol-induced apoptosis in liver cells through modulation of both the extrinsic and intrinsic pathways of apoptosis. Detailed mechanisms underlying inhibition of the intrinsic and extrinsic pathways of apoptosis by gAcrp-induced autophagy remain to be determined. Cyt. C: Cytochrome C, EtOH: Ethanol, gAcrp: Globular adiponectin.

with Fas apoptotic pathway thereby preventing cell death in lung epithelial cells [59]. This report suggests that LC3B can modulate apoptotic process through physical interaction with apoptotic inducer, as well as induction of autophagy. Although we don't have direct evidence for the interaction of LC3 with Fas and its functional role in the modulation of apoptosis in this experimental condition, we have used various pharmacological modulators of autophagy, such as bafilomycin A1, rapamycin and 3-methyl adenine, apart from LC3B silencing experiments, to dissect the involvement of autophagy induction by gAcrp in the suppression of ethanol-induced apoptosis. Further, gene silencing of Atg5 also restored suppression of caspase-3 activity by gAcrp (manuscript in preparation). All these data suggest that autophagy induction by gAcrp plays a critical role in the suppression of ethanol-induced apoptosis.

In conclusion, the data presented here demonstrate for the first time that globular adiponectin protects liver cells from ethanol-induced apoptosis through modulation of autophagy and this protective effect is mediated by AMPK and FoxO3A signaling (Fig. 8). The fate of the cells is governed by the balance between apoptosis and autophagy. In accordance with these findings, the pro-survival function of autophagy induced by globular adiponectin likely plays a key protective role in ethanol-induced cell death. Based on these findings, we suggest that induction of autophagy by adiponectin would be a novel mechanism for protection from chronic alcohol-induced liver injury. The aim of the present study is to understand the mechanisms underlying suppression of apoptosis by adiponectin in ethanol-treated liver cells, thus we have performed initial and critical experiments using primary rat hepatocytes, but not all the experiments. Further studies are now required to validate our findings in HepG2 cells into normal hepatocytes and its role in the prevention of alcoholic liver disease in an in vivo model.

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