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Original Article

Ghrelin inhibition of ethanol-induced gastric epithelial cell apoptosis is mediated by miR-21

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Abstract: Aim: To investigate the underlying mechanism of ghrelin-induced gastro-protection in a cell culture model of ethanol-induced gastric epithelial cell injury. Methods: The human gastric epithelial cell line GES-1 was incubated with ghrelin (0.01-1 μM), 1 μM ghrelin and 1 μM D-Lys3-growth hormone releasing peptide-6 (GHRP-6), or 1 μM ghrelin and 400 nM antagomiR-21 for 24 h, followed by treatment with 8% ethanol for 3 h to induce apoptosis. Cell viability was determined by MTT assays and flow cytometry was used for detection of apoptosis rates. miR-21 transcription was analyzed by qRT-PCR and Akt, Bcl-2, Bax and caspase 3 expressions were measured by Western blot. Results: Flow cytometry and a quantitative RT-PCR analysis of the expression of miR-21 showed that ghrelin inhibited apoptosis in a dose dependent manner through a signaling pathway that was both growth hormone secretagogue receptor (GHS-R) and miR-21 dependent, as the antiapoptotic effect of ghrelin was blocked by both D-Lys3-GHRP-6 and antagomiR-21, respectively. Western blotting of Akt, Bcl-2, Bax, and caspase 3 showed that the levels of the antiapoptotic proteins, Akt and Bcl-2, in the cells pretreated with ghrelin alone were higher than those in the cells pretreated with D-Lys3-GHRP-6 or antagomiR-21. By contrast, the levels of the proapoptotic proteins, Bax and caspase 3, in the cells pretreated with ghrelin alone were lower than those in the cells pretreated with D-Lys3-GHRP-6 or antagomiR-21. Conclusion: Ghrelin inhibits GES-1 cell apoptosis through GHS-R-dependent signaling in which miR-21 activates the PI3K/Akt pathway, which upregulates Bcl-2 and downregulates Bax and caspase 3 expression.

Keywords: Ghrelin, miR-21, PI3K/Akt, apoptosis, gastric epithelial cells

Introduction

Gastric injury is a common effect of ethanol exposure. Ethanol-induced gastric mucosal injury can cause hemorrhagic ulceration of the gastric epithelium, and the intragastric application of ethanol in experimental animals has long been used as a model for the study of gastric lesions. The mucosal damage that occurs in ethanol-induced gastric injury has been shown to involve various mechanisms, including direct toxic effects, inflammation, and prostaglandin and nitric oxide synthesis [1]. Recent studies have shown that ethanol also induces apoptosis in gastric epithelial cells [1, 2].

Ghrelin is a 28-aa growth hormone-releasing peptide [3] that functions as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [4]. Ghrelin is primarily produced in the gastric mucosa by X/A-like cells [5, 6], and exists in two major forms, acyl ghrelin and des-acyl ghrelin [3]. Acyl ghrelin binds the GHS-R-1a protein [7], and previous studies have shown that ghrelin influences neuroendocrine, cardiovascular, and gastrointestinal physiology [8, 9]. Recent studies have suggested that ghrelin plays an important role in gastroprotection [10-12] through the activation of the phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) signaling pathway [13].

MicroRNAs (miRNAs) are small (18-22 nt) endogenous, noncoding RNA molecules that suppress gene expression posttranscriptionally through base-pairing to complementary sequences in mRNAs, which mediates the subsequent Argonaute-catalyzed cleavage of the mRNA [14, 15]. Previous studies have shown that miRNAs regulate various cellular processes, including cell differentiation, proliferation, and apoptosis [16, 17]. The miR 21 miRNA has been implicated in the tumorigenesis of a variety of cancers [18]. The phosphatase and ten-

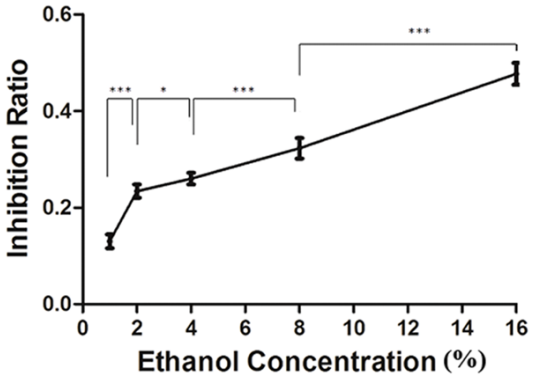


Figure 1. Inhibition of the proliferation of GES-1 cells following treatment with ethanol. The cells were treated with concentrations of ethanol ranging from 1% to 16% for 3 h, and the effect of each ethanol treatment on cell viability was quantified using an MTT assay. Values represent the mean ± SE (n = 5; $*P < 0.05$, $***P < 0.001$).

sin homolog deleted on chromosome 10 (PTEN), B-cell lymphoma/leukemic-2 (Bcl-2), and cysteine and aspartic proteinase 3 (caspase 3) mRNAs are important regulatory targets of miR-21 [19], and the antiapoptotic effects of miR-21 are associated with the PTEN-mediated upregulation of the PI3K/Akt signaling pathway [20].

Ghrelin has been shown to have protective effects against ethanol-induced gastric injury [10]. Although the mechanism by which ghrelin effects ethanol-induced lesions in the gastric epithelium is unclear, miR-21 has been shown to stimulate antiapoptotic pathways in a human gastric cancer cell line [21]. Therefore, we hypothesized that miR-21 mediates the effect of ghrelin on ethanol-induced gastric epithelial cell apoptosis. We investigated the relationship between ghrelin and miR-21 in a cellular model of ethanol induced gastric epithelial cell apoptosis. We also investigated whether the intracellular levels of Bcl-2, Bax, Caspase 3, and Akt were altered during the miR-21-mediated inhibition of ethanol-induced apoptosis.

Materials and methods

Cell culture

The human gastric epithelial cell line, GES-1 (KeyGen Biotech, Nanjing, Jiangsu, China), was maintained in 90% DMEM (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS; ExCell Biology, Shanghai, China) supplemented with penicillin and streptomycin (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2.

Ethanol-induced apoptosis of gastric epithelial cells

To establish a model of gastric epithelial cell apoptosis [22], GES-1 cells were treated with various concentrations of ethanol, and subsequent cell viability was assessed. The GES-1 cells were seeded in DMEM at a cell density of $1 \times 10^4/cm^2$, and grown to 70% to 80% confluence. To determine the optimal concentration of ethanol for inducing apoptosis, the GES-1 cells were incubated in 1%, 2%, 4%, 8%, or 16% ethanol in DMEM for 3 h. The normal control cells were treated using an equivalent volume of deionized water.

Cell viability assay

The ethanol-treated GES-1 cells and the normal control cells were collected, and plated in 96-well plates in DMEM. A 20-μL aliquot of 5 mg/mL 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Amresco, Solon, OH, USA) was added to each well, and the cells were incubated for 4 h. The medium was aspirated, and 150 μL of DMSO was added to each well. The plates were shaken gently for 10 min at 37°C. The optical density of the contents of each well was measured at 490 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA). The inhibition rate (%) was calculated as (1 - [experimental group/negative control group]) × 100% to represent the inhibition of cell proliferation caused by the ethanol treatment. Each assay was repeated three times.

Knockdown of endogenous miR-21

To demonstrate the effect of antagomi R-21 on the intercellular level of endogenous miR-21, GES-1 cells were transfected with antagomiR-21 (Guangzhou RiboBio, Guangdong, China), a chemically modified oligonucleotide complementary to the miR-21 sequence that mediates the degradation of miR21 in cells. The cells were grown to 70% to 80% confluence in Opti-MEM (Invitrogen) reduced serum medium, and transfected with 400 nM antagomiR-21 or vehicle control using Lipofectamine 2000 (Invitrogen), according to the manufacturer's

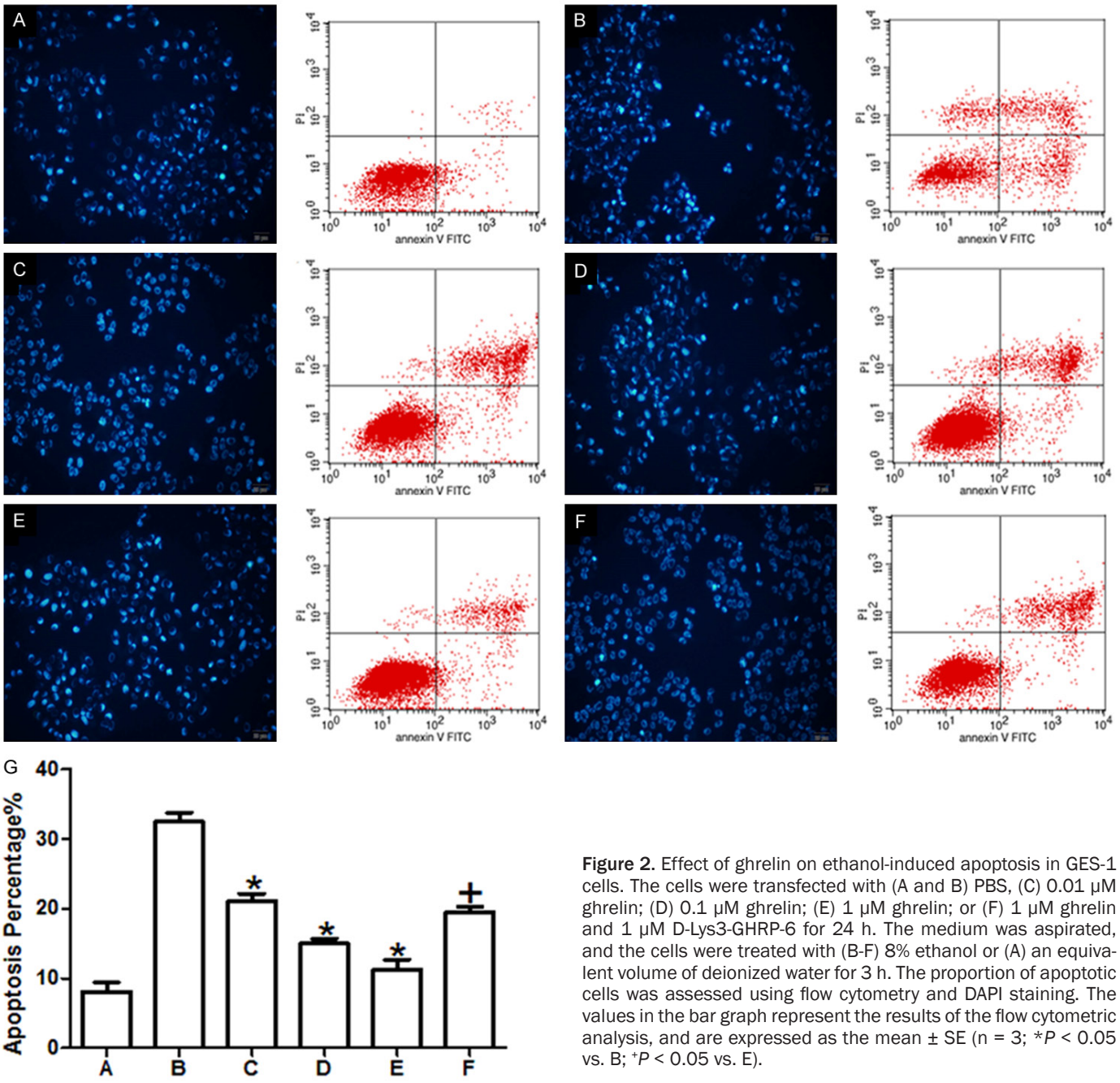


Figure 2. Effect of ghrelin on ethanol-induced apoptosis in GES-1 cells. The cells were transfected with (A and B) PBS, (C) 0.01 μM ghrelin; (D) 0.1 μM ghrelin; (E) 1 μM ghrelin; or (F) 1 μM ghrelin and 1 μM D-Lys3-GHRP-6 for 24 h. The medium was aspirated, and the cells were treated with (B-F) 8% ethanol or (A) an equivalent volume of deionized water for 3 h. The proportion of apoptotic cells was assessed using flow cytometry and DAPI staining. The values in the bar graph represent the results of the flow cytometric analysis, and are expressed as the mean ± SE (n = 3; $^{*}P < 0.05$ vs. B; $^{+}P < 0.05$ vs. E).

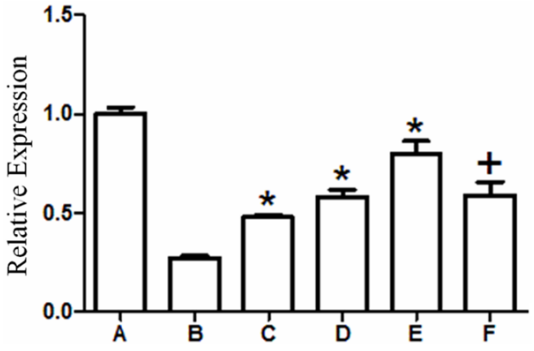


Figure 3. Expression of miR-21 in GES-1 cells following ethanol treatment to induce apoptosis. The cells were transfected with (A and B) PBS, (C) 0.01 μM ghrelin; (D) 0.1 μM ghrelin; (E) 1 μM ghrelin; or (F) 1 μM ghrelin and 1 μM D-Lys3-GHRP-6 for 24 h. The medium was aspirated, and the cells were treated with (B-F) 8% ethanol or (A) an equivalent volume of deionized water for 3 h. The intracellular level of miR-21 was quantified using qRT-PCR. The values are expressed as the mean ± SE (n = 3; $^{*}P < 0.05$ vs. B; $^{+}P < 0.05$ vs. E).

instructions. For transfection, the antagomiR-21 was gently mixed with the Lipofectamine 2000 in Opti-MEM, and the mixture was added to the cells. After 6 h, the medium was aspirated, and replenished with fresh Opti-MEM. At 24 h post-transfection, the level of miR-21 in the cells transfected with antagomiR-21 was compared to that of the vehicle control cells and nontransfected (normal control) cells.

Quantitative reverse transcription and real-time PCR (qRT-PCR)

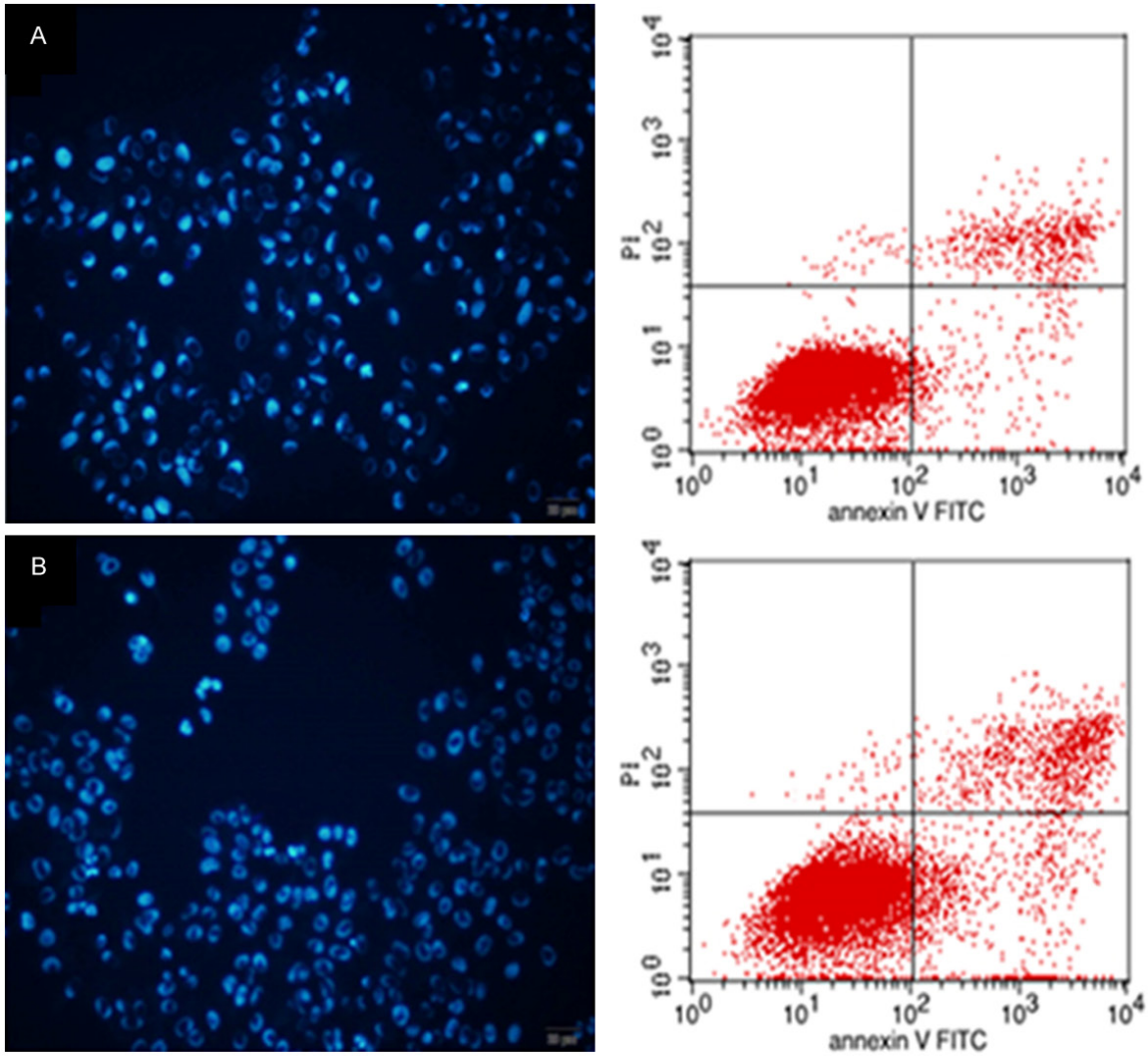
The intracellular level of miR-21 was quantified using qRT-PCR. Total RNA was extracted from the GES-1 cells using the Trizol reagent (Invitrogen). The concentration of the purified RNA was quantified using an ultraviolet spectrophotometer, and the ratio of the optical density at 260 nm to that at 280 nm was between 1.8 and 2.0 for all of the samples. The level of miR-21 in each sample was determined by qRT-PCR using SYBR Green stem-loop primers (Invitrogen) and the NCode miRNA qRT-PCR Kit (Applied Biosystems, Carlsbad, CA, USA). The level of the U6 small nuclear RNA (snRNA) was measured as an internal control for the relative quantification of miR-21. The sequence of the stem-loop primer used for the reverse transcription of miR-21 was 5'-GTCGTATCCAGTGC AGGGTCCGAGGTATTCGCACTGGATACGACTCAACA-3'. The primers used to amplify the miR-21 cDNA were 5'-CGCGCTAGCTTATCAGACT-3' and 5'-GTGCAGGGTCCGAGGT-3'. The sequence of the primer used for the reverse transcription of the Hsa-U6 snRNA was 5'-GTCGTATCCAGTGC-AGGGTCCGAGGTATTCGCACTGGATACGAC AAAATA-3'. The primers used to amplify the Hsa-U6 snRNA cDNA were 5'-GCGCGTCGTG AAGCGTTC-3' and 5'-GTGCAGGG TCCGAGGT-3'. Thermal cycling was performed at 94°C for 10 min, followed by 40 cycles of 94°C for 30 s and 60°C for 30 s. The relative change in the level of miR-21 was calculated using the 2-ΔΔCT method [23].

Analysis of the effects of ghrelin on ethanol-induced gastric epithelial cell apoptosis

Ghrelin (GenScript, Piscataway, NJ, USA) and D-Lys3-GHRP-6 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 100% DMSO, and diluted in phosphate-buffered saline (PBS; KeyGen Biotech) for use in the cell culture experiments. The GES-1 cells were washed with PBS, and incubated in DMEM containing 0.5% FBS for 24 h. The medium was aspirated, and replaced with DMEM containing 0.01 μM ghrelin, 0.1 μM ghrelin, 1 μM ghrelin, 1 μM ghrelin and 1 μM D-Lys3-GHRP-6, or 1 μM ghrelin and 400 nM antagomiR-21. The negative control cells (no ghrelin, antagomiR-21, or D-Lys3-GHRP-6) were treated using an equivalent volume of PBS. The cells were incubated for an additional 24 h. The medium was aspirated, and the cells were treated with 8% ethanol for 3 h, as described above. The control cells were treated using an equivalent volume of deionized water. The cells were collected for flow cytometric analysis, staining with 4', 6-diamidino-2-phenylindole (DAPI), qRT-PCR, and western blotting.

Flow cytometry

The cells were treated using the Apoptosis Assay Kit (KeyGen Biotech) before being subjected to the flow cytometric analysis. The cells were washed twice with ice-cold PBS, and suspended in 500 μL binding buffer before adding 5 μL of Annexin V-fluorescein isothiocyanate and 5 μL of propidium iodide. The mixture was placed in the dark, and incubated for 15 min. The percentage of apoptotic cells was analyzed in a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).


A
B
PI
annexin V FITC
C
Apoptosis (%)
0
10
20
30
40
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a
b
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d

Figure 4. Effects of antagomir-21 on ghrelin-mediated inhibition of ethanol-induced apoptosis in GES-1 cells. The cells were transfected with (A) 1 μM ghrelin or (B) 1 μM ghrelin and 400 ng of antagomiR-21 for 24 h, followed by treatment with 8% ethanol for 3 h, and the cells were analyzed using flow cytometry and DAPI staining. The proportion of apoptotic cells in (a) the normal control cells (b) the negative (ethanol) control cells, (c) the ghrelin-treated cells, and (d) the cells treated with ghrelin and antagomiR-21 was determined based on the results of the flow cytometric analysis, and are presented as the mean ± SE (n = 3; $^{**}P < 0.01$, $^{***}P < 0.001$).

Fluorescence microscopy

The cells were washed three times with PBS, and fixed in 4% paraformaldehyde for 15 min at 37°C. The cells were washed three times with PBS. The cells were stained using DAPI (KeyGen Biotech), and placed in the dark for 15 min at 37°C. The cells were washed twice with PBS to remove background staining. Apoptotic cells were indentified using fluorescence microscopy with an excitation wavelength of 359 nm.

Western blotting

The cells were washed three times with ice-cold PBS, and suspended in lysis buffer containing 150 mg/L protease inhibitor. The mixture was centrifuged at 4°C at 15000 × *g* for 15 min. The supernatant was collected, and stored at -80°C. The concentration of total protein in the supernatant was determined using a bicinchoninic acid reagent (Beyotime, Shanghai, China). The protein samples were adjusted to equivalent concentration by the addition of lysis buffer, and were mixed with sample buffer. An aliquot of each sample containing 100 μg of total protein was subjected to SDS-PAGE in a 12.5% acrylamide gel. The resolved protein bands were transferred onto a nitrocellulose membrane. Immunoblotting was performed using anti-Akt, anti-Bcl-2, anti-Bax, anti-Caspase 3, or anti-β-actin (control) primary antibodies (all from KeyGen Biotech). The secondary antibodies consisted of goat anti-rabbit IgG or goat anti-mouse IgG conjugated to alkaline phosphatase (KeyGen Biotech), and primary antibody reactivity was visualized using the BeyoECL Plus enhanced chemiluminescence kit (Beyotime). The relative level of protein expression was quantified based on band intensity using a Kodak Image Station 2000R (Rochester, NY, USA).

Statistical analysis

All of the data are presented as the mean ± standard error (SE). Intergroup differences in the continuous data sets were evaluated using an analysis of variance. All statistical analyses were performed using the Prism, version 5.0, software (GraphPad, La Jolla, CA, USA). The level of statistical significance was set at $P < 0.05$.

Results

Antagomir-21-mediated knockdown of miR-21

After transfection with antagomiR-21 for 24 h, the intracellular level of endogenous miR-21 was assessed using qRT-PCR. The level of miR-21 in the cells transfected with 400 nM antagomiR-21 was significantly lower (0.14 ± 0.03) than that of the negative control cells (1.00 ± 0.54) and the normal control cells (1.07 ± 0.47; $P < 0.01$).

Ethanol-induced GES-1 apoptosis

To establish a model of gastric epithelial cell apoptosis, the effect of ethanol on the viability of GES-1 cells was assessed using an MTT assay. The inhibition of GES-1 proliferation increased as the concentration of ethanol increased. The optimal concentration of ethanol for inducing apoptosis in the GES-1 cells was determined to be 8%, which resulted in an inhibition rate of 33% (**Figure 1**). In all of the subsequent experiments, a 3-hour treatment using 8% ethanol was used to induce apoptosis in the GES-1 cells.

Effect of ghrelin on apoptosis in GES-1 cells

Flow cytometry showed that the percentage of apoptotic cells among the GES-1 cells treated with ethanol only (negative control) was significantly higher (32.49% ± 1.29%; $P < 0.01$; **Figure 2B**) than that of the cells pretreated with 0.01, 0.1, or 1 μM ghrelin (21.05% ± 1.13%, 14.97% ± 0.76%, and 11.19% ± 1.53%, respectively; **Figure 2C-E**), and that the percentage of apoptotic cells gradually decreased as the concentration of ghrelin increased. Staining with DAPI also showed that the proportion of apoptotic cells in the cells treated with ethanol only

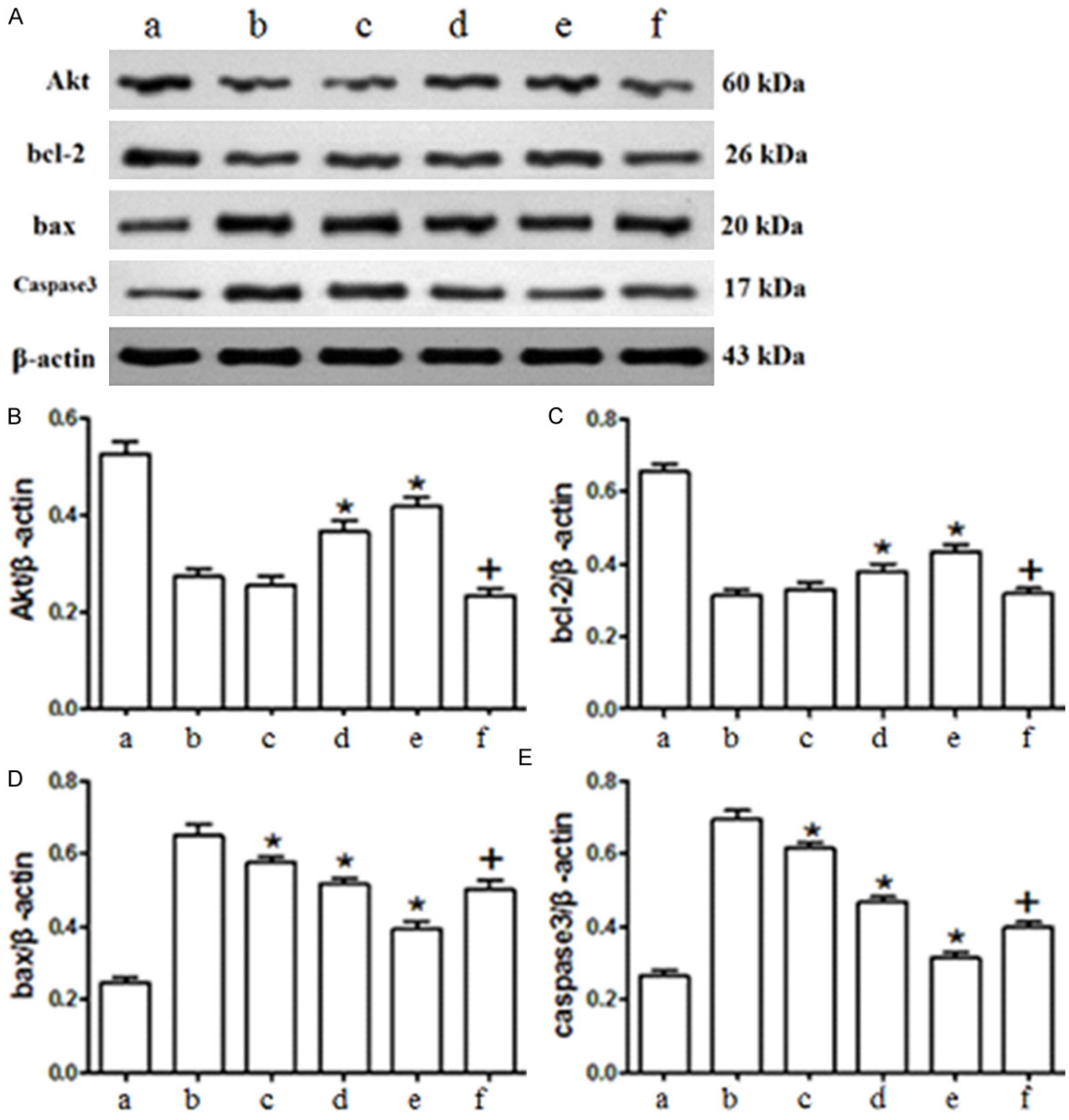

Figure 5. Levels of the Akt, Bcl-2, Bax, and Caspase 3 proteins in GES-1 cells pretreated with ghrelin before treatment with ethanol to induce apoptosis. The cells were transfected with (a and b) PBS, (c) 0.01 μM ghrelin; (d) 0.1 μM ghrelin; (e) 1 μM ghrelin; or (f) 1 μM ghrelin and 400 ng of antagomiR-21 for 24 h. The medium was aspirated, and the cells were treated with (b-f) 8% ethanol or (a) an equivalent volume of deionized water for 3 h. The intracellular levels of Akt, Bcl-2, Bax, and Caspase 3 were quantified by western blotting. The values in the bar graphs are expressed as the mean ± SE (n = 3; **P* < 0.05 vs. b; +*P* < 0.01 vs. e).

(negative control) was greater than that of the cells pretreated with ghrelin. Pretreatment with both ghrelin and the GHS-R blocker, D-Lys3-GHRP-6, significantly increased the percentage of apoptotic cells following ethanol treatment (19.44% ± 1.12%; *P* < 0.01; **Figure 2F**), compared with that of the cells pretreated with ghrelin only (11.19% ± 1.53%; **Figure 2E**).

Effect of ghrelin-mediated inhibition of apoptosis on miR-21 expression in GES-1 cells

The qRT-PCR analysis showed that pretreatment with 0.01, 0.1, or 1 μM ghrelin significantly increased the level of miR-21 in GES-1 cells following treatment with ethanol (0.48 ± 0.01, 0.58 ± 0.04, 0.80 ± 0.04, respectively; *P* <

0.01; **Figure 3**), compared with that of the negative control cells (0.27 ± 0.01). Pretreatment with both ghrelin and D-Lys3-GHRP-6 significantly reduced the level of miR-21 following ethanol treatment (0.59 ± 0.04; $P < 0.05$; **Figure 3**), compared with that of the cells pretreated with ghrelin alone.

Effect of antagomir-21 on ghrelin-mediated inhibition of apoptosis in GES-1 cells

Flow cytometry showed that pretreatment with ghrelin and antagomiR-21 significantly increased the percentage of apoptotic GES-1 cells following treatment with ethanol (18.32% ± 0.73%; $P < 0.01$; **Figure 4**), compared with that of the cells pretreated with ghrelin alone (11.19% ± 1.53%). Staining with DAPI also showed that, following treatment with ethanol, the cells pretreated with ghrelin and antagomiR-21 had a higher proportion of apoptotic cells, compared with the cells pretreated with ghrelin alone (**Figure 4**).

Effects of ghrelin on intracellular levels of Akt, Bcl-2, Bax, and Caspase 3

The western blotting analysis showed that pretreatment with 1 μM ghrelin significantly increased the intracellular levels ofAkt and Bcl-2 following ethanol treatment (0.38 ± 0.01 and 0.43 ± 0.01, respectively; $P < 0.05$), compared with the levels of Akt and Bcl-2 in the negative control cells (0.31 ± 0.01). Pretreatment with 0.1 μM ghrelin also increased the levels of Akt and Bcl-2 significantly (0.37 ± 0.02 and 0.42 ± 0.02, respectively; $P < 0.05$), compared with the levels of Akt and Bcl-2 in the negative control cells (0.27 ± 0.01). By contrast, pretreatment with 0.01, 0.1, or 1 μM ghrelin significantly reduced the intracellular level of Bax following ethanol treatment (0.58 ± 0.01, 0.52 ± 0.01, and 0.39 ± 0.02, respectively; $P < 0.05$), compared with that of the negative control cells (0.65 ± 0.02). Pretreatment with 0.01, 0.1, or 1 μM ghrelin also reduced the intracellular level of Caspase 3 significantly (0.62 ± 0.01, 0.47 ± 0.01, and 0.31 ± 0.01, respectively; $P < 0.05$), compared with that of the negative control cells (0.70 ± 0.01).

Pretreatment with ghrelin and antagomiR-21 significantly reduced the intracellular levels of Akt and Bcl-2 following ethanol treatment (0.23 ± 0.01 and 0.32 ± 0.01, respectively; $P < 0.01$), compared with those of the cells pretreated with ghrelin alone (0.42 ± 0.02 and 0.43 ± 0.02, respectively). By contrast, pretreatment with ghrelin and antagomiR-21 significantly increased the intracellular levels of Bax and Caspase 3 (0.50 ± 0.02 and 0.40 ± 0.01, respectively; $P < 0.01$), compared with those of the cells pretreated with ghrelin alone (0.39 ± 0.01 and 0.31 ± 0.01) (**Figure 5**).

Discussion

Alcohol use is a common etiological factor in acute gastric mucosal injury, and Sibilia *et al.* showed that ghrelin confers a protective effect of ghrelin against ethanol-induced gastric mucosal injury in animal models [11, 12]. Recent studies have also shown that ethanol can induce apoptosis in human gastric epithelial cells [24]. However, the mechanisms through which ghrelin inhibits alcohol-induced apoptosis in human gastric epithelial cells has remained unclear.

In our current study, we found that ghrelin significantly inhibited apoptosis in a cell culture model of ethanol-induced gastric epithelial cell injury. We pretreated GES-1 cells with ghrelin before treating them with ethanol to induce apoptosis, and our flow cytometric analysis showed that the percentage of apoptotic cells gradually decreased as the concentration of ghrelin increased. Staining with DAPI also showed that the cells treated with ethanol only had a greater proportion of apoptotic cells than those pretreated with ghrelin before the ethanol treatment.

To investigate the cellular mechanism through which ghrelin exerted its protective effect against apoptosis, we pretreated GES-1 cells with ghrelin and D-Lys3-GHRP-6, a GHS-R blocker, before inducing apoptosis using ethanol. Our flow cytometric analysis showed that the D-Lys3-GHRP-6 blocked the protective effects of ghrelin, increasing the proportion of apoptotic cells after ethanol treatment. This result indicated that the antiapoptotic effect of ghrelin was transduced via a GHS-R mediated pathway.

Because miR-21 has been shown to stimulate antiapoptotic pathways in a human gastric cancer cell line [21], we also investigated whether miR-21 mediated the protective effects of ghre-

lin on ethanol-induced apoptosis in GES-1 cells. Our qRT-PCR analysis showed that, following ethanol treatment, the level of miR-21 in the GES-1 cells pretreated with ghrelin was higher than that in the cells pretreated with ghrelin and D-Lys3-GHRP-6 and those that were not pretreated with ghrelin (negative control). These results indicated that the expression of miR-21 was upregulated by the GHS-R mediated pathway through which ghrelin inhibited apoptosis.

The PI3K/Akt signaling pathway has been shown to participate in antiapoptotic processes [25, 26] through the regulation of the expression of a number of different apoptosis-associated proteins, including Bcl-2, Bax, and Caspase 3 [27]. Previous studies have shown that Bcl-2 mediates antiapoptotic processes in a variety of cell types [28], and that the overexpression of Bax induces apoptosis [29]. A member of the cysteine-protease family, Caspase 3 is a central regulator of apoptosis. Once activated, Caspase 3 cleaves cytoskeletal and nuclear proteins, which induces apoptosis [30].

To identify the signaling pathway through which ghrelin inhibited apoptosis, we pretreated GES-1 cells with ghrelin, and examined the levels of theAkt, Bcl-2, Bax, and Caspase 3 proteins following ethanol treatment to induce apoptosis. Our western blotting analysis showed that the levels of Akt and Bcl-2 in the cells pretreated with ghrelin were higher than those in the cells that were not subjected to the ghrelin pretreatment (negative control). By contrast, the levels of Bax and Caspase 3 in the cells pretreated with ghrelin were higher than those in the negative control cells. These results indicate that the interaction of ghrelin and GHS-R resulted in Akt, the upregulation of Bcl-2 expression, and the downregulation of the expression of Bax and Caspase 3, suggesting that changes in the intracellular levels of these proteins contributed to the protective effect of ghrelin against ethanol-induced apoptosis.

The PI3K/Akt pathway is negatively regulated by PTEN [31, 32], and reduced PTEN expression is associated with increased cell proliferation and reduced apoptosis. The PTEN, Bcl-2, and Caspase 3 mRNAs are important regulatory targets of miR-21 [33, 34]. Therefore, we investigated whether the protective effect of ghrelin against ethanol-induced apoptosis was mediated by miR-21. We pretreated GES-1 cells with ghrelin and antagomir-21, which directs the degradation of miR-21, and examined the percentage of apoptotic cells and the levels of Akt, Bcl-2, Bax, and Caspase 3 following ethanol treatment. Flow cytometry and DAPI staining showed that miR-21 knockdown reduced the antiapoptotic effect of ghrelin. The western blotting analysis showed that the loss of miR-21 reduced the levels of antiapoptotic proteins, Akt and Bcl-2, and increased the levels of the proapoptotic proteins, Bax and Caspase 3. Our results collectively suggested that the protective effect of ghrelin on ethanol-induced apoptosis in gastric epithelial cells was brought about by miR-21-mediated changes in the levels of phospho-Akt, Bcl-2, Bax, and Caspase 3.

In conclusion, the results of our current study showed that ghrelin inhibits ethanol-induced apoptosis in human gastric epithelial cells through a GHS-R-dependent signal transduction pathway that upregulates the expression of miR-21, and that the upregulation of miR-21 expression activates the PI3K/Akt pathway, which upregulates the expression of Bcl-2 and downregulates the expression of Bax and Caspase 3, resulting in the inhibition of proapoptotic processes.

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Disclosure of conflict of interest

None.

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