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AMP-activated protein kinase activation protects gastric epithelial cells from *Helicobacter pylori*-induced apoptosis

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ABSTRACT

Helicobacter pylori (H pylori), infecting half of the world's population, causes gastritis, duodenal and gastric ulcer, and gastric cancers. AMP-activated protein kinase (AMPK) is a highly conserved regulator of cellular energy and metabolism. Recent studies indicated an important role for AMPK in promoting cell survival. In this study, we discovered that H Pylori induced AMPK activation in transformed (GEC-1 line) and primary human gastric epithelial cells (GECs). Inhibition of H Pylori-stimulated AMPK kinase activity by AMPK inhibitor compound C exacerbated apoptosis in transformed and primary GECs. Meanwhile, downregulation of AMPK expression by targeted shRNAs promoted apoptosis in H pylori-infected GECs. In contrast, A-769662 and resveratrol, two known AMPK activators, or AMPKα1 over-expression, enhanced H Pylori-induced AMPK activation, and inhibited GEC apoptosis. Our data suggested that transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) could be the upstream kinase for AMPK activation by H pylori. Partial depletion of TAK1 by shRNAs not only inhibited AMPK activation, but also suppressed survival of H pylori-infected GECs. Taken together, these results suggest that TAK1-dependent AMPK activation protects GECs from H pylori-Induced apoptosis.

1. Introduction

Helicobacter pylori (H pylori), which infects half of the world's population, is a gram-negative bacterium that selectively colonizes at gastric mucosa, leading to gastritis, duodenal and gastric ulcer, and gastric cancers [1,2]. The interplay between bacterial factors and host signal transduction pathways determines the fate of host cells, which can manifest as increased proliferation, and either increased or decreased apoptosis at different stages of infection [1,2]. Both *in vivo* and *in vitro* studies have shown that several H pylori virulence factors could stimulate gastric epithelial cell (GEC) apoptosis [3–5]. The cellular mechanisms by which H pylori induces apoptosis are not fully understood, although activation of major histocompatibility complex class II [6], p53 [5] Fas/FasL system [7], and tumor necrosis factor–related apoptosis-inducing ligand [8] has been proposed. Meanwhile, H pylori increases reactive oxygen species (ROS) production [9] and enhances Bax expression as well as mitochondrial translocation [10], causing GEC apoptosis. Anti-apoptotic mechanisms induced in H pylori – infected epithelium may ensure persistence of infection and GEC proliferation, leading to carcinogenesis [1,11]. Thus, understanding the mechanisms through which H pylori regulates cell fate is critical for establishing therapeutic approaches to potentially prevent H pylori-induced gastric diseases.

AMP-activated protein kinase (AMPK) is an evolutionarily conserved master regulator of energy metabolism. When facing energy crisis, increased AMP or ADP binds to AMPK's γ regulatory subunit to induce an allosteric conformational change, causing AMPKα catalytic subunit phosphorylation at Thr-172, which leads to AMPK activation [12]. Recent studies indicated an important role for AMPK activation in cell survival. For example, AMPK was shown to activate autophagy via binding and phosphorylation of the autophagy initiation kinase ULK1, Beclin 1, and Vps34 [13–15]. AMPK-activated autophagy generates new cellular components and energy to help cell survival [13–15]. Jeon et al. indentified the antioxidant ability of AMPK, and activated AMPK was shown to inhibit oxidative stress through inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) depletion and facilitating NADPH synthesis [16]. AMPK also activates tuberous sclerosis complex 2 (TSC2) to inhibit mammalian target of rapamycin (mTOR), and

Abbreviations: AMPK, AMP activated protein kinase; ACC, acetyl-CoA carboxylase; GECs, gastric epithelial cells; H pylori, *Helicobacter pylori*; ROS, reactive oxygen species; shRNA, short hairpin RNA; TGF-β, transforming growth factor-β; TAK1, activated kinase 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end.

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promotes cell survival under energy starvation [17,18]. However, whether AMPK could be activated by H pylori, and if so, its role in H pylori-induced GEC apoptosis have not be defined.

In this study, we discovered that AMPK signaling plays an important role in the regulation of the interaction between H pylori and GECs. Our results provide evidence to support that AMPK might be a previously uncharacterized component of the host response to H pylori.

2. Materials and methods

2.1. H pylori culture

H pylori 26695 (ATCC, Manassas, VA) were maintained on blood agar plates (Becton Dickinson and Company, Franklin Lakes, NJ). The bacteria were cultured overnight at 37 °C in Brucella broth (Becton Dickinson) with 10% fetal bovine serum (FBS, Gibco, Shanghai, China) under microaerophilic conditions before infection. H pylori was added to the cells at a multiplicity of infection (MOI) of 100:1 and co-cultured with cell lines for the indicated times.

2.2. GES-1 cell culture

The GES-1 immortalized human GECs were obtained from Shanghai Institute of Biological Science. GES-1 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco), 50 units/ml penicillin and 50 μg per streptomycin in a humidified atmosphere of 5% CO_2. Cultures were trypsinized upon confluence and sub-cultured into 6-well plates for further experiments.

2.3. Human GECs isolation and culture

Gastric biopsy specimens from the antral gastric mucosa were collected from adult patients undergoing esophagogastroduodenoscopy. The antral gastric mucosa were minced and incubated with 10 mM dithiothreitol and 1 mM EDTA at room temperature for 1 h with vigorous shaking. Cells were filtered through 70-μm nylon mesh, pelleted by centrifugation, and washed with PBS. The GECs were isolated, and resuspended in RPMI 1640 containing 20% FBS with antibiotics. The gastric epithelial population was tested by staining cells using an antibody against cytokeratin (Cell Signaling Tech, Shanghai, China), a specific epithelial marker, and flow cytometry. More than 90% of isolated cells were positive for cytokeratin. A total of 5×10^5 cells were plated in 12-well plates, allowed to adhere for 5 h, and then infected with H pylori or with indicated treatments. All patients enrolled provided individual informed consent with Wuxi Third People's Hospital and Nantong University Review Board – approved protocols. The experiments conformed to the principles set out in the Declaration of Helsinki and the NIH Belmont Report.

2.4. Western blots

The lysates (20 μg) from each sample were subjected to electrophoresis through 10% SDS–polyacrylamide gels and transferred onto PVDF membranes (Millipore). The membrane was blocked overnight with 5% nonfat milk at 4 °C and incubated with indicated primary antibody overnight. After three to five washes with TBST, the secondary antibody was added according to standard procedures. The membrane was washed another three times and developed using the ECL system (Amersham Pharmacia, GE Healthcare Bio-Sciences AB, Shanghai, China). Intensity of each band was quantified through ImageJ software, and value was normalized to corresponding loading.

2.5. Antibodies and reagents

Anti-AMPKα1, acetyl-CoA carboxylase (ACC), transforming growth factor-β(TGF-β) activated kinase 1 (TAK1), and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho(p)-AMPKα1 (Thr 172) and p-ACC (Ser 79) were purchased form Cell Signaling Tech. (Denver, MA, USA). Compound C, A-769662 and resveratrol were purchased from Sigma Chemicals (Shanghai, China).

2.6. Flow cytometry detecting Annexin V positive cells

GEC apoptosis was detected by the Annexin V In Situ Cell Apoptosis Detection Kit (Molecular Probes, Shanghai, China) according to the manufacturer's instructions. After treatment, cells were stained with propidium iodide (PI) and Annexin V. Annexin V^+ GECs (apoptotic cells) were recorded through FACS (BD Bioscience).

2.7. TUNEL staining

GEC apoptosis was also detected by the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) In Situ Cell Death Detection Kit (Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. GECs were stained simultaneously with 4′,6′-diamino-2-phenylin-dole (DAPI, Molecular Probes) to visualize the cell nuclei. Cell apoptosis was determined by TUNEL percentage, which was calculated under the confocal microscopy. At least 500 cells in 10 random view in each treatment were counted.

2.8. Histone-DNA enzyme-linked immunosorbent assay (ELISA) assay

GEC apoptosis was further quantified by Histone-DNA ELISA PLUS kit (Roche Applied Science, Shanghai, China) according to the manufacturer's protocol. Briefly, the cytoplasmic Histone/DNA fragments from GECs were extracted and bound to the immobilized anti-Histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was added for the detection of immobilized Histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined using a plate reader at a test wavelength of 400 nm. The OD value was utilized as an quantitative result of cell apoptosis.

2.9. Knockdown of targeted protein by short hairpin RNA (shRNA)

The lentiviral particles containing scramble shRNA or shRNAs of targeted protein were synthesized, verified and provided by Kaiji Biotech (Shanghai, China). The hairpin sequences for AMPKα1 were as follows. 5′-GCAGAAGTTTGTAGGGCAATT-3′(AMPKα1 shRNA-1) [19], 5′-GCATAATAAGTCACAGCCAAA-3′ (AMPKα1 shRNA-2) [20] and 5′CTCCAAGACCAGGAAGTCAT-ACAATAGAA-3′(AMPKα1 shRNA-3) [21]. To silence the expression of TAK1, two human TAK1 shRNA sequences were chosen: 5′-GAGGAAAGCGTTTATTGTATT-3′(TAK1-shRNA-1) [22] and 5′-CCCAATGGCTTATCTTACATT-3′(TAK1-shRNA-2) [22]. GECs were seeded in a 6-well plate in growth medium with 50% confluence. The lentiviral particles (20 μl/ml) were added to the cells, after 12 h, the lentiviral particles containing medium was replaced by cell culture medium, and cells were further cultured for another 24 h. Puromycin (Sigma, 0.25 μg/ml) was

added to select resistant stable colonies. The expression of target protein in stable cells was tested by Western blots.

2.10. AMPKα overexpression

AMPKα1 expression pcDNA3-EGFP vector was gift from Dr. Dong [23]. The plasmid was amplified. For transfection, GEC-1 cells were cultured in antibiotic- and serum-free medium with 50–60% confluence, AMPKα1 plasmid (1 μg/ml) or the empty vector (1 μg/ml) was transfected into GEC-1 cells with Lipofectamine™ 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. 48 h after transfection, AMPKα1 expression in transfected cells was examined by Western blots. We make sure that more than 60% of green fluorescence cells was seen after each transfection.

2.11. Statistical analysis

All statistical analyses were performed using SPSS statistical software (SPSS version 16.0, Chicago, USA). The values were presented as the mean ± SD of three independent experiments. The result's comparisons among multiple groups were performed using analysis of variance (ANOVA) followed by Student's *t*-tests. $^{*}P < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of H Pylori-stimulated AMPK kinase activity increases apoptosis in GECs

We first tested the effect of H Pylori on AMPK activation in cultured GECs. As shown in Fig. 1A, H Pylori induced phosphorylation of AMPKα1 (Thr 172) and its downstream effector ACC (Ser 79) in GEC-1 cells, which confirmed AMPK activation. As expected, compound C (CC), the AMPK kinase inhibitor, blocked H Pylori-induced AMPK activation. However, the expected increase in apoptosis-initiated by H pylori in GEC-1 cells was further enhanced by compound C, which was detected by Annexin V FACS assay (Fig. 1B), Histone DNA ELISA assay (Fig. 1C) and TUNEL staining assay (Fig. 1D). Similarly, in primary human GEC-1 cells, compound C blocked H Pylori-stimulated AMPK activation (AMPKα1/ACC phosphorylation) (Fig. 1E), while aggravating cell apoptosis (Fig. 1F). Due to the limitation of sources, all primary GECs were only tested for Annexin V FACS assay to examine cell apoptosis. Taken together, the results indicate that inhibition of H Pylori-stimulated AMPK kinase activity could enhance GEC apoptosis.

3.2. Suppression of AMPK expression promotes apoptosis in H pylori-infected GECs

To further investigate a potential role for AMPK in suppressing H pylori-induced apoptosis, we used shRNAs directed against AMPKα1 cDNA to knockdown its expression in GEC-1 cells. Western blot results in Fig. 2A showed that three non-overlapping AMPKα1 shRNAs (−1, −2 and −3) all reduced AMPKα1 expression in GEC-1 cells. Of which, AMPKα1-shRNA-2 and AMPKα1-shRNA-3 showed much higher efficiency, resulting over 90% knockdown activity (Fig. 2A). Consistent with the results of experiments in which AMPK kinase activity was inhibited, loss of AMPKα1 expression by targeted-shRNAs (−2/−3) significantly enhanced H pylori-stimulated apoptosis in GEC-1 cells, compared with non-transfection control ("Trans") group or non-targeting scramble shRNA transfection group (Fig. 2B and C). Similar results were also observed in primary human GECs, and suppression of AMPKα1

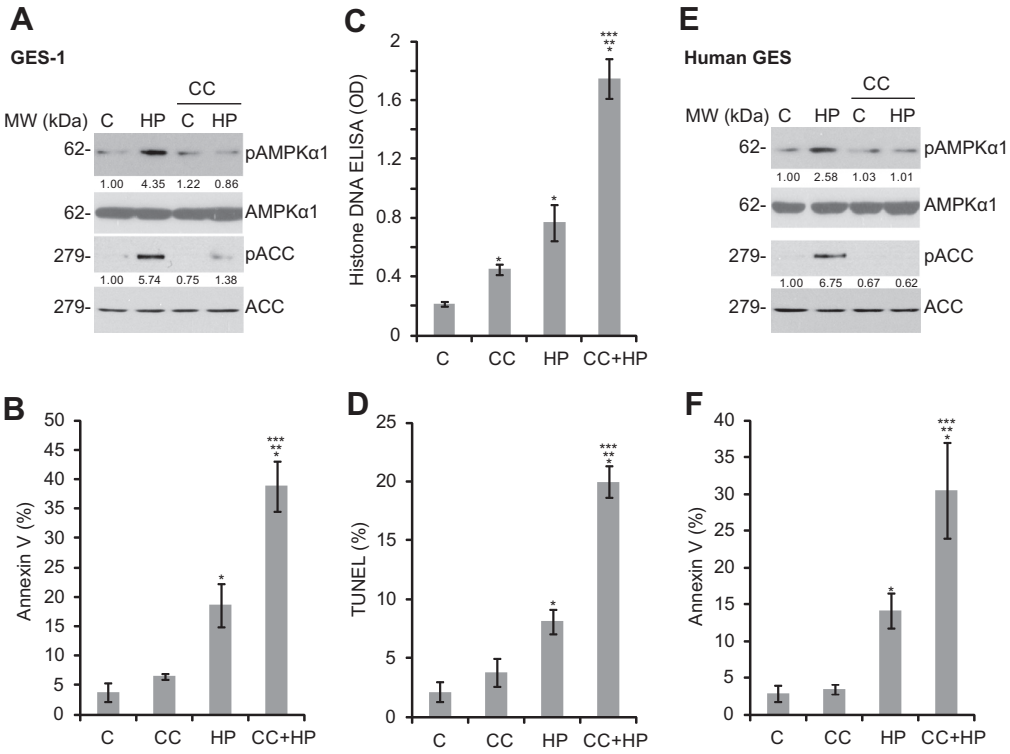

Fig. 1. Inhibition of H Pylori-stimulated AMPK kinase activity enhances apoptosis in GECs-GEC-1 cells or primary human GECs were infected with the H pylori (HP, bacteria: cells = 100:1) for 90 min (A, E) or 24 h (B–D, E), pretreated for 30 min with compound C (CC, 10 μM) or not pretreated. Phospho- or regular AMPK and ACC were tested by Western blots (A and E); Cell apoptosis was tested by Annexin V FACS assay (B and F), Histone DNA ELISA assay (C) and TUNEL staining assay (D). Data in this and subsequent figures are representative of at least 3 separate experiments. "C" stands for cell alone. AMPK and ACC phosphorylation was quantified and normalized to "C" group (A and E). $^{*}P < 0.05$ *vs.* "C", $^{**}P < 0.05$ *vs.* CC alone, $^{***}P < 0.05$ *vs.* HP alone.

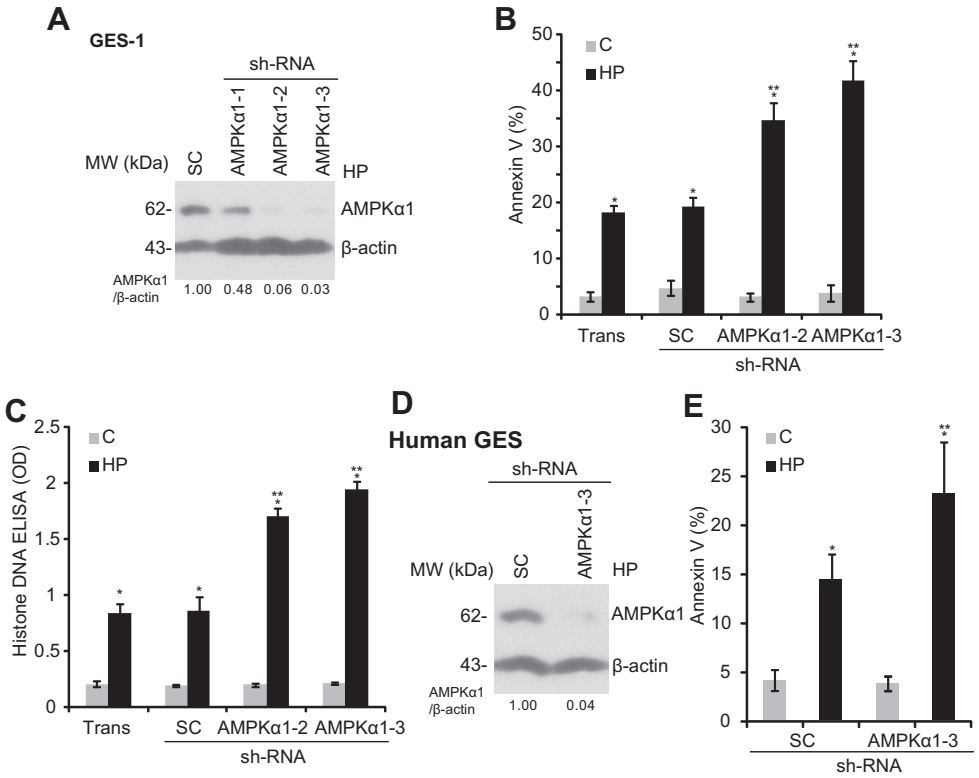

Fig. 2. Suppression of AMPK expression promotes apoptosis in H pylori-infected GECs-Stable GEC-1 cells or primary human GECs infected with scramble shRNA (SC) or AMPKα1 shRNA (−1/−2/−3) lentiviral particles (20 μl/ml) were co-cultured with H pylori (HP, bacteria: cells = 100:1) for 24 h, AMPKα1 expression was analyzed by Western blots, an anti-β-actin antibody was used as the loading control (A and D); Cell apoptosis was detected by Annexin V FACS assay (B and E) and Histone-DNA ELISA assay (C). "C" stands for cell alone. AMPK expression (*vs.* β-actin) was quantified. $^{*}P < 0.05$ *vs.* "C" (no HP infection), $^{**}P < 0.05$ *vs.* scramble shRNA group.

expression (Fig. 2D) increased apoptosis by H pylori (Fig. 2E). Taken together, these results suggest that AMPK expression is important for GEC survival after H pylori infection.

3.3. Increasing AMPK activation or expression inhibits H pylori-induced apoptosis in GECs

Next, we tested the effect of AMPK activators on H pylori-induced GEC apoptosis. As shown in Fig. 3A, A–769662 [24] and resveratrol [25], two known AMPK activators, further enhanced H pylori-induced AMPK activation in GEC-1 cells. As a consequence, H pylori-induced cell apoptosis, detected by Annexin V FACS assay (Fig. 3B) and Histone-DNA ELISA assay (Fig. 3C), was inhibited by A-769662 and resveratrol. Meanwhile, over-expression of AMPKα1 (Fig. 3D) inhibited apoptosis by H pylori (Fig. 3E and F). In consistent with these findings, we found that both A-769662 and resveratrol inhibited H pylori-induced apoptosis in primary human GECs (Fig. 3G). Taken together, these results demonstrate that AMPK activators or AMPKα1 over-expression inhibits H pylori-induced apoptosis in GECs.

3.4. TAK1 mediates AMPK activation and cell survival in H pylori-infected GECs

Next we studied the potential upstream kinase for AMPK activation by H pylori in GECs. TAK1, a member of the mitogen-activated protein kinase (MAPK) family, is involved in various biological responses, and can be activated by H pylori [26,27]. Recent studies have indicated that TAK1 could activate AMPK [28–30]. Next, we examined the role of TAK1 in H pylori-induced AMPK activation and apoptosis in GECs. We again utilized TAK1 shRNAs (−1/−2) to selectively knockdown TAK1 in GEC-1 cells (Fig. 4A). Results showed that TAK1 knockdown dramatically inhibited H pylori-induced AMPK activation in GEC-1 cells (Fig. 4A). As a result, cell apoptosis by H pylori was increased (Fig. 4B). Similar results were also seen in primary human GECs (Fig. 4C and D). Thus, these results suggest that TAK1 might be the upstream kinase to mediate AMPK activation and cell survival in H pylori-infected GECs.

4. Discussions

H pylori infection remains one main risk factor in the development of chronic gastritis, peptic ulceration, and gastric carcinoma [1,31,32]. The host GECs play a key role in H pylori – mediated pathogenesis and disease manifestations. Imbalances between cell apoptosis and proliferation may lead to peptic ulcer and gastric carcinogenesis [32]. As well as the numerous known metabolic targets of AMPK, it is becoming increasingly clear that AMPK has many other downstream signaling effectors to regulate gene transcription, cell mitosis, cell survival, apoptosis and autophagy [33,34]. In this study, our data showed that AMPK activation might be a key regulatory step in promoting an anti-apoptotic response in GECs infected with H pylori. Inhibition of AMPK by its kinase inhibitor (Compound C) or targeted shRNAs enhanced cell apoptosis by H pylori, while forced activation of AMPK by its activators (A769662 and resveratrol), or by over-expression of AMPKα1, promoted survival of H pylori-infected GECs. We thus suggest that activation of AMPK might be a previously uncharacterized "molecular switch" determining the survival response of H pylori-infected GECs.

Elucidating the upstream signal kinase for AMPK activation by H pylori is important for understanding the cellular responses induced by H pylori. Depending on the type of stimuli, different

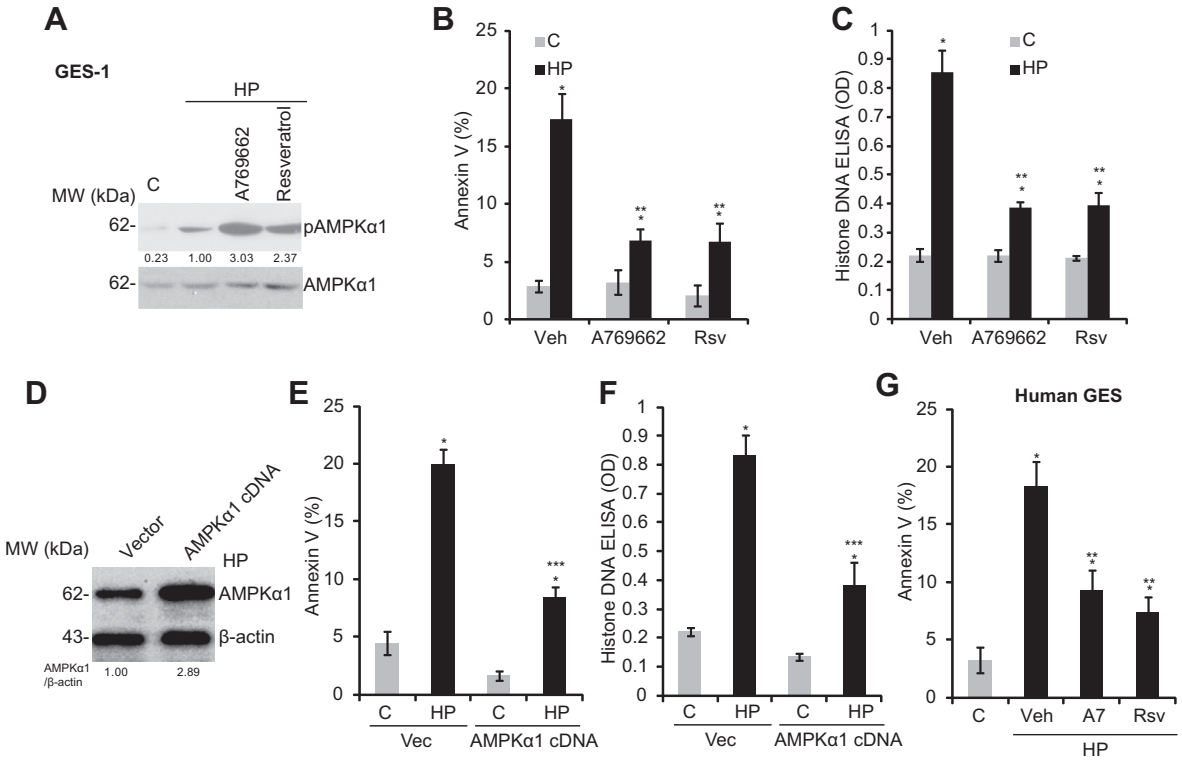

Fig. 3. Increasing AMPK activation or expression inhibits H pylori-induced apoptosis in GECs-GEC-1 cells were infected with the H pylori (HP, bacteria: cells = 100:1) for 90 min (A) or 24 h (B and C), pretreated for 30 min with A-769662 (A769662, 10 μM), resveratrol (Rsv, 50 μM) or not pretreated. Phospho- or regular AMPK were tested by Western blots (A), cell apoptosis was tested by Annexin V FACS assay (B) and Histone-DNA ELISA assay (C). GEC-1 cells transfected with 1 μg/ml of AMPKα1 cDNA or empty vector (Vec, pcDNA3-EGFP) were infected with the H pylori (HP, bacteria: cells = 100:1) for 24 h, AMPK and β-actin were tested (D), cell apoptosis was tested by Annexin V FACS assay (E) and Histone-DNA ELISA assay (F). Human GECs were infected with the H pylori (HP, bacteria: cells = 100:1) for 24 h, pretreated for 30 min with A-769662 (A7, 10 μM), resveratrol (Rsv, 10 μM) or not pretreated, cell apoptosis was tested by Annexin V FACS assay (G). "C" stands for cell alone. "Veh" stands for DMSO control. AMPK phosphorylation or expression (*vs.* β-actin) was quantified. $^{*}P < 0.05$ *vs.* "C" (no HP infection), $^{**}P < 0.05$ *vs.* "Veh" group. $^{***}P < 0.05$ *vs.* Vec group.

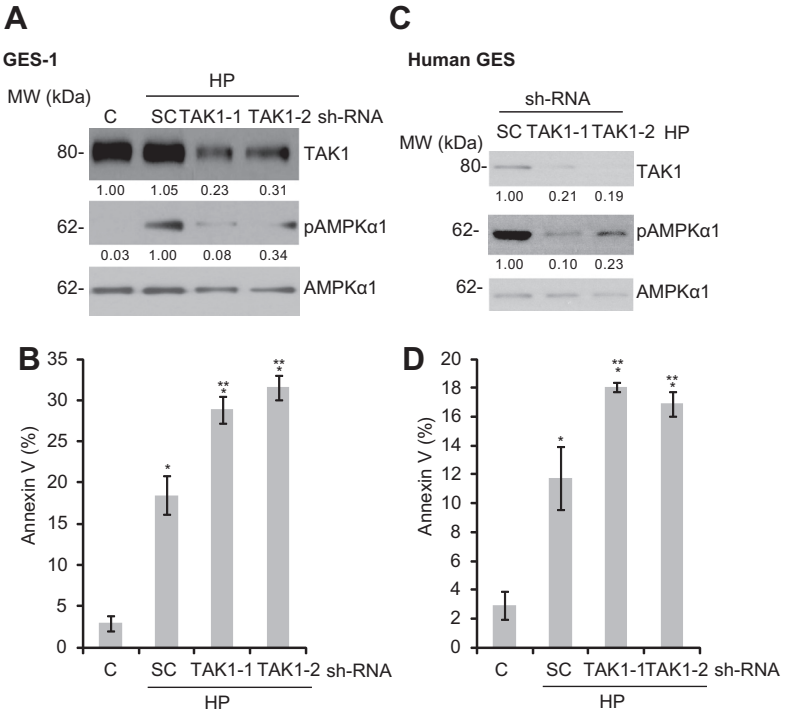

Fig. 4. TAK1 mediates AMPK activation and cell survival in H pylori-infected GECs-Stable GEC-1 cells or primary human GECs infected with scramble shRNA (SC) or TAK1 shRNAs (−1/−2) lentiviral particles (20 μl/ml) were infected with *H pylori* for 90 min (A and C) or 24 h (B and D), expression of indicated proteins was analyzed by Western blots (A and C); Apoptosis was detected through Annexin V FACS assay (B and D). AMPK phosphorylation and TAK1 expression (*vs.* AMPKα1) were quantified. "C" stands for cell alone. $^{*}P < 0.05$ *vs.* "C" (no HP infection), $^{**}P < 0.05$ *vs.* "SC" group.

studies have identified several potential upstream kinases for AMPK [35–37], including the best characterized LKB1 as well as ataxia telangiectasia mutated (ATM), Ca^{2+}/Calmodulin-dependent Kinase Kinase-β (CaMKK-β) [38] and TAK1 [28,29]. Here we provided evidence to support that TAK1 might be the upstream signal for AMPK activation by H pylori in GECs. We found TAK1 knockdown by shRNAs significantly inhibited H pylori-induced AMPK activation and GEC cell survival. These data are consistent with findings showing that AMPK activation by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) depends on TAK1 and TAK1-binding subunit 2, but not LKB1 or CaMKK-β [29]. Further, TAK1 directly associates and phosphorylates AMPK in H_2O_2-treated cardiomyocytes [28]. Since TAK1 was shown to be activated by H pylori [26,27], we thus propose that TAK1 activation mediates AMPK activation in H pylori-infected GECs.

In summary, we have shown that apoptosis associated with H pylori infection is negatively regulated by TAK1-AMPK signaling. These results suggest that AMPK may play an important role in the pathogenesis associated with H pylori infection.

Conflict of interests

No conflict of interests were stated by authors.

Acknowledgments

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