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Ampelopsin-induced reactive oxygen species enhance the apoptosis of colon cancer cells by activating endoplasmic reticulum stress-mediated AMPK/MAPK/XAF1 signaling

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Abstract. Ampelopsin (Amp) is bioactive natural product and exerts anti-cancer effects against several cancer types. The present study investigated the anti-colon cancer activity of Amp and explored its mechanism of action. The treatment of colon cancer cells with Amp resulted in the dose- and time-dependent induction of apoptosis via the activation of endoplasmic reticulum (ER) stress, 5' adenosine monophosphate-activated protein kinase (AMPK), and c-Jun N-terminal protein kinase (JNK)/p38 mitogen-activated protein kinases (MAPKs). Salubrinal, an ER stress inhibitor, prevented the upregulation of ER stress-associated proteins, including phosphorylated protein kinase RNA-like ER kinase, phosphorylated eukaryotic translation initiation factor 2α, glucose-regulated protein 78, and CCAAT/enhancer-binding protein homologous protein, as well as suppressing AMPK activation and the MAPK signaling pathway. Knockdown of AMPK by RNA interference failed to block ER stress. Additionally, SP600125 (a JNK inhibitor) and SB203580 (a p38-MAPK inhibitor) effectively inhibited apoptosis and attenuated the expression of X-linked IAP-associated factor 1 (XAF1) and apoptotic Bcl-2 family proteins (BCL2 antagonist/killer 1 and BCL2-associated X protein) in Amp-treated colon cancer cells. Furthermore, reactive oxygen species (ROS)-mediated ER stress/AMPK apoptotic signaling pathway in Amp-treated colon cancer cells were markedly inhibited by treatment with N-acetyl-L-cysteine, a ROS scavenger. These results demonstrate that treatment with Amp induces the apoptotic death of colon cancer cells through ER stress-initiated AMPK/MAPK/XAF1 signaling. These results also provide experimental information for developing Amp as therapeutic drug against colon cancer.

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Introduction

5' Adenosine monophosphate-activated protein kinase (AMPK) is a master regulator of cellular energy metabolism in normal and transformed cells (1,2). Activation of AMPK suppresses adenosine triphosphate (ATP)-dependent metabolic functions, and induces ATP generation via glycolysis and fatty acid oxidation (3). AMPK is activated by cellular stress, including glucose deprivation, hypoxia, and ischemia. During carcinogenesis and tumor progression, cancer cells generate and promote metabolically strained microenvironments (4,5). The phosphorylation of AMPK by the tumor suppressor serine/threonine kinase 11 (liver kinase B1) regulates cell growth and proliferation in response to stress (6). AMPK also supports the growth of aggressive orthotopic tumors prepared from MDA-MB-231 and DU4475 breast cancer cells (7); however, paradoxically, activation of AMPK promotes cellular apoptosis through the downstream signal mediators c-Jun N-terminal protein kinase (JNK) and p53 (8,9). Treatment of HCT-116 colon cancer cells with quercetin induces apoptosis under hypoxic conditions by suppressing AMPK activity (10), whereas activation of AMPK by plumbagin inhibits HT-29 colon cancer cell growth via the JNK-p53 signal axis (11). Based on the aforementioned results, the role of AMPK in colon cancer remains controversial.

The endoplasmic reticulum (ER) is the principal organelle responsible for protein synthesis and folding. It also serves as a Ca^{2+} reservoir. Conditions of cellular stress, such as hypoxia or exposure to chemotherapeutic drugs, change the redox state of proteins as well as the Ca^{2+} concentration in the ER, resulting in the induction of the unfolded protein response (UPR) and the release of Ca^{2+} from the ER (12,13). Ca^{2+} leakage from the ER into other cellular compartments stimulates autophagy through Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ) and subsequent activation of AMPK (14,15). In addition, the activation of AMPK by metformin triggers ER

stress via an AMPK-dependent mechanism in acute lymphoblastic leukemia (16). The induction of AMPK activity by metformin also mediates the anti-cancer effect of dasatinib in head and neck squamous cell carcinoma by activating AMPK-dependent ER stress (17). Despite supporting evidence that patients with metabolic syndrome exhibit decreased AMPK activity and increased cancer-associated mortality (18), the association between AMPK activity and ER stress in chemotherapy-treated colon cancer cells remains unclear.

Numerous types of cancer aberrantly express inhibitors of apoptosis (IAPs) (19). X-linked IAP (XIAP)-associated factor 1 (XAF1), a novel antagonist of XIAP (20), interacts with endogenous XIAP to suppress its anti-caspase activity (21). XAF1 expression is not only associated with colon cancer cell survival, but is also upregulated by various anti-cancer therapies (22-24). However, the connection between XAF1, AMPK, and ER stress in colon cancer remains to be elucidated.

Ampelopsin (Amp), which is extracted from *Ampelopsis grossedentata*, is reported to have various biological functions, including anti-oxidative (25) and hepatoprotective effects (26). This flavonoid, also called dihydromyricetin, has potent anti-cancer activities in several types of malignancy (27,28). For example, treatment of breast cancer cells with Amp decreases cell viability and induces apoptosis in a dose-dependent manner through the generation of reactive oxygen species (ROS) and induction of ER stress (29). In contradiction, another previous study reported that Amp-induced ER stress leads to the activation of autophagy, which protects breast cancer cells from apoptosis (30). In addition, treatment with Amp protects human umbilical vein endothelial cells from hyperglycemia-induced cell damage by upregulating autophagy via the enhancement of AMPK activity (31). Based on these reports, the effects of Amp on cancer cells are controversial.

In the present study, the chemotherapeutic effect of Amp on colon cancer cells was evaluated, and the relationship between ER stress, AMPK activation and downstream signaling was determined. In addition, the effects of AMPK activation or ER stress responses on the induction of XAF1 in Amp-treated colon cancer cells were examined.

Materials and methods

Cell culture. The human colon cancer cell lines HCT-116, HCT-8 and HT-29 were purchased from ATCC (Manassas, VA, USA). These cells were maintained in RPMI-1640 medium (Corning Inc., Corning, NY, USA), supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), antibiotics and glutamine (HyClone; GE Healthcare Life Sciences) at 37˚C in a humidified atmosphere with 5% CO_2.

Drugs and chemicals. Amp and N-acetyl-L-cysteine (NAC; a ROS scavenger) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Amp was dissolved in sterile dimethyl sulfoxide (DMSO) as a 200-mM stock solution, and diluted in medium to the indicated concentrations prior to use. Salubrinal (an ER stress inhibitor), SB203580 [a p38 mitogen-activated protein kinase (MAPK) inhibitor] and SP600125 (a JNK inhibitor) were purchased from Calbiochem (EMD Millipore, Billerica, MA, USA).

Analysis of apoptotic cells by flow cytometry. The percentages of human colon cancer HCT-116, HCT-8 or HT-29 cells undergoing apoptosis were determined by flow cytometry using fluorescein isothiocyanate (FITC)-labeled Annexin V and 7-aminoactinomycin D (7-AAD). To determine optimal conditions, experiments were performed using different concentrations of Amp (0, 10, 20, 50, 100, and 200 μM) and different periods of incubation (2, 4, 8, 16 and 24 h). For comparison, DMSO (0.05%) was used as a vehicle control and the negative group represented untreated cells. These cells were maintained in RPMI-1640 medium (Corning Inc.), supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences), antibiotics and glutamine (HyClone; GE Healthcare Life Sciences) at 37˚C in a humidified atmosphere with 5% CO_2. For staining, cells were harvested, rinsed with PBS, and resuspended in 100 μl 1X Annexin V binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$]. Following resuspension, 3 μl Annexin V-FITC and 3 μl of 7-AAD (both purchased from BD Biosciences, San Diego, CA, USA) were added, and the cells were incubated at room temperature for 15 min in the dark with gentle vortexing. The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuestpro software version 5.1 (BD Biosciences).

Measurement of mitochondrial membrane potential ($\Delta\psi_m$) and intracellular ROS production. The changes in $\Delta\psi_m$ were assessed using 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were treated with Amp or DMSO for 24 h, harvested, washed twice in PBS, resuspended in PBS supplemented with $DiOC_6$ (20 nM), incubated at 37˚C for 15 min in the dark, and immediately analyzed with a flow cytometer using the FL-1 filter. The intracellular accumulation of ROS was monitored by flow cytometry after staining with the fluorescent probe 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA; 10 μM; Molecular Probes; Thermo Fisher Scientific, Inc.) as previously described (32), with slight modification. DCFH-DA is deacetylated in cells by esterases to a nonfluorescent compound, DCFH, which remains trapped within the cell and is cleaved and oxidized by ROS in the presence of endogenous peroxidase to yield a highly fluorescent compound, 2',7'-dichlorofluorescein (DCF). Cells were pre-incubated with 10 μM DCFH-DA for 30 min at 37˚C, seeded in 6-well plates ($5x10^5$ cells/ml), and treated with or without Amp for 24 h. Cells were then washed, resuspended in PBS, and ROS levels were determined using a FACSCalibur flow cytometer.

Western blot analysis. Cells were washed in PBS and lysed in NP-40 buffer (Elpis Biotech, Daejeon, Korea) supplemented with a protease inhibitor cocktail (Sigma-Aldrich; Merck Millipore). To evaluate phosphorylation events, phosphatase inhibitors (Cocktail II; Sigma-Aldrich; Merck Millipore) were added to the NP-40 buffer. The protein concentration was determined using a Pierce BCA Assay kit (Thermo Fisher Scientific, Inc.). Proteins (10 μg/sample) were resolved through SDS-PAGE and then transferred to nitrocellulose membranes (EMD Millipore). The membranes were blocked

with 5% skim milk for 1 h at room temperature and then western blot analysis was performed. Following primary and secondary antibody staining, chemiluminescence was detected using an ECL kit (Advansta Corp., Menlo Park, CA, USA) and the LAS-3000 imaging system (Fujifilm, Tokyo, Japan). Primary antibodies against the following antigens were used: Caspase-8 (pro-caspase-8 and active caspase-8; cat. no. 9746; 1:1,000), caspase-3 (pro-caspase-3 and active caspase-3; cat. no. 9665; 1:1,000), caspase-9 (pro-caspase-9 and active caspase-9; cat. no. 9502; 1:1,000), poly ADP-ribose polymerase (PARP; cleaved PARP p89; cat. no. 9542; 1:1,000), β-actin (cat. no. 4967; 1:1,000), Bcl-2 (cat. no. 4967; 1:1,000), BCL2-associated X protein (Bax; cat. no. 2772; 1:1,000), myeloid cell leukemia sequence 1 (BCL2-related; Mcl-1; cat. no. 4572; 1:1,000), BCL2 antagonist/killer 1 (Bak; cat. no. 6947; 1:1,000), X-linked inhibitor of apoptosis protein (XIAP; cat. no. 2045; 1:1,000), phospho (p-)AMPK (Thr^{174}; cat. no. 2531; 1:1,000), AMPK (cat. no. 5832; 1:1,000), eukaryotic translation initiation factor 2α (eIF2α; cat. no. 9722; 1:1,000), p-eIF2α (Ser^{51}; cat. no. 9721; 1:1,000), p-JNK (Thr^{183}/Tyr^{185}; cat. no. 4671; 1:1,000), JNK (cat. no. 9258; 1:1,000), p-p38-MAPK (Thr^{180}/Tyr^{182}; cat. no. 9211; 1:1,000), p38-MAPK (cat. no. 9212; 1:1,000), extracellular signal-regulated kinase 1/2 (ERK1/2; cat. no. 9102; 1:1,000), and p-ERK1/2 (Thr^{202}/Tyr^{204}; cat. no. 9101; 1:1,000; all from Cell Signaling Technology, Inc., Beverly, MA, USA); protein kinase RNA-like ER kinase (PERK; cat. no. sc-13073; 1:500), p-PERK (Thr^{981}; cat. no. sc-32577; 1:500), glucose-regulated protein 78 (GRP78; cat. no. sc-1051; 1:500), and CCAAT/enhancer-binding protein homologous protein (CHOP; cat. no. sc-575; 1:200; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); and XIAP-associated factor 1 (XAF1; cat. no. 81353; 1:500; Abcam, Cambridge, UK). The membrane was probed with primary antibodies overnight at 4˚C, followed by the following specific secondary antibodies; horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; cat. no. K0211589; 1:3,000) or HRP-conjugated goat anti-rabbit IgG (cat. no. K0211708; 1:3,000; both from KOMABiotech, Seoul, Korea) for 1 h at room temperature.

Small interfering RNA (siRNA) transfection. Experimentally verified human AMPK-siRNA duplex (cat. no. 1121714; GCA UAU GCU GCA GGU AGA UdTdT) and negative control-siRNA (cat. no. SN-1003) were obtained from Bioneer Corporation (Daejeon, Korea). Cells were seeded at a concentration of $1x10^5$ per well in a 6-well plate and grown overnight. Cells were treated with 100 μM Amp for 6 h and then transfected with 200 nM siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were used for further experiments at 36 h after transfection.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analyses were conducted using one-way analysis of variance (ANOVA) using SigmaPlot software (version 10.0; Systat Software, Inc., San Jose, CA, USA). Bonferroni post hoc analysis was performed following one-way ANOVA for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Amp treatment of colon cancer cells enhances the activation of ER stress and the AMPK signaling pathway. It was first investigated whether Amp has an anti-colon cancer effect. Annexin V/7-AAD staining and $DiOC_6$ staining assays were adopted to measure cytotoxicity and $\Delta\psi_m$, respectively, in Amp-treated colon cancer cells. Following treatment with Amp, dose- and time-dependent induction of apoptosis was observed in HCT-116 colon cancer cells (Fig. 1A and B; $P<0.05$; DMSO-treated group vs. Amp-treated group). In addition, Amp treatment disrupted the $\Delta\psi_m$ of HCT-116 colon cancer cells in a dose- and time-dependent manner (Fig. 1A and B; $P<0.05$; DMSO-treated group vs. Amp-treated group). The treatment of colon cancer cells (HCT-116, HCT-8, and HT-29) with Amp also induced the cleavage and activation of caspase-9, caspase-3, and PARP, but not of caspase-8 (Fig. 1C). These results suggested that Amp-induced apoptosis is required for the mitochondrial apoptosis pathway to initiate and for caspases to activate.

Subsequently, the association of ER stress with Amp-induced colon cancer cell death was investigated. Amp-treated colon cancer cells exhibited upregulated expression of ER stress-associated proteins, including p-PERK, p-eIF2α, GRP78, and CHOP (Fig. 1D). It was also examined whether the apoptotic effect of Amp on colon cancer cells was associated with AMPK or XAF1 expression. Expression of p-AMPK and XAF1 in colon cancer cells was increased following treatment with 100 μM Amp for 24 h (Fig. 1E). The expression of Bcl-2 family proteins in colon cancer cells was also modulated by Amp treatment: Bcl-2 and Mcl-1 were easily detectable in untreated and vehicle-treated colon cancer cells, but markedly reduced following Amp treatment. In addition, the expression of Bak and Bax was induced in Amp-treated colon cancer cells (Fig. 1E). These results suggest that the cell death of Amp-treated colon cancer cells is closely associated with ER stress-mediated apoptosis and changes in AMPK-mediated signaling.

ER stress-dependent signaling directs Amp-induced apoptosis in colon cancer cells. To investigate the role of ER stress in Amp-induced apoptosis, colon cancer cells were pre-treated with salubrinal (a selective inhibitor of eIF2α) prior to Amp treatment. Pretreatment with salubrinal markedly inhibited the expression of p-PERK, p-eIF2α, GRP78, and CHOP in Amp-treated colon cancer cells (Fig. 2A). Amp-induced cleavage of caspase-9, caspase-3, and PARP were also attenuated by pretreatment with salubrinal in colon cancer cells (Fig. 2B). As ER stress activates JNK and p38-MAPK (33,34), the effect of salubrinal pretreatment on these kinases was assessed. Amp treatment of colon cancer cells downregulated the phosphorylation of ERK, whereas it upregulated the phosphorylation of JNK and p38-MAPK (Fig. 2C). Salubrinal pretreatment of colon cancer cells also effectively attenuated Amp-induced phosphorylation of JNK and p38-MAPK (Fig. 2D). Furthermore, inhibition of ER stress with salubrinal markedly reduced the Amp-dependent phosphorylation of AMPK and expression of XAF1 in colon cancer cells (Fig. 2D). These results indicate that ER stress-dependent AMPK/MAPK signaling is important for Amp-mediated apoptosis in colon cancer cells.

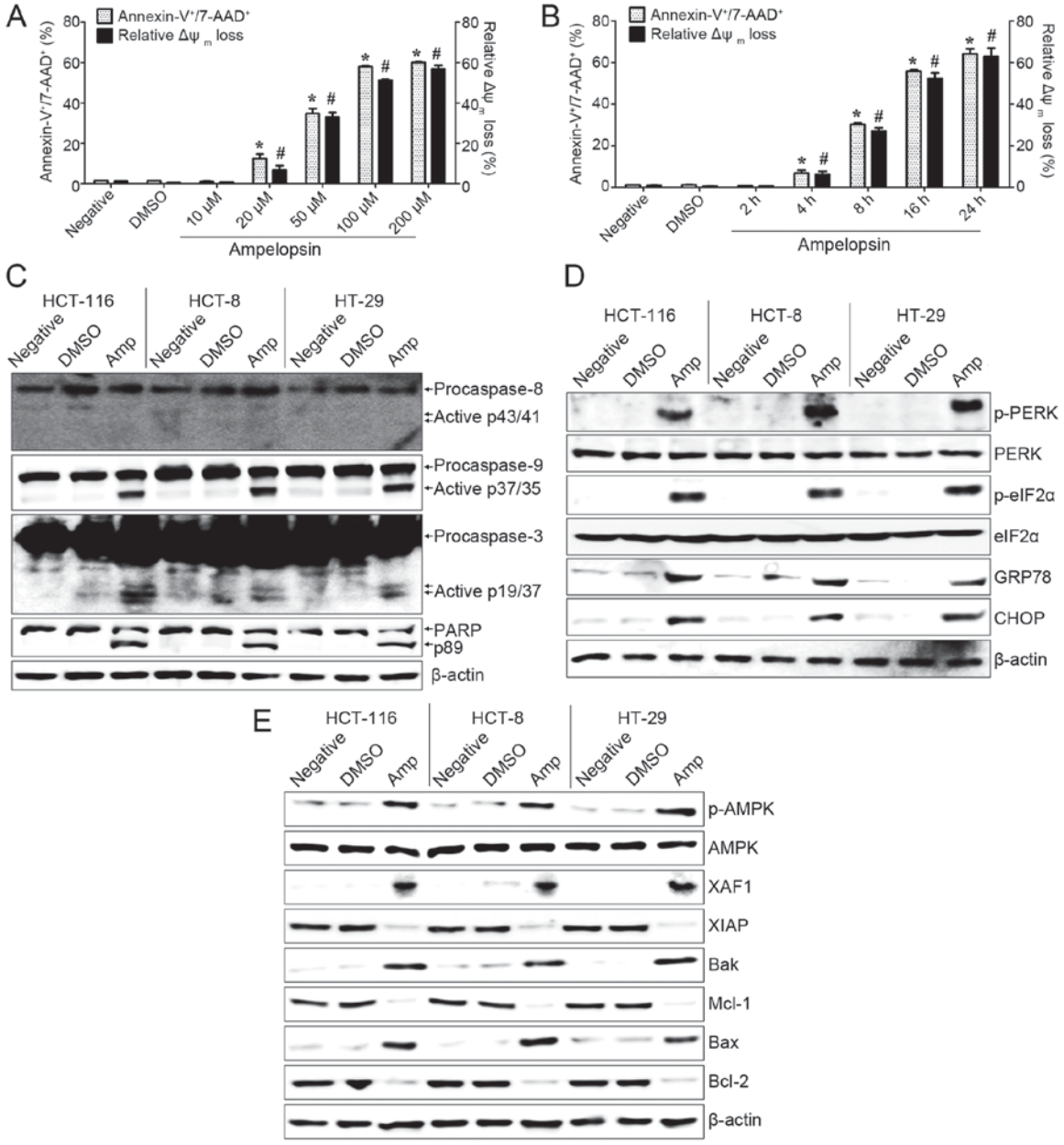

Figure 1. Amp induces apoptosis and enhances the activation of ER stress and AMPK signaling in colon cancer cells. (A) HCT-116 cells were treated with 10, 20, 50, 100, or 200 μM of Amp for 24 h. (B) HCT-116 cells were treated with 100 μM of Amp for 2, 4, 8, 16, or 24 h. To detect the degree of apoptosis, cells were stained Annexin V-fluorescein isothiocyanate and 7-AAD and analyzed by flow cytometry. The percentage of apoptotic cells signifies Annexin $V^+/7\text{-}AAD^+$. To measure disruption of $\Delta\psi_m$, cells were stained with DiOC6. Diminished DiOC6 fluorescence (%) indicates $\Delta\psi_m$ disruption. Each value is presented as the mean ± standard deviation of three determinations. (C-E) Cells were treated with 100 μM Amp for 24 h. Total cell lysates were western blotted with antibodies against (C) caspase-8 [pro-caspase-8 and active caspase-8 (p43/41)], caspase-9 [pro-caspase-9 and active caspase-9 (p37/35)], caspase-3 [pro-caspase-3 and active caspase-3 (p19/17)], or PARP [(PARP and cleaved PARP (p89)]; (D) p-PERK, PERK, p-eIF2α, eIF2α, CHOP, or GRP78; and (E) p-AMPK, AMPK, XAF1, XIAP, Bak, Bcl-2, Bax, or Mcl-1. β-actin was used as a loading control. The results are representative of three independent experiments. Untreated cells (negative) and vehicle-treated cells (DMSO) were used as the control groups. $^*P<0.05$ and $^\#P<0.01$, DMSO-treated cells vs. Amp-treated cells. Amp, ampelopsin; ER, endoplasmic reticulum; AMPK, 5' adenosine monophosphate-activated protein kinase; 7-AAD, 7-aminoactinomycin D; $\Delta\psi_m$, mitochondrial membrane potential; PARP, poly ADP-ribose polymerase; p-, phosphorylated; PERK, protein kinase RNA-like ER kinase; eIF2α, eukaryotic translation initiation factor 2α; CHOP, CCAAT/enhancer-binding protein homologous protein; GRP78, glucose-regulated protein 78; XAF1, XIAP-associated factor 1; XIAP, X-linked inhibitor of apoptosis protein; Bak, BCL2 antagonist/killer 1; Bax, BCL2-associated X protein; Mcl-1, myeloid cell leukemia sequence 1 (BCL2-related); DMSO, dimethyl sulfoxide.

ER stress triggers AMPK/MAPK-dependent apoptosis signaling in Amp-treated colon cancer cells. Expression of AMPK was downregulated by siRNA to study the association with ER stress in the Amp-induced apoptosis of colon cancer cells. Although AMPK-knockdown in HCT-116 and HT-29 cells produced no change in the Amp-dependent induction of the ER stress-related proteins p-PERK and CHOP, the levels of XAF1, p-p38, and p-JNK were markedly reduced in the two cell lines (Fig. 3A). AMPK knockdown also noticeably suppressed the activation (cleavage) of caspase-9, caspase-3 and PARP (Fig. 3B). In addition, downregulation of AMPK with siRNA in HCT-116 and HT-29 colon cancer cells reduced the Amp-induced expression of the apoptotic Bcl-2 family proteins Bak and Bax, while enhancing the expression of

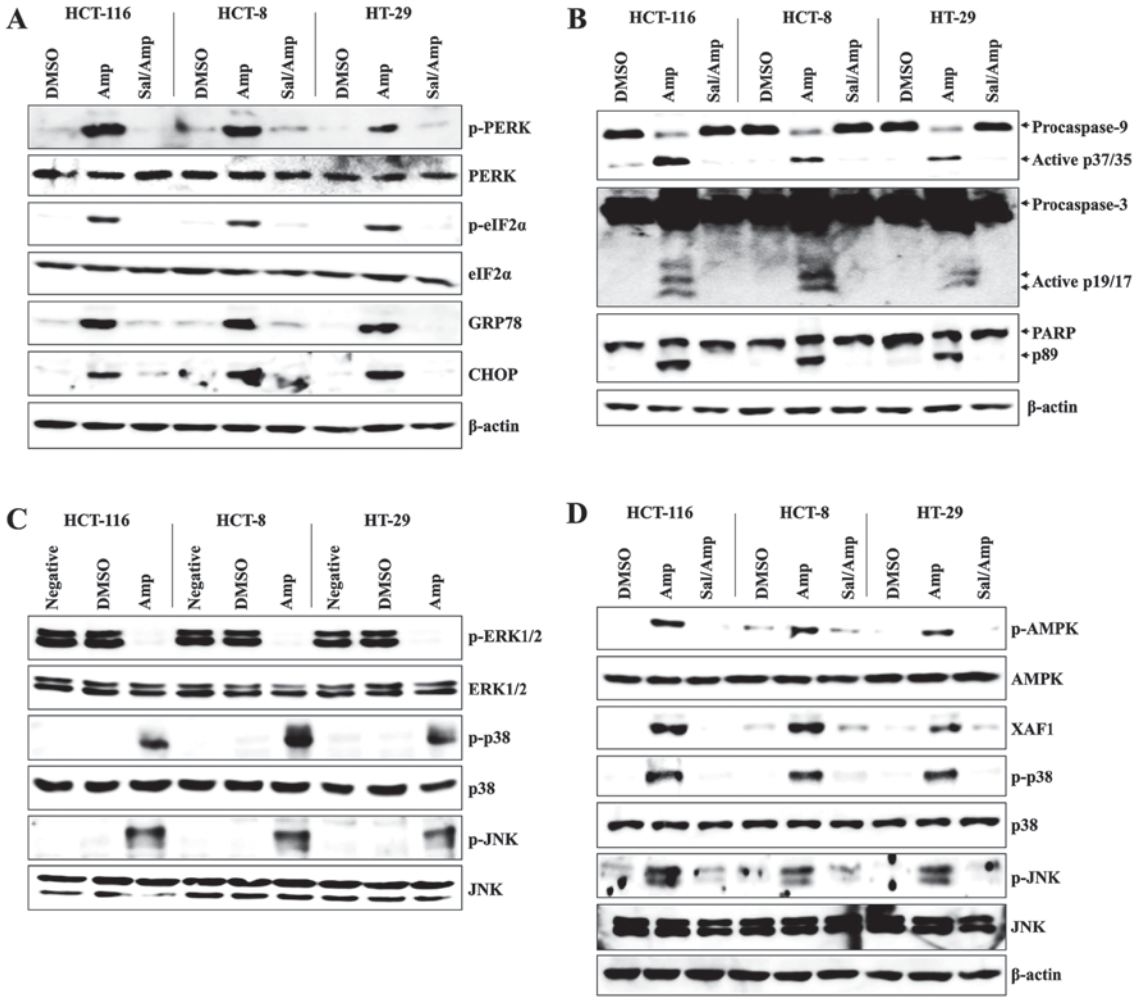

Figure 2. ER stress signaling is closely related to Amp-induced apoptosis of colon cancer cells. (A, B and D) Cells were pretreated with 2 μM salubrinal for 1 h and then treated with 100 μM Amp or DMSO at 37˚C for 24 h. Total protein was extracted from cell lysates and blotted for (A) p-PERK, PERK, p-eIF2α, eIF2α, CHOP, or GRP78 protein; (B) caspase-8 [pro-caspase-8 and active caspase-8 (p43/41)], caspase-9 [pro-caspase-9 and active caspase-9 (p37/35)], caspase-3 [pro-caspase-3 and active caspase-3 (p19/17)] or PARP [PARP and cleaved PARP (p89)]; (D) and p-AMPK, AMPK, XAF1, p-JNK, JNK, p-p38-MAPK or p38-MAPK protein. β-actin was used as a loading control. (C) Cells were treated with 100 μM Amp for 24 h. Western blotting for p-ERK1/2, ERK1/2, p-p38 MAPK, p38 MAPK, p-JNK, or JNK was performed. The results are representative of three independent experiments. Vehicle-treated cells (DMSO) were used as the control group. ER, endoplasmic reticulum; Amp, ampelopsin; DMSO, dimethyl sulfoxide; p-, phosphorylated; PERK, protein kinase RNA-like ER kinase; eIF2α, eukaryotic translation initiation factor 2α; CHOP, CCAAT/enhancer-binding protein homologous protein; GRP78, glucose-regulated protein 78; PARP, poly ADP-ribose polymerase; AMPK, 5' adenosine monophosphate-activated protein kinase; XAF1, XIAP-associated factor 1; JNK, c-Jun N-terminal protein kinase; p38-MAPK, p38 mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; Sal, salubrinal.

the anti-apoptotic Bcl-2 family proteins Bcl-2 and Mcl-1 (Fig. 3B). These results demonstrate that AMPK is one of the downstream signaling targets of ER stress and mediates Amp-induced colon cancer cell apoptosis.

MAPK activation leads to the expression of XAF1 in Amp-treated colon cancer cells. The roles of JNK and p38-MAPK activation in ER stress and AMPK expression were investigated using SB203580, an inhibitor of p38 phosphorylation, and SP600125, an inhibitor of JNK. HCT-116 and HT-29 cells were pretreated with either SB203580 or SP600125, followed by Amp treatment. Although the Amp-induced p-PERK, CHOP, and AMPK expression levels were unaffected in the two cell lines by either SB203580 or SP600125, each of these inhibitors led to a marked attenuation of Amp-mediated XAF1 expression (Fig. 4A). In addition, SB203580 and SP600125 effectively prevented Amp-induced activation and cleavage of caspase-9, caspase-3, and PARP. The levels of the anti-apoptotic proteins Bcl-2 and Mcl-1 were also restored by MAPK inhibition (Fig. 4B). These results reveal a critical role for JNK and p38-MAPK-dependent XAF1 induction in Amp-treated colon cancer cells.

Amp-induced ROS promote the ER stress/AMPK-mediated apoptosis of colon cancer cells. ROS are implicated in a variety of biological functions and are an upstream signal activating AMPK in epigallocatechin-3-gallate-mediated colon cancer cell death (35). In addition, Amp treatment reportedly triggers ROS generation in breast cancer cells (29). To assess the effect of ROS on ER stress-induced apoptosis in Amp-treated colon cancer cells, the ROS scavenger NAC was used. Amp treatment induced ROS production in HCT-116 cells in a time-dependent manner ($P<0.05$; non-treated cells vs. Amp-treated cells; $P<0.01$; non-treated cells vs. Amp-treated cells; Fig. 5A). Pretreatment with NAC efficiently quenched the Amp-induced generation of ROS ($P<0.01$; Amp-treated

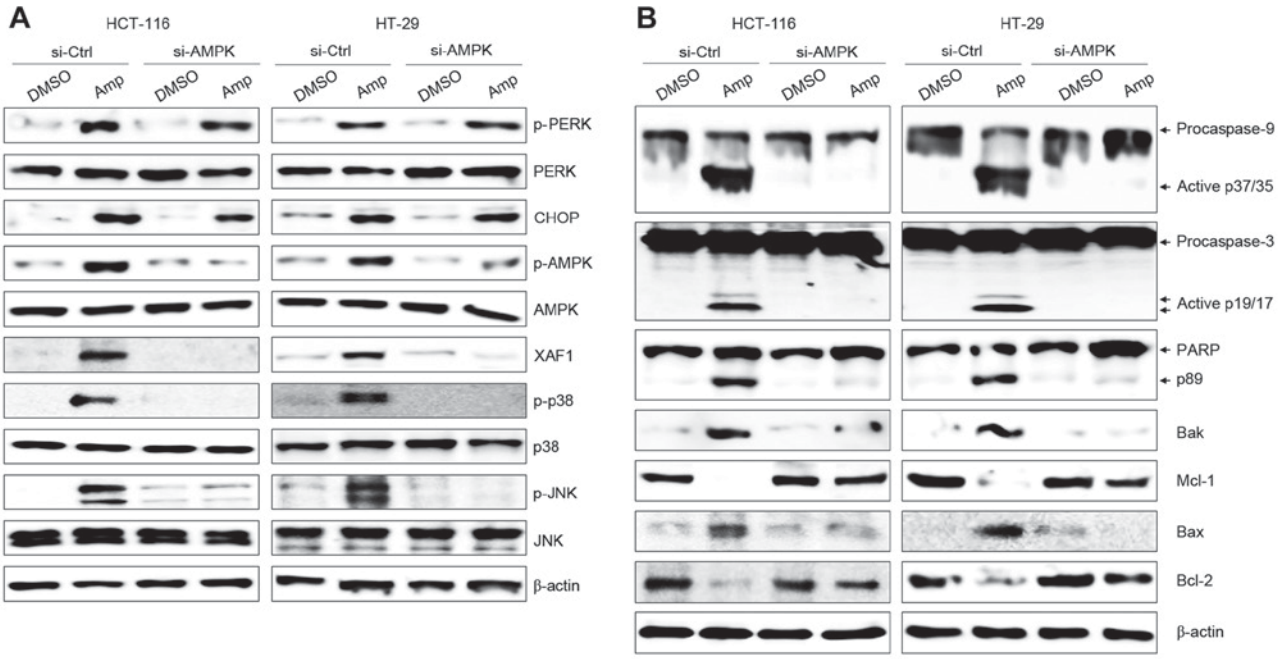

Figure 3. ER stress triggers AMPK-dependent apoptosis signaling in Amp-treated colon cancer cells. Cells were treated with 100 μM Amp for 6 h then transfected with 200 nM si-AMPK using the Lipofectamine RNAiMAX reagent according to the manufacturer's protocol. Cells were used for further experiments 36 h after transfection. Whole-cell lysates were subjected to western blotting using (A) p-PERK, PERK, CHOP, p-AMPK, AMPK, XAF1, p-p38-MAPK, p38-MAPK, p-JNK, or JNK, and (B) caspase-9 [pro-caspase-9 and active caspase-9 (p37/35)], caspase-3 [pro-caspase-3 and active caspase-3 (p19/17)] or PARP [PARP and cleaved PARP (p89)], Bcl-2, Bax, Bak, or Mcl-1 antibodies. β-actin was used as a loading control. The results are representative of three independent experiments. Vehicle-treated cells (DMSO) were used as the control group. ER, endoplasmic reticulum; AMPK, 5' adenosine monophosphate-activated protein kinase; Amp, ampelopsin; p-, phosphorylated; PERK, protein kinase RNA-like ER kinase; CHOP, CCAAT/enhancer-binding protein homologous protein; XAF1, XIAP-associated factor 1; p38-MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal protein kinase; PARP, poly ADP-ribose polymerase; Bax, BCL2-associated X protein; Bak, BCL2 antagonist/killer 1; Mcl-1, myeloid cell leukemia sequence 1 (BCL2-related); DMSO, dimethyl sulfoxide; si-Ctrl, control small interfering RNA; si-AMPK, small interfering RNA targeting AMPK.

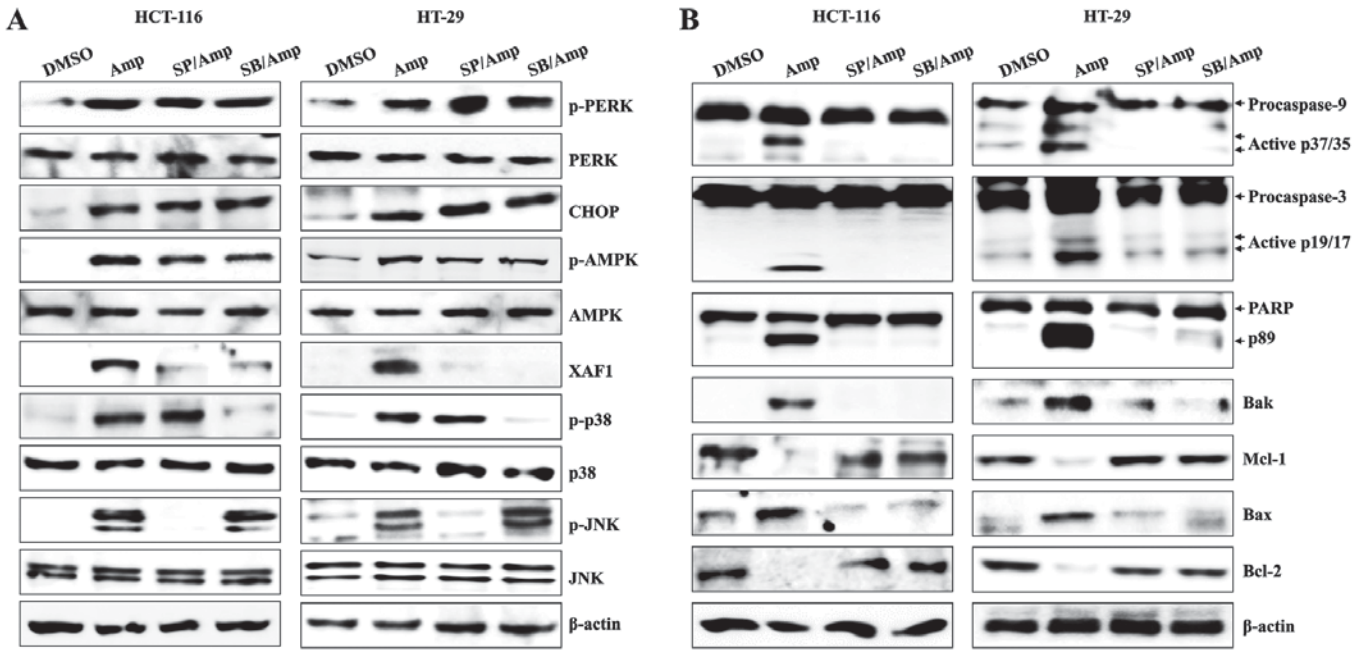

Figure 4. MAPK activation leads to apoptosis of Amp-treated colon cancer cells. Cells were pretreated with 25 μM SP600125 or 10 μM SB203580 at 37˚C for 1 h and then treated with 100 μM Amp or DMSO (control) at 37˚C for 24 h. Total protein was extracted from cell lysates, and western blotting for (A) p-PERK, PERK, CHOP, p-AMPK, AMPK, XAF1, p-p38-MAPK, p38-MAPK, p-JNK, or JNK protein, and (B) caspase-9 [pro-caspase-9 and active caspase-9 (p37/35)], caspase-3 [pro-caspase-3 and active caspase-3 (p19/17)], PARP [PARP and cleaved PARP (p89)], Bcl-2, Bax, Bak or Mcl-1 protein were performed. β-actin was used as a loading control. The results are representative of three independent experiments. MAPK, mitogen-activated protein kinase; Amp, ampelopsin; DMSO, dimethyl sulfoxide; p-, phosphorylated; PERK, protein kinase RNA-like endoplasmic reticulum kinase; CHOP, CCAAT/enhancer-binding protein homologous protein; AMPK, 5' adenosine monophosphate-activated protein kinase; XAF1, XIAP-associated factor 1; JNK, c-Jun N-terminal protein kinase; PARP, poly ADP-ribose polymerase; Bax, BCL2-associated X protein; Bak, BCL2 antagonist/killer 1; Mcl-1, myeloid cell leukemia sequence 1 (BCL2-related); SP, SP600125; SB, SB203580.

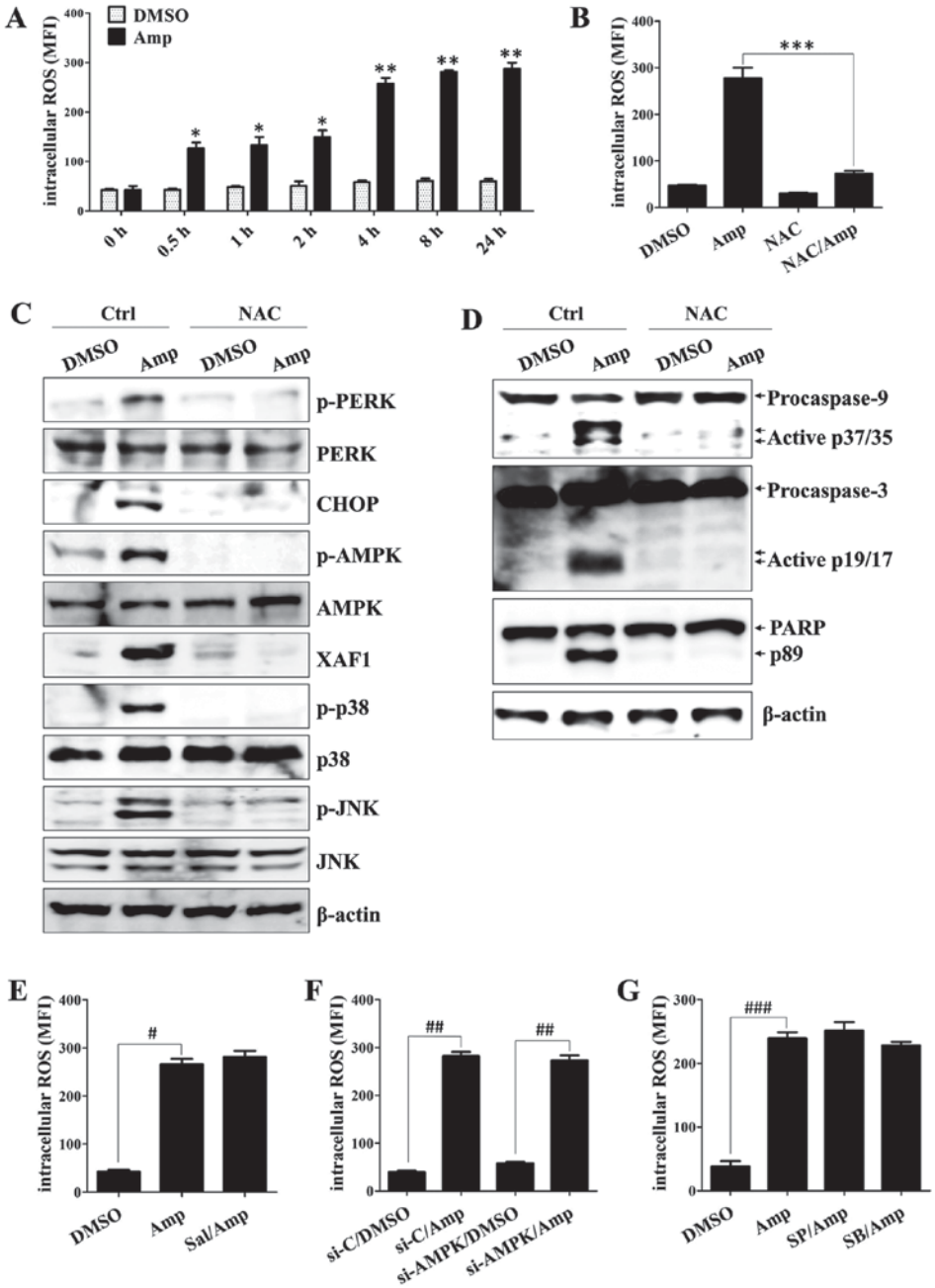

Figure 5. Amp promotes ER stress/AMPK-mediated apoptosis through ROS generation in colon cancer cells. (A, B and E-G) Cells were pretreated with 10 μM 2',7'-dichlorodihydro-fluorescein diacetate for 30 min and then treated with 100 μM Amp or DMSO at 37˚C for (A) 0-24 h, or (B and E-G) 8 h. The values in the 2',7'-dichlorofluorescein histograms indicate the MFI. (B-D) To quench ROS, cells were pretreated with 10 mM NAC for 1 h. Cells were then washed with PBS and treated with 100 μM Amp for (B) 8 h or (C and D) 24 h. (B) The effect of NAC on Amp-induced ROS production was measured using flow cytometry. Whole-cell lysates were subjected to western blotting using (C) p-PERK, PERK, CHOP, p-AMPK, AMPK, XAF1, p-p38-MAPK, p38-MAPK, p-JNK, and JNK antibodies, and (D) caspase-9 [pro-caspase-9 and active caspase-9 (p37/35)], caspase-3 [pro-caspase-3 and active caspase-3 (p19/17)] or PARP [PARP and cleaved PARP (p89)] antibodies. β-actin was used to normalize protein content. (E) To block ER stress, cells were pretreated with salubrinal (2 μM) for 1 h and then treated with 100 μM Amp for 8 h. (F) To prevent AMPK activation, cells were treated with 100 μM Amp for 6 h, then transfected with 200 nM si-AMPK using the Lipofectamine RNAiMAX Reagent according to the manufacturer's protocol. Cells were used for further experiments 36 h after transfection. (G) To inhibit the JNK or p38-MAPK cascade, cells were pretreated with 25 μM SP600125 or 10 μM SB203580 at 37˚C for 1 h and then treated with 100 μM Amp or DMSO for 8 h. The results are representative of three independent experiments. *P<0.05 and **P<0.01, untreated cells vs. Amp-treated cells; ***P<0.01, Amp-treated cells vs. cells co-treated with NAC and Amp; $^{\#}$P<0.01, DMSO-treated cells vs. Amp-treated cells; $^{\#\#}$P<0.01, DMSO-treated cells vs. Amp-treated cells; $^{\#\#\#}$P<0.01, DMSO-treated cells vs. Amp-treated cells. Vehicle-treated cells (DMSO) were used as the control group. Amp, ampelopsin; ER, endoplasmic reticulum; AMPK, 5' adenosine monophosphate-activated protein kinase; ROS, reactive oxygen species; DMSO, dimethyl sulfoxide; NAC, N-acetyl-L-cysteine; p-, phosphorylated; PERK, protein kinase RNA-like ER kinase; CHOP, CCAAT/enhancer-binding protein homologous protein; XAF1, XIAP-associated factor 1; p38-MAPK, p38-mitogen-activated protein kinase; JNK, c-Jun N-terminal protein kinase; PARP, poly ADP-ribose polymerase; MFI, mean fluorescence intensity; Sal, salubrinal; Ctrl, control; si-AMPK, small interfering RNA targeting AMPK; si-C, control small interfering RNA; SP, SP600125; SB, SB203580.

cells vs. cells co-treated with NAC and Amp; Fig. 5B), attenuated Amp-dependent induction of p-PERK, CHOP, p-AMPK and XAF1, and reduced the activation of JNK and p38-MAPK by Amp (Fig. 5C). NAC pretreatment also suppressed the

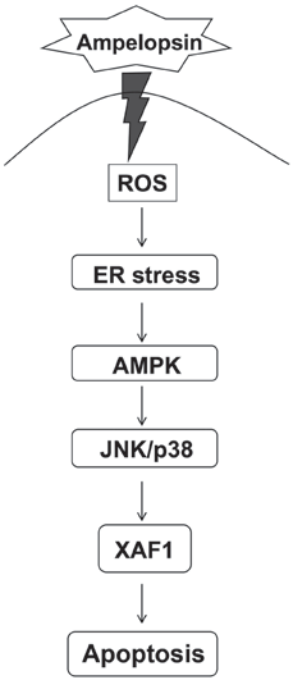

Figure 6. Schematic diagram of the intracellular signaling mechanism during Amp-induced apoptosis in colon cancer cells. Amp treatment triggers intracellular ROS generation and leads to the activation of ER stress. ER stress results in the phosphorylation of AMPK and JNK/p38-MAPK, causing progressive upregulation of XAF-1. XAF-1 elicits the disruption of the mitochondrial membrane potential, resulting in the induction of apoptosis. Amp, ampelopsin; ROS, reactive oxygen species; ER, endoplasmic reticulum; AMPK, 5' adenosine monophosphate-activated protein kinase; JNK, c-Jun N-terminal protein kinase; p38/p38-MAPK, p38 mitogen-activated protein kinase; XAF1, XIAP-associated factor 1.

Amp-induced cleavage and activation of caspase-9, caspase-3, and cleaved PARP in colon cancer cells (Fig. 5D). ROS levels in Amp-treated colon cancer cells were unaffected by salubrinal ($P<0.01$; DMSO-treated cells vs. Amp-treated cells; Fig. 5E), AMPK siRNA ($P<0.01$; DMSO-treated cells vs. Amp-treated cells; Fig. 5F), SB203580, or SP600125 ($P<0.01$; DMSO-treated cells vs. Amp-treated cells; Fig. 5G). These results suggest that ROS generated by Amp treatment are responsible for initiating the ER stress-mediated apoptosis pathway in colon cancer cells.

Discussion

Amp, a major component of *Ampelopsis grossedentata*, is reported to possess not only important pharmacological activities, such as anti-inflammatory and hepatoprotective properties (25,26), but also anti-cancer activity. The anti-tumor activity of Amp is associated with the induction of apoptosis (27-30) and the inhibition of metastasis (36). Amp causes ER stress, resulting in the inhibition of cell growth and induction of apoptosis in human breast cancer cells (29). However, the anti-tumor effects of Amp on colon cancer have not been studied previously, and its underlying mechanism of action remains to be investigated. ER stress, initiated by various stress conditions, results in the release the Ca^{2+} from the ER, leading to CaMKKβ-mediated AMPK activation (12,14). Severe ER stress triggers apoptotic cell death by inducing CHOP, activating caspase-12, and disrupting the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family (13,37). Amp-mediated ROS activate ER stress and promote signaling pathways that lead to breast cell death (29). However, the association between ER stress and AMPK activation in apoptotic signaling is not fully understood. The present study demonstrated that Amp activates a pro-apoptotic signaling pathway in colon cancer cells by promoting ROS generation, which causes ER stress. Amp-induced ER stress subsequently triggers the activation of AMPK to induce XAF1-mediated apoptosis through the JNK/p38-MAPK signaling pathway (Fig. 6).

AMPK activation promotes catabolic processes, resulting in the inhibition of lipid, glycogen, and protein synthesis, leading to the concomitant inhibition of cell growth and proliferation (1,2). Metformin, which activates AMPK, induces ER stress-mediated apoptosis of acute lymphoblastic leukemia (16). These reports are consistent with results that metabolic stress impairs protein-processing capacity to induce the UPR in the ER (38). When UPR in the ER is persistent and unresolved, cell survival processes are subverted by pro-apoptotic signaling pathways (39). When PERK is activated by stressful conditions, it phosphorylates eIF2α to attenuate protein synthesis, and upregulated eIF2α regulates CHOP expression, thereby mediating apoptosis (40). Although Amp-induced AMPK activity reportedly protects endothelial cells through autophagy (31), those studies also indicate that AMPK activation may be associated with the induction of ER stress to induce its anti-cancer effect (31,41). In accordance with those results, the results of the present study indicated that ER stress in Amp-treated colon cancer cells regulates the activation of AMPK. Salubrinal, an inhibitor of ER stress, effectively prevented the phosphorylation of AMPK and inhibited the apoptosis of colon cancer cells. Knockdown of AMPK with siRNA, however, did not attenuate ER stress, indicating that ER stress precedes AMPK activation, and also that increases in intracellular Ca^{2+} concentrations released from the ER likely activate AMPK.

XAF1 suppresses cancer through two principal mechanisms. To enhance apoptosis, XAF1 interacts directly with endogenous XIAP, suppressing its anti-caspase activity (21). XAF1 also localizes to the mitochondria and disrupts the $\Delta\psi_m$, leading to cancer cell death and suppression of tumor growth (42-44). XAF1 expression is upregulated by the inhibition of ERK signaling (24). The nuclear translocation of XAF1 and Bax are dependent on the phosphorylation of p38-MAPK in melphalan-induced apoptosis of Epstein-Barr virus-transformed B cells (45). The present study showed that Amp treatment of colon cancer cells could induce apoptosis in a dose-dependent manner, increase the expression of XAF1 and pro-apoptotic Bcl-2 family proteins (Bak and Bax), and increase the levels of phosphorylated JNK and p38-MAPK. Amp-induced XAF1 expression and apoptosis were inhibited by salubrinal, SB203580 (p38-MAPK inhibitor), or SP600125 (JNK inhibitor), as well as by the knockdown of AMPK. These results suggest an essential function for ER stress-mediated JNK/p38-MAPK signaling in the control of XAF1 expression in Amp-induced apoptosis in colon cancer cells.

ROS exert significant effects on multiple cellular functions (46). ROS cause ER stress, which results in the generation of more ROS (45,47). Although Amp-mediated ROS trigger ER stress/AMPK activation and JNK/p38-MAPK signaling, leading

to apoptosis in colon cancer cells, it is important to consider that Amp is not necessarily pro-ROS in all circumstances (25,48). In a previous study of lipopolysaccharide-activated macrophages, low doses of Amp effectively suppressed ROS generation and the release of inflammatory cytokines (25). Low-dose Amp has also been demonstrated to inhibit phosphoinositide 3-kinase activation without attenuating MAPK (ERK, JNK and p38-MAPK) activation (25), and Amp-mediated ROS reduction has been identified to trigger the apoptosis of hepatocellular carcinoma cells (48). These results indicate that effective chemotherapeutic doses will need to be carefully determined and that mechanism(s) of action must be elucidated within each dose range and for each cell type to mitigate the risk of pleiotropic effects. Taken together, the results of the present study suggested that the ER stress-mediated AMPK signaling pathway could induce the apoptosis of ampelopsin-exposed colon cancer cells and that the AMPK-mediated apoptosis signaling pathway with ampelopsin could be a promising strategy for improving the clinical outcomes of patients with colon cancer.

Acknowledgements

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