Cucurbitacin B induces apoptosis in colorectal cells through reactive oxygen species generation and endoplasmic reticulum stress pathways

JIAN-LAN HUANG, LI LIANG, PEI-EN XIE, WEI-LIANG SUN, LI WANG and ZHENG-WEN CAI

Department of Medical Oncology, The Second Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region 530007, P.R. China

Abstract. Cucurbitacin B (CuB) is a member of the cucurbitacin family, which has shown potent anticancer pharmacological activity. Prolonged or severe endoplasmic reticulum stress (ERS) induces apoptosis; therefore, the present study investigated whether CuB may activate the ERS pathway to induce apoptosis. HT-29 and SW620 colorectal cancer (CRC) cells were treated with a range of concentrations of CuB for 48 h, and the viability and proliferation of cells were determined using Cell Counting Kit 8 (CCK8) and colony formation assays. Subsequently, the appropriate CuB concentration (5 μM) was selected for treatment of CRC cells for 48 h. Western blot analysis was used to measure the expression levels of ERS-related proteins, flow cytometry was used to evaluate apoptosis, the dichlorodihydrofluorescein diacetate fluorescent probe was used to detect reactive oxygen species (ROS) production, and the relationship between ROS and ERS was determined by western blot analysis. Furthermore, flow cytometry was used to evaluate apoptosis after treatment with the ERS inhibitor 4-phenylbutyric acid, the ROS inhibitor N-acetylcysteine and following knockdown of CHOP expression. In addition, western blot analysis was performed to measure Bax and Bcl2 protein expression levels, and a CCK8 assay was performed to evaluate the viability of cells following knockdown of CHOP. Notably, CuB treatment increased apoptosis and inhibited cell proliferation in CRC cell lines, and these effects were mediated by ROS and ROS-regulated activation of the PERK and XBP1 ERS pathways. In conclusion, CuB may induce apoptosis in HT-29 and SW620 CRC cells via ROS and ERS.

Correspondence to: Professor Zheng-Wen Cai, Department of Medical Oncology, The Second Affiliated Hospital of Guangxi Medical University, 166 Daxue East Road, Nanning, Guangxi Zhuang Autonomous Region 530007, P.R. China
E-mail: 420346@sr.gxmu.edu.cn

Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide, after lung and breast cancer, and is one of the leading causes of cancer death, accounting for 9.4% of global cancer deaths (1,2). Although targeted therapies and immunotherapies have improved the treatment outcomes of advanced CRC, the therapeutic efficacy is still limited, especially because the development of drug resistance affects the further improvement of therapeutic efficacy (3,4). Therefore, there is an urgent need to identify and develop safer and more effective therapeutic agents with anticancer activity.

Natural compounds and their derivatives may be promising sources of therapeutic agents. Paclitaxel, for example, which has been used in clinical cancer treatment, is a natural substance derived primarily from the natural plant yew that frequently triggers cancer cell apoptosis (5). Cucurbitacins are a class of natural tetracyclic triterpenoids extracted from plants, which exhibit a variety of pharmacological activities, such as anti-inflammatory, anticancer and hepatoprotective activities (6). Previous studies have reported that cucurbitacins have a broad anticancer spectrum and can inhibit various types of cancer, such as breast, lung and laryngeal cancer (7-9). Cucurbitacin B (CuB) has received attention in cancer research as one of the most abundant and potent members of this family (10). It has been reported that CuB can target the Notch signalling pathway to induce apoptosis in CRC HCT116 and SW480 cells (11). Promkan *et al* (12) also reported that CuB induced apoptosis in colon cancer Caco-2 and SW620 cells while triggering autophagy. In addition, CuB may regulate the polarization of tumour-associated M2-type macrophages to inhibit the metastasis and invasion of colon cancer cells through suppression of JAK2/STAT3 signalling (13).

The endoplasmic reticulum (ER) is an intracellular organelle with important physiological functions, which is involved mainly in protein synthesis, folding and calcium storage (14). Aggregation of misfolded or unfolded proteins in the ER caused by various factors triggers ER stress (ERS) and the unfolded protein response (UPR), with the aim of maintaining ER homeostasis (15,16). Reactive oxygen species (ROS) is a general term describing molecular oxygen derivatives, including the superoxide anion, hydrogen peroxide and the

hydroxyl radical, which are produced during cellular respiration. These ROS act as agents of cellular damage, reacting with lipids, proteins and DNA to cause injury (17-19). ERS is further initiated when the intracellular environment is stimulated by ROS. When ERS is prolonged or intensified, apoptosis is induced (20-22). In cancer research, ROS have been reported to be able to regulate ERS to induce cancer cell apoptosis or death (23,24). Therefore, ERS may be a target for the treatment of cancer.

Notably, it remains to be elucidated whether CuB can induce apoptosis in CRC cells through ROS-activated ERS; therefore, the present study investigated the role of ROS and ERS in CuB-mediated induction of apoptosis in CRC cells.

Materials and methods

Cell treatment. CuB (Dalian Meilun Biology Technology Co., Ltd.) was dissolved in DMSO, and the stock solution (20 mM) was prepared and stored at -80°C. In all experiments, the final concentration of DMSO was <0.1%, and different concentrations of CuB working solution were prepared. The HT-29 and SW620 cells (Procell Life Science & Technology Co., Ltd.) used in the experiment were cultured in DMEM (Biological Industries) containing 10% FBS (Biological Industries) and 1% penicillin (Biological Industries) in a 5% CO_2 incubator at 37°C. In the experiments, HT-29 and SW620 cells were incubated with the CuB (5 μM) for 48 h at 37°C, and the concentration of DMSO in the control group was 0.1%. The human CRC HT-29 cell line used in the present study was verified by STR profiling.

Cell Counting Kit 8 (CCK8) assay. HT-29 and SW620 cells were seeded in a 96-well plate with complete culture medium ($5x10^3$ cells/well). After the cells attached to the plate, they were treated with different concentrations of CuB (0-100 μM) working solution and cultured in a 37°C incubator for 48 h. Subsequently, the drug-containing culture medium was removed, CCK8 reagent (100 μl; 1:100; Biosharp) was added to each well, and the 96-well plate was incubated at 37°C for 2 h. The absorbance was measured at 450 nm using a microplate reader. Calculate each group's cell proliferation rate was calculated using the formula follows: Cell viability=1-[(control group OD value-experimental group OD value)/(control group OD value-blank group OD value) x100].

Colony formation assay. HT-29 and SW620 cells were evenly seeded in 6-well cell culture plates (300 cells/well). After the cells were treated with different concentrations of CuB (0, 0.5, 1.0, 5.0 μM) at 37°C for 48 h, and the cell culture media was changed. The cells were cultivated for 14 days in a 5% CO_2 incubator at 37°C, with the media changed every three days. Subsequently, the original medium was removed, and 4% paraformaldehyde was added to fix the cells for 30 min and the cells were stained with 0.5% crystal violet at room temperature (25°C) for 10 min. The total number of colonies (>50 cells) formed was calculated by ImageJ 1.53t (National Institutes of Health)

Dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence probe ROS detection kit. HT-29 and SW620 cells were seeded in 12-well cell culture plates ($1x10^4$ cells/well),

cultured for 24 h and then treated with CuB (5 μM) in a 37°C cell incubator for 48 h. The original culture medium was aspirated and DCFH-DA working solution (MedChemExpress) was added at a final concentration of 10 μM in a 37°C incubator was 30 min, and the cells were observed and imaged under a fluorescence microscope. The average fluorescence intensity of each image was determined using ImageJ 1.53t software.

Flow cytometric apoptosis assay. HT-29 and SW620 cells were seeded into 6-well cell culture plates ($5x10^5$ cells/well), incubated for 24 h, and then treated with CuB (5 μM) alone or in combination with 4-phenylbutyric acid (4-PBA; 800 μM; MedChemExpress) or N-acetylcysteine (NAC; 5 mM; MedChemExpress) in a 37°C incubator for 48 h. The cells were collected and resuspended with PBS. Cells were stained using Annexin V-PE and 7-AAD (for nucleic acid staining) from a PE Annexin V Apoptosis Detection Kit (BD Pharmagen), both 5 μl. After gentle shaking, the solutions were incubated at room temperature (25°C) in dark for 15 mins. The cells were subjected to flow cytometry (CytoFLEX S; Beckman Coulter) and then the data were analysed (FlowJo; version 9.0.4; BD Pharmagen) with total apoptosis calculated as follow: Total apoptosis rate=early apoptosis rate + late apoptosis rate.

Establishment of stable cells. CHOP was knocked down in SW620 cells using a short hairpin RNA (shRNA). For the experiment, OBiO Technology (Shanghai) Co., Ltd. produced the lentivirus plasmid pCLenti-U6-shRNA (CHOP)-CMV Uro WPRE. 293T (American Type Culture Collection) cells that were transfected grew well. The transfected virus vector plasmid was 32 μg (skeleton plasmid: shuttle plasmid, 1:1) in the case of a 100 mm disk, packing the virus. Cells were cultured in a cell culture compartment and 48 h after transfection, the virus was collected and purified. Then, SW620 cells in logarithmic growth phase were seeded in a 6-well plate ($5x10^4$ cells/well) and cultured in an incubator at 37°C. When the cell confluence reached ~30%, viral particles (multiplicity of infection, 10) were added for infection for 12 h, and medium containing puromycin (Beijing Solarbio Science & Technology Co., Ltd.; 2 μg/ml) was added to screen for positive cells 12 h later. The screened positive cells were subcultured, and the purinomycin concentration remained constant at 2 μg/ml. Following each successful infection, SW620 cells from the first to third generations were subjected to subsequent experimentation. The cells with CHOP knockdown were denoted as the shRNA-CHOP group and the cells transduced with the negative control (NC) vector were denoted the shRNA-NC group. The shRNA targeting sequences were as follows: shRNA-NC, 5'-TTCTCCGAACGTGTCACGT-3'; shRNA-CHOP, 5'-TGA ACGGCTCAAGCAGGAAAT-3'.

Protein isolation and western blotting. CRC cell lines (HT-29 and SW620) were detached, suspended and seeded into a 100-mm Petri dish. After adhered cells were 70% confluent, CuB working solution (5 μM) combined with the ROS scavengers NAC (5 mM, 1 h pretreatment) and 4-PBA (800 μM, 2 h pretreatment) were used to treat the cells. The cells were incubated in a suitable cell incubator at 37°C for 48 h and the cells were collected immediately after incubation. RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), which was

precooled in advance and mixed with protease and phosphatase inhibitors, was used to fully lyse the cells. Subsequently, the protein concentration in each group was determined using a BCA assay. All protein samples were separated by SDS-PAGE (30 μg/lane) on 12.5 (proteins >30 kDa) and 15% (proteins <30 kDa) gels with a molecular weight marker (cat. no. WJ102; Ipsen Biopharmaceuticals, Inc.), and the separated proteins were transferred to PVDF membranes with a constant current electrophoretic transfer system. PVDF membranes were placed in 1X rapid blocking solution (New Cell & Molecular Biotech Co., Ltd.) for 30 min on a shaker at room temperature. Primary antibodies against the following proteins were used: GAPDH (1:10,000; 36 KDa; cat. no. 10494-1-AP; Wuhan Sanying Biotechnology); Bax (1:5,000; 21 kDa; cat. no. GR3275117-D; Abcam) and Bcl2 (1:2,000; 26 kDa; cat. no. GR3325129-9; Abcam); glucose regulated protein 78 (GRP78; 1:1,000; 78kDa; cat. no. 11587-1-AP; Wuhan Sanying Biotechnology), CHOP (27 kDa; cat. no. 44266; 1:1,000; GeneTex, Inc.), activating transcription factor 4 (ATF4; 42 kDa; cat. no. 39568; 1:1,000; GeneTex, Inc.) and X-box binding protein 1 splicing form (XBP1-s; 55 kDa; cat. no. 44286; 1:1,000; GeneTex, Inc.); IRE1 (1:1,000; 130 kDa; cat. no. 3294S; Cell Signaling Technology, Inc.); phosphorylated (p)-protein kinase R-like ER kinase (PERK; 1:1,000; 125 kDa; cat. no. 6N04; SAB Biotherapeutics, Inc.); PERK (1;1,000; 115 kDa; cat. no. 5683S; Cell Signaling Technology, Inc.); p-eukaryotic translation initiation factor 2α (eIF2α; 1:1,000; 32 kDa; cat. no. AP0692; ABclonal Biotech Co., Ltd.) and eIF2α (1:1,000; 38 kDa; cat. no. 42774; GeneTex, Inc.). After incubation overnight at 4°C, the membranes were incubated with a fluorescent secondary antibody (anti-rabbit IgG; 1:10,000; cat. no. 5151P Cell; Signaling Technology, Inc.) at room temperature for 2 h. The LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences) was used to scan and visualize protein bands. Following that, the relative protein expression was determined by evaluating the band densities with ImageJ software.

Statistical analysis. SPSS 25.0 statistical software (IBM Corp.) was used for analysis and GraphPad Prism 5.0 software (Dotmatics) was used for graph generation. The results of at least three independent experiments were used for statistical analysis in all experiments, and the experimental data are expressed as the mean ± SD. The data were analysed using independent samples t-test, or one-way ANOVA and Tukey post hoc test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CuB inhibits CRC cell viability and proliferation. The present study initially assessed the viability of human CRC cell lines (HT-29 and SW620) using a CCK8 assay. A series of concentrations of CuB (Fig. 1A) were prepared, which were then used to treat cells for 48 h. In the first experiment, the appropriate concentration of CuB was selected for subsequent experiments. The results of the CCK8 assay showed that 0.005 μM CuB inhibited the proliferation of CRC cells, and the inhibitory effect became more obvious with increasing concentrations (Fig. 1B). At 5 μM, the inhibition rate of CuB-treated HT-29 and SW620 cells was close to 50%. These results indicated

that CuB may have a marked inhibitory effect on CRC cell viability. In addition, to study the effect of CuB on the proliferation of CRC cells, a cell plate colony formation assay was performed, and the results confirmed that the colony formation ability of cancer cells was significantly inhibited by treatment with 0.5-5 μM CuB compared with that in the control group (P<0.05; Fig. 1C and D). These results indicated that CuB may have strong antitumour activity against CRC cells and 5 μM was selected as the concentration for the follow-up experiments.

CuB induces ERS in CRC cells. Western blot analysis was performed to determine whether CuB (5 μM) could activate ERS. The present study detected the ER stress markers GRP78 and CHOP, and their corresponding activated UPR signalling pathways were assessed by measuring the levels of proteins related to the PERK/eIF2α/ATF4 and IRE1/XBP1 signal transduction pathways. Cells exposed to CuB for 48 h exhibited an upregulation of ER stress markers, and increased expression levels of the UPR-related proteins p-PERK, p-eIF2α, ATF4, IRE1α and XBP1, compared with those in the control group (P<0.05; Fig. 2).

CuB mediates ROS production in CRC cells and induces ERS. To determine whether CuB can promote the production of ROS in CRC cells, the DCFH-DA fluorescent probe was used to detect the production of ROS in CRC cells after continuous treatment with CuB. Compared with those in the untreated group, following exposure to CuB-mediated toxicity, the amounts of ROS produced in the two CRC cell lines were significantly increased with increasing CuB concentration (P<0.05; Fig. 3A and B). These results confirmed that CuB can promote the production of ROS in CRC cell lines. Furthermore, the ROS inhibitor NAC (5 mM) was used to examine the relationship between CuB-induced ROS production and ERS. The results showed that NAC pretreatment significantly inhibited the CuB-induced increases in the protein expression levels of GRP78, CHOP, p-PERK, p-eIF2α, ATF4, XBP1 and IRE1 (P<0.05; Fig. 3C-F). These findings suggested that ROS produced in CRC cells mediated by CuB treatment may induce ERS.

CuB can induce apoptosis in CRC cells. Following treatment with CuB (5 μM), apoptosis in HT-29 and SW620 cells was significantly increased compared with that in the control groups (P<0.05; Fig. 4A-C). By contrast, when HT-29 and SW620 cells were pretreated with the ERS inhibitor 4-PBA (800 μM) for 2 h, CuB-induced apoptosis was significantly inhibited (P<0.05) compared with that in the groups treated with CuB alone, as determined by flow cytometry (Fig. 4A-C). In addition, CuB induced significant upregulation of the proapoptotic protein Bax and significant downregulation of the antiapoptotic protein Bcl2 compared with that in the control group. Furthermore, when compared to the CuB alone group, the protein expression levels of Bax in the CuB combined 4-PBA group were reduced, while those of Bcl2 were increased (P<0.05; Fig. 4D-F).

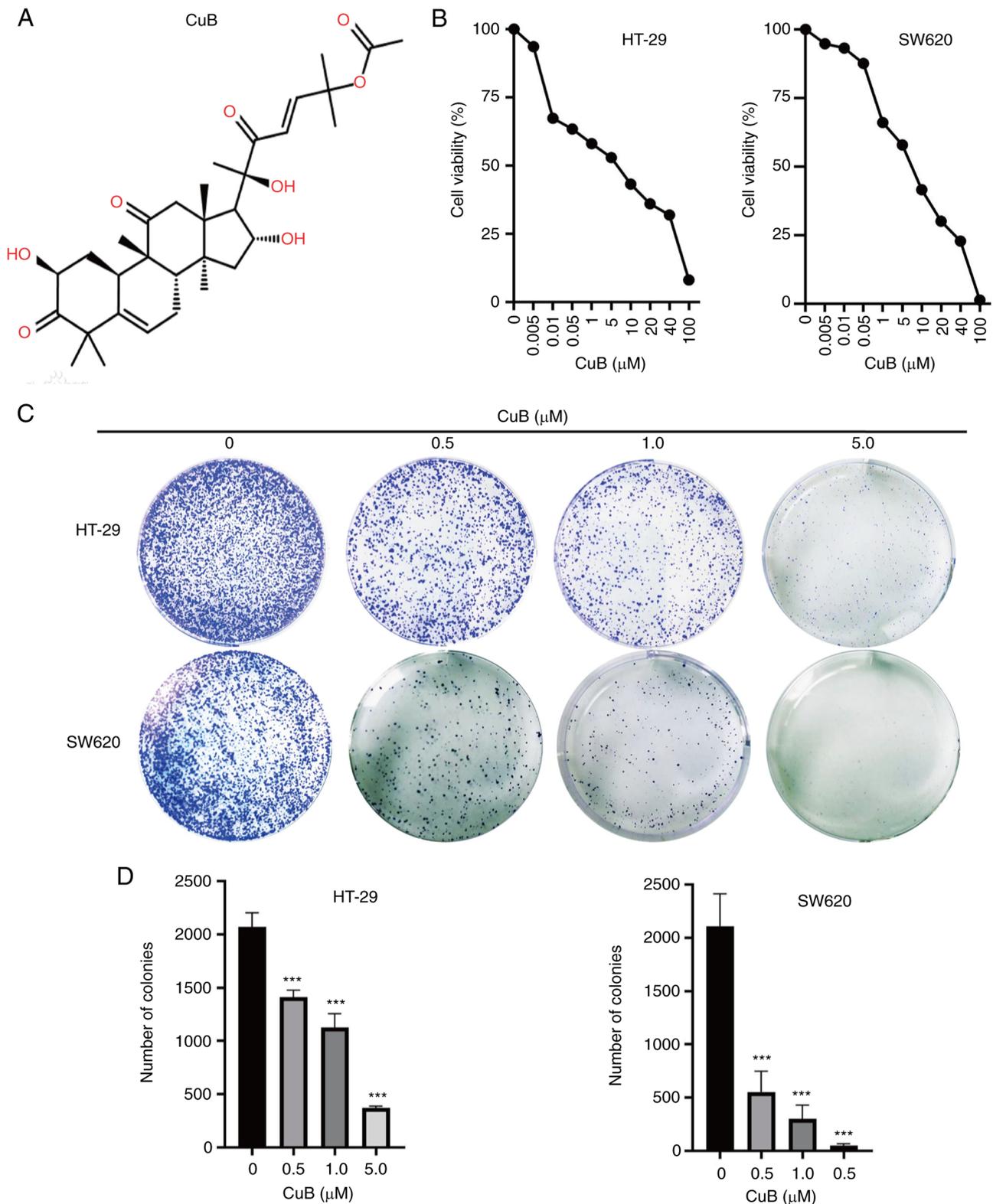*CuB induces apoptosis via ERS and its signal transduction pathways.* To further explore the effect of CuB-induced

Figure 1. CuB inhibits the proliferation of CRC cells. (A) Chemical structure of CuB. (B) CRC cells were treated with CuB (0-100 μM) for 48 h, and the cell viability inhibition rate was measured using a Cell Counting Kit 8 assay. (C) Colony formation assay of CRC cells treated with CuB (0-5 μM) for 14 days. (D) Number of colonies was determined. Data are presented as the mean ± SD of three independent experiments. ***P<0.001 vs. control (0 μM CuB). CRC, colorectal cancer; CuB, cucurbitacin B.

activation of ERS on apoptosis, a model of CHOP knockdown was established in SW620 cells. By comparing the CHOP knockdown group with the NC group, the successful establishment of cells with CHOP knockdown was confirmed

(P<0.05; Fig. 5A and B). When CuB (5 μM) working solution was added to the shRNA CHOP group, it was revealed that CuB treatment could still promote the expression of CHOP; however, the expression of CHOP was significantly
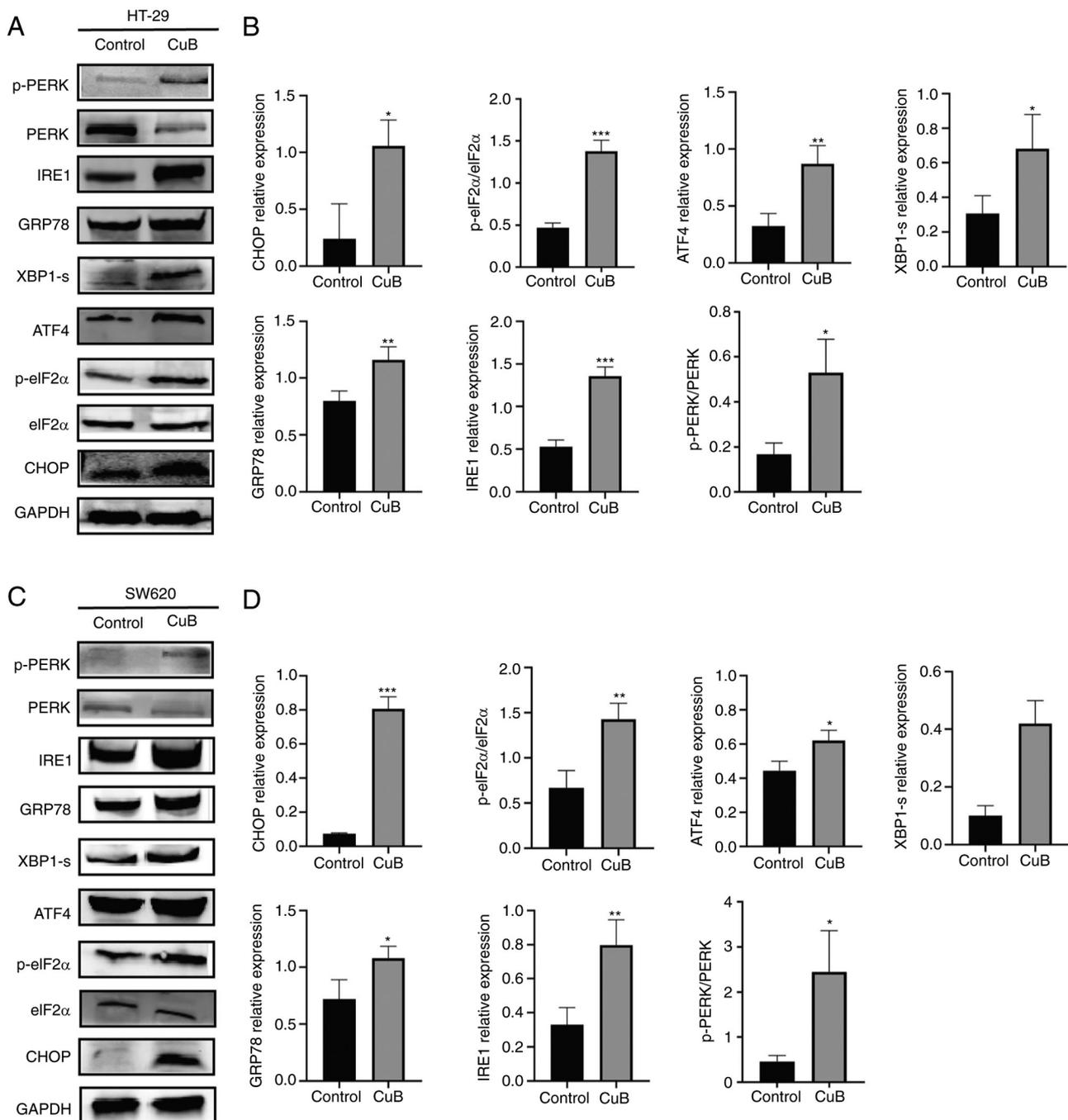
Figure 2. CuB induces ER stress in CRC cells. After CRC cells were treated with CuB (5 μM) for 48 h, the expression levels of ER stress-related proteins was evaluated by western blotting. (A and C) The expression of ERS-related proteins in different groups of HT-29 and SW620 cells. (B and D) Quantification of expression levels of ERS-related proteins. Data are presented as the mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control. ATF4, activating transcription factor 4; CRC, colorectal cancer; CuB, cucurbitacin B; eIF2α, eukaryotic translation initiation factor 2α; GRP78, glucose regulated protein 78; p-, phosphorylated; PERK, protein kinase R-like ER kinase; XBP1-s, X-box binding protein 1.

decreased compared with that in the group treated with CuB alone (P<0.05; Fig. 5A and B). Subsequently, the effects of CHOP knockdown were assessed on the inhibition of cell viability and apoptosis. The results revealed that when CuB was activated, the CHOP knockdown group increased cell viability relative to the NC group; by contrast, the apoptosis rate in the shRNA CHOP group exposed to CuB was significantly lower than that in the group treated with CuB alone (P<0.05; Fig. 5C-E). The aforementioned data suggested that CuB may induce apoptosis in CRC cells by mediating ROS-induced activation of the PERK and IRE1 ERS signalling pathways.

CuB mediates ROS production and induces apoptosis in CRC cells. It has been shown that ROS overload can induce apoptosis (25). Therefore, the present study hypothesized that the production of ROS mediated by CuB (5 μM) may also promote apoptosis. Notably, pretreatment with the ROS inhibitor NAC (5 mM) for 1 h significantly inhibited apoptosis in cancer cells compared with that in cells treated with CuB alone (P<0.05;
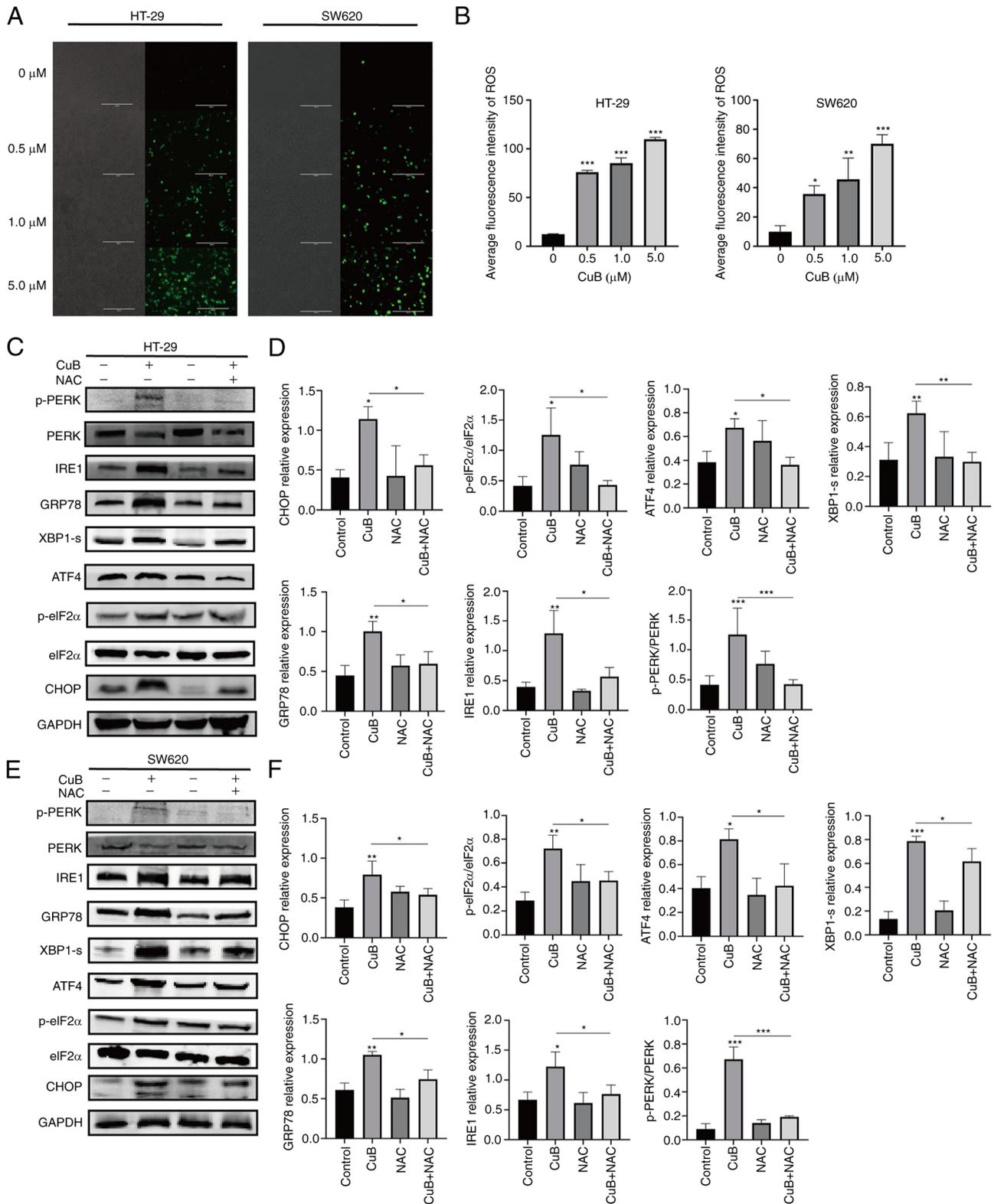
Figure 3. CuB mediates ROS production in CRC cells and induces ERS. (A and B) After CRC cells were treated with CuB (0-5 μM) for 48 h, the dichlorodihy-drofluorescein diacetate fluorescent probe was used to evaluate the amount of ROS produced. Scale bars, 200 μm. CRC cells were pretreated with NAC (5 mM) for 1 h before adding CuB (final concentration of 5 μM). After 48 h of treatment, the expression of ERS-related proteins was evaluated by western blotting. (C and E) The expression of ERS-related proteins in different groups of HT-29 and SW620 cells. (D and F) Quantification of expression levels of ERS-related proteins. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control or as indicated. CRC, colorectal cancer; CuB, cucurbitacin B; eIF2α, eukaryotic translation initiation factor 2α; ERS, endoplasmic reticulum stress; GRP78, glucose regulatory protein 78; NAC, N-acetylcysteine; p-, phosphorylated; PERK, protein kinase R-like ER kinase; ROS, reactive oxygen species; XBP1-s, X-box binding protein 1.

Fig. 6A-C). Moreover, the upregulation of Bax and the down-regulation of Bcl2 induced by CuB were reversed by NAC

pretreatment (P<0.05; Fig. 6D-F). These findings indicated that CuB may also induce apoptosis in cancer cells through ROS.
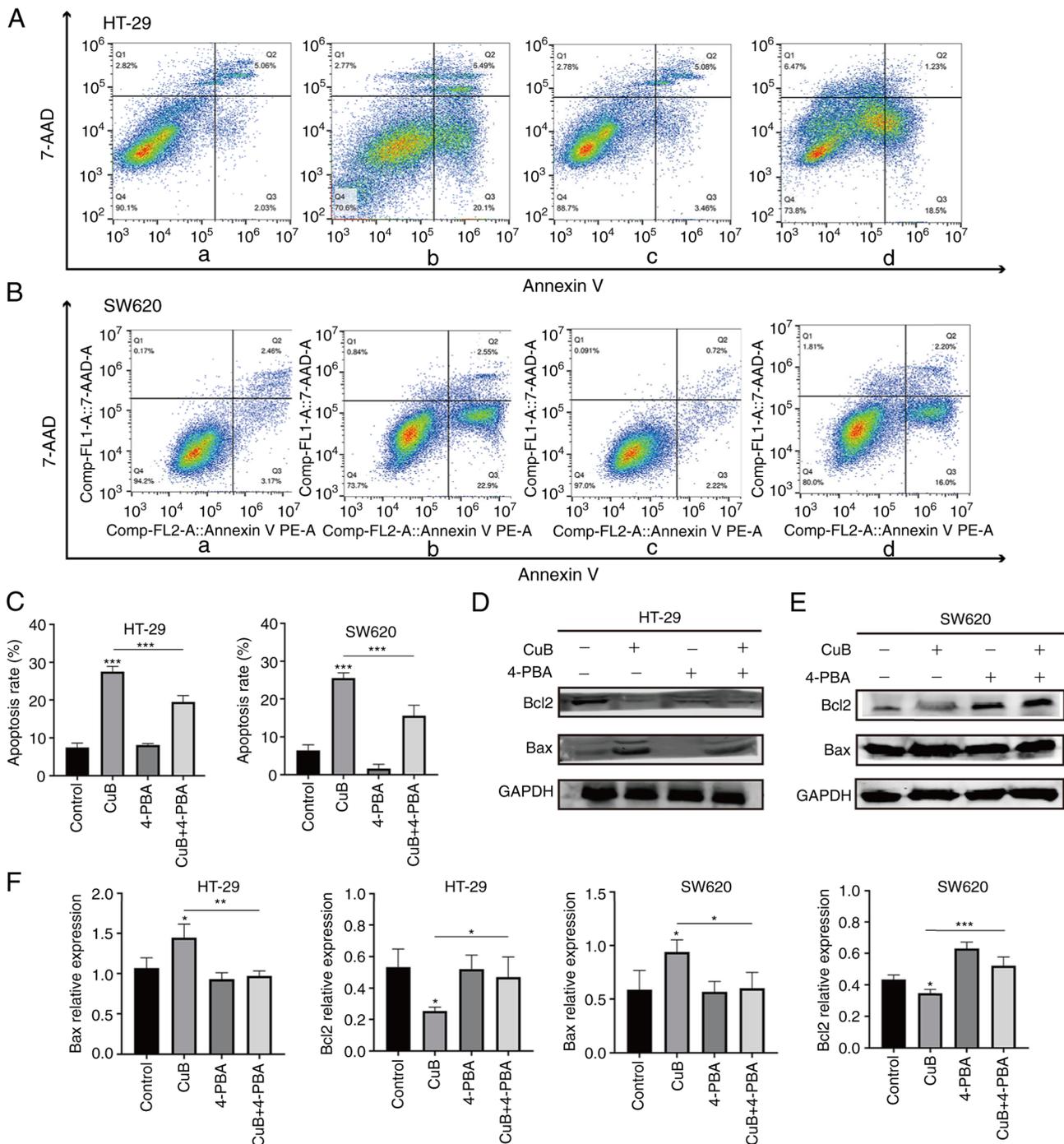
Figure 4. CuB can induce apoptosis in CRC cells. (A-C) CRC cells were pretreated with 4-PBA (800 μM) for 2 h and treated with CuB (5 μM) for 48 h. The apoptosis rates in the (Aa and Ba) control group, (Ab and Bb) CuB-treated group, (Ac and Bc) 4-PBA group and (Ad and Bd) 4-PBA + CuB-treated group were determined by flow cytometry. (A and B) Representative maps of apoptosis rates in HT-29 and SW620 cells detected by flow cytometry. (C) Quantification of apoptosis rates. Western blotting was used to evaluate the expression of the apoptosis-related Bax and Bcl2 proteins in CRC cells pretreated with 4-PBA to inhibit endoplasmic reticulum stress pathways. (D and E) The expression of Bax and Bcl2 proteins in various groups in HT-29 and SW620 cells. (F) Quantification of Bax and Bcl2 protein expression. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control or as indicated. CRC, colorectal cancer; CuB, cucurbitacin B; 4-PBA, 4-phenylbutyric acid.

Discussion

To date, a number of natural compounds have been shown to have significant anticancer activity and act on a variety of targets. Polydatin, for example, inhibits liver cancer cell migration and invasion by targeting c-MYC and Shikonin stimulates the MAPK pathway and promotes death in melanoma cells (26,27). As aforementioned, CuB exhibits significant anticancer activity in various types of cancer; however, the underlying mechanisms of its significant anticancer effects have not been fully elucidated. In the present study on CuB in HT-29 and SW620 CRC cells, it was revealed that CuB was able to inhibit the proliferation of these cells in a concentration-dependent manner and could increase their
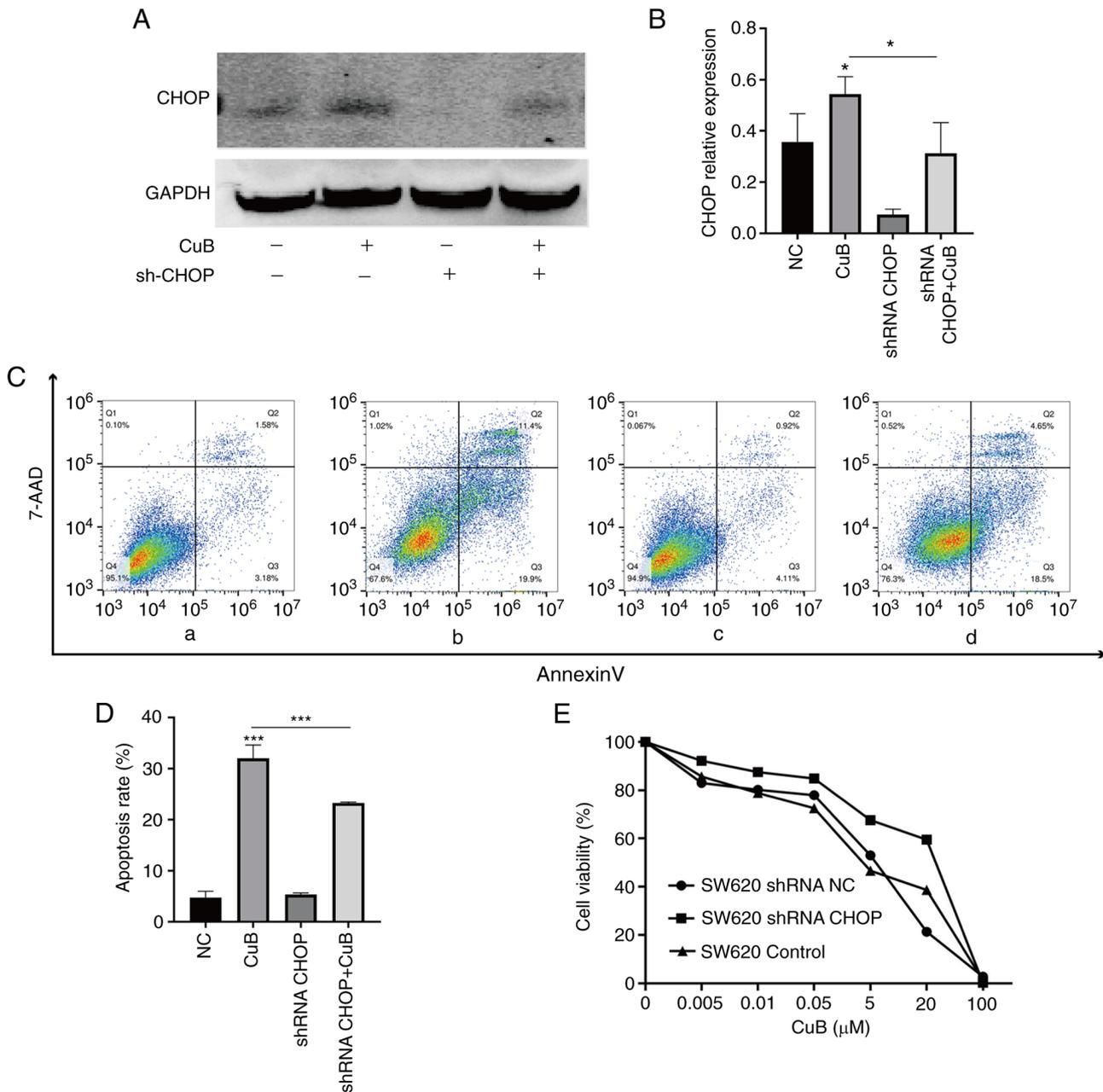
Figure 5. CuB induces apoptosis via endoplasmic reticulum stress and its signal transduction pathways. Lentivirus-mediated knockdown of CHOP was performed in SW620 cells, and the expression of CHOP was measured by western blotting after cells were treated with CuB (5 μM) for 48 h. (A) The expression of CHOP protein. (B) Quantification of CHOP protein expression. (C) After cells were treated with CuB (5 μM) for 48 h, the apoptosis rates in the (Ca) NC group, (Cb) CuB-treated group, (Cc) shRNA CHOP group and (Cd) CuB-treated shRNA CHOP group were determined by flow cytometry. (D) Quantification of apoptosis rates. (E) SW620 cells were treated with CuB (0-100 μM) for 48 h, and the cell viability inhibition rates in the shRNA CHOP group, shRNA NC group and control group were determined using a Cell Counting Kit 8 assay. Data are presented as the mean ± SD of three independent experiments. *P<0.05, ***P<0.001 vs. control or as indicated. CuB, cucurbitacin B; NC, negative control; shRNA, short hairpin RNA.

apoptosis. The main mechanism of action of these effects was related to the CuB-mediated increase in ROS production and ERS pathway activation.

Apoptosis is a stable and conservatively controlled process of autonomous and orderly cell death, which serves an important role in maintaining the stability of the internal environment of an organism (28). From the perspective of tumour therapy, evasion of apoptotic signalling confers a survival advantage on tumour cells, creating conditions suitable for angiogenesis, invasive metastasis and even drug resistance (29). Previously, it has been reported that CuB may have a role in proliferation

inhibition and apoptosis in Burkitt lymphoma Ramos cells by inhibiting the phosphorylation of STAT3, thereby downregulating Bcl-2 and upregulating Bax (30). The present study also revealed that CuB was effective in increasing the apoptosis rates of HT-29 and SW620 cells, increasing the expression levels of Bax and decreasing those of Bcl-2 in CRC cells.

GRP78 is a molecular chaperone in the lumen of the ER, which has an important role in the correct folding of proteins and the maintenance of luminal homeostasis. Elevated GRP78 expression indicates the presence of ERS (31). It has been demonstrated that the IRE1/XBP1
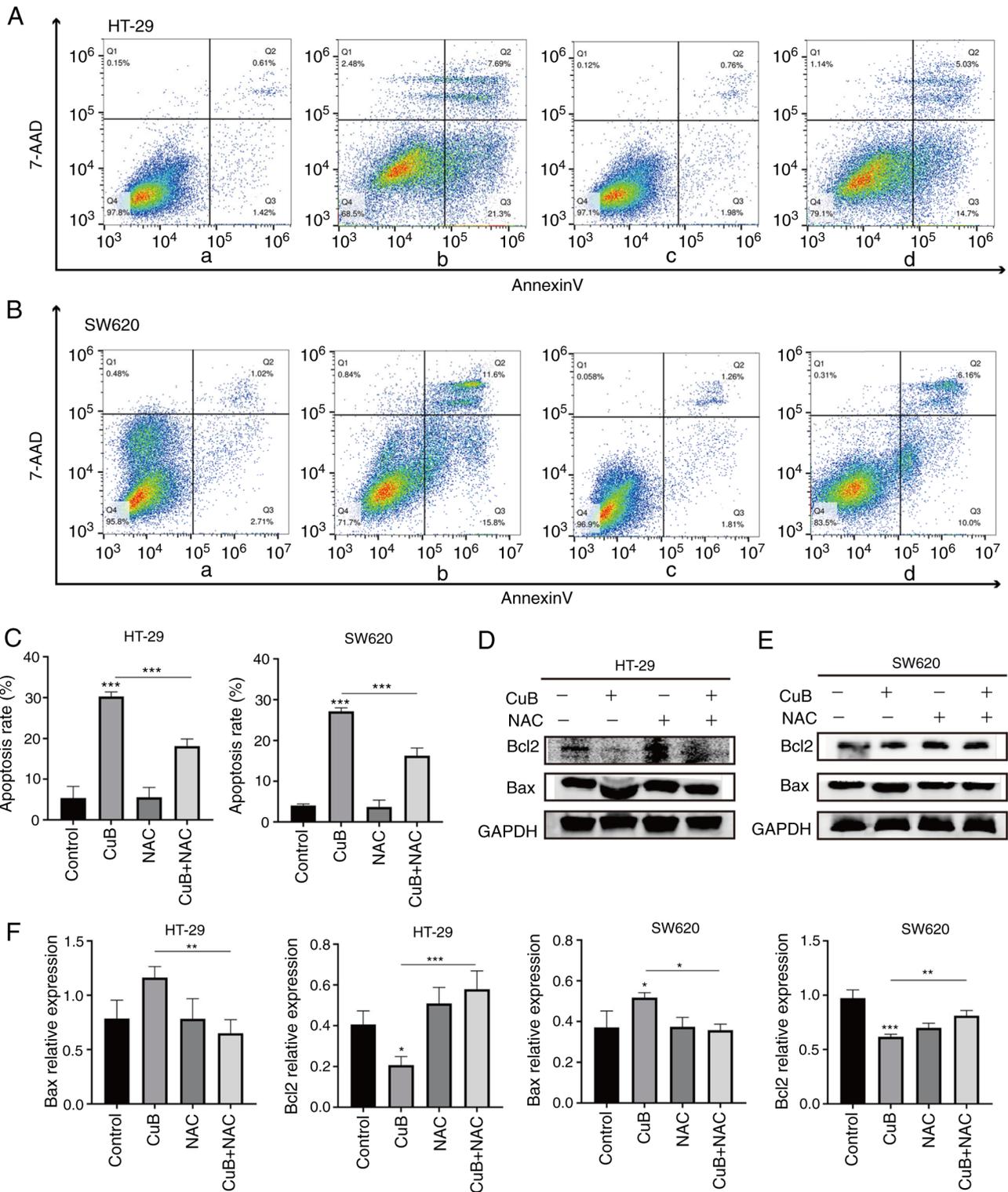
Figure 6. CuB mediates ROS production and induces apoptosis in CRC cells. After CRC cells were pretreated with NAC (5 mM) for 1 h and treated with CuB (5 µM) for 48 h, the apoptosis rates in the (Aa and Ba) control group, (Ab and Bb) CuB-treated group, (Ac and Bc) NAC group and (Ad and Bd) NAC + CuB-treated group were determined by flow cytometry. (A and B) Representative maps of apoptosis rates in HT-29 and SW620 cells detected by flow cytometry. (C) Quantification of apoptosis rates. Western blotting was used to evaluate the expression of the apoptosis-related Bax and Bcl2 proteins in CRC cells pretreated with NAC to inhibit ROS production. (D and E) The expression of Bax and Bcl2 proteins in various groups in HT-29 and SW620 cells. (F) Quantification of Bax and Bcl2 protein expression. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control or as indicated. CRC, colorectal cancer; CuB, cucurbitacin B; NAC, N-acetylcysteine.

and PERK/eIF2α/ATF4/CHOP signalling pathways, in the presence of long-term sustained ERS activation, induce apoptosis (32,33). In the present study, treatment of

HT-29 and SW620 cells with CuB resulted in increased GRP78 expression, and activation of the IRE1/XBP1 and PERK/eIF2α/ATF4/CHOP signalling pathways in the UPR,

a result that demonstrates the ability of CuB to induce ERS in CRC cells.

It has been reported that ROS at low and appropriate concentrations regulate and maintain normal physiological functions, whereas intracellular ROS overload has destructive effects (34). In cancer research, Lai *et al* (35) reported that crassolide, a diterpenoid derived from soft coral, could trigger ERS to induce apoptosis and autophagy through the production of ROS in lung cancer cells. The present experimental results demonstrated that ROS production in HT-29 and SW620 cells was gradually increased with increasing CuB concentration. This is consistent with the findings of increased ROS production induced by CuB in a related study in hepatocellular carcinoma (36). Furthermore, the present study revealed that after treatment with the ROS inhibitor NAC, the expression of ERS-related proteins was suppressed, suggesting that CuB may mediate the regulation of both IRE1 and PERK ERS via ROS production in HT-29 and SW620 cells. In addition, when HT-29 and SW620 cells were pretreated with the ERS inhibitor 4-PBA, CuB-induced apoptosis, Bcl2 downregulation and Bax upregulation were reversed, emphasizing that CuB-induced apoptosis in CRC cells may be associated with ERS.

CHOP is a marker of ERS-induced apoptosis and ultimately induces apoptosis through signalling cascades (37-39). Therefore, the present study also assessed the effects of blocking the main target of ERS-induced apoptosis by downregulating CHOP expression in SW620 cells. The results revealed that knockdown of CHOP reduced CuB-induced apoptosis in SW620 cells compared with that in control cells. Thus, these results suggested that CuB induces apoptosis in CRC cells through ROS-mediated activation of the IRE1 and PERK ERS signalling pathways. When the viability of cells was examined using a CCK8 assay, knockdown of CHOP was found to increase the viability of SW620 cells, further demonstrating that CuB inhibits the viability of CRC through ROS-mediated activation of ERS.

According to a previous study, ROS can cause apoptosis (40). The present results showed that the CuB-induced apoptosis of CRC cells was decreased after pretreatment with NAC, and that CuB-induced Bcl2 downregulation and Bax upregulation were also reversed, indicating that CuB can induce apoptosis through ROS. These findings demonstrated that CuB can induce apoptosis in CRC cells through ROS and ERS; furthermore, CuB may induce apoptosis and inhibit cancer cell proliferation through ROS-mediated activation of ERS.

Although the present results confirmed the mechanism underlying the effects of CuB on the apoptosis of HT-29 and SW620 CRC cells, the present study still has limitations. First, the present study successfully verified the role of ERS in the CuB-induced apoptosis of CRC cells in the SW620 cell line, in which CHOP was knocked down, but did not validate the results in the HT-29 cell line. Second, to the best of our knowledge, just one *in vivo* investigation of CRC has validated the antitumour effectiveness of CuB (11). As a result, whether CuB has a similar curative effect in animal models, and whether it is associated with ERS and ROS should be investigated in further animal studies.

In conclusion, the results of the present study demonstrated that ROS and ERS have important roles in CuB-induced apoptosis in CRC cells, and specifically demonstrated that CuB can induce apoptosis in CRC cells through ROS-mediated activation of ERS. These findings may provide new ideas for the clinical development of novel drugs for the treatment of CRC.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JH was mainly responsible for experiments, data analysis and draft writing. LL visualized and analysed the data. PX performed experiments and data visualization. WS was responsible for data collation and analysis. LW was responsible for analysing the data. ZC was responsible for project conceptualization and design, management, validation, and review and editing of the manuscript. JH and ZC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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