Icariin sensitizes human colon cancer cells to TRAIL-induced apoptosis via ERK-mediated upregulation of death receptors

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Abstract. Tumor necrosis factor-associated apoptosis-inducing ligand (TRAIL) is considered to be a potential therapeutic target for various types of cancer. However, colon cancer is difficult to treat due to its resistance to TRAIL. Therefore, various trials have been conducted to overcome TRAIL resistance in colon cancer. The present study aimed to determine whether icariin (ICA) may sensitize human colon cancer cells to TRAIL-induced apoptosis *in vitro* and *in vivo*. In the investigation of the effect of ICA on TRAIL-induced apoptosis, the LIVE/DEAD assay results demonstrated that TRAIL plus ICA synergistically induced apoptosis in 49% of HCT116 colon cancer cells. These results were confirmed using long-term colony formation assay. ICA potentiated TRAIL-induced apoptosis by modulating the expression of apoptotic proteins and the induction of cell surface death receptors (DRs) 4 and 5. Upregulation of DRs by ICA was also observed at the transcriptional level by RT-PCR. The expression of DR by ICA was increased through the production of reactive oxygen species (ROS). The results also suggested that increased expression of DR by ICA may be due to the activation of ERK and induction of the transcription factor CCAAT enhancer-binding protein homologous protein (CHOP). NAC, a ROS scavenger, reduced the effect of ICA on ERK activation, DR induction and sensitization of TRAIL-induced apoptosis. In addition, ICA enhanced the effects of TRAIL to reduce tumor growth in an *in vivo* xenograft mouse model. Overall, the present study provided evidence that ICA sensitized tumor cells to TRAIL-induced apoptosis via ROS-, ERK- and CHOP-mediated upregulation of DR5 and DR4. Based on

these results, it is suggested that the antitumor activity of ICA and TRAIL co-treatment *in vitro* and *in vivo* may be used as an effective therapeutic agent in chemotherapy.

Introduction

Colon cancer is the third most common cancer and a leading cause of cancer-associated death in the world (1). Despite the availability of effective screening, chemopreventive and lifestyle strategies that have resulted in a decline in the mortality rate, about one-fifth of patients with colon cancer present with metastatic disease at diagnosis, and one-fifth develop metastasis during the course of treatment (2). Therefore, new colon cancer therapies are urgently needed.

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is considered to be a potential target in anticancer therapy due to its specific ability to induce apoptosis of cancer cells, but not normal human cells (3). TRAIL interacts with the extracellular domain of death receptors (DRs), DR4 and DR5, which in turn activate intracellular apoptotic signaling (3). Mutations or decreased levels of DR4 and DR5 induce cancer cell resistance to TRAIL, whereas upregulation of DR4 and DR5 expression using co-treatment with subtoxic doses of chemotherapeutic drugs is an effective strategy to overcome TRAIL resistance (4,5). TRAIL recombinant protein and agonistic antibodies for DR4 and DR5 have been developed as potential treatments for cancer (6,7). However, advances in pharmacotherapy may limit the effectiveness of agonistic antibodies by monotherapy, and it is imperative to identify molecules that promote TRAIL-induced cell death or sensitize resistant cancer cells to TRAIL.

TRAIL induces cell death via two main apoptotic pathways: Death receptor-mediated extrinsic pathway and mitochondria-mediated intrinsic apoptotic pathways (8). In the extrinsic pathway, TRAIL binds with death TRAIL receptor (DR4 or DR5) to stimulate the recruitment of Fas-associated death domain (FADD) and pro-caspase-8, known as the death-inducing signaling complex (DISC). Subsequently, pro-caspase-8 is cleaved to caspase-8, which directly activates caspase-3 (3). In the intrinsic pathway, caspase-8 cleaves BH3 interacting-domain death agonist (Bid), and truncated Bid (tBid) is translocated to the mitochondria to initiate mitochondrial apoptosis; mitochondrial disruption leads to the release of cytochrome *c* from the mitochondria

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into the cytoplasm, which binds with apoptotic peptidase-activating factor 1 to form an apoptosome and activates caspase-9, as well as downstream caspases (9).

In addition, reactive oxygen species (ROS) are upstream signaling molecules that induce DR expression in various cancer cells and are involved in the regulation of TRAIL signaling (10). High ROS activity in cancer cells compared with normal cells is known to induce apoptosis by damaging the DNA, proteins and lipid membranes (11). ROS induces apoptosis through the regulation of pro- and anti-apoptotic proteins by mitogen-activated protein kinase (MAPK) phosphorylation (12). Based on this, ROS are highly dependent on the sustained MAPK signaling activity (12). Thus, TRAIL and its receptor DR4 and DR5 agonists targeting the apoptotic pathway have been actively studied as potential therapeutic methods for inhibiting the proliferation of various types of cancer cells (6,7).

Icariin (ICA), a prenylated flavonol glycoside derived from the Chinese herb *Epimedium sagittatum*, exhibits a variety of pharmacological properties including antioxidant (13), antitumor (14,15) and estrogen-like (16,17) activities. In particular, ICA exhibits a broad spectrum of anticancer effects, such as tumor growth inhibition (14), suppression of tumor cell invasion and migration (18) and induction of the S-phase in cell cycle arrest and apoptosis (19). Based on the effects of ICA reported in these previous studies, it was hypothesized that ICA may activate TRAIL-induced apoptosis through the induction of DR expression. Therefore, this study investigated whether ICA can sensitize colon cancer cells to TRAIL-induced apoptosis and which mechanism is involved in this pathway.

Materials and methods

Reagents. ICA was prepared by Professor Ki Yong Lee (College of Pharmacy, Korea University). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, Antibiotic-antimycotic and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. 2',7'-dichlorofluorescein diacetate (DCFH-DA), Lipofectamine® 2000, TRIzol® reagent and kits for the LIVE/DEAD assay were purchased from Invitrogen; Thermo Fisher Scientific, Inc. AccuPower Rocketscript cycle RT premix and AccuPower PCR PreMix were purchased from Bioneer Corporation. Soluble recombinant human TRAIL/Apo2L (10 mg/ml; cat. no. 310-04) was purchased from PeproTech, Inc. Antibodies against CCAAT enhancer-binding protein homologous protein (CHOP; 1:1,000; cat. no. 2895), Bcl-xL (1:1,000; cat. no. 2764), cIAP-1 (1:1,000; cat. no. 4952), poly (ADP-ribose) polymerase (PARP; 1:1,000; cat. no. 9542), caspase-3 (1:1,000; cat. no. 9662), caspase-9 (1:1,000; cat. no. 9502), cleaved PARP (1:1,000; cat. no. 5625), cleaved caspase-3 (1:1,000; cat. no. 9661), cleaved caspase-8 (1:1,000; cat. no. 9496), cleaved caspase-9 (1:1,000; cat. no. 7237), ERK (1:1,000; cat. no. 9102), phospho-ERK (1:1,000; cat. no. 9101), p38 (1:1,000; cat. no. 9212), phospho-p38 (1:1,000; cat. no. 9211), phospho-JNK (1:1,000; cat. no. 9255), survivin (1:1,000; cat. no. 2808), JNK (1:1,000; cat. no. 9252), X-linked inhibitor or apoptosis proteins (XIAP; 1:1,000; cat. no. 2042), cytochrome *c* (1:1,000; cat. no. 11940), DR5 (1:1,000; cat. no. 3696) and β-actin (1:1,000; cat. no. 3700) antibodies,

and anti-rabbit (1:5,000; cat. no. 7074) and anti-mouse (1:5,000; cat. no. 7076) secondary antibodies were obtained from Cell Signaling Technology, Inc. Bcl-2 associated X protein (BAX; 1:1,000; cat. no. sc-493), Bcl-2 (1:1,000; cat. no. sc-492) and DR4 (1:1,000; cat. no. sc-7863) were obtained from Santa Cruz Biotechnology, Inc. N-acetyl-L-cysteine (NAC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich; Merck KGaA. RNAiMAX transfection reagent was purchased from Invitrogen; Thermo Fisher Scientific, Inc. The control small interfering (si)RNA (scRNA) and CHOP siRNA were obtained from Santa Cruz Biotechnology, Inc. The PD98059, SB202190, SP600125 and ERK1/2-MAPK siRNA were obtained from Cell Signaling Technology, Inc.

Cell culture. Cell lines were obtained from ATCC. The human cell lines HCT-116 (colon adenocarcinoma), MDA-MB-231 (breast adenocarcinoma), HPAC, PANC-1 and BxPC3 (pancreatic adenocarcinoma) were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. The human cell line MCF-7 (breast adenocarcinoma) was cultured in RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin. The human colon cancer cell line HT-29 was cultured in RPMI-1640 medium containing 25 mM HEPES, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained at 37°C in an atmosphere of 5% CO_2.

Cytotoxicity assay. MTT assay was used to test the effects of ICA on the cytotoxic potential of TRAIL. To investigate the synergy between ICA and TRAIL, HCT116 and HT-29 cells ($5x10^3$) were treated with ICA alone (5, 10, 20, 40 and 80 μM), TRAIL alone (5, 10, 20,40 and 80 ng/ml) and ICA (5, 10, 20, 40 and 80 μM) in combination with 20 ng/ml TRAIL for 24 h at 37°C. Subsequently, 10 μl MTT solution (5 mg/ml) was added to each well and cultured for 4 h at 37°C. The medium was removed, formazan was dissolved in DMSO, and cell viability was measured at 560 nm using a microplate reader (Tecan Group, Ltd.). The results were described as the relative percentage compared with untreated cells.

LIVE/DEAD assay. The LIVE/DEAD assay, which is a two-color fluorescence assay that determines numbers of live and dead cells, was used to measure apoptosis. Briefly, $1x10^6$ HCT116 cells were incubated with 10 μM ICA, 20 ng/ml TRAIL or 10 μM of ICA in combination with 20 ng/ml TRAIL for 24 h at 37°C. Cells were stained with the LIVE/DEAD reagent (5 μM ethidium homodimer and 5 μM calcein-AM) and incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (x200 magnification; Nikon Corporation). The percentage value was derived by calculating the number of red and green cells.

Colony formation assays. Colony formation assays can be used to analyze colony formation *in vitro* and determine cell viability. For the anchorage-dependent colony formation assay, HCT-116 cells ($5x10^4$) were seeded on top of 1 ml of 0.9% agar containing 10 ml each of 10 μM ICA, 20 ng/ml TRAIL and 10 μM ICA in combination with 20 ng/ml TRAIL. The plates were incubated in complete DMEM for 14 days. Images of the stained colonies were acquired using a digital camera

(Canon, Inc.), and the number of colonies was counted using ImageJ bundled with 64-bit Java 1.6.0_ 20 software (National Institutes of Health).

Measurement of intracellular ROS. Intracellular ROS levels were measured using the fluorescent probe 2,7-dichloro-fluorescein diacetate (DCFH-DA). HCT116 cells ($5x10^5$) were pre-exposed to NAC for 1 h, treated with ICA for 24 h, washed and labeled with 25 μM DCFH-DA. Following incubation for 30 min at 37°C in a 5% CO_2 incubator, the cells were washed twice with PBS, and intracellular ROS was detected under fluorescence microscopy (x200 magnification; Nikon Corporation) at an excitation of 488 nm and emission of 525 nm. Image-Pro Plus 4.5 software (Media Cybernetics, Inc.) was used for analysis. The mean fluorescence intensity of the images was assessed and normalized to obtain relative ratios that were compared between the experimental groups.

MAPK inhibitor treatment. HCT116 cells ($1x10^6$) were pretreated with MAPK inhibitors, such as ERK1/2-specific inhibitor PD98059 (20 μM), JNK-specific inhibitor SP600125 (20 μM) and p38-specific inhibitor SB202190 (10 μM). After 30 min at 37°C, the cells were treated with ICA (10 μM) for 24 h.

Western blot analysis. The protein extracts from cells and xenograft tumors were subjected to western blot analysis as previously described (20). Cells and tissues were harvested and lysed with RIPA buffer, and the protein samples were quantified using a Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amount of protein extracts was denatured by boiling at 100°C for 5 min in sample buffer (5X SDS-PAGE buffer). The proteins (30 μg) were separated by 8-12% SDS-PAGE and transferred to a PVDF membrane (Roche Diagnostics GmbH). The membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween-20 (TBS-T; 10 mM Tris, 150 mM NaCl, pH 7.5; 0.1% Tween-20) for 1 h at room temperature. The membranes were washed three times for 10 min with TBS-T and incubated with primary antibodies at 4°C. After three washes of 10 min each in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 2 h and washed. The membranes were incubated with SuperSignal Pico Chemiluminescent substrate or Dura-Luminol substrate (Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction and visualized with Imagequant™ LAS 4000 (Fujifilm Life Science, Tokyo, Japan).

RNA analysis and reverse transcription-polymerase chain reaction (RT-PCR). mRNA levels were measured using RT-PCR as described in our previous study (20). The following primers were used: DR4 forward, 5'-AAGTCCCTG CACCACGAC-3' and reverse, 5'-CCACAACCTGAGCCG ATG-3'; DR5 forward, 5'-AAGACCCTTGTGCTCGTTGT-3' and reverse, 5'-GACACATTCGATGTCACTCCAG-3'; and GAPDH forward, 5'-CAGCCTCAAGATCATCAGCA-3' and reverse, 5'-GTCTTCTGGGTGGCAGTGAT-3'. The amplified products were analyzed by electrophoresis using a 1.5% agarose gel stained with ethidium bromide and photographed with ImageQuant LAS 4000 (Fujifilm Corporation).

Transfection with siRNA. HCT116 cells ($5x10^5$) were plated in 6-well plates and allowed to adhere for 24 h. On the day of transfection, 9 μl Lipofectamine RNAiMAX transfection reagent was added to 10 μM CHOP siRNA, ERK1/2 siRNA and scRNA in 150 μl Opti-MEM. After 24 h of transfection, the cells were treated with ICA alone or ICA in combination with TRAIL. CHOP siRNA (cat. no. sc-35437; Santa Cruz Biotechnology, Inc.) contained three unique 21mer siRNAs as follows: 35437A, sense, 5'-GAAGGCUUGGAGUAGACA ATT-3', antisense 3'-UUGUCUACUCCAAGCCUUCTT-5'; 35437B, sense 5'-GGAAAGGUCUCAGCUUGUATT-3', antisense 3'UACAAGCUGAGACCUUUCCTT-5'; 35437C, sense 5'-GUCUCAGCUUGUAUAUAGATT-3', antisense 3'-UCUAUAUACAAGCUGAGACTT-5'. ERK1/2 siRNA (cat. no. 6560; Cell Signaling Technology, Inc.) sequences were as follows: Sense 5'-CCUCCAACCUGCUCAUCAA-3', antisense 3'-UUGAUGAGCAGGUUGGAGG-5'. The negative control scRNA (cat. no. sc-37007; Santa Cruz Biotechnology, Inc.), which did not target any endogenous transcript, was used as a control.

Animals. Male BALB/c (nu/nu) mice (5 weeks old) were purchased from Orient Bio, Inc. All protocols were approved by the Institutional Animal Care and Use Committee of the Keimyung University (Daegu, South Korea; approval no. KM_2018-010; 1 August 2018) and were performed in accordance with the criteria outlined in the Institutional Guidelines for Animal Research. The mice were maintained in a room with no airborne pathogen under controlled illumination (12 h light/day) with free access to food and water. Humane endpoints were based on activity assessments such as hunching, lack of activity, poor grooming and ruffling or a 20% reduction in the overall body weight of the mice. No animals were sacrificed due to meeting these endpoints.

In vivo xenograft tumor model and treatment. HCT-116 cells ($5x10^5$/100 μl) were suspended in DMEM with 100 μl Matrigel and inoculated subcutaneously into the left flank of each mouse. When tumor masses were established and palpable (tumor volume >150 mm^3), mice were randomly divided into 4 groups (5 mice/group) for intraperitoneal injection as follows: i) Vehicle group, 0.9% sodium chloride + 1% DMSO; ii) ICA group, 10 mg/kg ICA dissolved in vehicle; iii) TRAIL group, 100 μg/kg TRAIN dissolved in vehicle; and iv) ICA and TRAIL group, ICA and TRAIL in combination three times per week for 3 weeks. Tumor volumes and body weights were measured three times per week. Tumor growth was monitored twice a week by measuring two axes of the tumor (L, longest axis; W, shortest axis) with a digital caliper during the treatment. Tumor volume was calculated as $V=LxW^2/2$. All mice were sacrificed by carbon dioxide 19 days after the first day of treatment, and cancer tissues were collected. Mice were euthanized by 100% carbon dioxide at 20-30% volume/min. for 5-6 min. Death was confirmed when no spontaneous breathing or blinking reflex was observed for 2-3 min.

Statistical analysis. Data are presented as the mean ± SEM of least three independent experiments. The statistical analyses were performed using GraphPad Prism 6 software package (GraphPad Software, Inc.). Statistical analyses were

performed using one-way ANOVA and Bonferroni's post hoc test to identify significant differences in MTT assay, western blot analysis, colony formation assay, RT-PCR and *in vivo* measurements. The Bonferroni's post hoc test was used for comparison with the control group. P<0.05 was considered to indicate a statistically significant difference.

Results

ICA enhances TRAIL-induced apoptosis in HCT-116 Cells. The present study investigated the effects of ICA on TRAIL-induced cytotoxicity by MTT assay. The viability of HCT-116 and HT-29 cells was significantly reduced by ICA and TRAIL co-treatment compared with the control cells (Fig. 1A and B). To determine the effect of ICA on TRAIL-induced apoptosis in HCT116 cells, apoptotic cell death was measured using the LIVE/DEAD assay. The results indicated that TRAIL alone exhibited a minimal effect (12%) on apoptosis, and ICA alone induced apoptosis in 6% of HCT-116 cells. However, co-treatment with TRAIL and ICA synergistically induced apoptosis in 49% of HCT-116 cells (Fig. 1C). Next, long-term colony formation assay was used to determine whether ICA enhanced the effect of TRAIL. The results demonstrated that ICA or TRAIL alone exhibited minimal effects on colony formation of HCT-116 cells, whereas the combination treatment completely suppressed the colony-forming ability of these cells compared with the control cells (Fig. 1D). The present study also investigated whether ICA enhanced TRAIL-induced activation of apoptosis markers caspase-9 and caspase-3, as well as consequent PARP cleavage. The results demonstrated that that ICA enhanced TRAIL-induced activation of caspases compared with the control group, leading to increased PARP cleavage (Fig. 1E). These results indicated that ICA enhanced TRAIL-induced apoptosis in HCT-116 cells.

ICA induces the expression of DR4 and DR5 in HCT-116 and HT-29 cells. To explore the mechanism underlying the increased levels of TRAIL-induced apoptosis following exposure to ICA, the effect of ICA on the expression of DRs was analyzed. When HCT-116 and HT-29 cells were treated with ICA, the expression levels of DR4 and DR5 were increased at 5 and 10 μM (Fig. 2A). In addition, ICA induced DR4 and DR5 at 24 h (Fig. 2B). RT-PCR results also demonstrated that ICA substantially upregulated DR4 and DR5 mRNA expression levels at 10 μM (Fig. 3A) and 24 h (Fig. 3B). ICA-induced expression of DR4 and DR5 was also examined in various cancer cell types; ICA induced the expression of DR4 and DR5 in pancreatic adenocarcinoma (HPAC, PANC-1 and BxPC3) and breast adenocarcinoma (MDA-MB-231 and MCF7) cells compared with untreated cells (Fig. 3C). These results suggested that ICA induced the upregulation of DR4 and DR5 in various types of cancer cells.

Co-treatment with TRAIL and ICA modulates the expression of apoptotic proteins. Whether ICA modulated the expression of proteins involved in apoptosis of colon cancer cells upon co-treatment with 20 ng/ml TRAIL was then examined. In HCT-116 and HT-29 cells, the expression of antiapoptotic proteins Bcl-2, Bcl-xL, c-IAP-1, survivin and XIAP was

reduced by 10 μM ICA (Fig. 4A). In addition, the results presented in Fig. 4B indicated that the expression levels of the pro-apoptotic proteins BAX and cytochrome c were significantly increased following co-treatment with ICA and TRAIL compared with those in the control group. These results suggested that ICA sensitized TRAIL-induced apoptosis by modulating the expression of apoptotic proteins.

ICA-induced DR5 upregulation is mediated by the induction of CHOP. Several studies have reported that the induction of DRs by various agents is mediated by the activation of CHOP (10,21-23). Therefore, the present examined whether ICA induced the expression of CHOP. Cells were pretreated with the indicated concentrations of ICA for 24 h or 10 μM ICA for the indicated times, and the expression of CHOP was measured. The results demonstrated that ICA induced CHOP expression in HCT-116 and HT-29 cells in a dose-(Fig. 5A) and time-dependent manner (Fig. 5B). To elucidate the functional role of CHOP in ICA-induced upregulation of DR4 and DR5, CHOP siRNA was transfected into HCT-116 cells. Transfection with CHOP siRNA significantly abrogated the ICA-mediated upregulation of DR5 compared with the control group, whereas DR4 and DR5 expression levels were increased by ICA in non-transfected and scrRNA-transfected cells. CHOP siRNA did not exhibit notable effects on ICA-induced DR4 expression. Knockdown of CHOP by siRNA also reduced PARP cleavage in HCT-116 cells co-treated with ICA and TRAIL compared with the control (Fig. 5C).

ROS are required for the upregulation of DR4 and DR5 by ICA. The role of ICA in the induction of CHOP and DRs was further investigated. Several studies have reported that ROS serve a role in the induction of DR4 and DR5 (24-26). Therefore, the present study investigated whether ROS mediated ICA-induced upregulation of DRs. Whether ICA triggered ROS generation in HCT-116 cells was first examined; as demonstrated in Fig. 6A, treatment with ICA increased the ROS generation compared with the control cells, which was reversed by pretreatment with the ROS scavenger NAC. To examine whether ROS production was required for the expression of DRs by ICA, HCT-116 cells were pretreated with the indicated concentrations of NAC and subsequently supplemented with ICA. The results demonstrated that ICA induced DR4 and DR5 expression compared with the control group, which was suppressed by NAC in a concentration-dependent manner (Fig. 6B).

Whether NAC pretreatment affected the sensitization of TRAIL-induced apoptosis by ICA was also determined. ICA enhanced TRAIL-induced the cleavage of caspase-8, caspase-3, caspase-9 and PARP compared with the control cells, whereas NAC abrogated this increase (Fig. 6C).

ICA activates MAPK signaling. As reported in previous studies, MAPK activation serves an important role in DR upregulation (27,28). Therefore, to further investigate the upstream signaling pathways mediating CHOP and DR expression regulation by ICA, HCT-116 cells were treated for 24 h with the indicated concentrations of ICA, and the expression of the MAPK pathway proteins was analyzed by western
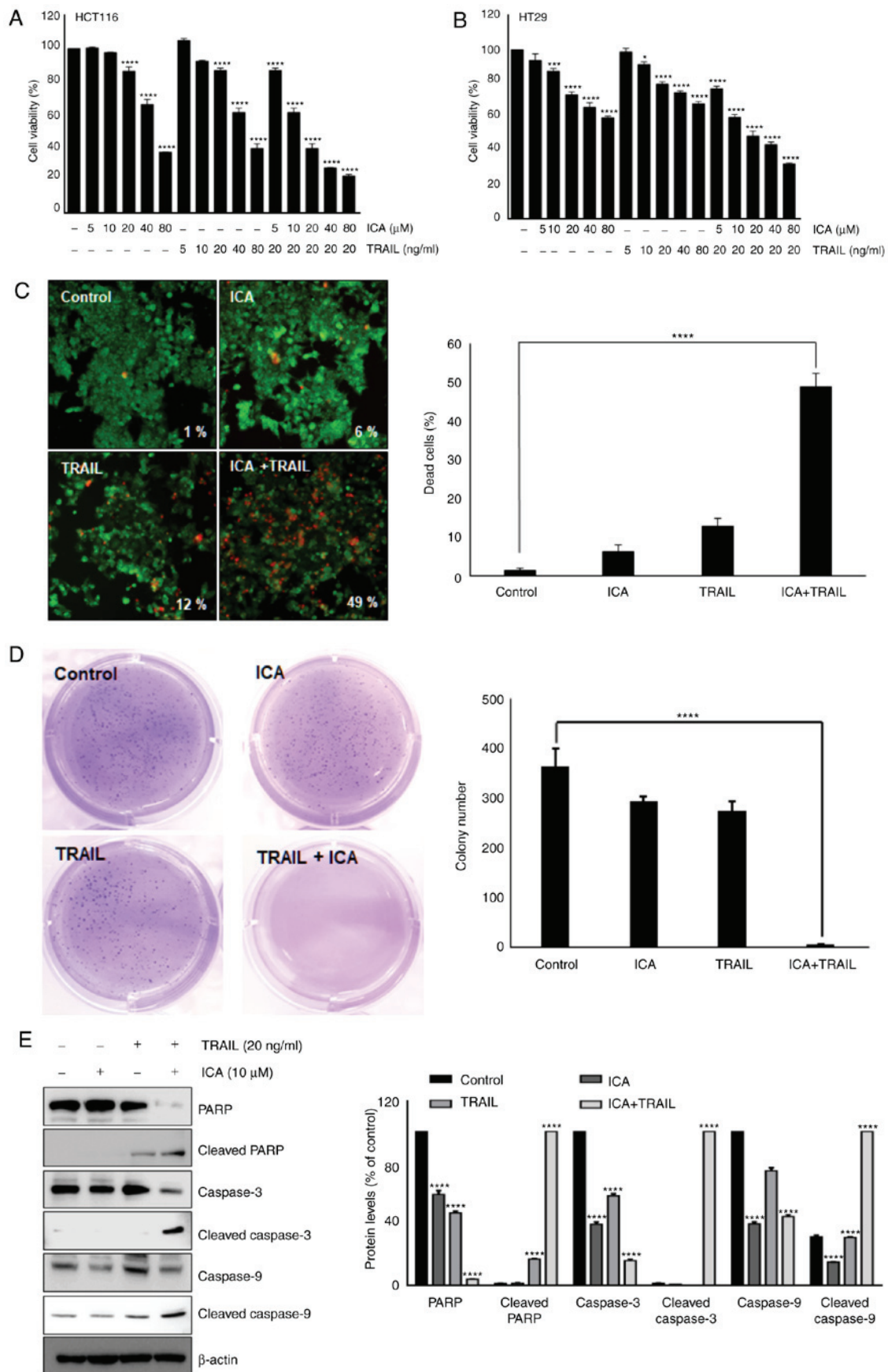
Figure 1. ICA sensitizes human cancer cells to TRAIL. (A) HCT-116 and (B) HT-29 cells were treated with the indicated concentrations of ICA or TRAIL alone or ICA + 20 ng/ml TRAIL for 24 h. Cell viability was measured by MTT assay. (C) HCT116 cells were treated with 10 μM ICA or 20 ng/ml TRAIL alone or 10 μM ICA + 20 ng/ml TRAIL for 24 h. A LIVE/DEAD assay was performed, and the percentages of dead cells (red) are presented. (D) HCT-116 cells were washed and allowed to form colonies for 14 days. The colonies were then stained with crystal violet and counted. (E) Whole cell extracts from treated cells were analyzed by Western blotting using the indicated antibodies. Data are expressed as the mean ± SEM and represent three independent experiments. [*]P<0.05, [***]P<0.001 and [****]P<0.0001 vs. control. ICA, icariin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.
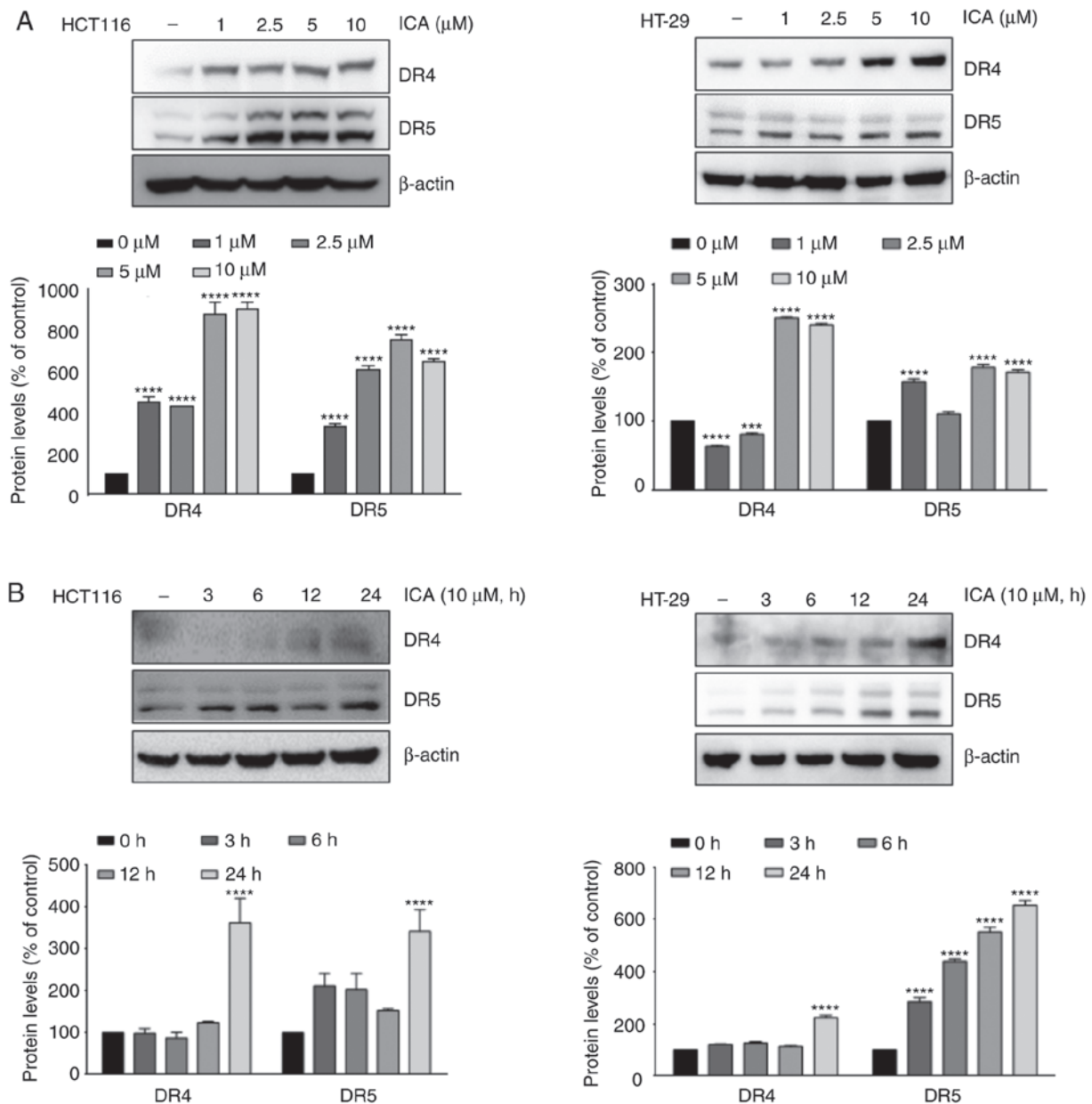
Figure 2. ICA induces the protein expression of TRAIL receptors DR4 and DR5. (A) HCT-116 and (B) HT-29 cells were treated with the indicated concentrations of ICA for 24 h or 10 μM ICA for the indicated time. DR4 and DR5 protein expression was measured by western blotting. The results are expressed as mean percentage of control ± SEM and represent three independent experiments. ***P<0.001 and ****P<0.0001 vs. control. ICA, icariin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; DR, death receptor.

blotting. ICA markedly induced the phosphorylation of ERK and p38 MAPK in HCT-116 cells (Fig. 6D). In addition, it has been reported that ROS are involved in the activation of MAPK (29). Therefore, the present study investigated whether the activation of MAPK proteins by ICA was mediated by ROS production. Cells were exposed to 20 mM NAC for 1 h, and treated with the indicated concentrations of ICA. Pretreatment with NAC did not affect the activation of p38 and JNK; however, NAC inhibited ICA-induced ERK phosphorylation (Fig. 6E). These results suggested that the activation of the ERK signaling pathway induced by ICA was mediated by ROS synthesis.

Activation of ERK is required for ICA-induced upregulation of DR4 and DR5. To elucidate the association between DR

expression and ERK activation, HCT-116 cells were pretreated with 20 μM PD98059 (ERK inhibitor), 10 μM SB202190 (p38 inhibitor) and 20 μM SP600125 (JNK inhibitor) prior to treatment with 10 μM ICA. The results revealed that PD98059 markedly suppressed ICA-induced expression of DR4 and DR5 (Fig. 7A). To demonstrate that ICA served an important role in promoting TRAIL induced apoptosis by increasing ERK phosphorylation and death receptor expression, ERK siRNA was used to inhibit the activity of ERK. As presented in Fig. 7B, the knockdown of ERK notably inhibited ICA-induced DR4, DR5 and CHOP expression in HCT-116 cells. In addition, ERK knockdown reduced the cleavage of PARP induced by ICA and TRAIL co-treatment in HCT-116 cells (Fig. 7C). These results indicated that the activation of ERK by ICA was essential for the upregulation of CHOP, DR4 and DR5
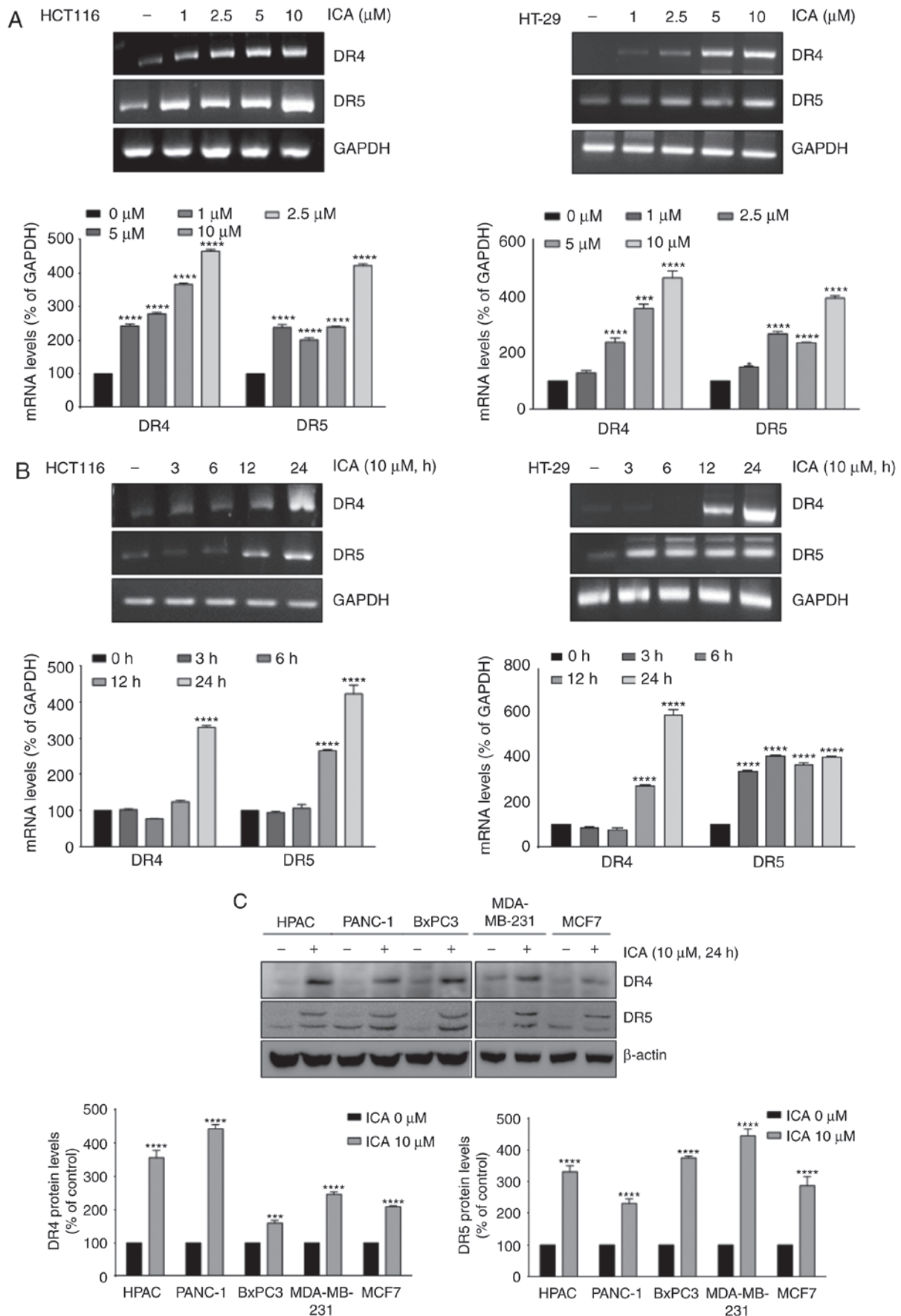
Figure 3. ICA induces the mRNA expression of TRAIL receptors DR4 and DR5. (A) HCT-116 and (B) HT-29 cells were treated with the indicated concentrations of ICA for 24 h or 10 μM ICA for the indicated time. Total RNA was extracted, and mRNA levels were analyzed by RT-PCR. GAPDH was used as an internal control. (C) Human pancreatic cancer (HPAC, PANC-1, BxPC3) and breast cancer (MDA-MB-231, MCF7) cells were treated with 10 μM ICA for 24 h. The expression of DRs was analyzed by western blotting. The results are expressed as the mean percentage of control ± SEM and represent three independent experiments. [***]P<0.001 and [****]P<0.0001 vs. control. ICA, icariin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; DR, death receptor.
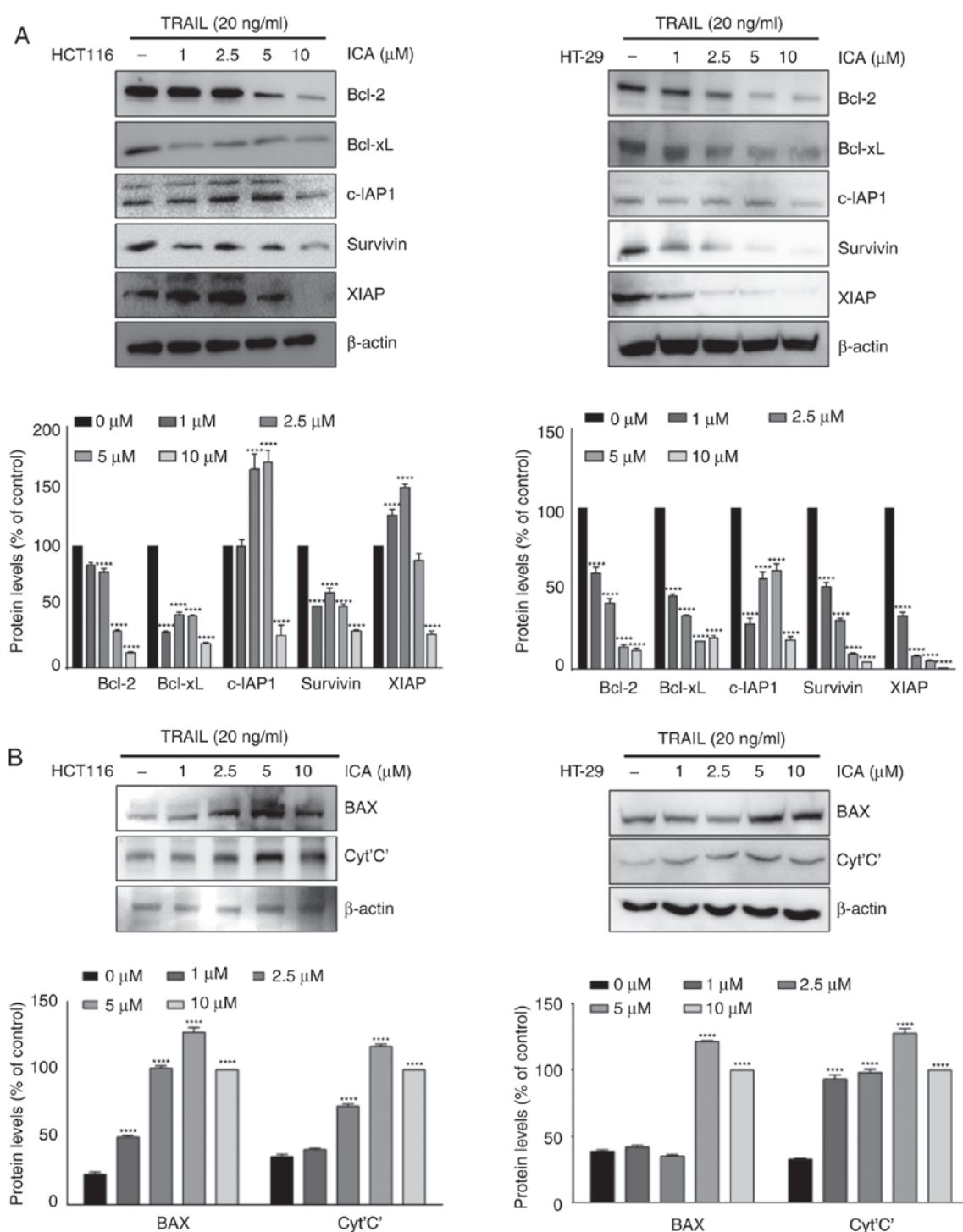
Figure 4. Combination of TRAIL and ICA modulates the expression of apoptotic proteins. (A) HCT-116 and (B) HT-29 cells were treated with the indicated concentrations of ICA and 20 ng/ml TRAIL for 24 h. The whole cell extracts were subjected to western blot analysis. The results are expressed as mean percentage of control ± SEM and represent three independent experiments. ****P<0.0001 vs. control. ICA, icariin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; c-IAP1, cellular inhibitor of apoptosis proteins; XIAP, X-linked inhibitor or apoptosis proteins; Cyt'C', cytochrome *c*.

expression and sensitization of TRAIL-induced apoptosis in colon cancer cells.

ICA enhances the effects of TRAIL to reduce tumor growth in vivo. Based on the aforementioned results, the present study evaluated the therapeutic effect of ICA using an *in vivo* xenograft mouse model. Since a previous study reported that administration of ICA with 10 mg/kg to mice contributed to

the improvement of colitis (30), the same dose was selected in the present study. Treatment was initiated when tumor size reached 150 mm^3, and mice received intraperitoneal injections of 10 mg/kg ICA and 100 μg/kg TRAIL. Tumor volumes were calculated to evaluate the tumor suppression efficacy of the drugs. The largest volumes of the subcutaneous tumors in the study were as follows: Control group, 303.5 mm^3; ICA group, 135.5 mm^3; TRAIL group, 121.6 mm^3; and ICA + TRAIL
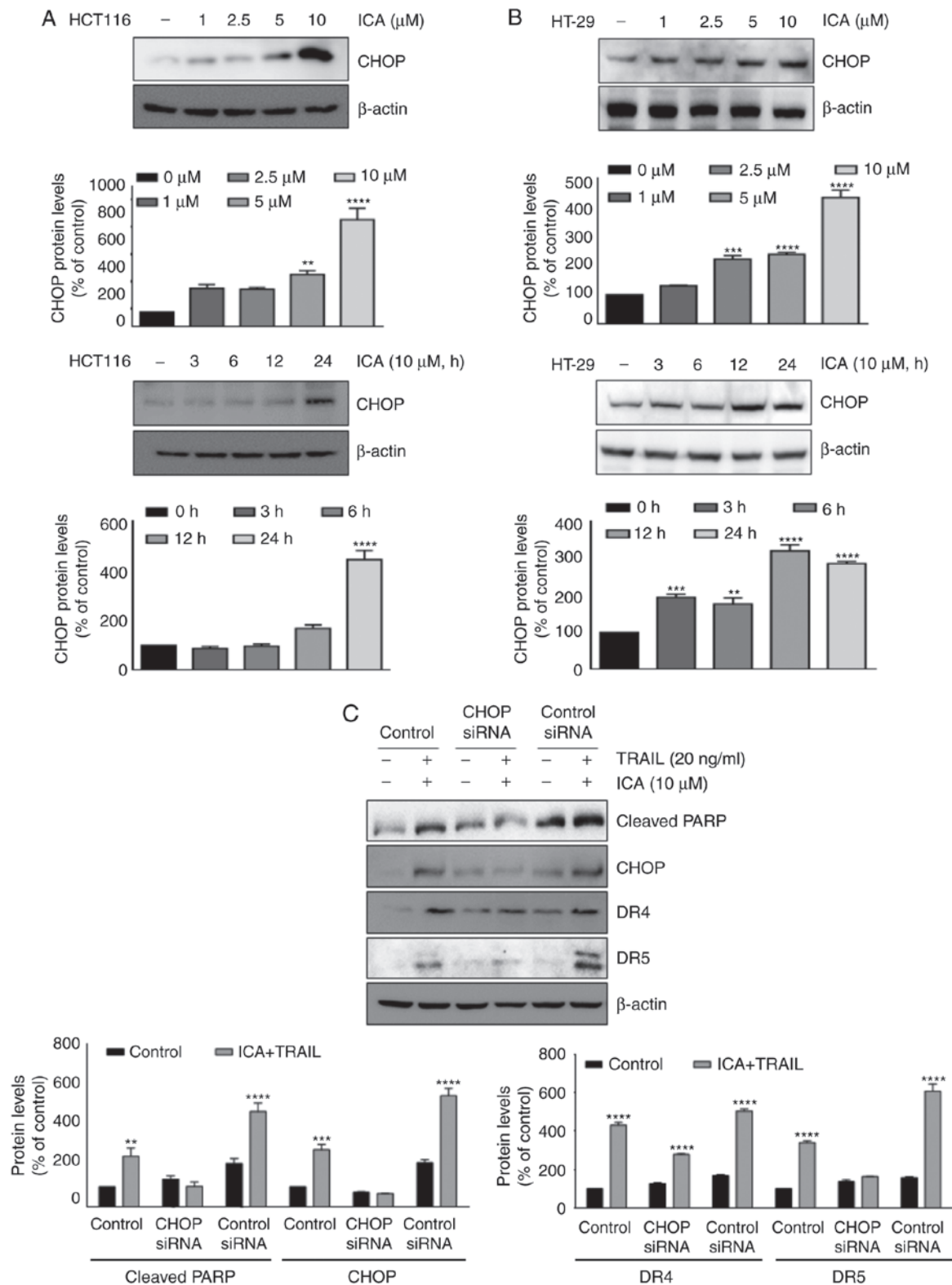
Figure 5. ICA-induced DR5 upregulation is mediated by the induction of CHOP. (A) HCT-116 cells and (B) HT-29 cells were treated with the indicated concentrations of ICA for 24 h or 10 μM ICA for the indicated time. CHOP protein expression was analyzed by western blotting. (C) Cells were transfected with CHOP siRNA or control siRNA for 24 h and treated with 10 μM ICA for 24 h. Whole cell extracts were subjected to western blot analysis. The results are expressed as mean percentage of control ± SEM and represent three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ vs. control. ICA, icariin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; DR, death receptor; CCAAT enhancer-binding protein homologous protein; PARP, poly (ADP-ribose) polymerase.

group, 91.6 mm³. As presented in Fig. 8B and C, co-treatment with ICA and TRAIL significantly suppressed the xenograft tumor growth compared with ICA or TRAIL treatment alone.

In addition, no apparent loss of body weight was detected in mice during the experiment (Fig. 8A). These results demonstrated that *in vivo* tumor co-treatment with ICA and TRAIL
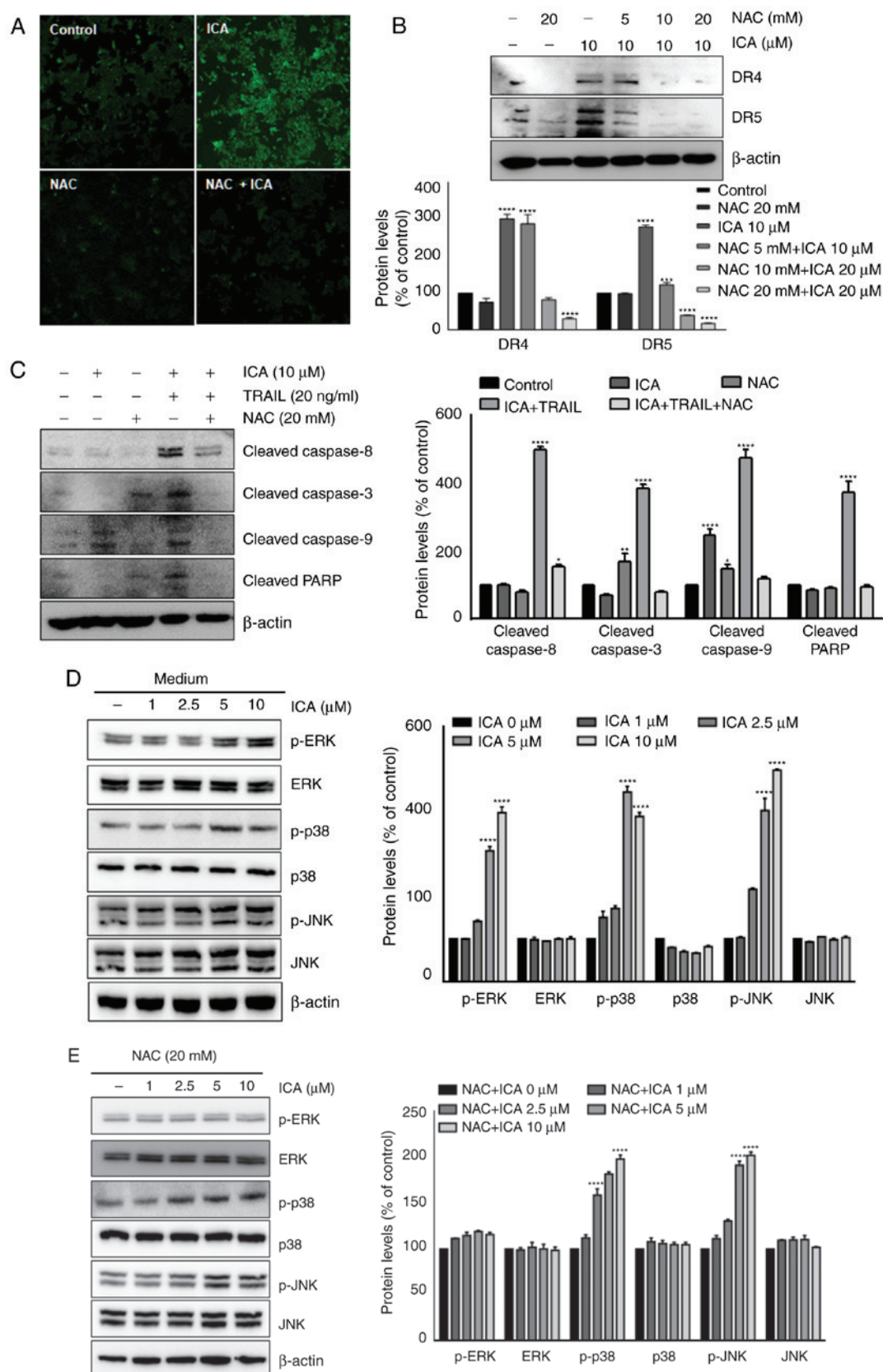
Figure 6. ROS are required for ICA-induced upregulation of DR4 and DR5. (A) HCT-116 cells were pre-exposed to NAC for 1 h, treated with ICA for 24 h, washed and labeled with DCFH-DA. The levels of ROS were observed by fluorescence microscopy. (B) HCT-116 cells were treated with the indicated concentrations of NAC for 1 h and 10 μM ICA for 24 h. The whole cell extracts were analyzed by western blotting using DR5 and DR4 antibodies. (C) Cells were pretreated with NAC for 1 h, washed, treated with 10 μM ICA and 20 ng/ml TRAIL for 24 h. Whole cell extracts were analyzed by western blotting using the indicated antibodies. (D) HCT116 cells were treated with the indicated concentrations of ICA for 24 h. Whole cell extracts were analyzed by western blotting using the indicated antibodies. (E) HCT116 cells were pretreated with NAC for 1 h and exposed to the indicated concentrations of ICA for 24 h. Whole cell extracts were analyzed by western blotting using the indicated antibodies. The results are expressed as mean percentage of control ± SEM and represent three independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control. ICA, icariin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; DR, death receptor; NAC, N-acetyl-L-cysteine; PARP, poly (ADP-ribose) polymerase; p, phosphorylated.
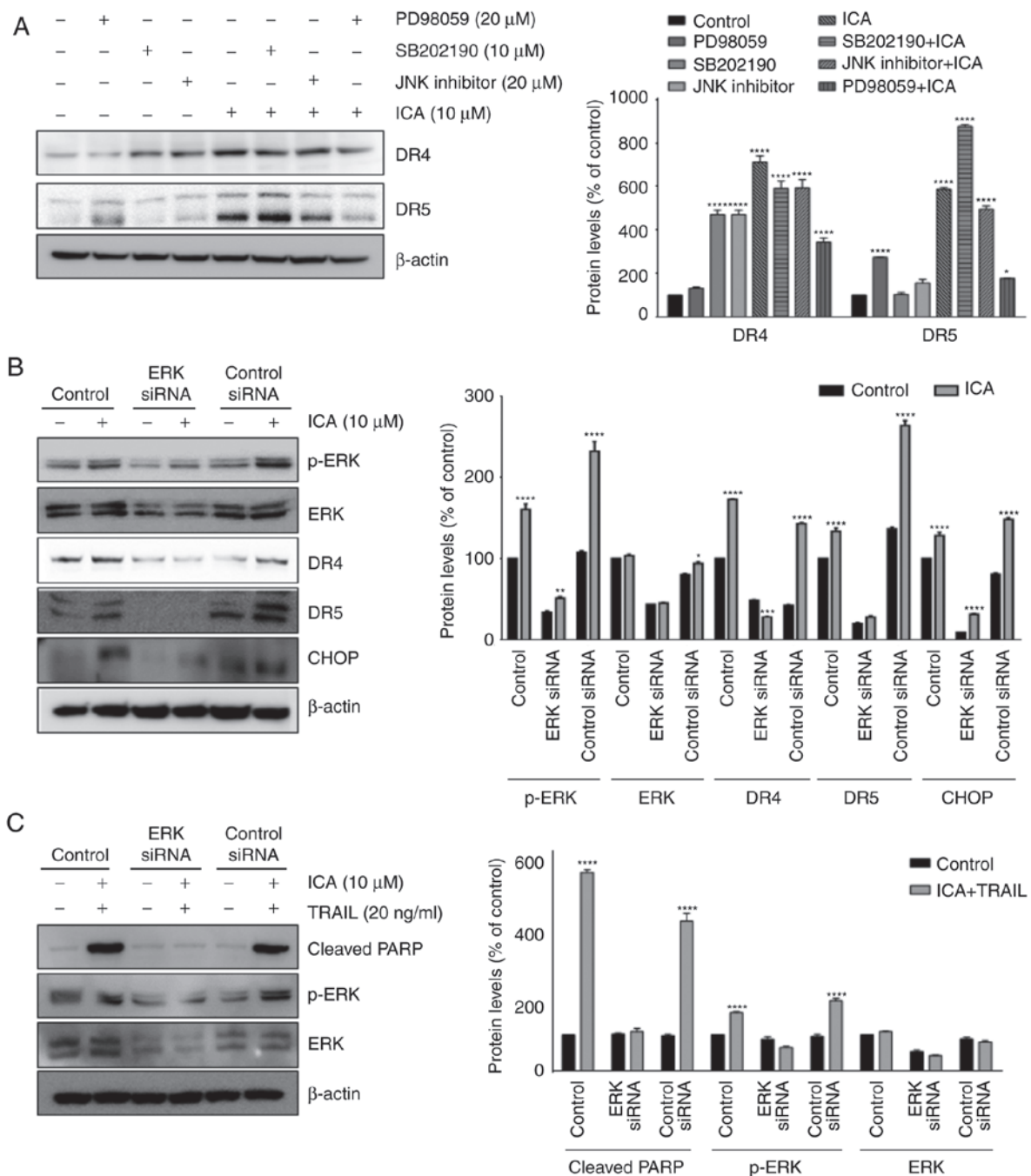
Figure 7. ICA upregulates DR4 and DR5 in human cancer cells through the MAPK pathway (A) HCT-116 cells were pretreated with ERK inhibitor (PD98059), p38 MAPK inhibitor (SB202190) and JNK inhibitor for 1 h and subsequently treated with 10 μM ICA for 24 h. DR4 and DR5 protein expression was analyzed by western blotting. (B) HCT116 cells were transfected with ERK siRNA or control siRNA for 24 h and treated with 10 μM ICA for 24 h. The expression of each protein was analyzed by western blotting. (C) HCT116 cells transfected with ERK siRNA or control siRNA were treated with 10 μM ICA and 20 ng/ml TRAIL for 24 h. Whole cell extracts were subjected to western blot analysis. The results are expressed as mean percentage of control ± SEM and represent three independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control. ICA, icariin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; DR, death receptor; MAPK, mitogen-activated protein kinase.

inhibited tumor growth more efficiently compared with either drug alone. This suggested that ICA may help treat cancer with TRAIL resistance.

ICA augments TRAIL-induced apoptosis by upregulating DRs and CHOP in vivo. The present study demonstrated that ICA enhanced TRAIL-induced apoptosis by upregulating various molecules that are involved in apoptosis *in vitro*. Western blot analysis of mouse xenograft samples confirmed

a strong induction of DR4, DR5 and CHOP protein expression by ICA compared with the control group (Fig. 8D). In addition, the cleaved forms of PARP, caspase-3, caspase-8 and caspase-9 were markedly increased in the group co-treated with TRAIL and ICA compared with the control group (Fig. 8E). In conclusion, these results suggested that co-treatment with TRAIL and ICA significantly promoted apoptosis *in vivo*, resulting in the suppression of colon cancer growth.
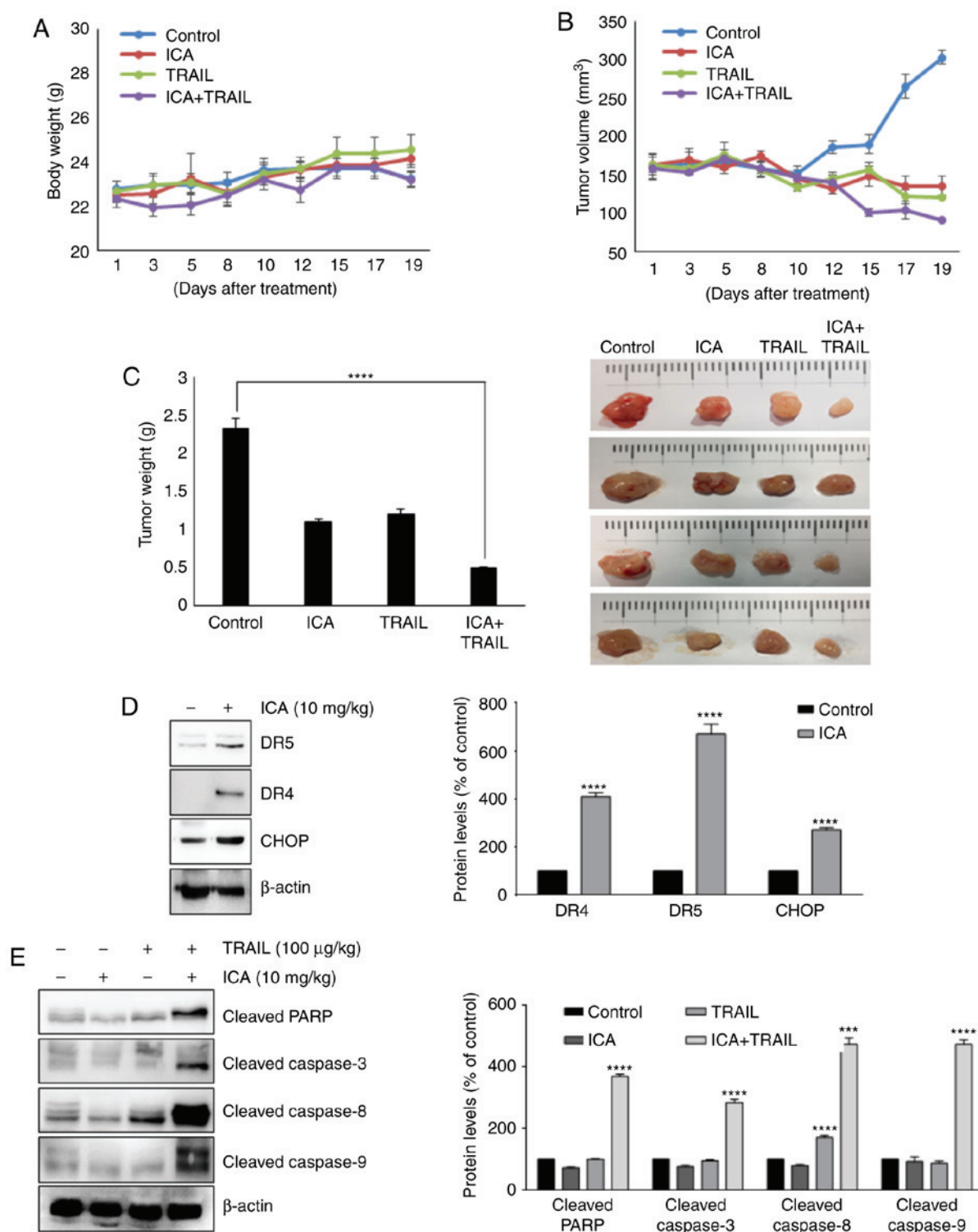
Figure 8. ICA enhances TRAIL-induced apoptosis *in vivo*. Mice were subcutaneously injected with 5×10^5 HCT-116 cells; when the diameter of established tumors reached ~150 mm³, the mice were treated with the vehicle, 10 mg/kg ICA, 100 μg/kg TRAIL or their combination intraperitoneally three times a week. (A) The body weight was calculated. Tumor (B) volume and (C) weight were assessed. (D and E) Tumor samples isolated from mice were subjected to western blot analysis. The results are expressed as mean percentage of control ± SEM and represent three independent experiments. ***P<0.001 and ****P<0.0001 vs. control. ICA, icariin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; DR, death receptor; CCAAT enhancer-binding protein homologous protein; PARP, poly (ADP-ribose) polymerase.

Discussion

To the best of our knowledge, among the various apoptosis-inducing cytokines, TRAIL is currently the only protein under active clinical investigation as a promising anticancer drug (3). However, increasing TRAIL resistance in various types of cancer is a major issue limiting its therapeutic efficacy. Therefore, agents that can overcome TRAIL resistance in tumor cells can be useful in effective cancer treatment. The present study provided evidence that ICA sensitized

cancer cells to TRAIL-induced apoptosis by downregulating cell survival proteins, upregulating cell death proteins and inducing DR4 and DR5 expression through the activation of the ROS-ERK-CHOP signal transduction pathway.

Overexpression of DRs in TRAIL-resistant cancer cells has been reported to restore TRAIL sensitivity (24,31). The results of the present study demonstrated that ICA significantly increased the protein levels of DR4 and DR5 in HCT-116 and HT-29 colon cancer cells, which led to the activation of caspase-3, -9 and PARP in cells treated with TRAIL and ICA. These results suggested that ICA promoted TRAIL-induced apoptosis via caspase activation followed by DR4 and DR5 upregulation. Overexpression of survival proteins such as survivin, XIAP, Bcl-2, c-IAP-1 and Bcl-xL has also been reported as a cause of TRAIL resistance in tumor cells (32). The results of the present study revealed that ICA down-regulated the expression of these survival proteins when the apoptosis of colon cancer cells was stimulated by TRAIL. Therefore, the sensitivity of tumor cells to TRAIL may be attributed to the downregulation of cell survival proteins by ICA. However, the downregulation of these survival proteins by ICA is still unclear. The majority of the antiapoptotic proteins described above are regulated by NF-κB. Since ICA has been demonstrated to inhibit NF-κB activation (20), it is possible that the downregulation of these proteins is mediated by the inhibition of NF-kB. The present results also indicated that ICA significantly upregulated the expression of BAX and cytochrome c, both of which have been identified to be critical for TRAIL-induced apoptosis (33). Thus, the upregulation of BAX and cytochrome c by ICA may also contribute to TRAIL-induced apoptosis.

The results of the present study also revealed that ICA induced TRAIL receptor expression through the induction of CHOP. ICA treatment upregulated CHOP expression, whereas CHOP silencing diminished the effects of ICA on DR5 upregulation and TRAIL-induced apoptosis. In previous studies, a number of cancer chemopreventive agents upregulated DRs through ROS generation (27,34,35). Consistent with these studies, the results of the present study demonstrated that ICA induced ROS production, and the loss of ROS by the antioxidant reagent NAC abrogated ICA-induced DR4 and DR5 upregulation, which attenuated TRAIL-induced apoptosis by ICA. These results suggested that ROS was the most important upstream regulator in TRAIL-induced apoptosis. Based on previous studies reporting that MAPK signaling regulates CHOP, DR4 and DR5 expression (27,28,36), the present study also investigated the involvement of MAPK signaling during TRAIL-induced apoptosis; the results demonstrated that ICA stimulated ERK activation, and a specific ERK inhibitor or siRNA targeting ERK abolished the ability of ICA to increase CHOP and DR expression and sensitize TRAIL-induced apoptosis in colon cancer cells.

The present study also investigated whether these results were applicable to *in vivo* conditions. In the tumor xenograft model, the combination of ICA and TRAIL effectively inhibited tumor growth. These results suggested that the ICA-induced sensitivity to TRAIL was increased *in vivo*. ICA increased DR4, DR5 and CHOP expression, as well as promoted TRAIL-induced apoptosis via the activation of the caspase pathway *in vivo*. Therefore, ICA may be useful to overcome TRAIL resistance during cancer treatment. However, further animal study with oral administration is urgently needed to verify these results.

In conclusion, the present study provided evidence that ICA increased DR5 and DR4 expression through ROS-, ERK-, and CHOP-mediated pathways, suggesting that ICA may overcome TRAIL resistance in tumor cells. ICA and TRAIL exhibited synergistic anticancer effects *in vitro* and *in vivo*, suggesting that ICA may be a potent therapeutic agent for chemotherapy.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

BP and BK conceived the study. BK developed the methodology, obtained and validated the data. BK and BP performed the experiments. BP, KL and JS analyzed and interpreted the data. BK and BP prepared the original draft. BP, KL and JS revised the draft.

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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