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Phloroglucinol Derivative MCPP Induces Cell Apoptosis in Human Colon Cancer

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ABSTRACT

This study is the first to investigate the anticancer effects of the new phloroglucinol derivative (3,6-bis(3-chlorophenylacetyl)phloroglucinol; MCPP) in human colon cancer cells. MCPP induced cell death and antiproliferation in three human colon cancer, HCT-116, SW480, and Caco-2 cells, but not in primary human dermal fibroblast cells. MCPP-induced concentration-dependent apoptotic cell death in colon cancer cells was measured by fluorescence-activated cell sorter (FACS) analysis. Treatment of HCT-116 human colon cancer cells with MCPP was found to induce a number of signature endoplasmic reticulum (ER) stress markers; and up-regulation of CCAAT/enhancer-binding protein homologous protein (CHOP) and glucose-regulated protein (GRP)-78, phosphorylation of eukaryotic initiation factor-2α (eIF-2α), suggesting the induction of ER stress. MCPP also increased GSK3α/β(Tyr270/216) phosphorylation and reduced GSK3α/β(Ser21/9) phosphorylation time-dependently. Transfection of cells with GRP78 or CHOP siRNA, or treatment of GSK3 inhibitor SB216163 reduced MCPP-mediated cell apoptosis. Treatment of MCPP also increased caspase-7, caspase-9, and caspase-3 activity. The inhibition of caspase activity by z-DEVE-FMK or z-VAD-FMK significantly reduced MCPP-induced apoptosis. Furthermore, treatment of GSK3 inhibitor SB216763 also dramatically reversed MCPP-induced GRP and CHOP up-regulation, and pro-caspase-3 and pro-caspase-9 degradation. Taken together, the present study provides evidences to support that GRP78 and CHOP expression, and GSK3α/β activation in mediating the MCPP-induced human colon cancer cell apoptosis. J. Cell. Biochem. 112: 643–652, 2011.

KEY WORDS: GSK3α/β; CHOP; GRP78; COLON CANCER; ER STRESS

The endoplasmic reticulum (ER) is an organelle in the secretory pathway, and serves as a central role in lipid synthesis, protein folding and modification prior to transportation to Golgi bodies for export. Proteins are correctly folded in the ER under normal conditions. However, protein folding in the ER is impaired under various of toxic insults, including hypoxia, failure of protein synthesis, misfolding, transport or degradation, and Ca^{2+} overload, can result in ER stress-related events [Abcouwer et al., 2002;

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Soboloff and Berger, 2002]. There is increasing evidence that ER stress plays a crucial role in the regulation of apoptosis. It has been reported that ER stress triggers several specific signaling pathways, such as ER-associated protein degradation and the unfolded protein response (UPR) [Feldman et al., 2005; Moenner et al., 2007]. In addition, glucose-regulated proteins (GRPs) are the most abundant glycoproteins in the ER and play a critical role in ER regulation. The UPR induces the expression of ER-resident chaperones, such as GRP78 and GRP94 [Lee, 2001]. The protective functions of GRP protein have also been observed in resistance to radiation in cancer cells [Kubota et al., 2005]. On the other hand, several pro-apoptotic factors like CHOP/GADD153, and pro-apoptotic Bcl-2 family members, have been also shown to be involved in ER stress-induced cell death. CHOP/GADD153 is apparently a pro-apoptotic transcription factor induced during ER stress [Wang et al., 1996; Harding et al., 2002; Rao et al., 2004]. Deletion of the CHOP gene leads to an attenuation ER stress-induced cancer cell death [Friedman, 1996], but overexpression of CHOP gene promotes cell [McCullough et al., 2001]. Furthermore, it has been reported that Rotterlin and resveratrol-induced apoptosis in human colon cancer through GRP78 and CHOP up-regulation [Park et al., 2007; Lim et al., 2008, 2009].

GSK3 kinase plays a central role in ER stress-induced apoptosis [Song et al., 2002; Kim et al., 2005]. Previous report showed that a synthetic GSK3 inhibitor, lithium and valproate through inhibit GSK3β to protect cells from ER stress-induced dysfunction [Chen et al., 1999; Tatebayashi et al., 2004; Kim et al., 2005]. GSK3 kinase has two phosphorylation sites that influence the catalytic activity of the protein. The phosphorylation site of Ser9 inactivates GSK3β. On the other hand, phosphorylation of Tyr216, located on the activation loop, increases the catalytic activity. In addition, GSK3 substrates have grown to encompass a broad array of proteins that are involved in several important regulatory and developmental pathways including translation initiation factor eIF2β ε and various transcription factors [Doble and Woodgett, 2003].

Numerous naturally occurring substances are recognized to be antioxidants, cancer preventive agents, or even antineoplastic agent such as paclitaxel. There are various kinds of natural phloroglucinol derivatives have been identified as possessing anticancer activity. In example, Rottlerin isolated from *Hypericum drummondii* have shown cytotoxic activity in human fibrosarcoma [Song et al., 2008]. Bullatenone is a triketone phloroglucinol, the main cytotoxic component compound in *Lophomyrtus bullata* [Larsen et al., 2005]. Hyperforin, a new lead compound extracts from the herbaceous plant *Hypericum perforatum* L., has been reported against the progression of cancer in leukemia [Quiney et al., 2006]. Moreover, thouvenol, the alkyl phloroglucinol, isolated from *Protorhus thouvenotii*, has shown cytotoxicity in ovarian cancer cells [Cao et al., 2004]. Although the effect of phloroglucinol derivatives on tumor apoptosis have been studied in various cancers. However, the cytotoxic activity of phloroglucinol derivatives in colon cancer remains largely unclear.

In this study, we synthesized the new phloroglucinol derivative 3,6-bis(3-chlorophenylacetyl)phloroglucinol (MCPP) (Fig. 1A) and investigated its anticancer activity in human colon cancer cells. Our data indicate that MCPP reduces survival and tumor growth of human colon cells.

MATERIALS AND METHODS

MATERIALS

The phloroglucinol derivatives (Fig. 1A; chemical purity ≥ 95%) were synthesized at the Graduate Institute of Pharmaceutical Chemistry, China Medical University (Taichung, Taiwan) following the general procedure. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and OPTI-MEM were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA).

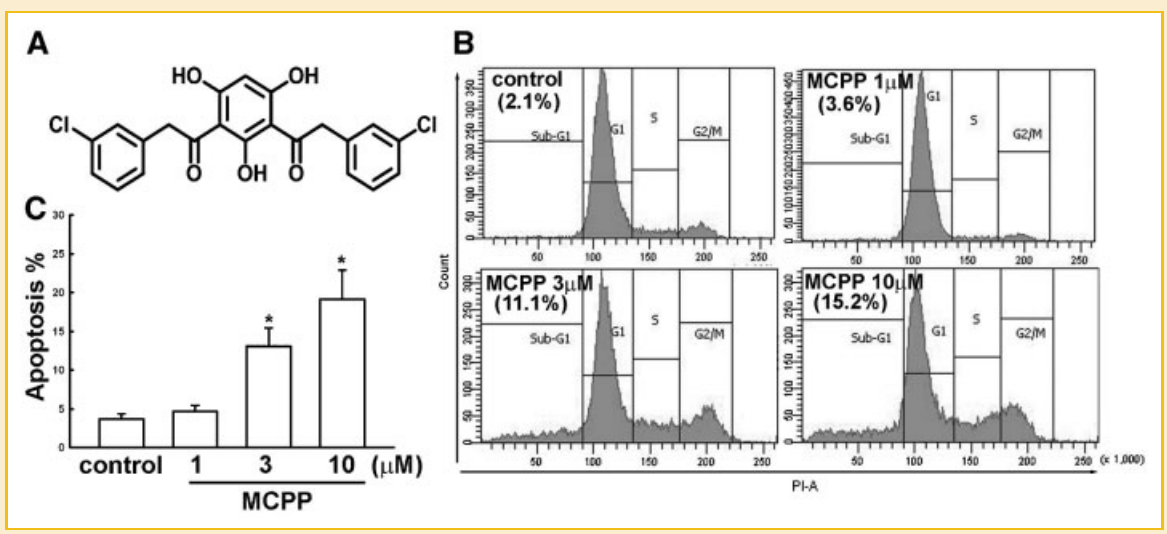

Fig. 1. MCPP induces cell apoptosis in HCT-116 human colon cancer cells. Cells were treated with vehicle or phloroglucinol derivative (3,6-bis(3-chlorophenylacetyl)phloroglucinol; MCPP) (A) for 24 h. B: The percentage of apoptotic cells was analyzed by flow cytometry of PI staining. The quantitative data of apoptosis were shown in (C) (n = 3–4). Results are expressed as the means ± SEM.

Fibroblast medium and fibroblast growth supplement were purchased from ScienCell (San Diego, CA). Primary antibody against phosphorylation of eIF-2α and GSK3α/β were purchased from Cell Signaling and Neuroscience (Danvers, MA). Goat antimouse and antirabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for GRP78, GRP94, PARP, caspase-3, caspase-9, caspase-7, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

CELL CULTURE

The human colon carcinoma cell lines HCT-116, SW480, and Caco-2 were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% FBS and maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were grown in plastic cell culture dishes in a humidified atmosphere of 95% air/5% CO_2 in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air.

The adult human dermal fibroblast cell line was obtained from ScienCell and maintained in fibroblast medium containing fibroblast growth supplement (undisclosed formulation) and 2% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air.

SULFORHODAMINE B ASSAY (SRB)

The SRB assay is based on the measurement of cellular protein content. After treatment with MCPP for 24 h, cells were fixed with 10% trichloroacetic acid and stained SRB at 0.4% (w/v) in 1% acetic acid for 30 min. Unbound SRB was washed out by 1% acetic acid and SRB-bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm using a microplate reader (Bio-Tek, Winooski, VT).

MTT ASSAY

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After treatment with MCPP for 24 or 48 h, cultures were washed with PBS. MTT (0.5 mg/ml) was then added to each well and the mixture was incubated for 2 h at 37 °C. Culture medium was then replaced with an equal volume of DMSO to dissolve formazan crystals. After the mixture was shaken at room temperature for 10 min, absorbance was determined at 550 nm using a microplate reader (Bio-Tek).

QUANTIFICATION OF APOPTOSIS BY FLOW CYTOMETRY

Apoptosis was assessed by binding of Annexin V protein to exposed phosphatidylserine (PS) residues at the surface of cells undergoing apoptosis, as previously described [Chen et al., 2010]. Cells were treated with vehicle or MCPP for indicated time periods. After treatment, cells were washed twice with PBS and re-suspended in staining buffer containing propidium iodide (PI, 1 μg/ml) and Annexin V-FITC (0.025 μg/ml). Double-labeling was performed at room temperature for 10 min in darkness before flow cytometric analysis. Cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson, Lincoln Park, NJ, USA).

Quantitative assessment of apoptotic cells was also conducted by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method, which examines DNA-strand breaks during apoptosis with the BD ApoAlert™ DNA Fragmentation Assay Kit (Lincoln Park, NJ). Cells were incubated with MCPP for the indicated time periods, trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After undergoing washing, the cells were incubated with the reaction mixture for 60 min at 37 °C. The stained cells were then analyzed by flow cytometry [Chen et al., 2010; Liu et al., 2010].

WESTERN BLOT ANALYSIS

Cells were treated with MCPP for various time periods and then washed with cold PBS that had been lysed for 30 min on ice with radioimmunoprecipitation assay buffer. Protein samples were separated by sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinyldifluoride (PVDF) membranes. The blots were blocked with 5% nonfat milk in PBS for 1 h at room temperature and then probed with primary antibodies for 1 h at room temperature. After three washes, the blots were subsequently incubated with a secondary antibody for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). The blots were subsequently stripped through incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 0.1 M β-mercaptoethanol) and reprobed for β-actin as a loading control. Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

CASPASE-3 ACTIVITY

The assay was based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate Ac-DEVD-pNA for caspase-3. The cell lysates were prepared and incubated with specific caspase-3 antibody. Immunocomplexes were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 2 h at 37°C. The release of *p*-nitroaniline was monitored at 405 nm. Results are represented as the fold change of the activity compared to the untreated control.

siRNA Transfection

The siRNAs against human GRP78, CHOP and control siRNA were purchased commercially from Santa Cruz Biotechnology. Cells were transiently transfected with the siRNA by Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the siRNA (at a final concentration of 100 nM) and LF2000 were premixed in OPTI-medium for 20 min and then applied to the cells. An equal volume of medium containing 20% FBS was added 6 h later. After transfection for 24 h, LF2000-containing medium was replaced with fresh serum-free medium and treated with MCPP for another 24 h.

REVERSE TRANSCRIPTASE-PCR (RT-PCR)

Total RNA was extracted from cells using a TRIzol kit (MDBio, Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA

using the oligo(dT) primer, then amplified using oligonucleotide primers:

CHOP: 5′-CAACTGCAGAGAATTCAGCTGA-3′ and
5′-ACTGATGCTCTAGATTGTTCAT-3′;
GRP78: 5′-GCTCGACTCGAATTCCAAAG-3′ and
5′-TTTGTC AGGGGTCTTTCACC-3′;
GAPDH: 5′-TGGGCTACACTGAGCACCAG-3′ and
5′-GGGTGTCGCTGTTGAAGTCA-3′.

Each PCR cycle was carried out for 30 s at 95°C, for 30 s at 55°C, and for 1 min at 72°C. PCR products were then separated electrophoretically in a 2% agarose gel and stained with ethidium bromide. The band intensity was quantified with a densitometric scanner and presented as the relative level of GAPDH.

STATISTICS

The values given are means ± SEM. The significance of difference between the experimental group and control groups was assessed by the Student's *t*-test. The difference was significant if the *P*-value was <0.05.

RESULTS

MCPP INDUCES CELL APOPTOSIS IN HUMAN COLON CANCER CELLS

To investigate the cytotoxicity of 3,6-bis(3-chlorophenylacetyl)phloroglucinol (MCPP, Fig. 1A) in human colon cancer cells, we examined the effects on cell viability in HCT-116 human colon cancer cells using PI staining by fluorescence-activated cell sorter (FACS) analysis (Fig. 1B). As shown in Figure 1C that MCPP induced HCT-116 cell death in a concentration-dependent manner. The SRB (for 24 h) and MTT (for 24 or 48 h) assays were further confirmed

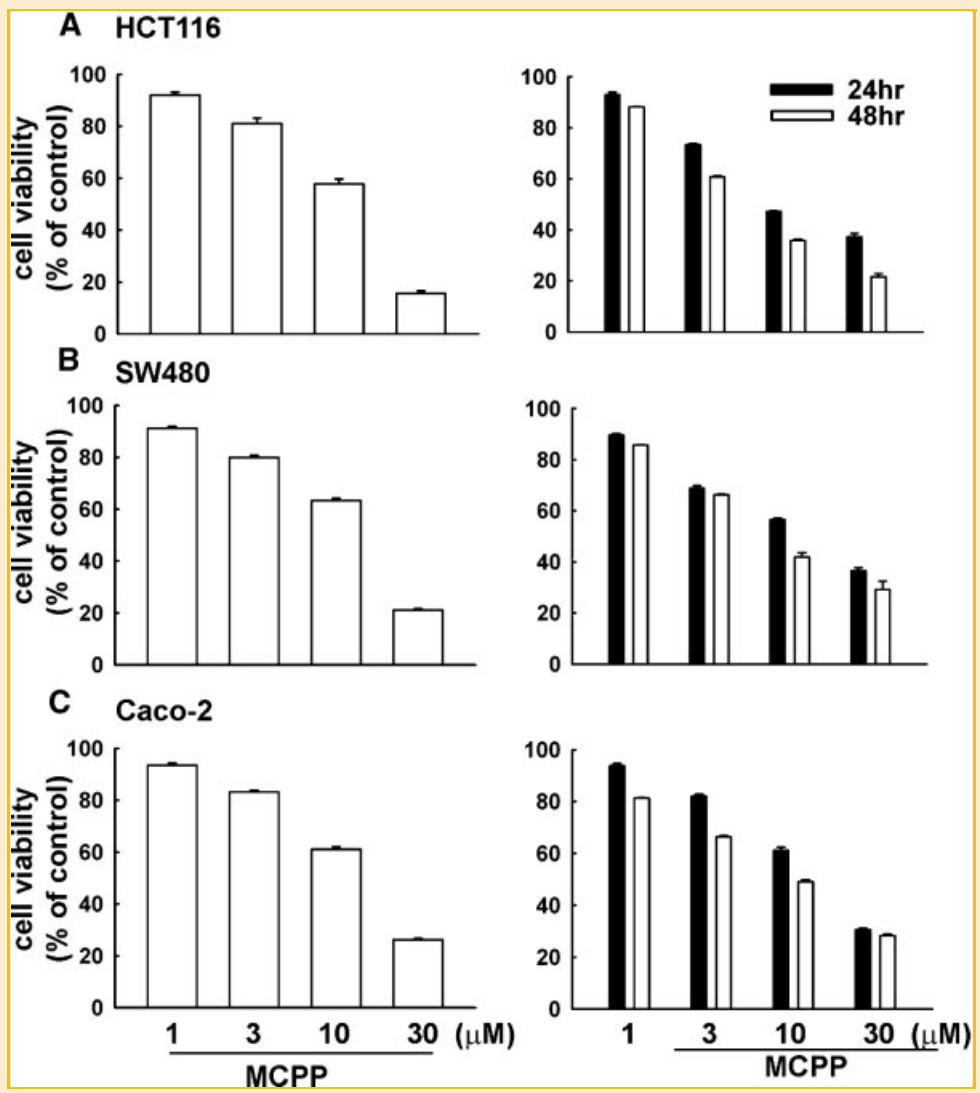

Fig. 2. MCPP induces cancer cell death and antiproliferation in human colon cells. MCPP induces apoptosis of human colon cells was showed in HCT-116 (A), SW480 (B), and Caco-2 (C). Cells were incubated with various concentrations of MCPP (1, 3. or 10 μM), the cell viability was examined by SRB assay (for 24 h, left panel) and MTT assay (for 24 or 48 h, right panel). Results are expressed as the means ± SEM of at least three independent experiments.

that MCPP induces cell death in three differences of human colon cancer cell line, HCT-116 (Fig. 2A), SW480 (Fig. 2B), and Caco-2 (Fig. 2C), respectively. The SRB assay determined that MCPP induces cell death in human colon cell lines HCT-116 (IC50 = 15.69 μM), SW480 (IC50 = 17.39 μM) and Caco-2 (IC50 = 18.5 μM) but not in primary human dermal fibroblast cells (IC50 > 30 μM). Treatment of three differences of human colon cancer cells with MCPP induced cell death in a concentration-dependent manner (Fig. 2A–C). Next, we investigated whether MCPP induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used

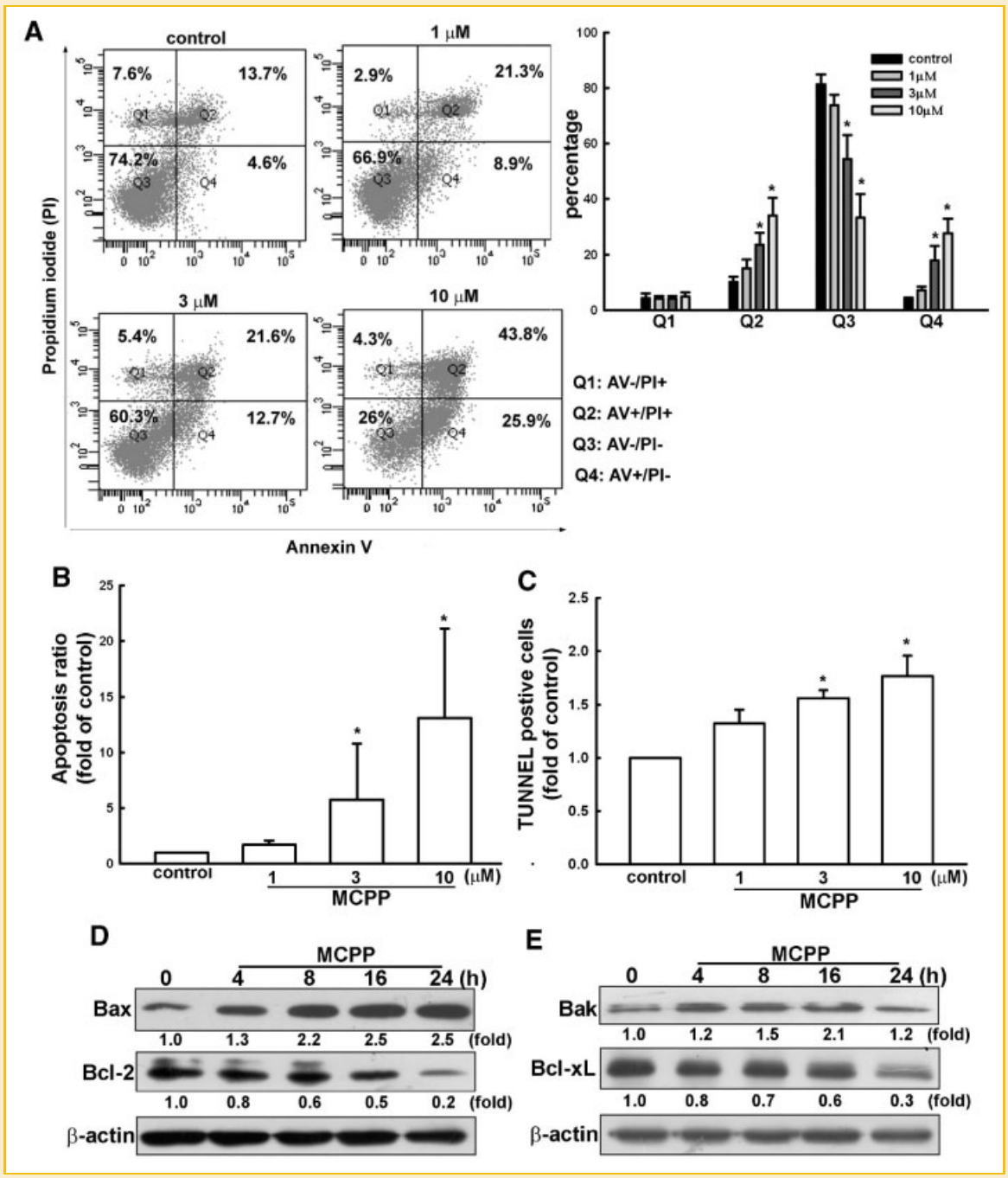

Fig. 3. MCPP induces cell apoptosis in HCT-116 human colon cancer cell. Cells were treated with various concentrations (1, 3, or 10 μM) of MCPP for 24 h. The percentage of apoptotic cells was analyzed by flow cytometry of Annexin V/PI double staining (n = 3–4) (A). The proportion of apoptosis cells (Q2 + Q4)/viable cells (Q3) was showed in (B). Results are expressed as the means ± SEM. C: Cells were treated with various concentrations (1, 3, or 10 μM) of MCPP for 24 h, the TUNEL positive cells were examined by flow cytometry. Results are expressed as the means ± SEM of at least three independent experiments. D,E: Cells were incubated with MCPP (10 μM) for indicated time periods, the Bax, Bcl-2, Bak, and Bcl-xL expressions were examined by Western blot analysis. Results are the representative of three independent experiments. Relative of each protein expression was normalized by β-actin. The statistical analysis was shown in Supplementary Data Figure S1.

for the detection of PS (phosphoserine) externalization, a hallmark of early phase of apoptosis. As shown in Figure 3A, MCPP induced cell apoptosis concentration-dependently in HCT-116 human colon cancer cells. As compared to vehicle-treated cells, a high proportion of apoptosis cells (Q2 + Q4)/viable cells (Q3) was significantly increased in cells treated with MCPP (Fig. 3B). We then investigated the effects of MCPP-induced apoptosis by using the TUNEL assay. Compared with vehicle-treated HCT-116 cells, those treatment with MCPP showed significant TUNEL positive activity (Fig. 3C). Next, to determine whether MCPP induces apoptosis by triggering the mitochondrial apoptotic pathway, we measured the change in the expression of Bcl-2 family proteins. Treatment of HCT-116 cells with MCPP induced Bax and Bak protein up-regulation (Fig. 3D,E). In addition, MCPP decreased the expression of Bcl-xL and Bcl-2, which led to an increase in the pro-apoptotic/antiapoptotic Bcl-2 ratio (Fig. 3D,E). These data indicate that MCPP induces cell apoptosis in HCT-116 human colon cancer cells.

MCPP INCREASES GRP78 AND CHOP EXPRESSION IN HCT-116 HUMAN COLON CANCER CELLS

ER stress, is generally characterized by up-regulation of GRP78 and CHOP, and phosphorylation of eukaryotic initiation factor-2α (eIF2α). MCPP exposure caused a significant increase in the expression of GRP78 (Fig. 4A) and CHOP (Fig. 4C) as well as the phosphorylation of eIF2α at Ser51 (Fig. 6A) in HCT-116 cells, indicating the occurrence of ER stress. However, MCPP treatment did not affect GRP94 protein expression (Fig. 4B). Stimulation of cells with MCPP also increased GRP78 and CHOP mRNA expression in a time-dependent manner (Fig. 4D). To further investigate whether MCPP induced cell apoptosis through GRP78

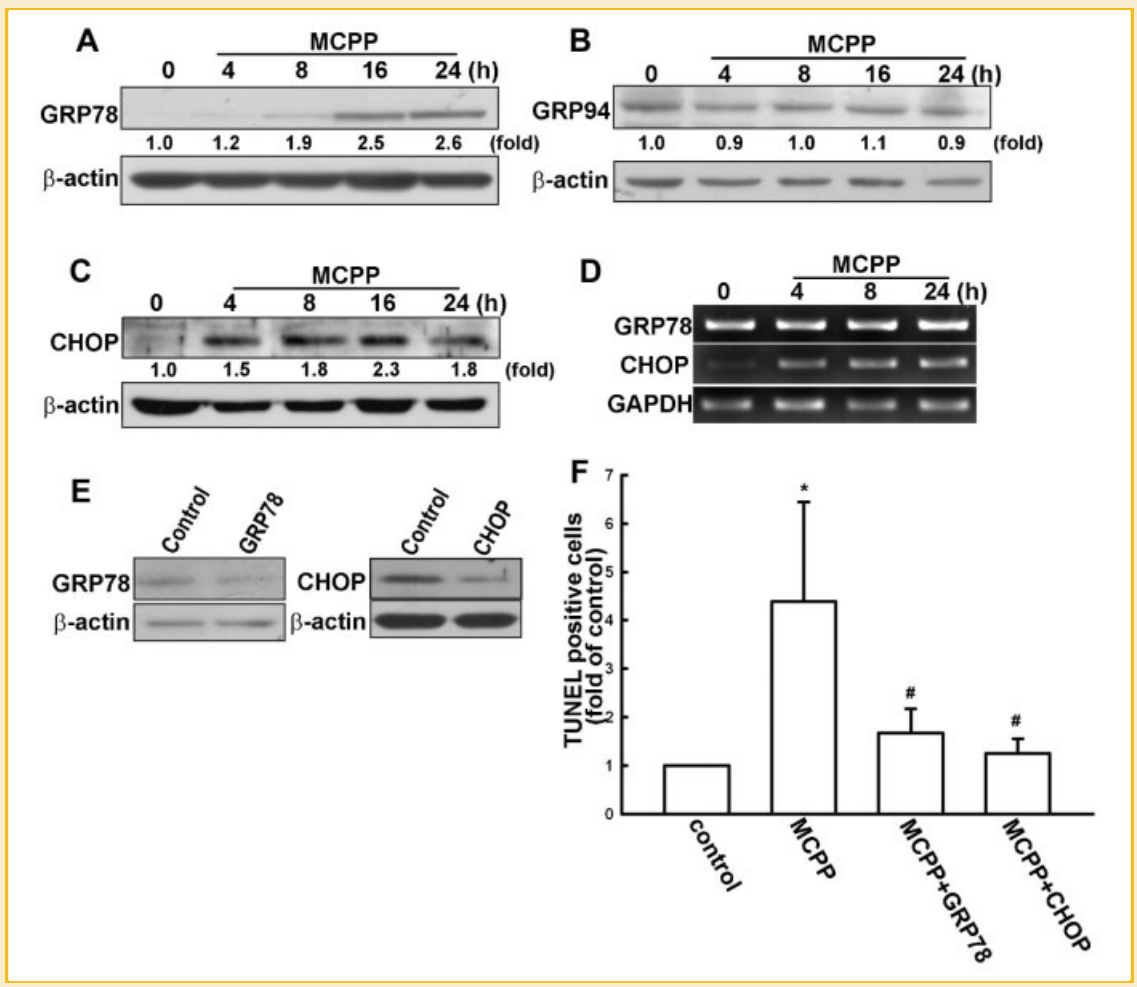

Fig. 4. ER stress-related proteins activation are involved in MCPP-mediated cell apoptosis in HCT-116 human colon cells. Cells were incubated with MCPP (10 μM) for indicated time periods. GRP78 (A), GRP94 (B), and CHOP (C) expression were examined by Western blot analysis. Results are the representative of three independent experiments. Relative of each protein expression was normalized by β-actin. The statistical analysis was shown in Supplementary Data Figure S2. D: Cells were incubated with MCPP for indicated time periods, mRNA expression of GRP78 and CHOP were examined by RT-PCR analysis. E: Cells were transfected with GRP78, CHOP, or control siRNA for 24 h. GRP78 and CHOP expression were examined by Western blot analysis. F: Cells were transfected with GRP78, CHOP, or control siRNA for 24 h, followed by incubation with MCPP for another 24 h. The proportion of apoptosis cells (Q2 + Q4)/viable cells (Q3) was analyzed by flow cytometry of Annexin V/PI double staining. Results are expressed as the means ± SEM of at least three independent experiments. $^{*}P < 0.05$ compared with control group. $^{\#}P < 0.05$ compared with MCPP treatment.

and CHOP activation, cell were transfected with GRP78 and CHOP siRNA, which specifically inhibited GRP78 and CHOP expression, respectively (Fig. 4E), but also reduced MCPP-induced cell apoptosis (Fig. 4F). Thus, our data suggest that GRP78 and CHOP activation are involved in MCPP-mediated cancer cell deaths.

MCPP INCREASES CASPASE-3 AND -9 EXPRESSION IN HCT-116 HUMAN COLON CANCER CELLS

One of the hallmarks of the apoptotic process is the caspases activation, which represent both initiators and executors of death signals. Treatment of MCPP decreased the pro-caspase-3 expression (Fig. 5A) and increased the caspase-3 activity in HCT-116 cells (Fig. 5B). Pretreatment of cells with the specific caspase-3 inhibitor z-DEVD-FMK and pan caspase inhibitor z-VAD-FMK reduced the MCPP-induced cell death (Fig. 5C). Notably, MCPP also increased cleaved-PARP expression (Fig. 5D). Upstream pro-caspase-9 expression also decreased upon treatment with MCPP in HCT-116 cells (Fig. 5A). It has been reported that ER stress-triggered apoptosis involves the activation of the intrinsic pathway of apoptosis involving the activity of caspase-9, caspase-3, and caspase-7 [Reddy et al., 2003; Masud et al., 2007]. Here, we also found that treatment of MCPP effectively decreased the pro-caspase-7 expression and increased the cleaved form expression in HCT-116 colon cancer cells (Fig. 5E). These results suggest that MCPP triggers ER stress and induces cancer cell apoptosis in HCT-116 cancer cells.

MCPP-INDUCED ER STRESS IS MEDIATED BY GSK3α/β ACTIVATION

We next further determined whether the eIF2α and GSK3β activation are involved in MCPP-induced ER stress in colon cancer cells. As shown in Figure 6A, MCPP induced eIF2α phosphorylation in a time-dependent manner. However, treatment of HCT-116 cells with eIF2α inhibitor did not reduce MCPP-mediated cell apoptosis (Fig. 6C). On the other hand, GSK3β is a kinase that plays an important role in various cellular functions in glycogen metabolism and regulation of cell survival [Grimes and Jope, 2001; Holmes et al., 2008]. It has been reported that ER stress may activate GSK3β by inducing dephosphorylation at Ser9 [Song et al., 2002; Chen et al., 2004] and phosphorylation at Tyr216 [Chen et al., 2004]. As shown in Figure 6B, MCPP reduced phosphorylation of GSK3α/β at Ser21/9, whereas it stimulated phosphorylation of

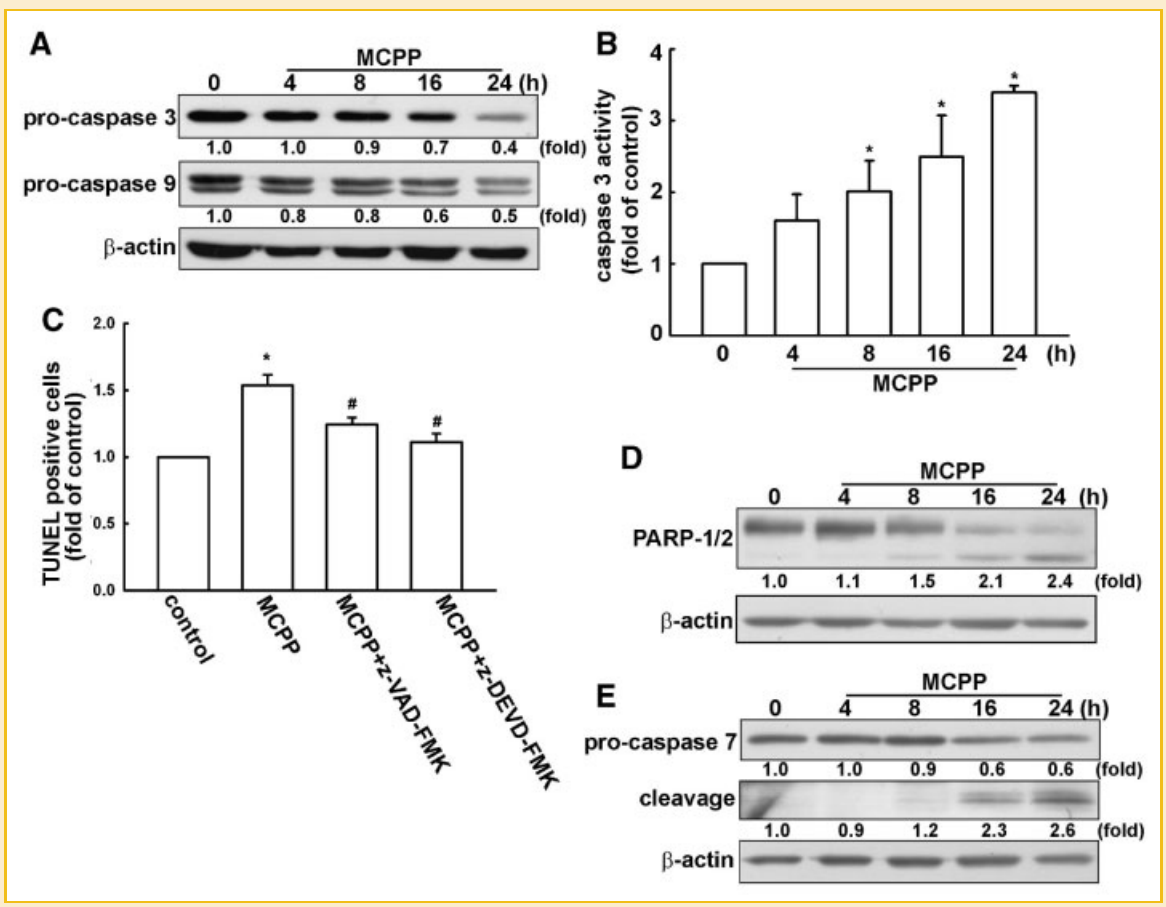

Fig. 5. MCPP induces the activation of caspases in HCT-116 human colon cancer cells. Cells were incubated with MCPP (10 μM) for different time periods, levels of caspase-3 and caspase-9 (A), PARP (C), and caspase-7 (E) expressions were examined by Western blot analysis. Results are the representative of three independent experiments. Relative of each protein expression or PARP clear form was normalized by β-actin. The statistical analysis was shown in Supplementary Data Figure S3. B: Cells were incubated with MCPP (10 μM) for indicated time periods, caspase-3 activity was examined by ELISA kit. D: Cells were pretreated with z-VAD-FMK or z-DEVD-FMK for 30 min, followed by stimulation with MCPP for 24 h. The quantitative data of apoptotic cells were analyzed by flow cytometry of TUNEL-stained cells. Results are expressed as the means $\pm$ SEM of at least three independent experiments. $^{*}P < 0.05$ compared with control group. $^{\#}P < 0.05$ compared with MCPP treatment group.

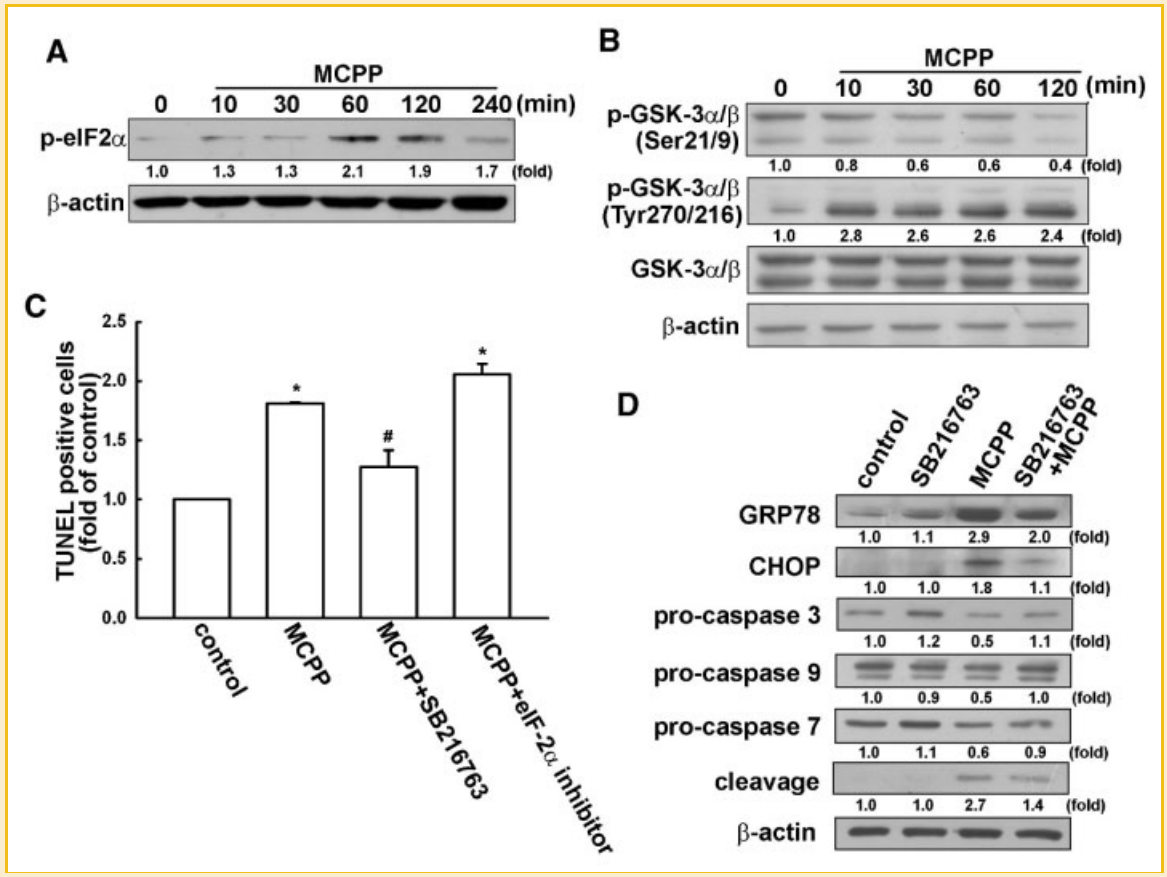

Fig. 6. MCPP induces ER stress through GSK3α/β activation in HCT-116 human colon cancer cells. Cells were incubated with MCPP (10 μM) for indicated time periods. The phosphorylation levels of eIF2α (A) and GSK3β (B) were examined by Western blot analysis. Results are the representative of three independent experiments. Relative phosphorylation levels of eIF2α and GSK3β expression were normalized by β-actin. C: Cells were pretreated with GSK3β inhibitor (SB216763) or eIF2α inhibitor for 30 min, followed by stimulation with MCPP for 24 h. The quantitative data of apoptotic cells were analyzed by flow cytometry of TUNEL-stained cells. Results are expressed as the means ± SEM of at least three independent experiments. $^{*}P < 0.05$ compared with control group. $^{\#}P < 0.05$ compared with MCPP treatment group. D: Cells were pretreated with GSK3β inhibitor (SB216763) for 30 min, followed by stimulation with MCPP for 24 h. The levels of GRP78, CHOP, caspase-3, caspase-9, and caspase-7 expressions were examined by Western blot analysis. Results are the representative of three independent experiments. Relative of each protein expression was normalized by β-actin. The statistical analysis was shown in Supplementary Data Figure S4.

GSK3α/β at Tyr270/216 in HCT-116 cells. MCPP had little effect on the expression of GSK3α/β. We next determined whether the GSK3 inhibition is involved in MCPP-induced colon cancer cell death. As shown in Figure 6C, treatment of cells with GSK3 inhibitor SB216763 reduced MCPP-mediated cell apoptosis. Furthermore, treatment of GSK3 inhibitor SB216763 also dramatically reversed MCPP-induced GRP and CHOP up-regulation, and pro-caspase-3 and pro-caspase-9 degradation (Fig. 6D). Pretreatment of SB216763 also reduced caspase-7 cleaved form expression (Fig. 6D). Thus, our data suggest that GSK3α/β activation but not eIF2α is involved in MCPP-mediated HCT-116 human colon cancer cell deaths.

DISCUSSION

In this study, we demonstrate the molecular mechanism by which MCPP triggered human colon cancer cells undergoing apoptosis. Our results demonstrate that MCPP inhibits cancer cell growth of three human colon cancer cell lines, such as HCT-116, SW480 and Caco-2. Our previous study [Liu, 2010] and presence data demonstrate that phloroglucinol derivatives induce human cancer cell death but not in primary human cells. The central finding in this study provides important evidence to support the involvement of ER stress in the induction of apoptosis by MCPP in HCT-116 colon cancer cells. The following experimental evidence in the present study demonstrates that the induction of ER stress-related proteins may be involved in MCPP induced apoptosis. (i) MCPP induces GRP78 and CHOP expression. CHOP/GADD153, is one of the highest inducible genes during ER stress; (ii) suppression of GRP78 and CHOP genes by specific siRNA attenuated MCPP-induced apoptosis; (iii) MCPP also induces phosphorylation of eIF-2α; (iv) GSK3β kinase plays a central role in ER stress-induced apoptosis [Song et al., 2002; Kim et al., 2005]. MCPP increases GSK3α/β activity, treatment of GSK3β inhibitor SB216763 attenuated MCPP-induced apoptosis. Taken together, MCPP induces up-regulation of GRP78 and CHOP and phosphorylation of GSK3β and eIF-2α, all of which are indicative of ER stress. Here, we do not observe eIF-2α inhibitor reveres the MCPP-induced apoptosis, the effects of eIF-2α

involved in MCPP-induced apoptosis require further experiments to investigate. In conclusion, these observations suggest that MCPP induces cancer cell death-mediated apoptosis. Indeed, treatment with MCPP caused the activation of caspase-9, -3, and -7, associated with the degradation of PARP, which preceded the onset of apoptosis.

Bcl-2 family proteins play an important role in cancer cells apoptosis [Cotter, 2009; Leber et al., 2010]. The Bcl-2 family can regulate mitochondrial membrane permeabilization. Bax and Bak proteins are mediate mitochondrial membrane permeabilization, but Bcl-2 and Bcl-XL are inhibitory proteins. In present study, MCPP induces PS externalization, an early phase of apoptosis. Furthermore, MCPP also increases TUNEL positive expression, the last phase of apoptosis, in HCT-116 cancer cells. Moreover, treatment of HCT-116 cells with MCPP induced Bax and Bak protein levels increase within a period 4 h and prolonged its expression at 24 h. On the other hand, MCPP-decreased the expression of Bcl-xL and Bcl-2 initiated at 4 h and sustained at 24 h, which led to an increase in the pro-apoptotic/antiapoptotic Bcl-2 ratio. These data indicate that MCPP induces cell apoptosis by triggering the mitochondrial apoptotic pathway in HCT-116 human colon cancer cells.

GSK3 kinase plays a central role in ER stress-induced apoptosis [Song et al., 2002; Kim et al., 2005]. Inhibition of GSK3 kinase by a synthetic GSK3 inhibitor, lithium or valproate reduces ER stress-induced cellular dysfunction [Chen et al., 1999; Tatebayashi et al., 2004; Kim et al., 2005]. Here, we show that MCPP increases GSK3α/β phosphorylation at Tyr270/216 and dephosphorylation at Ser21/9. Our results also show that treatment a GSK3 inhibitor SB216763 reduces MCPP-induced apoptosis in HCT-116 cells, suggests that GSK3 plays a central role in signaling from the ER stress.

Caspase-7 activation may be a general characteristic of the ER-stress pathway. It has been reported that ER stress stimulates caspase-7 activation in human neuroblastoma [Dahmer, 2005], embryonic kidney cell HEK293T [Rao et al., 2001], and liver Huh7 cell [Xie et al., 2002]. It has also been reported that caspase-7 is found in a complex with caspase-12 and the chaperone protein GRP78 [Rao et al., 2002; Reddy et al., 2003], suggest that this effector caspase may play a role in ER stress-induced cell death. Further studies will be required to determine whether caspase-7 forms complexes with GRP78 and CHOP in human colon cancer cells.

Our results show that down-regulation of antiapoptotic proteins, loss of mitochondrial function and activation of caspases may be involved in MCPP-induced apoptotic cell death in human colon cancer, and with its ability to cause ER stress. In conclusion, the novel phloroglucinol derivative MCPP human colon cancer cell death is mediated by GSK3α/β phosphorylation, which subsequently induces GPR78 and CHOP expression, increases caspase-7, caspase-9, and caspase-3 activity, resulting in apoptosis. We hope that this study for the molecular basis will provide valuable strategies of target signal transducers for the development of effective chemotherapy.

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