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Isoobtusilactone A Sensitizes Human Hepatoma Hep G2 Cells to TRAIL-Induced Apoptosis via ROS and CHOP-Mediated Up-regulation of DR5

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ABSTRACT: Hepatoma cells are relatively resistant to TRAIL. We have previously shown that isoobtusilactone A (IOA), a potent anticancer agent isolated from *Cinnamomum kotoense*, induced mitochondria-mediated apoptosis in hepatoma cells. Here, we report that IOA could potentiate TRAIL-induced apoptosis in Hep G2 cells. The combined treatment with IOA and TRAIL significantly induced caspase-dependent apoptosis. This correlated with the up-regulation of C/EBP homologous protein (CHOP) and death receptor 5 (DR5) protein levels. Gene silencing of the DR5 by small interfering RNA abrogated the apoptosis induced by the combined regimen of IOA and TRAIL, suggesting that the sensitization to TRAIL was mediated through DR5. By analyzing the DR5 promoter, we found that IOA induced a CHOP-dependent DR5 transactivation. DR5 expression after IOA treatment was accompanied by provoking intracellular reactive oxygen species (ROS) generation. Pretreatment with *N*-acetyl-L-cysteine (NAC) attenuated IOA-induced CHOP and DR5 expression and inhibited TRAIL-induced apoptosis. Taken together, our data suggested that ROS-dependent and CHOP-regulated DR5 expression played a pivotal role in the synergistic enhancement of TRAIL-induced apoptosis instigated by IOA in Hep G2 cells.

KEYWORDS: *apoptosis, IOA, DR5, reactive oxygen species, TRAIL, CHOP*

INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing factor ligand (TRAIL) is considered as a promising cancer-specific agent due to its ability to selectively induce apoptosis in a variety of malignant cells with little or no toxicity to nontransformed cells.[1] However, recent studies have shown that some cancer cells, including hepatoma cells, can acquire resistance to TRIAL-induced apoptosis.[2,3] Hence, new strategies to overcome the resistance of hepatoma cells are urgently needed. Until recently, many studies have demonstrated that TRAIL-resistant cancer cells can be sensitized by chemotherapeutic drugs through the induction of death receptor 5 (DR5) expression. Therefore, sensitization to TRAIL-induced apoptosis by the up-regulation of DR5 may underscore a potential strategy for treating TRAIL-resistant hepatoma cells.[4−7]

TRAIL cross-links with DR4 or DR5, resulting in DR4 or DR5 trimerization and intracellular death domain clustering, leading to the formation of death-inducing signaling complex.[8,9] This complex can then recruit the Fas-associated death domain adaptor molecule and subsequently activates caspase-8. The activated caspase-8 further activates downstream "effector" caspase, such as caspase-3.[10]

The CCAAT/enhancer-binding protein homologous protein (CHOP) is an endoplasmic reticulum (ER) stress-associated protein involved in the regulation of ER stress-induced apoptosis.[11] Recent studies also demonstrated that CHOP directly modulated DR5 expression via binding to a CHOP-binding site in the 5′-flanking region of DR5 gene and sensitized tumor cells to TRAIL-induced apoptosis.[12,13]

Isoobtusilactone A (IOA, Figure 1), a butanolide constituent isolated from the leaves of *Cinnamomum kotoense*, has been

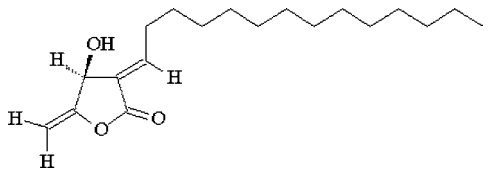

Figure 1. Chemical structure of IOA.

shown to induce apoptosis in various human cancer cell lines including Hep G2 cells, MCF 7 cells, MDA-MB-231 cells, and A549 cells.[14−17] The mechanisms underlying the IOA-induced apoptosis on these cancer cells were documented to be involved in mitochondrial-mediated apoptotic pathway. However, the question as to whether the ER stress is involved in the biological effects of IOA remains to be clarified.

In the present study, we unveiled for the first time that IOA could evoke ER stress and sensitized human hepatoma Hep G2 cells to TRAIL-induced apoptosis through CHOP-mediated up-regulation of DR5. These findings suggest that combined treatment

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of IOA and TRAIL can provide an effective therapeutic strategy to preferential eradication of human hepatoma Hep G2 cells.

MATERIALS AND METHODS

Reagents. Procurement of IOA from the leaves of *C. kotoense* plant was described previously.[14] The purity of IOA (>95%) was confirmed by high-performance liquid chromatography (HPLC) analysis. Stock solutions of IOA (1 M) were made by dissolving this compound in DMSO; various aliquots of diluted preparations were then frozen at −20 °C until use. The cell-permeable pan-caspase inhibitor Z-Val-Ala-Asp (OMe)-CH_2F (Z-VAD.fmk) was purchased from Calbiochem (San Diego, CA) and dissolved in DMSO at 50 mM. Propidium iodide was acquired from Molecular Probes (Eugene, OR). Soluble recombinant human TRAIL/Apo2L and DR5 (TRAIL-R2)/Fc chimera protein were separately purchased from BIOMOL International (Plymouth Meeting, PA) and R&D systems (Minneapolis, MN). Rabbit polyclonal antibody specific for DR5 was purchased from ProSci (Poway, CA). Antibodies against caspase-3 and caspase-8 were from New England Biolabs (Ipswich, MA). Mouse monoclonal antibody specific for β-actin was from Sigma-Aldrich. Antibody against CHOP was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 reagent was obtained from Invitrogen (Carlsbad, CA).

Cell Culture. The human hepatoma Hep G2, Hep 3B, and SK-Hep-1 cell lines were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown at 37 °C and 5% CO_2 in a humidified environment. IOA alone at 20 or 40 μM, which caused little or less apoptotic cell death as compared to the combined regimen of IOA and TRAIL, was used to determine its sensitized effect on TRAIL-induced apoptosis. In addition, we chose DMSO as a vehicle control to test its apoptosis-inducing effect, because IOA was dissolved in DMSO.

Apoptosis Assay. Hep G2 cells were grown and treated with vehicle (0.1% DMSO), IOA (20 or 40 μM) alone, or combined with TRAIL (40 ng/mL) for 24 h. After drug treatment, cells were subsequently collected and suspended in 30 μL of ice-cold Tris-EDTA (pH 8.0), to which were added 12 volumes of 6 M guanidine-HCl, 1 volume of 7.5 M ammonium acetate, 1 volume of 20% sodium dodecyl sulfate, and 1 volume of proteinase K (3 mg/mL). Lysate was incubated at 50 °C for 2 h, and genomic DNA was extracted using phenol/chloroform/isoamylalcohol (25:24:1) and precipitated with absolute alcohol. DNA samples were electrophoresed on a 1.8% agarose gel at 100 V for 40 min and visualized with ethidium bromide staining under UV illumination.

Quantitative analysis of apoptotic cells was determined by the assessment of the percentage of hypo-diploid DNA (sub-G1). Briefly, cells following incubation with the indicated agents were harvested by trypsinization and fixation in PBS−MeOH (1:2, v/v) at 4 °C for at least 18 h. After a wash with PBS, the cell pellets were suspended in PBS (500 μL) and incubated with 2.4 μL of RNase A (10 μg/mL) and the same volume of propidium iodide (10 μg/mL) in the dark for 30 min. The stained cells were analyzed using a Becton-Dickinson FACS-Calibur flow cytometer.

Cell Lysate Preparation and Immunoblotting. Total cell lysates were prepared as described previously.[14] Briefly, control (0.1% DMSO) and IOA-treated cells were collected by centrifugation, and then, the cell pellets were resuspended in a lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride] at 4 °C for 1 h. For Western blotting, equal amounts of proteins were resolved on 12% polyacrylamide gel and transferred to nitrocellulose membrane. After blocking with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with the appropriate primary antibodies. The immunoreactive bands were detected using enhanced chemiluminescence kit with Hyper-film (Amersham). Quantitative data normalized with internal control were obtained by using the computing densitometer and Multi Gauge v. 3.0 software (FujiFilm Life Science, Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from cells using TRIzol reagent (Invitrogen). For first strand cDNA synthesis, 2 μg of RNA was used as a template and subjected to reverse transcription using Superscript II (Invitrogen) and oligo (dT) primer. An equal amount of cDNA was then amplified by PCR using primers specific for GRP78 (sense, 5′-gttcttcaatggcaaggaaccatc-3′; antisense, 5′-ccatcctttcgatttcttcaggtg-3′), XBP1 (sense, 5′-ccttgtagttgagaaccagg-3′; antisense, 5′-ggggcttggtatatatgtgg-3′), DR5 (sense, 5′-gacctagctccccagcagagag-3′; antisense, 5′-cggctgcaactgtgactcctat-3′), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (sense, 5′-accacagtccatg ccatcac-3′; antisense, 5′-tccaccaccctgttgctgta-3′). On the basis of their sequences, the expected PCR product sizes from these sets of primers were 442 and 416 bp for XBP1u and XBP1s, respectively, 403 and 490 bp for DR5, 494 bp for GRP78, and 452 bp for GAPDH. The 50 μL PCR reaction contained 2 μL of cDNA, 1 μL of dNTP (10 mM), 1 μL each of the sense and antisense primer (10 μM), 1 μL of Amplitag DNA polymerase (Applied Biosystems, Foster City, CA), 5 μL of 10× reaction buffer, and sterile H_2O. The amplification reaction was initialized by incubation of PCR samples at 94 °C for 2 min, followed by a cyclic program at 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 40 s. DNA products were electrophoresed on a 1.8% agarose gel at 100 V for 40 min and visualized with ethidium bromide staining under UV illumination.

Establishment of DR5-Knockdown (Hep G2/DR5-shRNA) and Nonsilencing Control (Hep G2/Luci-shRNA) Cell Lines. DR5 short-hairpin RNA (DR5-shRNA) and luciferase shRNA (Luci-shRNA) in pLKO.1 plasmids were purchased from the RNAi Consortium at the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Luci-shRNA was used as a nonsilencing control. To establish stable cell lines, Hep G2 cells were separately transfected with DR5-shRNA and Luci-shRNA plasmids using lipofectamine 2000 reagent according to the manufacturer's instructions. Stable cell lines were selected with fresh media containing 1 mg/mL puromycin. The silencing of DR5 in Hep G2/DR5-shRNA cells and Hep G2/Luci-shRNA cells was confirmed by Western blotting.

Construction of DR5 Promoter Plasmid, Transient Transfection, and Luciferase Assay. The pDR5/Sac I [containing DR5 promoter sequence (−2500/+3)], pDR5/-605 [containing DR5 promoter sequence (−605/+3)] and pGVB2 plasmids were kindly provided by Dr. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). Using pDR5/-605 plasmid as a template, a series of deletion constructs ranging from −605 bp to translation start site of DR5 gene were generated by PCR. The deleted PCR products were individual subcloned into the pGVB2 vector through the *Bgl*II and *Kpn*I restriction site. For PCR amplification, the reverse primer 5′-CTTAAGATCTGGCGGTAGGGAACGCTCTTATAGTC-3′ was used to make all deletion constructs. The following forward primers used were 5′-CTTAGGTACCACCCAGAAACAAACCACAGCCCGGG-3′ (−373), 5′-CTTA GGTACCACTTGGACGCGCTTGCGGAGG-3′ (−293), and 5′-CTTAGGTACCCTT ATTTATTGTCACCAA-CCTGTGG-3′ (−255). These reporter constructs were named pDR5/-373, pDR5/-293, and pDR5/-255. To investigate the effect of IOA on DR5 transcriptional activation, cells were plated onto 24-well plate at a density of 3×10^4 per well. The next day, cells were cotransfected with 1 μg of various reporter plasmids and 50 ng of pSV-β-galactosidase plasmid using Lipofectamine 2000 reagent. Twenty-four hours later, cells were treated with IOA. After 12 h, luciferase and β-galactosidase activities were assayed using a luciferase assay system (Promega). The luciferase activity was normalized to β-galactosidase activity in the same lysates and expressed as an average of three independent experiments.

Statistical Analysis. All assay were carried out in triplicate. Data were analyzed to determine statistical significance of difference between the control and test group by Student's *t*-test. A p value of <0.05 was considered significant.

RESULTS

IOA Enhanced TRAIL-Induced Apoptosis in Hep G2 Cells through Caspase-Dependent Pathway. Hep G2 cells, the human hepatoma cells, are resistant to the apoptotic effect of TRAIL.[2] In this study, we initially examined whether IOA could sensitize Hep G2 cells to TRAIL-induced apoptosis. Hep G2 cells were treated with IOA alone (20 or 40 μM) or combined with TRAIL (40 ng/mL). Apoptotic fractions (sub-G_1, subdiploid peak)

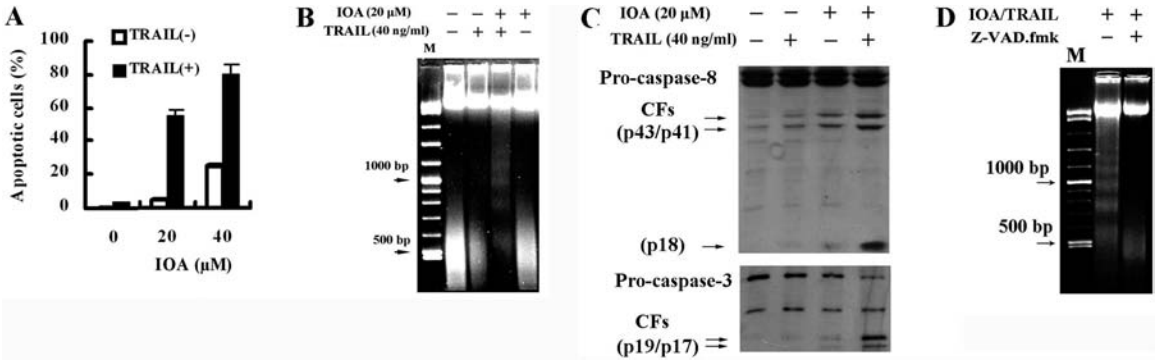

Figure 2. IOA combined with TRAIL-induced caspase-dependent apoptosis. (A) Significant induction of apoptosis caused by the combined treatment of IOA and TRAIL. Hep G2 cells were treated with IOA alone (20 or 40 μM) or combined with TRAIL (40 ng/mL) for 24 h. Apoptotic fractions (sub-G_1, subdiploid peak) were measured by flow cytometry. Data were expressed as means ± SDs from three independent experiments. (B and C) DNA fragmentation and caspase activation were induced by the combined regimen of IOA and TRAIL. Hep G2 cells were treated with IOA alone (20 μM) or combined with TRAIL (40 ng/mL) for 24 h. (B) DNA fragmentation was analyzed by agarose gel electrophoresis. M, size marker DNA. Data were representative of three independent experiments. (C) Cleaved enzyme products of caspase-3 and caspase-8 were measured by Western blotting. CFs, cleaved forms. (D) Suppression of DNA fragmentation induced by IOA combined with TRAIL by a pan-caspase inhibitor. Hep G2 cells were pretreated with Z-VAD.fmk (50 μM) for 1 h, followed by the treatment with a combined regimen of IOA (20 μM) and TRAIL (40 ng/mL) for 24 h. DNA fragmentation was determined by agarose gel electrophoresis. Data were representative of three independent experiments.

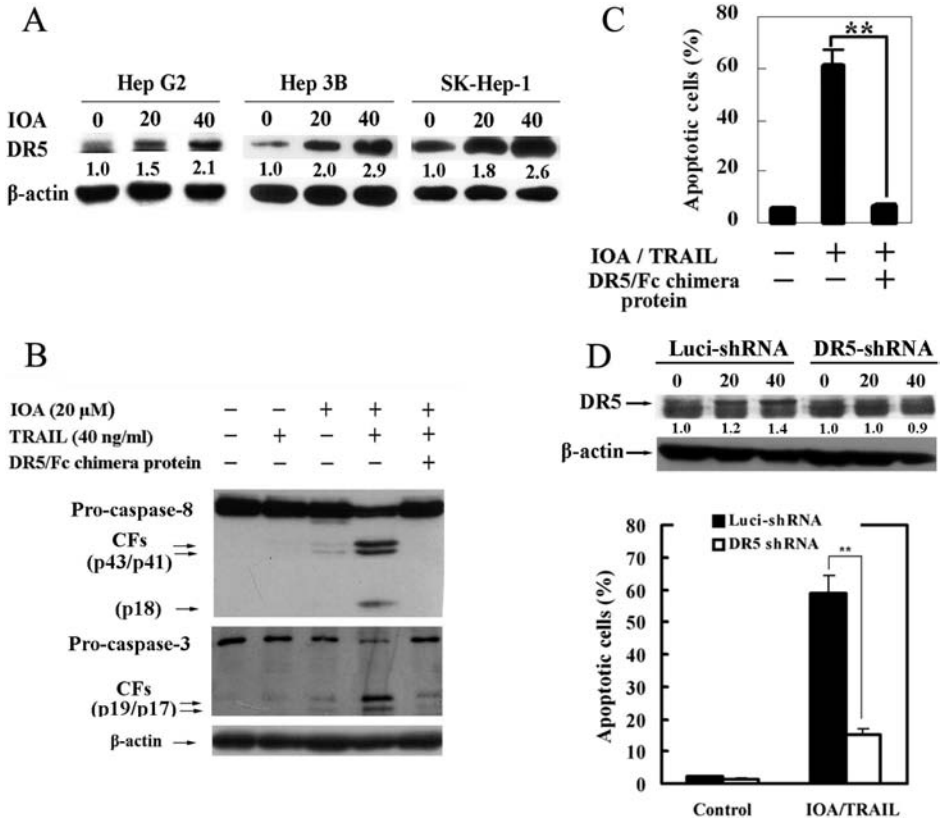

Figure 3. DR5 was involved in the sensitization to TRAIL-induced apoptosis in IOA-treated Hep G2 cells. (A) IOA induced DR5 expression. Human hepatoma cells (Hep G2, Hep 3B, and SK-Hep-1) were treated with various concentrations of IOA (20 and 40 μM) or DMSO (0.1%) for 24 h. Total cell lysates were prepared and subjected to Western blotting using antibody against DR5. (B) DR5/Fc chimera protein inhibited caspase-8 and caspase-3 activation. Hep G2 cells were pretreated with DR5/Fc chimera protein (1 μg/mL) for 1 h, followed by the treatment with IOA alone (20 μM) or combined with TRAIL (40 ng/mL) for 24 h. Caspase-8 and caspase-3 activation were determined by the detection of their cleavages by Western blotting. CFs, cleaved forms. (C) DR5/Fc chimera protein inhibited apoptosis. Hep G2 cells were pretreated with DR5 chimera/Fc protein (1 μg/mL) for 1 h, followed by the treatment with a combined regimen of IOA (20 μM) and TRAIL (40 ng/mL) for 24 h. Sub-G_1 fraction was determined by flow cytometry. Data were expressed as means ± SDs from three independent experiments. ** denoted the data that were significantly different between the two groups at $p < 0.01$. (D) Hep G2/Luci-shRNA cells and Hep G2/DR5-shRNA cells were separately treated with a combined regimen of IOA (20 μM) and TRAIL (40 ng/mL) for 24 h. Apoptotic death was assessed by the determination of sub-G_1 cell population. Data were expressed as means ± SDs from three independent experiments. ** denoted the data that were significantly different between the two groups at $p < 0.01$.

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were measured by flow cytometry. As shown in Figure 2A, apoptosis was greatly enhanced when IOA was combined with TRAIL. To further demonstrate that the demise of these cells by the combined regimen of IOA and TRAIL was apoptotic in nature, we also performed DNA fragmentation studies. As shown in Figure 2B, oligonucleosomal DNA fragmentation was found in Hep G2 cells exposing to the combined regimen of IOA (20 μM) and TRAIL (40 ng/mL) but not IOA or TRAIL alone. Next, we examined whether caspases activation were involved in the apoptotic cell death induced by the combined regimen, and caspase-8 and caspase-3 activation were first evaluated by Western blotting using monoclonal antibodies that specifically recognized their active forms of caspase. As shown in Figure 2C, increments of active caspase-8 and caspase-3 cleavage products were observed in cells exposed to the combined regimen. To confirm the critical role of caspases in this synergistic induction of apoptosis, Hep G2 cells were pretreated with Z-VAD.fmk (50 μM) for 1 h, followed by the treatment with a combined regimen of IOA (20 μM) and TRAIL (40 ng/mL). Our data showed that Z-VAD.fmk could completely inhibit oligonucleosomal DNA fragmentation induced by the combined regimen in Hep G2 cells (Figure 2D).

Up-regulation of DR5 by IOA Contributes to the Enhancement of TRAIL-Induced Apoptosis in Hep G2 Cells. It is known that DR5 functions as a cell surface receptor to facilitate cell death induced by TRAIL.[18] Thus, we were interested to determine whether this synergistic apoptosis caused by the combined regimen of IOA and TRAIL was attributed to IOA-induced DR5 up-regulation. Western blotting was used to examine DR5 expression in Hep G2 cells treated with various concentrations of IOA (20 and 40 μM). As expected, IOA could provoke the up-regulation of DR5 expression in a concentration-dependent manner. In addition, we also demonstrated that IOA could dose dependently up-regulate the expression of DR5 in another TRAIL-resistant HCC cell lines, such as Hep 3B and SK-Hep-1 cells (Figure 3A).

To clarify the functional role of DR5 in the sensitization of TRAIL-induced apoptosis instigated by IOA, Hep G2 cells were pretreated with DR5/Fc chimera protein (1 μg/mL), which has a dominant-negative function against DR5, followed by the treatment with a combined regimen of IOA (20 μM) and TRAIL (40 ng/mL). By using DR5/Fc chimera protein, we found that the formation of caspase-8 and caspase-3 cleaved products and sub-G_1 fraction induced by the combined regimen were inhibited (Figure 3B,C). Meanwhile, DR5-knockdown Hep G2 cells (Hep G2/DR5-shRNA) and nonsilencing control cells (Hep G2/Luci-shRNA) were separately treated with the combined regimen of IOA (20 μM) and TRAIL (40 ng/mL). As illustrated in Figure 3D, the combined regimen remarkably enhanced the accumulation of sub-G_1 in Hep G2/Luci-shRNA cells but only slightly in Hep G2/DR5-shRNA cells. These data clearly indicated that IOA-instigated up-regulation of DR5 was a contributing factor for sensitizing Hep G2 cells to TRAIL-induced apoptosis.

DR5 Transcriptional Activation. As illustrated in Figure 3A, IOA could up-regulate DR5 protein expression. To determine whether IOA-induced DR5 protein up-regulation was regulated at the transcriptional level, we detected DR5 mRNA levels in Hep G2 cells exposed to various concentrations of IOA (20 and 40 μM) using RT-PCR. As shown in Figure 4A, IOA was demonstrated to be capable of increasing DR5 mRNA levels in a dose-dependent manner. Furthermore, we also examined the effect of IOA on DR5 promoter activity using reporter constructs carrying DR5 5′-flanking regions (pDR5/Sac I and pDR5/-605). After transient transfection with these two constructs and IOA treatment (20 and 40 μM),

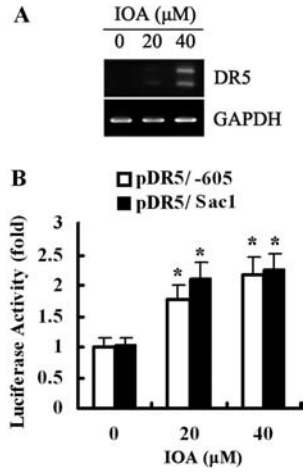

Figure 4. DR5 mRNA expression dose dependently increased at transcriptional level in IOA-treated Hep G2 cells. (A) IOA up-regulated DR5 mRNA expression. Cells were treated with IOA (20 and 40 μM) or DMSO (0.1%) for 24 h. Cellular total RNA was prepared for the detection of DR5 mRNA by reverse transcription-PCR. GAPDH levels were used as an internal control. (B) IOA transactivated DR5 gene expression. Cells were transiently cotransfected with pDR5/SacI or pDR5/-605 plasmid with pSV-β-galactosidase plasmid for 24 h and then treated with various concentrations of IOA (20 and 40 μM) for an additional 12 h. The cells were subjected to a luciferase assay. Data were expressed as means ± SDs from three independent experiments. *, $p < 0.05$ as compared with vehicle control.

luciferase activities were assayed. As shown in Figure 4B, IOA significantly increased the promoter activities of both two plasmids in a dose-dependent manner, indicating that IOA enhanced DR5 transcriptional activation.

IOA Induced DR5 Expression through a CHOP-Dependent Transcriptional Activation. CHOP was reported to be responsible for DR5 up-regulation by some therapeutic agents.[12,13,19] Therefore, we set out to examine whether IOA could provoke CHOP expression. Western blotting was used to examine CHOP expression in Hep G2 cells treated with IOA (20 and 40 μM). As shown in Figure 5A, IOA dose dependently increased CHOP expression, which was accompanied by DR5 up-regulation. Along this same vein, we also investigated whether CHOP was involved in the up-regulation of DR5 induced by IOA, and the transactivation of reporter constructs with different lengths of DR5 5′-flanking regions was first examined. In this transient transfection and luciferase assay, IOA failed to increase the luciferase activity of pDR5/-255 while significantly increasing pDR5/-293, pDR5/-373, and pDR5/-605, indicating that the region between −255 and −293 contains essential element(s) responsible for DR5 transactivation (Figure 5B). This region has been demonstrated to contain a CHOP-binding site.[12] Thus, we unequivocally established that the CHOP-binding site in the DR5 promoter region was required for IOA-mediated DR5 transactivation.

Given that CHOP is an ER stress-associated protein.,[11] we decided to further examine whether IOA could alter the expression of GRP78 and XPB-1, two ER stress-related markers. Semi-quantitative RT-PCR was used to examine GRP78 and XPB-1 expression in Hep G2 cells treated with IOA (20 and 40 μM). As shown in Figure 5C, IOA dose dependently increased the mRNA levels of spliced forms of XPB-1 (XPB-1s) but not GRP78. Taken together, our data showed that CHOP induction, derived from IOA-induced ER stress, played a critical role in the up-regulation of DR5.

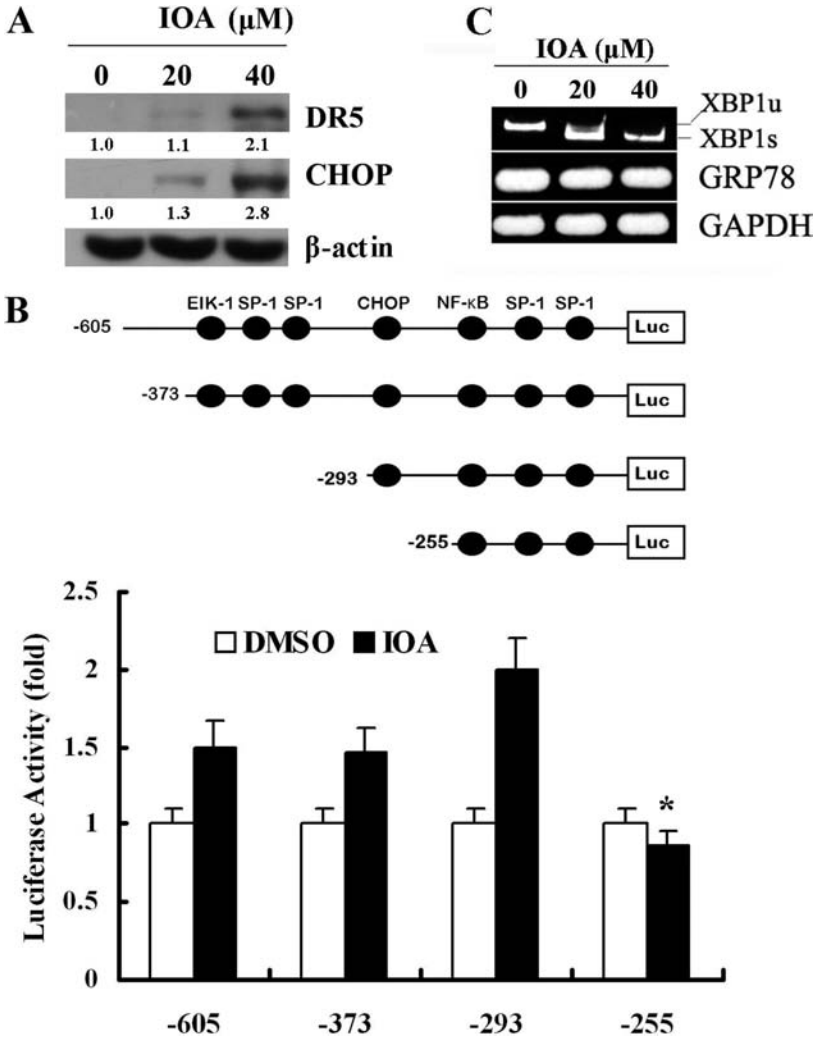

Figure 5. CHOP mediated IOA-induced DR5 up-regulation. (A) Effect of IOA on protein levels of CHOP and DR5 expression. Hep G2 cells were treated with various concentrations of IOA (20 and 40 μM) or DMSO (0.1%) for 24 h. The protein levels of CHOP and DR5 were determined by Western blotting. (B) Identification of the DR5 5′-flanking region that is responsible for DR5 transcription. Luciferase reporter plasmid containing different length of 5′-flanking region of the DR5 promoter was cotransfected with pSV-β-galactosidase plasmid into Hep G2 cells. After 24 h, cells were treated with DMSO (0.1%) or IOA (40 μM) for 12 h and then subjected to luciferase assay. Data were expressed as means ± SDs from three independent experiments performed in triplicate. *, $p < 0.05$ as compared with cells transfected with reporter plasmids (pDR5/-293, pDR5/-373, or pDR5/-605). (C) Effect of IOA on mRNA expression of GRP78 and XBP-1. Hep G2 cells were treated with various concentrations of IOA (20 and 40 μM) or DMSO (0.1%) for 24 h. mRNA levels of GRP78 and XPB-1 were determined by semiquantitative RT-PCR. Data were representative of three independent experiments.

Reactive Oxygen Species (ROS) Generation Was Involved in IOA-Enhanced TRAIL-Induced Apoptosis. Our previous data demonstrated that IOA could increase ROS production in Hep G2 cells.[13] In addition, recent studies have shown that ROS play a critical role in DR5 up-regulation and sensitization to TRAIL-induced apoptosis.[4−6,20] Therefore, we examined whether ROS was involved in the up-regulation of DR5 and sensitization to TRAIL-induced apoptosis instigated by IOA. Hep G2 cells were pretreated with 10 mM *N*-acetyl-L-cysteine (NAC) for 1 h followed by IOA (20 and 40 μM) treatment for 24 h. The protein levels of CHOP and DR5 were determined by Western blotting. It was found that pretreatment with NAC attenuated IOA-induced up-regulation of CHOP and DR5 (Figure 6A). In addition, the effect of NAC on caspase-8 activation, caspase-3 activation, and sub-G_1 accumulation in Hep G2 cells treated with a combined regimen of IOA (20 μM) and TRAIL (40 ng/mL) were also evaluated by Western blotting and flow cytometry, respectively. The results showed that caspase-8 and caspase-3 activation (Figure 6B) and sub-G_1 accumulation (Figure 6C) induced by the combined regimen were also reduced by NAC pretreatment. Collectively, these results indicated that ROS generation induced by IOA was a prerequisite for the up-regulation of CHOP and DR5, leading to TRAIL-induced apoptosis.

DISCUSSION

Our previous study has demonstrated that IOA at 50 μM could induce both caspase-dependent and -independent apoptosis in Hep G2 cells.[15] In this study, we further showed that IOA at 20 μM, which caused little apoptosis in Hep G2 cells, could potentiate TRAIL lethality through DR5-modulated and caspase-dependent apoptotic pathway.

An untagged recombinant human TRAIL (amino acids 114−281) has been proven to be nontoxic to normal human hepatocytes and is currently being developed as a clinical agent

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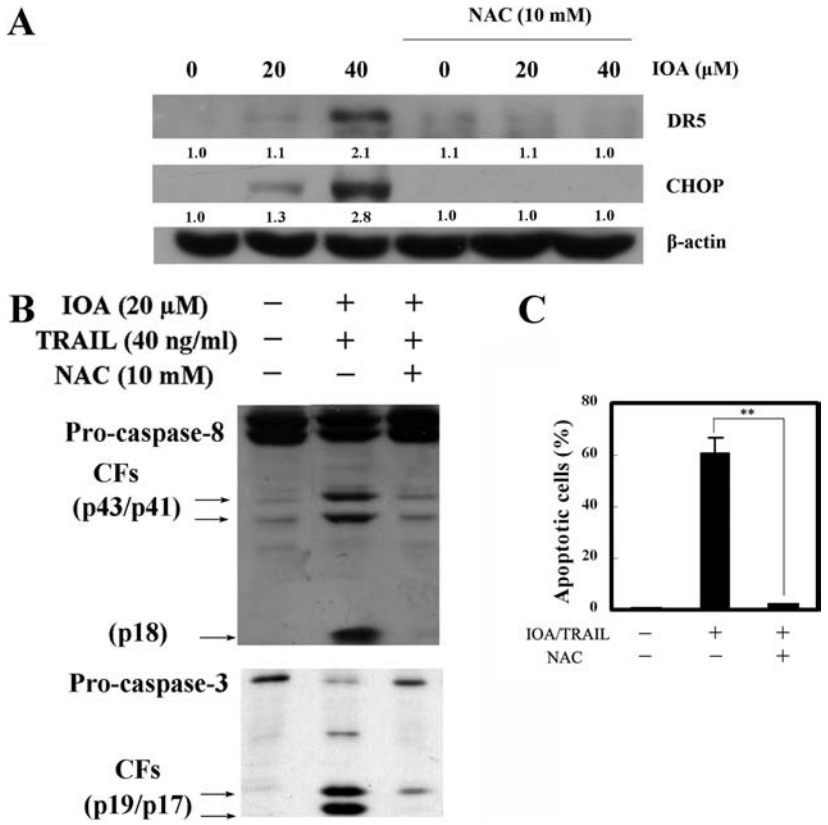

Figure 6. ROS generation was critical for IOA-induced DR5 expression and sensitization to TRAIL-induced apoptosis. (A) NAC inhibited CHOP and DR5 expression. Hep G2 cells were pretreated with 10 mM NAC for 1 h followed by IOA (20 and 40 μM) treatment for 24 h. The protein levels of CHOP and DR5 were determined by Western blotting. (B and C) Hep G2 cells were pretreated with 10 mM NAC for 1 h, followed by the treatment with a combined regimen of IOA (20 μM) and TRAIL (40 ng/mL) for 24 h. (B) Caspase-8 and caspase-3 activation were determined by the detection of their cleavages by Western blotting. (C) Sub-G_1 fraction was analyzed by flow cytometry. Data were expressed as means ± SDs from three independent experiments. ** denoted the data that were significantly different between the two groups at $P < 0.01$.

for the treatment of some liver diseases.[21] However, recent studies have shown that some human hepatoma cells were resistant to TRAIL-induced apoptosis.[22,23] Thus, TRAIL sensitizers that are capable of overcoming TRAIL resistance are currently being investigated. Several studies have shown that DR5 up-regulation maybe a promising candidate for sensitizing tumor cells to TRAIL-induced apoptosis. In the present study, we showed for the first time that IOA could sensitize human hepatoma Hep G2 cells, a TRAIL-resistant and characteristic strong drug-resistant subline,[24] to TRAIL-induced apoptosis through the up-regulated expression of DR5.

In the present study, we showed that combined treatment with IOA and TRAIL could enhance caspase-8 and caspase-3 activation and DNA fragmentation in Hep G2 cells (Figure 2). Z-VAD-fmk pretreatment could effectively inhibit TRAIL-induced apoptosis sensitized by IOA. In addition, IOA-induced DR5 up-regulation was associated with the enhancement of apoptosis induced by TRAIL. By using DR5/Fc chimera protein or DR5 short hairpin RNA (DR5-shRNA) to inhibit DR5 function, we found that both treatments could effectively inhibit apoptosis induced by the combined regimen. These results firmly established that DR5 up-regulation induced by IOA enhanced TRAIL-induced apoptosis executed through a caspase-dependent mechanism.

It has been well documented that CHOP modulates DR5 expression through the CHOP binding site in the DR5 promoter region.[12,13] In the current study, we presented the evidence that IOA could increase DR5 mRNA expression at the transcriptional level (Figure 4A). The deletion analyses of DR5 promoter region unveiled that the region containing the CHOP-binding site was essential for IOA-mediated DR5 transactivation (Figure 5B). Collectively, our results indicated that IOA induced DR5 expression through a CHOP-dependent manner. Further studies indicated that IOA not only up-regulated CHOP protein levels but also induced XBP-1s mRNA expression (Figure 5C). XBP-1s mRNA, modulated by IRE1 signaling pathway during ER stress, encodes active XBP-1s transcription factor that triggers transcription of a set of ER chaperones.[25] These results indicated that IOA is an ER stresser in Hep G2 cells. The underlying mechanism associated with IOA-mediated CHOP expression warrants further investigation.

Several chemotherapeutic agents and proteasome inhibitors have been found to induce ROS-dependent DR5 expression and enhancement of TRAIL-induced apoptosis.[4−6,20] In the present study, we found that ROS induced by IOA seemed to up-regulate CHOP and DR5 expression. Pretreatment with ROS scavenger NAC could significantly decrease CHOP and DR5 expression (Figure 6A). Moreover, our results also showed that NAC could reverse IOA-enhanced TRAIL-induced apoptosis (Figure 6C). Taken together, these results suggested that IOA-promoted TRAIL-induced apoptosis of Hep G2 cells through DR5 up-regulation and ER stress-related CHOP activation may be mediated through an oxidative stress mechanism.

In conclusion, we showed here for the first time that IOA effectively sensitized Hep G2 cells to TRAIL-induced apoptosis through DR5 up-regulation. The DR5 induction was regulated at the transcriptional level and via ROS-mediated CHOP

activation. Thus, we suggest that a combined regimen of IOA and TRAIL may offer a promising therapeutic strategy to preferential eradication of TRAIL-resistant human hepatoma cells.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; TRAIL, tumor necrosis factor-related apoptosis-inducing factor; IOA, isoobtusilactone A; DR5, death receptor 5; CHOP, CCAAT/enhancer-binding protein homologous protein

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