Coupling of Endoplasmic Reticulum Stress to CDDO-Me–Induced Up-regulation of Death Receptor 5 via a CHOP–Dependent Mechanism Involving JNK Activation

Wei Zou, Ping Yue, Fadlo R. Khuri, and Shi-Yong Sun

Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia

Abstract

The synthetic triterpenoid methyl-2-cyano-3,12-dioxoolean-1,9-dien-28-oate (CDDO-Me) is in phase I clinical trials as a novel cancer therapeutic agent. We previously showed that CDDO-Me induces c-Jun NH_2-terminal kinase (JNK)–dependent death receptor 5 (DR5) expression and augments death receptor–induced apoptosis. The current study focused on addressing how CDDO-Me induces JNK-dependent DR5 expression. Analysis of DR5 promoter regions defines that the CCAAT/enhancer binding protein homologous protein (CHOP) binding site is responsible for CDDO-Me–induced transactivation of the *DR5* gene. Consistently, CDDO-Me induced DR5 expression and parallel CHOP up-regulation. Blockade of CHOP up-regulation also abrogated CDDO-Me–induced DR5 expression. These results indicate that CDDO-Me induces CHOP-dependent DR5 up-regulation. Moreover, the JNK inhibitor SP600125 abrogated CHOP induction by CDDO-Me, suggesting a JNK-dependent CHOP up-regulation by CDDO-Me as well. Importantly, knockdown of CHOP attenuated CDDO-Me–induced apoptosis, showing that CHOP induction is involved in CDDO-Me–induced apoptosis. Additionally, CDDO-Me increased the levels of Bip, phosphorylated eukaryotic translation initiation factor 2α, inositol requiring kinase 1α, and activating transcription factor 4, all of which are featured changes during endoplasmic reticulum (ER) stress. Furthermore, salubrinal, an inhibitor of ER stress–induced apoptosis, inhibited JNK activation and up-regulation of CHOP and DR5 by CDDO-Me and protected cells from CDDO-Me–induced apoptosis. Thus, ER stress seems to be important for CDDO-Me–induced JNK activation, CHOP and DR5 up-regulation, and apoptosis. Collectively, we conclude that CDDO-Me triggers ER stress, leading to JNK-dependent, CHOP-mediated DR5 up-regulation and apoptosis. [Cancer Res 2008;68(18):7484–92]

Introduction

The novel synthetic triterpenoid methyl-2-cyano-3,12-dioxoolean-1,9-dien-28-oate (CDDO-Me) potently induces apoptosis of cancer cells and exhibits antitumor activity in animal models (1–6). Thus, CDDO-Me holds promise as a cancer therapeutic agent and is currently being tested in phase I clinical trials. It is well known that apoptosis can occur through two major apoptotic pathways: the intrinsic mitochondria-mediated pathway and the extrinsic death receptor–induced pathway. These two pathways are linked by the truncated proapoptotic protein Bid (7). Our previous studies have shown that CDDO-Me depletes intracellular glutathione (GSH), activates c-Jun NH_2-terminal kinase (JNK), and finally leads to up-regulation of death receptor 5 (DR5), which contributes to CDDO-Me–mediated activation of the extrinsic apoptotic pathway and augmentation of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptosis (8, 9). However, the detailed molecular mechanism by which CDDO-Me induces DR5 expression is unknown.

It is known that DR5 expression can be regulated at the transcriptional level through both p53-dependent (10, 11) and p53-independent mechanisms (12–15). Our previous study suggested that CDDO-Me induces DR5 expression independent of p53 status (8). Thus, we are particularly interested in p53-independent mechanisms. It has been documented that the CCAAT/enhancer binding protein homologous protein (CHOP), also known as growth arrest and DNA damage gene 153 (GADD153), directly regulates DR5 expression through a CHOP binding site in the 5-flanking region of the *DR5* gene (16, 17). Thus, certain drugs induce DR5 expression through CHOP-dependent transactivation of the *DR5* gene (16–20).

The endoplasmic reticulum (ER) is the primary organelle for proper protein folding and assembling as well as calcium storage. The accumulation of unfolded protein in the ER lumen leads to the ER stress response known as unfold protein response. This response ensures coordinate regulation of gene expression at various levels including transcription, translation, and protein degradation to shut down general protein synthesis and to induce the expression of genes including those encoding resident ER proteins with chaperone and folding functions for restoring proper protein folding and ER homeostasis. In addition, this response also activates specific apoptotic pathways to eliminate severely damaged cells, in which the protein folding defects cannot be resolved (21–23).

Inositol requiring kinase 1 (IRE1), double-stranded RNA-activated protein kinase–like ER kinase (PERK), and activating transcription factor (ATF)-6 are three critical transmembrane ER signaling proteins that regulate the unfold protein response through their respective signaling cascades. Under resting condition, the chaperone protein Bip binds to the luminal domains of IRE1, PERK, and ATF6, preventing their activation. However, accumulation of unfolded proteins will release Bip, allowing aggregation of these transmembrane signaling proteins and launching the unfold protein response (21, 23). Although ER stress–induced apoptotic signaling pathways have not been fully

Requests for reprints: Shi-Yong Sun, Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, 1365-C Clifton Road Northeast, C3088, Atlanta, GA 30322. Phone: 404-778-2170; Fax: 404-778-5520; E-mail: ssun@emory.edu.

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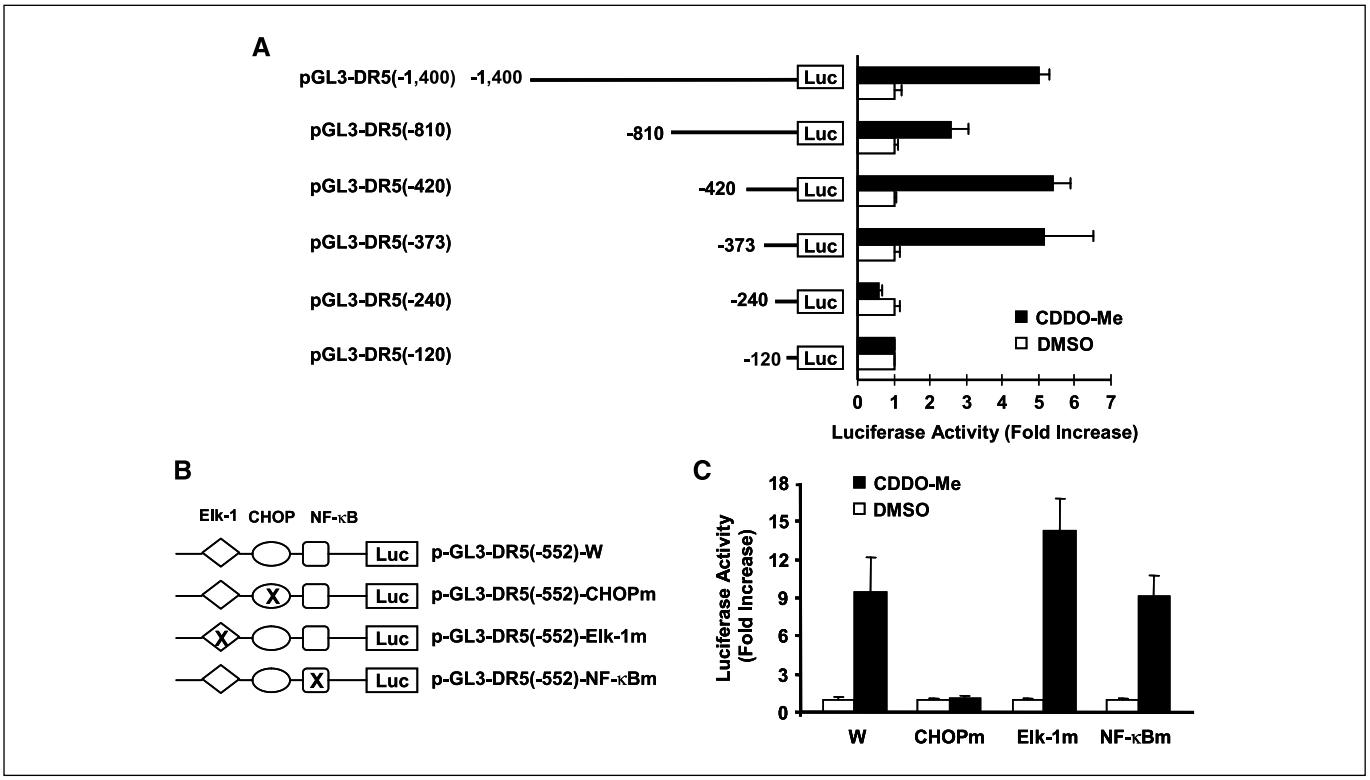

Figure 1. CDDO-Me induces CHOP-dependent transcription of the *DR5* gene. *A,* identification of the region in the DR5 5′-flanking region that is responsible for CDDO-Me–induced DR5 transactivation. The given reporter constructs with different lengths of the 5′-flanking region of the *DR5* gene were cotransfected with pCH110 plasmid into H1792 cells. After 24 h, the cells were treated with DMSO or 1 μmol/L CDDO-Me for 12 h and then subjected to luciferase assay. *Column,* mean of triplicate determinations; *bars,* SD. *B* and *C,* the CHOP binding site is required for CDDO-Me–induced DR5 transactivation. The given reporter constructs with and without different mutated binding sites (*B*) were cotransfected with pCH110 plasmids into H1792 cells. After 24 h, the cells were treated with DMSO or 1 μmol/L CDDO-Me for 12 h and then subjected to luciferase assay (*C*). *Column,* mean of triplicate determinations; *bars,* SD.

uncovered, several mechanisms have been suggested. These include transcriptional induction of CHOP through multiple signaling pathways including PERK/eukaryotic translation initiation factor 2α (eIF2α)–dependent mechanisms, IRE1-mediated JNK activation, and cleavage and activation of ER-specific caspase-12 (22–24).

Given that CDDO-Me induces JNK-dependent DR5 up-regulation (8, 9), the current study focused on addressing whether CHOP is involved in JNK-dependent DR5 induction by CDDO-Me and, if it does, whether CDDO-Me also induces ER stress. Using human non–small-cell lung cancer (NSCLC) cells as a model system, we have, for the first time, showed that CDDO-Me indeed induces ER stress and CHOP expression, which participate in JNK-dependent DR5 expression by CDDO-Me.

Materials and Methods

Reagents. CDDO-Me, which was previously described (25) and provided by Dr. M.B. Sporn (Dartmouth Medical School, Hanover, NH), was dissolved in DMSO at a concentration of 10 mmol/L, and aliquots were stored at −80°C. Stock solution was diluted to the desired final concentrations with growth medium just before use. The specific JNK inhibitor SP600125 was purchased from Biomol. The ER stress inhibitor salubrinal was purchased from EMD Chemicals, Inc. Rabbit polyclonal anti-DR5 antibody was purchased from ProSci, Inc. Mouse monoclonal anti-CHOP (B-3) and rabbit polyclonal ATF4 (CREB-2; C-20) antibodies were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti–caspase-3 was purchased from Imgenex. Rabbit polyclonal anti–phospho-c-Jun (Ser63), anti–c-Jun, anti-IRE1α, anti–phospho-elF2α (Ser^{51}), anti–caspase-8, anti–caspase-9, and anti-PARP antibodies were purchased from Cell Signaling Technology, Inc. Mouse monoclonal anti-Bip/GPR78 antibody was purchased from BD Biosciences. Rabbit polyclonal anti–β-actin antibody and *N*-acetylcysteine (NAC) were purchased from Sigma Chemical Co.

Cell lines and cell culture. The human NSCLC cell lines used in this study were purchased from the American Type Culture Collection. These cell lines were cultured in RPMI 1640 containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Western blot analysis. Whole-cell protein lysates were prepared and analyzed by Western blotting as described previously (26, 27).

Cell survival assay. Cells were seeded in 96-well cell culture plates and treated the next day with the agents indicated. The viable cell number was estimated using the sulforhodamine B assay, as previously described (28).

Detection of apoptosis. Apoptosis was evaluated by Annexin V staining using the Annexin V-phycoerythrin (PE) apoptosis detection kit purchased from BD Biosciences following the manufacturer's instructions or by measuring sub-G_1 populations using flow cytometry as described previously (29). We also detected caspase activation by Western blotting (as described above) as an additional indicator of apoptosis.

Detection of cell surface DR5. The procedure for direct antibody staining and subsequent flow cytometric analysis of cell surface proteins was described previously (19). The mean fluorescent intensity, which represents antigenic density on a per cell basis, was used to represent TRAIL receptor expression level. PE-conjugated mouse anti-human DR5 (DJR2-4) and PE-mouse IgG1 isotype control (MOPC-21/P3) were purchased from eBioscience.

Gene silencing using small interfering RNAs. Silencing of CHOP was achieved by transfecting CHOP small interfering RNA (siRNA) using RNAifect transfection reagent (Qiagen) following the manufacturer's

instructions. Control (i.e., nonsilencing) and CHOP siRNAs were described previously (19). Gene silencing effect was evaluated by Western blot analysis as described above after the indicated time of treatment.

Construction of DR5 reporter plasmids, transient transfection, and luciferase activity assay. The reporter constructs containing a 552-bp 5′-flanking region of the *DR5* gene with wild-type, mutated CHOP binding site, nuclear factor κB (NF-κB) binding site, or Elk binding sites were generously provided by Dr. H-G. Wang (University of South Florida College of Medicine, Tampa, FL; ref. 16). The pGL3-DR5(−1400), pGL3-DR5(−810), pGL3-DR5(−420), pGL3-DR5(−373), pGL3-DR5(−240), and pGL3-DR5(−120) reporter constructs were described previously (19, 30). The plasmid transfection and luciferase assay were the same as described previously (19).

Results

CDDO-Me activates DR5 transcription in a CHOP-dependent manner. We previously reported that CDDO-Me increases DR5 transcription (8). To determine how CDDO-Me increases DR5 transcription, we began our study by examining the effects of CDDO-Me on the transactivation of reporter constructs with different lengths of DR5 5′-flanking regions (Fig. 1*A*) to identify the region responsible for CDDO-Me–mediated DR5 transactivation. In this transient transfection and luciferase assay, CDDO-Me did not increase the luciferase activity of pGL3-DR5(−240) and pGL3-DR5(−120) while significantly increasing the luciferase activity of pGL3-DR5(−373), pGL3-DR5(−420), and pGL3-DR5(−1040; Fig. 1*A*), indicating that the region between −240 and −373 has essential element(s) responsible for CDDO-Me–induced DR5 transactivation. Because a CHOP binding site is located in this region, we further compared the effects of CDDO-Me on the transactivation of reporter constructs carrying wild-type and mutated CHOP binding sites, respectively. As controls, we also included constructs carrying mutated NF-κB and Elk binding sites, respectively (Fig. 1*B*). As presented in Fig. 1*C*, CDDO-Me increased

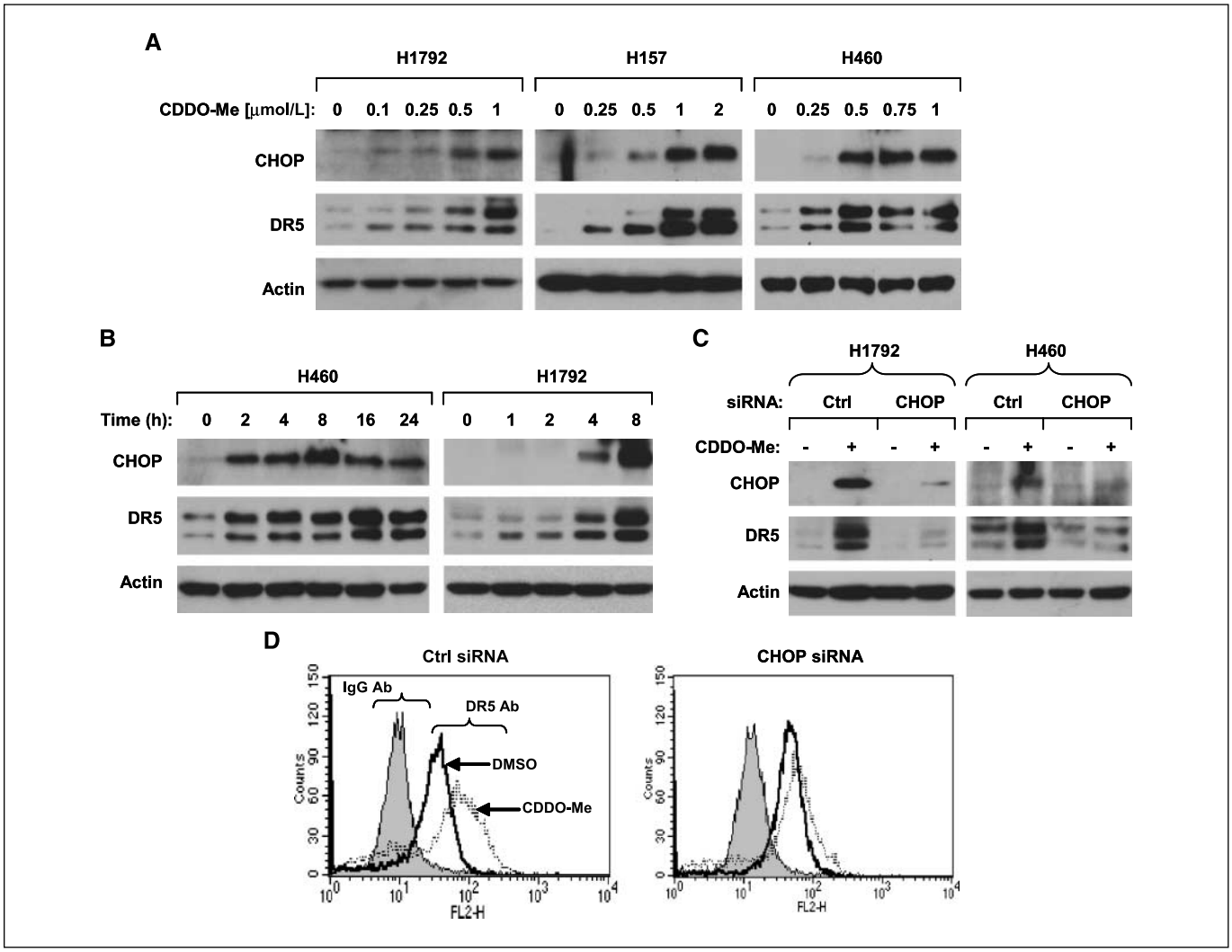

Figure 2. CDDO-Me induces CHOP-dependent DR5 expression. *A,* CDDO-Me induces dose-dependent DR5 expression. The indicated cell lines were treated with the indicated concentrations of CDDO-Me for 12 h. *B,* CDDO-Me induces time-dependent DR5 expression. H460 and H1792 cells were treated with 1 μmol/L CDDO-Me for the times indicated. *C,* blockade of CHOP induction abrogates CDDO-Me–induced DR5 up-regulation. Both H1792 and H460 cell lines were transfected with control (*Ctrl*) or CHOP siRNA. After 48 h, the cells were treated with 1 μmol/L CDDO-Me for 12 h (H1792) or with 0.5 μmol/L CDDO-Me for 8 h (H460). After the aforementioned treatments (*A–C*), the cells were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis. *D,* blockade of CHOP induction inhibits CDDO-Me–induced increase in cell surface DR5. H1792 cells were transfected with control or CHOP siRNA. After 48 h, the cells were treated with 1 μmol/L CDDO-Me for 12 h and then subjected to staining of cell surface DR5 and subsequent flow cytometry. *Ab,* antibody.

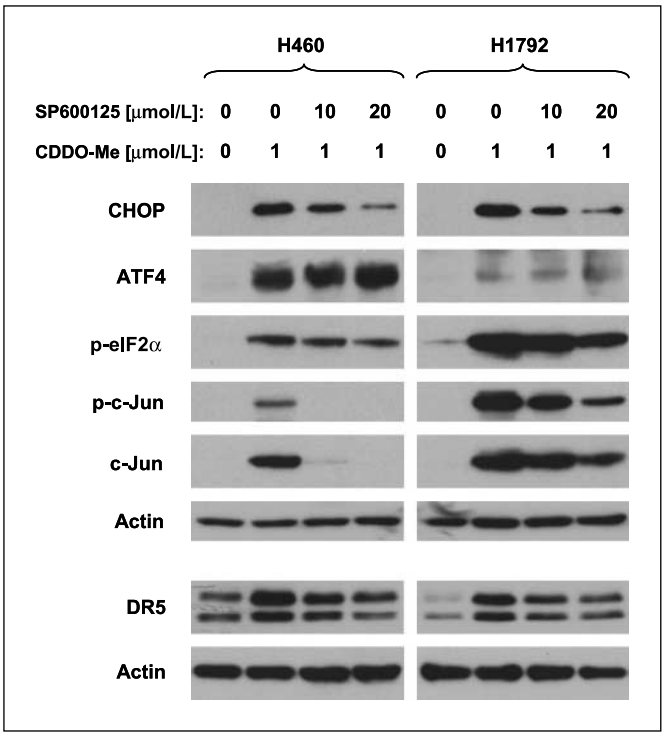

Figure 3. Blockade of JNK activation prevents CDDO-Me–induced up-regulation of CHOP and DR5 but not increases in ER stress marker proteins. The indicated cell lines were pretreated with 10 or 20 μmol/L SP600125 for 30 min and then cotreated with 1 μmol/L CDDO-Me for an additional 6 h. The cells were then harvested and subjected to preparation of whole-cell protein lysates for detection of the indicated proteins by Western blot analysis. SP600125 alone at the tested concentrations did not modulate the levels of CHOP, DR5, ATF4, and p-eIF2α.

the lucifease activity of the constructs carrying the wild-type DR5 promoter region or the DR5 promoter region with mutated NF-κB or Elk binding sites. However, CDDO-Me failed to increase the luciferase activity of the construct carrying the DR5 promoter region with a mutated CHOP binding site. These results clearly indicate that the CHOP binding site in the DR5 promoter region is responsible for CDDO-Me–induced DR5 transactivation.

CDDO-Me increases CHOP expression leading to DR5 up-regulation. We next determined whether CDDO-Me actually induces CHOP expression. By Western blot analysis, we detected a concentration- and time-dependent CHOP induction accompanied by DR5 up-regulation in cells exposed to CDDO-Me (Fig. 2*A* and *B*). To test whether CHOP induction is involved in mediating CDDO-Me–induced DR5 up-regulation, we silenced CHOP expression via siRNA transfection to block CDDO-Me–induced CHOP expression. As presented in Fig. 2*C*, we detected CHOP induction in cells transfected with control siRNA, but not or only minimally in CHOP siRNA–transfected cells after exposing to CDDO-Me, indicating a successful silencing of CHOP expression. Accordingly, we detected DR5 up-regulation only in control siRNA–transfected cells when treated with CDDO-Me. In agreement, CDDO-Me increased cell surface DR5 levels in control siRNA–transfected cells, but only minimally in CHOP siRNA–transfected cells (Fig. 2*D*). Thus, these data clearly indicate that CDDO-Me–induced DR5 up-regulation is secondary to CHOP induction.

We detected two bands for DR5 protein in the blots (Fig. 2). It is noteworthy that the two-band pattern of DR5 protein is consistent with the previously published results on DR5 protein (8, 27, 30–32). Their specificities were also confirmed by using DR5 siRNA (8, 19, 27) previously and in the current study (Fig. 2). The two bands of DR5 protein may correspond to the products of two isoforms of *DR5* gene as described previously (33).

CDDO-Me–induced CHOP expression is JNK dependent. We previously showed that CDDO-Me induces JNK-dependent DR5 up-regulation (8). Given that CHOP is also regulated by JNK activation (34), we then examined whether CHOP induction by CDDO-Me involves JNK activation. To this end, we treated cells with CDDO-Me in the absence and presence of the JNK inhibitor SP600125 and then analyzed CHOP and DR5 expression. In agreement with our previous finding (8), the presence of SP600125 abrogated the ability of CDDO-Me to increase p-c-Jun levels and DR5 expression. Concurrently, CDDO-Me–induced CHOP expression was also inhibited by SP600125 (Fig. 3). Thus, these results suggest that CDDO-Me induces CHOP-dependent DR5 up-regulation involving JNK activation.

CHOP induction contributes to CDDO-Me–induced apoptosis. We previously showed that DR5 up-regulation contributes to CDDO-Me–induced apoptosis (8). Given that CDDO-Me induces CHOP-dependent DR5 expression, it is plausible to speculate that CHOP induction should also contribute to CDDO-Me–induced apoptosis. Indeed, we found that CDDO-Me induced ~35% apoptotic cells in control siRNA–transfected H1792 cells, but only ~20% apoptotic cells in CHOP siRNA–transfected H1792 cells (Fig. 4*A*). In agreement, we detected a striking activation of caspase-8 and caspase-3 in CDDO-Me–treated H460 cells transfected with control siRNA evidenced by decreased levels of proforms of caspase-8, caspase-3, and PARP, accompanied by the appearance of the strong cleaved bands of these proteins. However, we detected only minimal reduction of the proforms of these proteins with weaker bands of their cleaved forms in CDDO-Me–treated H460 cells transfected with CHOP siRNA (Fig. 4*B*). By directly evaluating apoptosis using the Annexin V staining, we detected ~25% and 34% apoptosis, respectively, in control siRNA–transfected H460 cells but only 11% and 17% apoptosis, respectively, in CHOP siRNA–transfected cells when exposed to 0.5 and 1 μmol/L CDDO-Me (Fig. 4*C*). These results collectively indicate that blockade of CHOP induction attenuates CDDO-Me–induced apoptosis. Thus, we conclude that CHOP induction contributes to CDDO-Me–induced apoptosis.

CDDO-Me induces ER stress. Given that CHOP is known to be a featured ER stress marker protein involved in ER stress–mediated apoptosis, we further determined whether CDDO-Me induces ER stress. To this end, we detected the levels of several typical ER stress marker proteins, which are usually increased (e.g., Bip, IRE1α, p-eIF2α, and ATF4) during ER stress, in cells exposed to CDDO-Me. As presented in Fig. 5*A*, CDDO-Me increased the levels of Bip in three NSCLC cell lines (*left*). Moreover, CDDO-Me also elevated the levels of IRE1α, p-eIF2α, and ATF4 (*right*). The increase in Bip, IRE1α, p-eIF2α, and ATF4 occurred quickly, as early as 1 or 2 hours after CDDO-Me treatment. Together, these results suggest that CDDO-Me induces ER stress.

CDDO-Me–induced ER stress is associated with induction of apoptosis. To determine the effect of ER stress on CDDO-Me–induced apoptosis, we compared the effects of CDDO-Me on induction of apoptosis in the absence and presence of salubrinal, an inhibitor of ER stress–induced apoptosis. As shown in Fig. 5*B*, the presence of salubrinal (particularly at 50 and 75 μmol/L) significantly protected lung cancer cells from CDDO-Me–induced cell death. In agreement, CDDO-Me–induced cleavage of caspase-8,

caspase-9, and PARP and an increase in Annexin V–positive (i.e., apoptotic) populations were also substantially inhibited by salubrinal (Fig. 5*C* and *D*). Thus, it seems that ER stress is involved in CDDO-Me–induced apoptosis.

CDDO-Me–induced JNK activation and up-regulation of CHOP and DR5 are secondary to ER stress. To decipher the relationship between ER stress, JNK activation, and CHOP-dependent DR5 induction, we compared the effects of CDDO-Me on the levels of p-c-Jun, CHOP, and DR5 in the absence and presence of salubrinal. As presented in Fig. 6*A*, CDDO-Me increased the levels of p-c-Jun, CHOP, and DR5 in the absence of salubrinal, but these effects were substantially inhibited in the presence of salubrinal, suggesting that CDDO-Me–initiated increases in the levels of these proteins are all associated with ER stress. Consistently, the JNK inhibitor SP600125 had minimal inhibition on CDDO-Me–induced increase in ATF4 and p-eIF2α while preventing CHOP and DR5 up-regulation (Fig. 3), further supporting the notion that the activation of JNK and up-regulation of CHOP and DR5 are consequences of ER stress.

We noted that salubrinal alone strongly increased the levels of p-eIF2α and ATF4, but weakly elevated CHOP levels. When combined with CDDO-Me, salubrinal minimally inhibited the CDDO-Me–induced ATF4 increase while substantially abrogating CDDO-Me–induced CHOP up-regulation in both H460 and H1792 cell lines. Similarly, salubrinal did not affect (e.g., H1792) or weakly inhibited (e.g., H460) CDDO-Me–induced elevation of p-eIF2α levels (Fig. 6*A*).

CDDO-Me triggers ER stress through depletion of intracellular GSH. We and others previously showed that GSH depletion plays a critical role in mediating induction of apoptosis including JNK activation and DR5 up-regulation by CDDO-Me or its analogues (1, 9, 35, 36). Thus, we further examined the protective effects of NAC, which prevents GSH reduction with an antioxidative property, on CDDO-Me–induced ER stress, JNK activation, and up-regulation of CHOP and DR5 expression. In agreement with our previous findings (9), NAC attenuated the ability of CDDO-Me to increase p-c-Jun levels and DR5 expression. Besides, CDDO-Me–induced increases in CHOP and ATF4 and phosphorylation of elF2α were all substantially inhibited by NAC (Fig. 6*B*). Collectively, these results indicate that CDDO-Me induces ER stress through depletion of intracellular GSH.

Discussion

Our previous studies have shown that CDDO-Me induces DR5 up-regulation, resulting in induction of apoptosis and

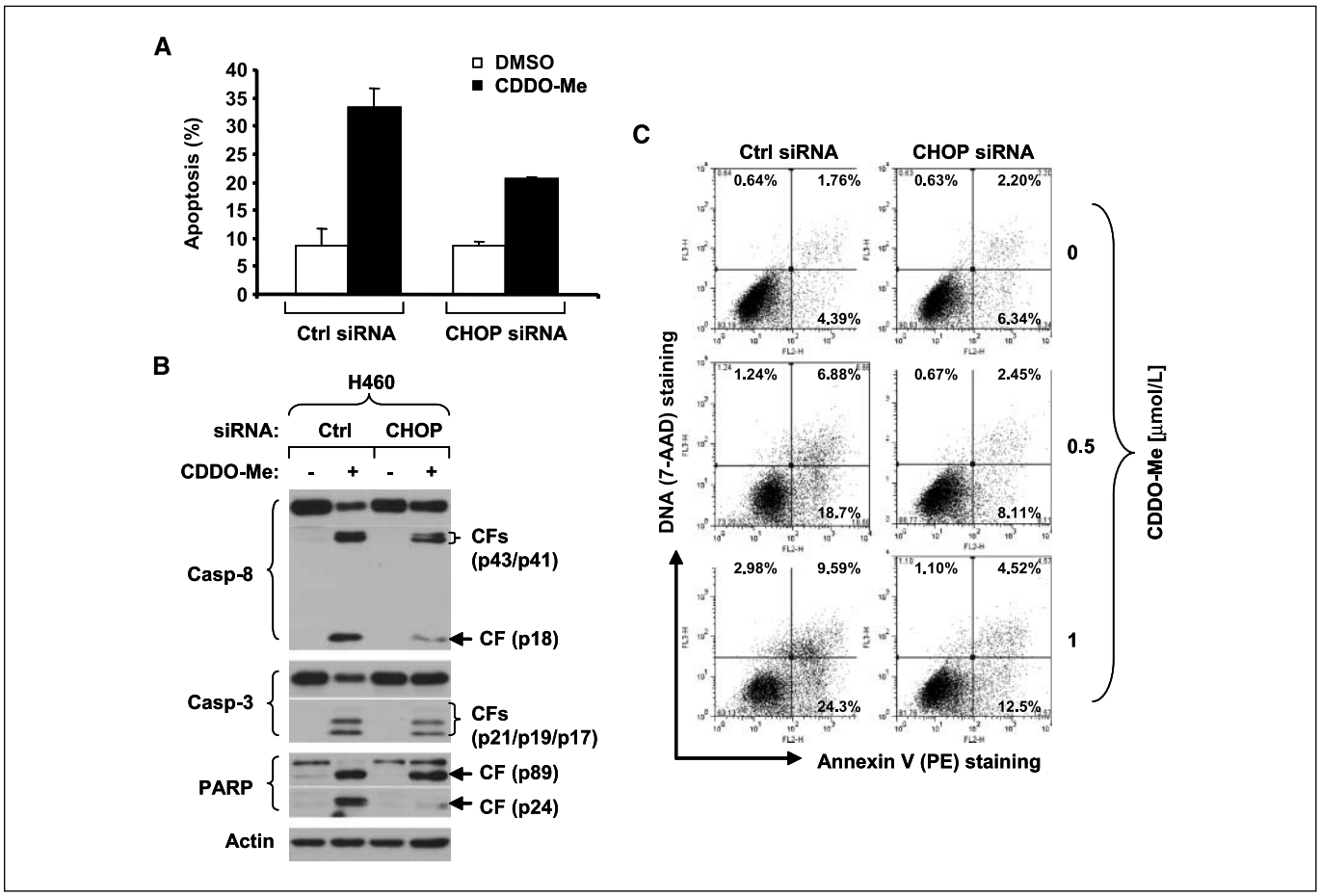

Figure 4. Blockade of CHOP induction diminishes the ability of CDDO-Me to induce apoptosis. *A,* H1792 cells were plated in six-well cell culture plates and transfected with control or CHOP siRNA. After 48 h, the cells were treated with 1 μmol/L CDDO-Me for 12 h and then subjected to flow cytometric analysis for sub-G_1 populations. *Columns,* means of duplicate assays; *bars,* SE. *B* and *C,* H460 cells were plated in six-well cell culture plates and transfected with control or CHOP siRNA. After 48 h, the cells were treated with 0.5 μmol/L CDDO-Me for 8 h and then subjected to preparation of whole-cell protein lysates and Western blot analysis (*B*). In addition, the cells were also harvested for Annexin V staining and flow cytometric analysis of apoptotic cells (*C*). The percent positive cells in the top right and bottom right quadrants were added to yield the total of apoptotic cells. *7-AAD,* 7-amino-actinomycin D.

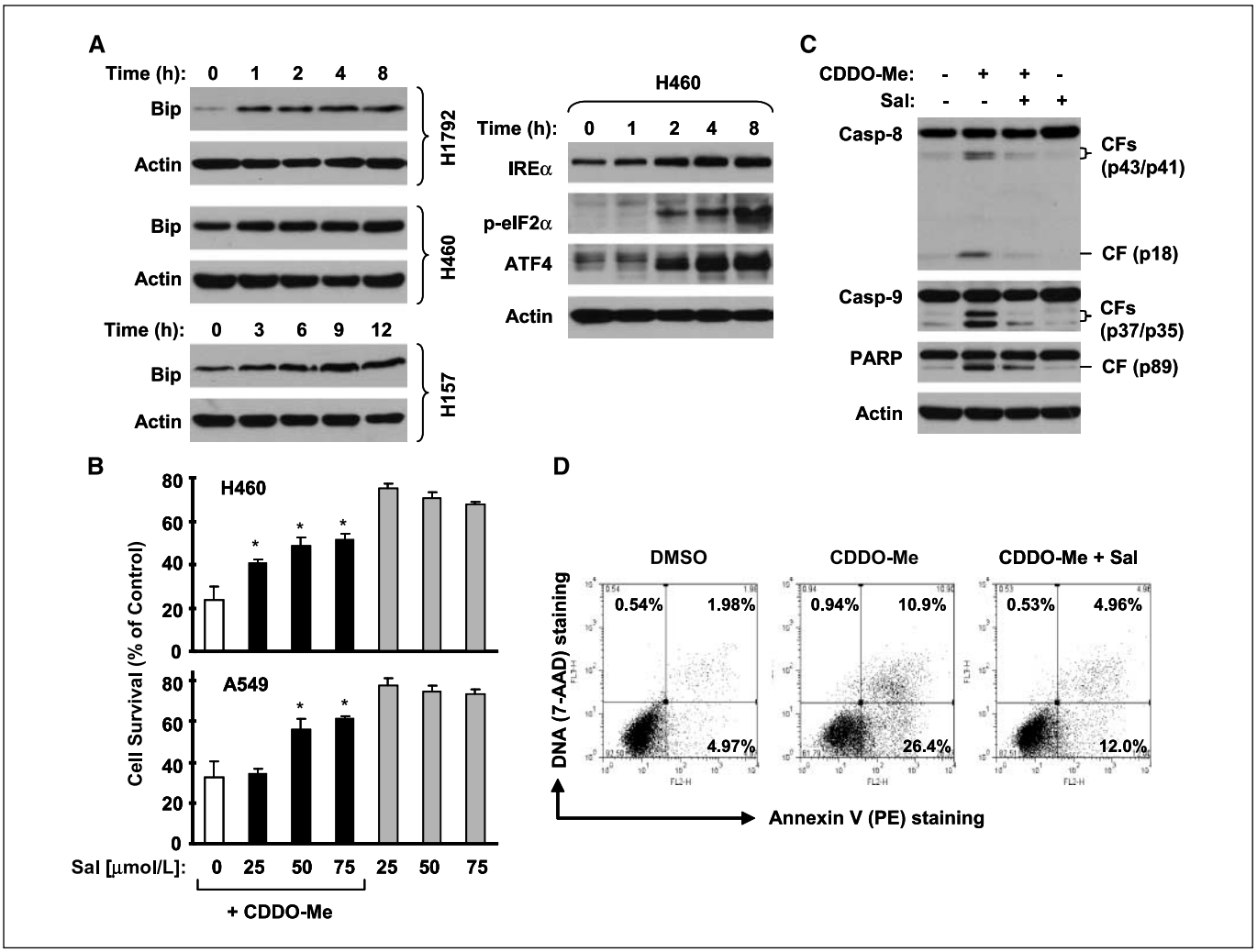

Figure 5. CDDO-Me increases the levels of ER stress marker proteins (*A*) and induces ER stress–mediated apoptosis (*B–D*). *A,* the indicated cell lines were treated with 1 μmol/L CDDO-Me. After the indicated times, the cells were harvested and subjected to preparation of whole-cell protein lysates for detection of the indicated proteins by Western blot analysis. *B,* the indicated cell lines were seeded in 96-well cell culture plates and treated the next day with DMSO, 1 μmol/L CDDO-Me alone, the given concentrations of salubrinal (*Sal*) alone, and the respective combination of CDDO-Me with each of the given concentrations of salubrinal. After 24 h, the cells were subjected to the sulforhodamine B assay for estimation of cell numbers. *Column,* mean of four replicate determinations; *bars,* SD. *, $P < 0.001$, compared with CDDO-Me alone by one-way ANOVA analysis using GraphPad InStat 3 software (GraphPad Software). *C* and *D,* H460 cells were seeded in six-well plates and treated the next day with DMSO, 1 μmol/L CDDO-Me alone, 75 μmol/L salubrinal alone, and their combination. After 6 h, the cells were harvested for preparation of whole-cell protein lysates for detection of the indicated proteins by Western blot analysis (*C*) or for detection of Annexin V–positive cells using flow cytometry (*D*). *CFs,* cleaved forms.

enhancement of TRAIL-induced apoptosis in human lung cancer cells (8, 9). Moreover, we have shown that CDDO-Me increases DR5 expression at the transcription level (8) secondary to GSH depletion–initiated JNK activation (8, 9). Here we further show that CDDO-Me increases CHOP-dependent transcription of the *DR5* gene. It has been well documented that CHOP is an important transcription factor that regulates DR5 expression (16, 17, 19, 20). Through the deletion and mutation analysis of the DR5 5′-flanking region, we revealed that the regions containing the CHOP binding site are essential for CDDO-Me–mediated DR5 transactivation (Fig. 1). Consistently, CDDO-Me induced a time- and dose-dependent CHOP expression, which was accompanied by DR5 up-regulation. Moreover, blockage of CDDO-Me–mediated CHOP induction by CHOP siRNA accordingly abrogated the ability of CDDO-Me to up-regulate DR5 expression (Fig. 2). Collectively, we conclude that CDDO-Me induces DR5 expression through a CHOP-dependent mechanism.

We previously showed that DR5 induction is involved in CDDO-Me–induced apoptosis (8). In this study, we found that blockade of CDDO-Me–induced CHOP up-regulation through siRNA-mediated gene silencing not only abrogated DR5 up-regulation by CDDO-Me but also diminished the ability of CDDO-Me to induce apoptosis (Fig. 4). Thus, we conclude that CHOP induction also participates in CDDO-Me–induced apoptosis. Thus, these findings further highlight the important role of DR5 up-regulation in CDDO-Me–induced apoptosis. We noted that blockage of CHOP did not abolish the ability of CDDO-Me to induce apoptosis despite substantially inhibiting DR5 induction (Figs. 2 and 4). Thus, it is possible that mechanisms other than CHOP/DR5 may also be involved in mediating CDDO-Me–induced apoptosis. It has been documented that NF-κB represses CHOP expression (37), whereas CDDO-Me inhibits NF-κB (38, 39). Thus, future study may investigate whether inhibition of NF-κB is involved in CDDO-Me–induced CHOP/DR5 expression.

It is well known that CHOP is a typical ER stress–regulated protein involved in ER stress–induced apoptosis (24). Thus, our finding on CHOP induction by CDDO-Me suggests that CDDO-Me may trigger ER stress. Indeed, CDDO-Me increased the levels of Bip, IRE1α, p-eIF2α, and ATF4 (Fig. 5), all of which are additional proteins accumulated or increased during ER stress (21, 22). Therefore, it seems that CDDO-Me induces ER stress. Salubrinal is a selective inhibitor of eIF2α dephosphorylation and ER stress–induced apoptosis (40). The presence of salubrinal apparently protected lung cancer cells from CDDO-Me–induced decrease in cell survival, caspase activation, and apoptotic death (Fig. 5). Thus, it seems that CDDO-Me induces apoptosis involving ER stress. To the best of our knowledge, this is the first study showing the involvement of ER stress in CDDO-Me–induced apoptosis.

Both JNK and CHOP are implicated in mediating ER stress–induced apoptosis (23). A recent study suggested that JNK activation during ER stress contributes to CHOP expression (34). We previously showed that CDDO-Me activates JNK leading to DR5 up-regulation and apoptosis (8, 9). In the present study, we detected both JNK activation and CHOP induction in cells exposed to CDDO-Me. Moreover, the presence of the JNK inhibitor SP600125 attenuated CDDO-Me–induced CHOP expression in addition to inhibition of DR5 expression, indicating that CDOO-Me-induced CHOP expression is also JNK dependent. Collectively, we conclude that JNK activation mediates CHOP-dependent DR5 induction. We noted that SP600125 inhibited CDDO-Me–induced c-Jun phosphorylation completely, but only partially prevented CHOP and DR5 up-regulation by CDDO-Me in H460 cells (Fig. 3). Thus, we cannot role out the possibility that other mechanisms also participate in CDDO-Me–induced, CHOP-dependent DR5 up-regulation.

Although SP600125 inhibited CDDO-Me–induced CHOP and DR5 up-regulation, it minimally prevented CDDO-Me–induced increases in p-eIF2α and ATF4 (Fig. 3), suggesting that JNK activation and subsequent up-regulation of CHOP and DR5 are secondary to ER stress. This notion was further supported by our findings that salubrinal attenuated the ability of CDDO-Me to increase c-Jun phosphorylation, CHOP induction, and DR5 up-regulation (Fig. 6*A*) while inhibiting CDDO-Me–induced apoptosis (Fig. 4). Taken together, we conclude that CDDO-Me induces ER stress, leading to JNK activation and subsequent CHOP-dependent DR5 up-regulation (Fig. 6*C*).

It is generally thought that the eIF2α/ATF4 signaling pathway is the primary mechanism for the induction of CHOP in ER stress (22, 24). However, other pathways also can regulate CHOP expression in ER stress (22, 24). It has been initially shown that salubrinal protects cells from ER stress–induced apoptosis through

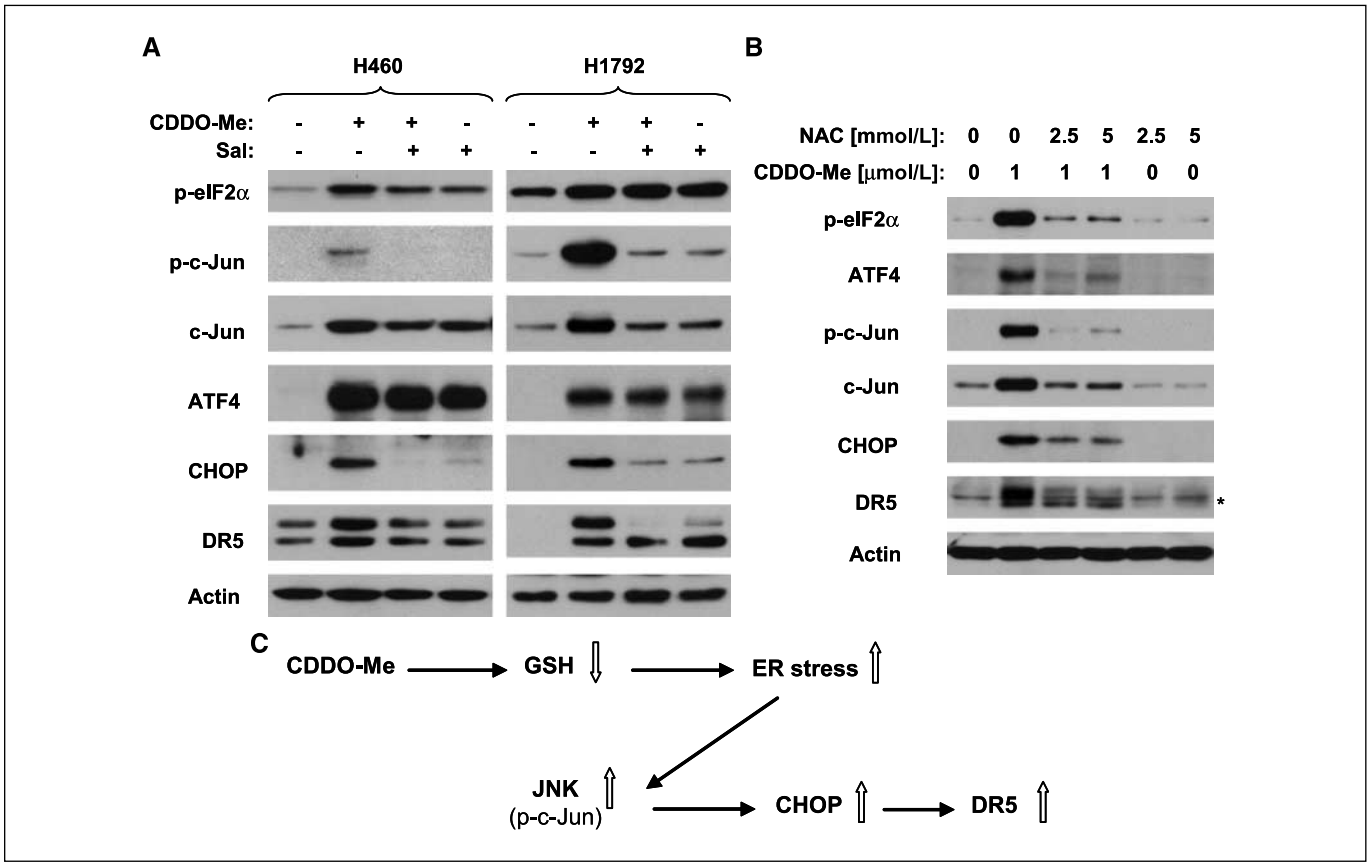

Figure 6. Effects of salubrinal (*A*) or NAC (*B*) on CDDO-Me–induced JNK activation, CHOP and DR5 up-regulation, and increase in the levels of ER stress marker proteins, and a schematic model for CDDO-Me–induced DR5 expression (*C*). *A* and *B,* both H460 and H1792 cells (*A*) or H1792 cells (*B*) were pretreated with 75 μmol/L salubrinal (*A*) or the indicated concentrations of NAC (*B*) for 30 min and then cotreated with 1 μmol/L CDDO-Me for an additional 6 h. The cells were then subjected to preparation of whole-cell protein lysates for detection of the indicated proteins by Western blot analysis. *Asterisk,* nonspecific bands. *C,* schematic model for CDDO-Me–induced DR5 expression. CDDO-Me induces ER stress via depletion of intracellular GSH, leading to JNK activation and subsequent CHOP-dependent DR5 up-regulation.

increasing p-eIF2α levels by inhibiting eIF2α dephosphorylation. Consequently, CHOP expression is also increased (40). However, salubrinal can also potentiate ER stress and induce apoptosis through such a mechanism involving up-regulation of ATF4 and CHOP under certain conditions (41). In our study, salubrinal apparently protects NSCLC cells from CDDO-Me–induced apoptosis as discussed above. Salubrinal itself strongly increased the levels of p-eIF2α and ATF4 while weakly inducing CHOP expression in human NSCLC cells. Interestingly, salubrinal minimally inhibits CDDO-Me–induced elevation of p-eIF2α and ATF4 levels, whereas it strongly abrogated the ability of CDDO-Me to increase p-c-Jun, CHOP expression, and DR5 up-regulation (Fig. 6*A*). These results imply that CDDO-Me is unlikely to induce CHOP expression through the p-eIF2α/ATF4-mediated signaling pathway in ER stress. Thus, the mechanism underlying CDDO-Me–induced, JNK-dependent CHOP up-regulation in ER stress needs further investigation.

It has been documented in several studies that depletion of intracellular GSH plays a critical role in initiating apoptosis by CDDO-Me or its analogues (1, 9, 35, 36). This is likely caused by the reversible interaction between CDDO-Me and GSH (42). We previously showed that CDDO-Me depletes intracellular GSH, resulting in JNK-dependent DR5 up-regulation and apoptosis in human NSCLC cells (9). In this study, we found that the presence of NAC abrogated the effects of CDDO-Me not only on activating JNK and inducing DR5 expression but also on increasing the levels of CHOP, ATF4, and p-eIF2α, all of which are typical ER stress marker proteins (Fig. 6*B*). Thus, it seems that CDDO-Me induces ER stress through depletion of intracellular GSH.

In summary, the present study has shown that CDDO-Me depletes intracellular GSH, resulting in ER stress. Subsequently, it activates JNK, leading to CHOP-dependent DR5 up-regulation and apoptosis (Fig. 6*C*).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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