Dehydrocostuslactone, a Medicinal Plant-Derived Sesquiterpene Lactone, Induces Apoptosis Coupled to Endoplasmic Reticulum Stress in Liver Cancer Cells

Ya-Ling Hsu, Ling-Yu Wu, and Po-Lin Kuo

Graduate Institute of Medicine (Y.-L.H., L.-Y.W.) and Institute of Clinical Medicine (P.-L.K.), College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

Received November 6, 2008; accepted January 30, 2009

ABSTRACT

This study is the first to investigate the anticancer effect of dehydrocostuslactone [DHE (3αS,6αR,9αR,9βS)-decahydro-3,6,9-tris(methylene) azulenol[4,5-b]furan-2(3H)-one)], a medicinal plant-derived sesquiterpene lactone, on hepatocellular carcinoma. Our results showed that DHE inhibits the proliferation of HepG2 and PLC/PRF/5 cells by inducing apoptosis. DHE induces up-regulation of Bax and Bak, down-regulation of Bcl-2 and Bcl-XL, and nuclear relocation of the mitochondrial factors apoptosis-inducing factor (AIF) and endonuclease G (Endo G). DHE triggered endoplasmic reticulum (ER) stress, as indicated by changes in cytosol-calcium levels, double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase phosphor- ylation, inositol-requiring protein 1 (IRE1) and CHOP/GADD153 up-regulation, X-box transcription factor-1 mRNA splicing, and caspase-4 activation. Enhancement of ER stress by DHE is through p38 and extracellular signal-regulated kinase 1/2-dependent manners and subsequently causes c-Jun NH2-terminal kinase activation, resulting in AIF and Endo G nuclear relocation. Both of IRE1 small interfering RNA transfection and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid-acetoxymethyl ester pretreatment inhibit DHE-mediated apoptosis, supporting the hypothesis that DHE induces cell death through ER stress. It is noteworthy that animal studies have revealed a dramatic 50% reduction in tumor volume after 45 days of treatment. This study demonstrates that DHE may be a novel anticancer agent for the treatment of liver cancer.

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm in the world, with increasing incidence in western countries (Parkin et al., 2005; Llovet et al., 2008). Chronic infection with hepatitis B virus is the predominant risk factor for HCC in Southeast Asia and Africa, and chronic infection with hepatitis C virus is the predominant risk factor for HCC in western countries and Europe (El-Serag and Mason, 1999; Parkin et al., 2005; Llovet et al., 2008). This pathology is currently controlled by surgery, transplantation, percutaneous ethanol injection, and radiotherapy and is frequently supported by adjuvant chemotherapies (Llovet et al., 2003). However, HCC is highly resistant to chemotherapy, and there is still no effective cure for patients with advanced stages of the disease (Raoul, 2008). Effective chemopreventive treatment for liver cancer would have a tremendous impact on liver cancer morbidity and mortality rates.

Endoplasmic reticulum (ER) is a central organelle engaged in lipid synthesis, protein folding, and maturation. A variety of toxic insults, including hypoxia, failure of protein synthesis, folding, transport or degradation, and Ca2+ overload, can...
disturb the ER function and result in ER stress (Abcouwer et al., 2002; Soboloff and Berger, 2002; Feldman et al., 2005; Moenner et al., 2007; Yung et al., 2007). There is increasing evidence that ER stress plays a crucial role in the regulation of apoptosis. That is, ER stress triggers several specific signaling pathways, including ER-associated protein degradation and unfolded protein response (UPR) (Feldman et al., 2005; Moenner et al., 2007). The UPR involves the activation of several proteins, including inositol-requiring protein 1 (IRE1), PERK and activating transcription factor 6 (ATF6) (Moenner et al., 2007). In turn, activation of PERK phosphorylates eukaryotic translation initiation factor-2 (eIF-2α), which suppresses protein synthesis (Jiang and Wek, 2005; Moenner et al., 2007). Activation of the RNase activity of IRE1 initiates splicing of X-box transcription factor-1 (XBP-1) into spliced variant XBP-1 mRNA, which is subsequently translated into a potent transcription factor (Shuda et al., 2003). A combination of ATF6 and the spliced variant of XBP-1 positively regulates a wide variety of UPR target gene expression, including several ER-resident chaperones (Shuda et al., 2003; Moenner et al., 2007). CHOP/GADD153 is a proapoptotic transcription factor that suppresses the transcription of Bcl-2, which can be induced by a combination of the PERK/ATF4 and ATF6 pathways (Hetz et al., 2006; Anding et al., 2007; Moenner et al., 2007).

Dehydrocostus lactone (DHE; Fig. 1A), a medicinal plant-derived sesquiterpene lactone, was extracted from *Saussurea lappa* and *Aucklandia lappa* (Sun et al., 2003; Li et al., 2005). DHE has been found to possess antifungal activity (Wedge et al., 2000) and also has been reported to exhibit a cytotoxic effect against OVCAR-3 and HeLa cell lines (Sun et al., 2003). However, the anticancer effect and mechanism of DHE in liver cancer remain unknown. The studies reported here was to establish an understanding of the mechanism of action of DHE in preclinical models of HCC. Direct effects of DHE in HCC tumor cells were evaluated in vitro in a poorly

![Fig. 1](https://i.imgur.com/12345.png)

**Fig. 1.** The effects of DHE on cell proliferation inhibition and colony formation in liver cancer cell lines HepG2 and PLC/PRF/5. A, chemical structure of DHE. B, cell proliferation inhibition effect of DHE in HepG2 and PLC/PRF/5. C, influence of HepG2 and PLC/PRF/5 on the number of colony-forming cells, as evaluated by clonogenic assay. Cell growth inhibition activity of DHE was assessed by XTT. For colony-forming assay, the clonogenic assay was performed as described under Materials and Methods. Results are expressed as the percentage of cell proliferation relative to the proliferation of the control. The data shown are the mean from three independent experiments. Each value is the mean ± S.D. of three determinations. *p*, significant difference between control and DHE-treated cells, as analyzed by Dunnett’s test (p < 0.05).
differentiated PLC/PRF/5 cell line (p53-mutant, K-Ras-mutant, hepatitis B virus integration) and a well-differentiated HepG2 (p53 wild type and K-Ras-mutant lack the hepatitis B virus integration) cell line. PLC/PRF/5 xenograph was also used to assess the anticancer effect of DHE in vivo. This present study sought: 1) to determine the antiproliferative effect of DHE in vitro and in vivo, 2) to assay the effect of DHE on cell cycle progression and apoptosis, 3) to investigate ER stress as a potential molecular target of DHE, and 4) to establish whether MAPK/ER stress signaling is involved in DHE-mediated apoptosis in HCC.

Materials and Methods

Test Compound. DHE was obtained from Wako Pure Chemicals (Osaka, Japan), dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. The purity was >98%, as assessed by high-performance liquid chromatography. Control cultures received the carrier solvent (0.1% DMSO).

Cell Proliferation and Clonogenic Assay. To measure the effect of DHE on cell proliferation, the HepG2 and PLC/PRF/5 (1 × 10^5 cells/well) were seeded into a 96-well plate. Cells were treated with vehicle (0.1% DMSO) and 1, 10, 20, and 40 μM DHE for 48 h. At the end of the assay period, cell proliferation was measured by XTT assay as described in detail (Kuo et al., 2008). The XTT cell proliferation assay is a colorimetric assay system that measures the reduction of a tetrazolium component (XTT) into soluble formazan product by the mitochondria of viable cells. This bioreduction occurs in viable cells only and is primarily related to glycolytic NAD(P)H production. The amount of color produced is directly proportional to the number of metabolically active cells. In this study, the drug concentration required to inhibit cell growth by 50% (IC50) was determined by interpolation from dose-response curves.

To determine long-term effects, cells were treated with DHE at various concentrations for 3 h. After being rinsed with fresh medium, cells were allowed to form colonies for 14 days, which were then stained with crystal violet (0.4 g/l; Sigma-Aldrich, St. Louis, MO). The stained colonies were allowed to form colonies for 14 days, which were then determined by interpolation from dose-response curves.

Materials and Methods

Small Interfering RNA Transfection. Cells were seeded in 12-well plates and allowed to reach ~50% confluence on the day of transfection. The ON-TARGET smart pool small interfering RNA (siRNA) of IRE1, p38, extracellular signal-regulated kinase (ERK) 1/2, and JNK were obtained from Dharmacon RNA Technologies (Lafayette, CO). The scrambled siRNA sequences for each ON-TARGET smart pool siRNA were also obtained from Dharmacon RNA Technologies.

Transmission Electron Microscopy. Cells or tumor sections were fixed with 1% glutaraldehyde and postfixed with 2% osmium tetroxide. The cell pellets or sections were embedded in epoxy resin. Representative areas were chosen for ultrathin sectioning and viewed with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan).

In Vivo Tumor Xenograft Study. Male nude mice (6 weeks old; BALB/cA-nu (nu/nu)) were purchased from the National Science Council Animal Center (Taipei, Taiwan) and maintained in pathogen-free conditions. PLC/PRF/5 cells were injected subcutaneously into the flanks of these nude mice (5 × 10^6 cells in 200 μl). Tumors were allowed to develop for ~45 days until they reached a size of approximately 100 mm³, when treatment was initiated. Thirty mice were randomly divided into two groups. The mice in the DHE-treated group were injected intraperitoneally daily with DHE dissolved in a clear solution containing 25% polyethylene glycol (10 mg/kg body weight), in a volume of 0.2 ml. The control group was treated with an equal volume of vehicle. After transplantation, tumor size was measured using calipers, and tumor volume was estimated according to the formula: tumor volume (millimeters cubed) = L × W²/2, where L is the length, and W is the width. Tumor-bearing mice were sacrificed after 45 days after dosing.

Statistical Analysis. Data were expressed as means ± S.E.M. Statistical comparisons of the results were made using analysis of variance. Significant differences (p < 0.05) between the means of test groups were analyzed by Dunnett's test.

Results

DHE Inhibits the Proliferation of Human Liver Cancer Cell Lines HepG2 and PLC/PRF/5. To investigate the potential cell proliferative inhibition activity of DHE in liver cancer, we first examined the effect of DHE on cell proliferation and clonogenic survival in HepG2 and PLC/PRF/5 cells. As shown in Fig. 1B, DHE inhibited cell proliferation in HepG2 and PLC/PRF/5 cancer cell lines in a concentration-
DHE was observed, with a dose-dependent inhibition in clonogenic assays of tumorigenicity in nude mice (Freedman and Shin, 1974). Both HepG2 and PLC/PRF/5 cells showed the ability to form clones in the untreated control wells. However, upon addition of DHE, a dose-dependent inhibition in clonogenic activity was observed, with a >50% inhibition at dosages as low as 5 μM DHE (Fig. 1C).

DHE Induces Apoptosis in HepG2 and PLC/PRF/5 Cells. We next assessed the effect of DHE on the induction of apoptosis in HepG2 and PLC/PRF/5 cells by DNA fragmentation and TUNEL assay. The results showed that DHE treatment results in the formation of DNA fragments in both cancer cell lines, as determined by agarose gel electrophoresis at the indicated times (Fig. 2A). In addition, a quantitative evaluation was then sought using TUNEL assay to detect DNA breaks. Compared with vehicle-treated cells, DHE induced apoptosis in HepG2 and PLC/PRF/5 cells after 48 h of treatment (Fig. 2B). TUNEL-positive cells were also visible using a fluorescence microscope (Fig. 2B).

We further confirmed mitochondrial apoptotic pathways triggered by DHE. Immunoblot analysis showed that treatment of HepG2 and PLC/PRF/5 cells with DHE increased Bax and Bak levels (Fig. 2C). In contrast, DHE decreased Bcl-2 and Bcl-XL levels in both cancer cell lines. The exposure of HepG2 and PLC/PRF/5 cells to DHE increased caspase-9 activity (Fig. 2D), whereas caspase-9-specific inhibitor Leu-Glu-His-Asp-CHO caused only a partial change in DHE-mediated apoptosis (Fig. 2E).

Given caspase-9 inhibitor, failure to completely halt DHE-induced cell death, we next explored whether DHE triggers caspase-9-independent apoptotic events and, in particular, whether it induces the release of the AIF and Endo G from the mitochondria into the nuclei. Immunoblot data showed that although control cells demonstrated a lack of nuclear expression of AIF and Endo G, DHE treatment induced demonstrable translocation of AIF and Endo G to the nuclei (Fig. 2F). We also addressed the role of AIF in DHE-induced apoptosis by using N-phenylmaleimide (50 μM) (Wang et al., 2007). Our data showed that N-phenylmaleimide (50 μM) inhibited DHE-mediated apoptosis (Fig. 2E).

DHE Raises Cytosol Ca2+ Level and Induces ER Stress. Depletion of luminal ER calcium stores is believed to reflect ER stress, which can promote induction of the ER stress (Benali-Furet et al., 2005). We assessed the effect of DHE on the mobilization of Ca2+. As shown in Fig. 3A, treatment with DHE resulted in a rapid increase in cytosolic calcium levels as early as 1 h after DHE treatment in both cancer cell lines.

Increased phosphorylation of PERK and eIF-2α, up-regulation of IRE1 and CHOP/GADD153 protein concentration, and splicing of XBP-1 mRNA are the major markers of ER stress (Moenner et al., 2007). As shown in Fig. 3B, DHE induced phosphorylation of PERK and eIF-2α. The amount of Bip, IRE1, and CHOP/GADD153 protein also increased. Furthermore, there was a gradual increase in the amount of spliced variant of XBP-1 mRNA upon DHE treatment. In addition, Bap31, an integral membrane protein of the ER, was cleaved into a p20 fragment after DHE treatment. Evidence of ER stress was further confirmed by transmission electron microscopy analysis. In control cells, a few strands of rough ER with narrow cisternae had been observed (Fig. 3C), but after 6 h of DHE treatment, almost all cisternae had dilated into circular shapes. These observations suggest that DHE triggers ER stress.

Several caspases have been reported to become activated during ER stress (Moenner et al., 2007), and examination of caspase activation in DHE-treated cells has shown that caspase-3 was significantly activated (Fig. 3D). In addition, DHE treatment also increased the activation of caspase-4, a potential homolog of murine caspase-12 that locates at the cytoplasmic side of ER (Moenner et al., 2007), and is activated after ER stress (Fig. 3E).

DHE Induces Activation of MAPK. We assessed the effect of DHE on MAPK activation. Exposure of HepG2 and PLC/PRF/5 cells to 30 μM DHE resulted in rapid and sustained phosphorylation of JNK, p38, and ERK1/2. On the other hand, expression of all three unphosphorylated MAPKs remained unaltered by DHE treatment (Fig. 4A). DHE-mediated activation of MAPK was confirmed additionally by determining phosphorylation of their substrates (c-Jun for JNK, ATF-2 for p38, and Elk-1 for ERK1/2). As shown in Fig. 4B, in comparison with the control, the Ser383 phosphorylation of Elk-1 increased after a 1-h exposure of HepG2 and PLC/PRF/5 cells to 30 μM DHE. Phosphorylation of Elk-1 increased relative to the control at all four points in time (Fig. 4B). Likewise, phosphorylation of ATF-2 at Thr71 increased in both DHE-treated HepG2 and PLC/PRF/5, in contrast to the control. In contrast, phosphorylation of c-Jun increased after 3 h of treatment.

The Role of MAPK on DHE-Induced ER Stress and Apoptosis. To analyze the roles of p38, JNK, and ERK1/2 on DHE-induced ER stress and apoptosis, we examined the effect of DHE on MAPK activation. Exposure of HepG2 and PLC/PRF/5 cells to 30 μM DHE resulted in rapid and sustained phosphorylation of JNK, p38, and ERK1/2 by siRNA on the ER stress and apoptosis induction. As shown in Fig. 5A, p38, JNK, and ERK1/2 siRNA significantly reduced p38, JNK, and ERK1/2, respectively. Genetic inhibition of p38 and ERK1/2 by siRNA significantly reduced CHOP/GADD153 and Bip up-regulation and caspase-4 activation in DHE-treated cells (Fig. 5, B and C). In contrast, knockdown of JNK by siRNA had little effect on the expressions of these ER stress-related factors (Fig. 5, B and C). These data suggest that activation of p38 and ERK1/2 is upstream of ER stress, whereas JNK activation is a downstream event.

We next assessed the role of MAPK on DHE-mediated apoptosis. As shown in Fig. 5D, p38, JNK, and ERK1/2 inhibition abrogated DHE-mediated apoptosis. These results suggest that apoptosis induction by DHE is due to MAPK activation.

We further investigated the mechanism that accounts for the actions of MAPKs in DHE-mediated ER stress and apoptosis in liver cancer cells by specific chemical inhibitors. As shown in Supplemental Fig. 1, A and B, pretreatment of HepG2 and PLC/PRF/5 cells with SB203580 (p38 inhibitor) or PD98059 (MEK inhibitor) completely blocked DHE-mediated GADD153 and Bip up-regulation and caspase-4 activation. These effects were not affected when cells were pretreated with SP600125 (JNK inhibitor). In addition, all MAPK inhibitors decreased DHE-induced apoptosis in HepG2 and PLC/PRF/5 cells (Supplemental Fig. 1A). These consequences of p38 and ERK1/2 inhibition by chemical in-
Fig. 2. DHE induces apoptosis in HepG2 and PLC/PRF/5 cells. A, DHE induced apoptosis in both cancer cell lines, as determined by agarose gel electrophoresis. B, quantitative evaluations of TUNEL assay by flow cytometry and fluorescent microscope. C and D, the effects of DHE on Bcl-2 family protein (C) and caspase-9 (D). E, effect of caspase-9 and AIF inhibitor on DHE-mediated apoptosis. F, effect of DHE on the translocation of AIF and Endo G. For A, cells were treated with vehicle alone (0.1% DMSO) and 30 μM DHE for the indicated times, and then DNA fragmentation was assessed by agarose gel electrophoresis. For B, cells were treated with vehicle alone (0.1% DMSO) and 15 and 30 μM DHE for the 48 h and then the TUNEL-positive cells were examined by flow cytometry and were examined visible by means of fluorescent microscope. For D, cells were treated with vehicle alone (0.1% DMSO) or 30 μM DHE for the indicated times. The activity of caspase-9 was assessed by caspase-9 activity kit. The expression
hibitors coincide with genetic inhibition, indicating that p38 and ERK1/2 may play upstream roles on DHE-mediated ER stress.

The Role of ER Stress on DHE-Mediated Apoptosis. To determine whether any of the known ER stressors play a role in DHE-mediated apoptosis, we silenced IRE1 by specific siRNA pools. Figure 6A shows that siRNA knockdown of IRE1 markedly inhibited its basal expression in both cancer cell lines. Selective genetic inhibition of IRE1 abrogated JNK phosphorylation (Fig. 6B), but not p38 and ERK1/2. In addition, siRNA knockdown of IRE1 also markedly reduced DHE-mediated apoptosis, in comparison with those transfected with the control siRNA (Fig. 6C).

To establish the hierarchy among calcium mobilization, MAPK activation, and AIF nuclear translocation, calcium was depleted by the calcium chelator BAPTA-AM (Benali-Furet et al., 2005). Pretreatment with BAPTA-AM (5 μM) not only completely inhibited DHE-mediated JNK activation and AIF nuclear translocation (Fig. 6D) but also partially blocked p38 and ERK1/2 activation. In addition, BAPTA-AM also blocked DHE-induced apoptosis (Fig. 6E). These findings suggest that Ca2+ mobilization is involved in DHE-induced mitochondrial apoptosis and JNK phosphorylation but only partially participates in p38 and ERK1/2 activation.

DHE Inhibits Tumor Growth in Nude Mice. We further used animal experiments to determine whether DHE of various proteins was assessed by immunoblot. For blocking experiments, cells were preincubated with Leu-Glu-His-Asp-CHO (20 μM) or N-phenylmaleimide (50 μM) for 1 h before the addition of 30 μM DHE for an additional 48 h. Results shown are representative of three independent experiments. *, significant difference between control and DHE-treated cells or two test groups, as analyzed by Dunnett's test (p < 0.05).
DHE slightly increases caspase-9 activity. However, our findings show that pretreatment of the cells with the specific caspase-9 inhibitor did not completely block DHE-induced apoptosis. These results suggest that DHE-induced apoptosis is through caspase-9 dependent and -independent manners. In contrast, our results also show that both AIF and Endo G were progressively released from the mitochondria to the nuclei after incubation of human liver cancer cells with DHE. Moreover, pretreatment of liver cancer cells with the AIF inhibitor N-phenylmaleimide prevented DHE-mediated apoptosis, further suggesting that the cooperation of AIF with Endo G also plays a crucial role in DHE-induced apoptosis.

Many ER-resident proteins display an altered expression pattern in cancers. Activation of the UPR provides a protective function to cells under ER stress (Ranganathan et al., 2008). However, prolonged activation of the UPR by excessive ER stress can activate multiple apoptotic pathways in mammalian cells (Ron and Walter, 2007). Elevation of cytosolic calcium levels or depletion of ER calcium stores represents typical responses of cells to various stimuli. The ER-resident transmembrane proteins IRE1, PERK, and ATF6 constitute the core stress regulator of the UPR, transducing signals from the ER to the cytoplasm and nucleus after ER stress (Shuda et al., 2003; Li et al., 2007; Lin et al., 2007; Moenner et al., 2007). In addition, the balance between Bip and CHOP/GADD153 expression is considered to lead to either cell survival or cell death in ER stress (Ron and Walter, 2007). Our study found that DHE induces a number of ER stress markers, including cytosolic-calcium level elevation, IRE1 and CHOP/GADD153 up-regulation, PERK and eIF-2α phosphorylation, XBP-1 mRNA splicing, and caspase-4 activation. The triggers of ER stress are also characterized by dilated ER cisternae after exposures to DHE. Furthermore, the calcium chelator BAPTA-AM blocked DHE-induced AIF nuclear translocation, which plays a major role in DHE-mediated apoptosis. IRE1 siRNA also declined DHE-mediated apoptosis. Together, these findings indicate that DHE induces apoptotic cell death through ER stress in human liver cancer cells.

Activation of MAPKs has been implicated in the regulation of gene expression in the ER stress signaling cascade and is involved in many aspects of the control of cellular proliferation and apoptosis (Hsu et al., 2005; Dhillon et al., 2007; Kuo et al., 2007). p38 has been shown to be involved in the triggering of ER stress by increasing the expressions of CHOP/GADD153 and Bip, rendering these cells responsive to various stimuli (Devries-Seimon et al., 2005; Yang et al., 2007). Although ERK1/2 activation is generally considered as a survival signaling, ERK1/2 phosphorylation has been reported to play a significant role in the induction of apoptosis in various types of cells during ER stress (Arai et al., 2004; Joo et al., 2007). The JNK pathway has also been shown to be a positive regulator of ER stress-induced apoptosis (Nieto-Miguel et al., 2007). ER stress-activated IRE1 recruits tumor necrosis factor receptor-associated factor 2, which in turn activates the apoptosis signal-regulating kinase 1, which in turn activates the JNK (Nishitoh et al., 2002; Lin et al., 2007; Nieto-Miguel et al., 2007). In this report, we have shown that treatment of HepG2 and PLC/PRF/5 cells with DHE resulted in the accumulation of phosphorylated p38, JNK, and ERK1/2. The calcium chelator BAPTA-AM also partially affects p38 and ERK1/2 activity, suggesting that the activation of p38 and ERK1/2 is through a calcium-dependent and
Fig. 5. The relation of MAPK and ER stress. A, genetic suppression of MAPKs by siRNA transfection. Inhibition of p38 and ERK1/2 decreased the regulation of CHOP/GADD153 and Bip (B) and caspase-4 activation (C). D, blockade of MAPKs reduced DHE-mediated apoptosis. Cells were transfected with control siRNA, p38, JNK, or ERK1/2 siRNA by Lipofectamine 2000 agents, then treated with 30 μM DHE for the indicated times (3 h for CHOP/GADD153 and Bip expression, 12 h for capase-4 activation, and 48 h for apoptosis assay). Protein expression was assessed by immunoblot analysis. The induction of apoptosis was assessed by TUNEL. Results shown are representative of three independent experiments. *, significant difference between the two test groups, as analyzed by Dunnett’s test (p < 0.05).
Fig. 6. The role of ER stress on DHE-mediated apoptosis. A, genetic suppression of IRE1 by siRNA transfection. B, inhibition of IRE1 decreased the activation of JNK. C, inhibition of IRE1 decreased DHE-induced apoptosis. BAPTA-AM pretreatment decreased JNK phosphorylation (D) and apoptosis (E) in DHE-treated cells. Cells were transfected with control siRNA or IRE1 by Lipofectamine 2000 agents and then treated with DHE (30 μM) for the indicated times (1 h for MAPK phosphorylation, 12 h for AIF translocation, and 48 h for apoptosis assay). Protein expression was assessed by immunoblot analysis. The induction of apoptosis was assessed by TUNEL. Results shown are representative of three independent experiments. *, significant difference between the two test groups, as analyzed by Dunnett’s test ($p < 0.05$).
-independent manner. Although the mechanism by which calcium activates p38 remains unclear, it was shown recently that synthetic peroxisome proliferator-activated receptor γ ligands increased p38 activation through the calcium/calmodulin-dependent kinase II pathway (Gardner et al., 2005). In addition, inhibition of p38 and ERK1/2 significantly lowered the CHOP/GADD153 enhancement and caspase-4 activation. These observations support the notion that DHE-mediated ER stress results from the cooperation of the p38 and ERK1/2 pathway. In contrast, inhibition of JNK by siRNA does not affect the ER stress trigger, whereas selectively knockdown IRE1 expression decreased the effects of DHE on the activation of JNK, but not p38 and ERK1/2, suggesting that IRE is an upstream activator of the JNK pathway. These results reveal that p38 and ERK1/2 activations act as the upstream activator, whereas JNK is a downstream event of UPR signaling in response to DHE.

In conclusion, the present study has demonstrated the
following. 1) DHE causes apoptosis in liver cancer in vitro and in vivo (Fig. 8). 2) DHE-induced cell death is mediated by increasing Ca$^{2+}$ mobilization and ERK1/2 and p38 activation, which subsequently triggers UPR. 3) DHE increases UPR response and, subsequently, JNK activation, resulting in apoptosis. 4) It is important to note that DHE inhibits tumor cell growth in a PLC/PRF/5 xenograft mouse model and is well tolerated by the nude mice. The proposed working models for the molecular basis would provide valuable insights for approaches to the development of effective chemotherapy by targeting appropriate signal transducers.

Acknowledgments

We thank the Division of Research Resource, Department of Medical Research, Kaohsiung Medical University Hospital for providing experimental space and experimental instruments.

References


Address correspondence to: Dr. Po-Lin Kuo, Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan. E-mail: kuopolin@seed.net.tw