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ω-Hydroxyundec-9-enoic acid induces apoptosis through ROS-mediated endoplasmic reticulum stress in non-small cell lung cancer cells

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ABSTRACT

ω-Hydroxyundec-9-enoic acid (ω-HUA), a hydroxyl unsaturated fatty acid derivative, is involved in the antifungal activity of wild rice (*Oryza officinalis*). Here, we investigated the anti-cancer activity of ω-HUA on a non-small cell lung cancer (NSCLC) cell line. ω-HUA increased apoptosis and induced cleavages of caspase-6, caspase-9, and poly (ADP-ribose) polymerase (PARP). ω-HUA treatment significantly induced endoplasmic reticulum (ER) stress response. Suppression of CHOP expression and inhibiting ER stress by 4-phenylbutyrate (4-PBA) significantly attenuated the ω-HUA treatment-induced activation of caspase-6, caspase-9, and PARP, and subsequent apoptotic cell death, indicating a role for ER stress in ω-HUA-induced apoptosis. In addition, cells subjected to ω-HUA exhibited significantly increased quantity of reactive oxygen species (ROS), and the ROS scavenger *N*-acetyl-L-cysteine (NAC) inhibited ω-HUA-induced apoptotic cell death and ER stress signals, indicating a role for ROS in ER stress-mediated apoptosis in ω-HUA-treated cells. Taken together, these results suggest that sequential ROS generation and ER stress activation are critical in ω-HUA treatment-induced apoptosis and that ω-HUA represents a promising candidate for NSCLC treatment.

1. Introduction

The endoplasmic reticulum (ER) in eukaryotic cells is a membranous network that is responsible for protein folding, lipid biogenesis, and calcium homeostasis. Different physiological and pathological conditions that impair these normal cellular functions of the ER can cause a condition known as ER stress. A variety of anti-cancer therapies have been linked to the induction of ER stress in cancer cells, making ER stress the subject of a great deal of attention for anti-cancer therapy. Several pathways have been directly implicated in ER stress-induced apoptosis. For example, C/EBP homologous protein (CHOP) is induced by ER stress at the transcription level and plays a key role in ER stress-mediated apoptosis [1].

Reactive oxygen species (ROS) have an important role in regulating cell signaling cascades. Although ROS are essential for cellular function, they are also potentially toxic. ROS are capable of damaging DNA, RNA, and proteins and degrading essential cellular molecules. In particular, ROS induce oxidative damage of DNA, including strand breaks and base- and nucleotide modifications. ROS-induced oxidative stress has been implicated in the multistage process of carcinogenesis by both genotoxic and non-genotoxic mechanisms contributing to malignant transformation. In contrast to tumor-promoting activity, ROS have been implicated in anti-cancer agent-induced cancer cell apoptosis. Anti-cancer agent-induced cancer cell apoptosis is often associated with damage of mitochondrial function that leads to increased intracellular ROS levels. ROS and ER stress are also closed linked, and ROS can be an essential component of the events leading to protein misfolding in ER and ER stress-induced apoptosis [2].

Fatty acids are carboxylic acids (or organic acids) with a long aliphatic tail, which is either saturated or unsaturated. They come in hundreds of varieties, distinguished primarily by structure, which, in turn, determines their physiological role. Various fatty acids are promising as anti-cancer agents or for biomedical applications. For example, omega-3 unsaturated fatty acids have anti-proliferative effects by inducing autophagy in breast cancer cells [3] and apoptosis through ROS accumulation, caspase-8 activation, and autophagy of human pancreatic cancer cells [4]. Linoleic acid

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induces ROS-mediated ER stress and cell death in human colon cancer cells [5]. Palmitic acid treatment leads to induction of ER stress and apoptosis of pancreatic β-cells, hepatocytes, and cardiomyocytes [6–8]. (R)-9-Hydroxystearic acts as a histone deacetylase inhibitor, exerting antiproliferative effect in colon cancer [9]. However, little is known about the biological function of hydroxyl fatty acids acquired from bioconversion.

ω-Hydroxyundec-9-enoic acid (ω-HUA), a new anti-fungal substance, was originally isolated from the leaves of wild rice (*Oryza officinalis*) and was found to make wild rice resistant to the rice blight fungus (*Pyricularia oryzae*) [10]. We have isolated ω-HUA, a hydroxyl unsaturated fatty acid acquired from bioconversion, through a multi-step biocatalysis in an *Escherichia coli* system [11]. ω-HUA may become an interesting therapeutic candidate as a new anti-cancer agent, because it is much more soluble in the aqueous environment and highly reactive compared to natural long chain fatty acids such as linoleic acid and palmitic acid. Therefore, we investigated its anti-cancer activity and underlying mechanisms using non-small cell lung cancer (NSCLC) cells.

2. Materials and methods

2.1. Preparation of ω-HUA

ω-HUA was obtained at a purity of >90% according to our previous study [11]. A stock solution (400 mM) was prepared by dissolving 50 mg ω-HUA in 100% pure ethanol.

2.2. Cell culture and fatty acid treatment

Human NSCLC H1299, A549, and HCC827 cells (American Type Culture Collection, ATCC; Manassas, VA, USA) were cultured in RPMI supplemented with 10% fetal bovine serum and 150 μg/mL penicillin/streptomycin. The cultures were maintained at 37 °C in 5% CO_2 incubator. Cells were incubated with ω-HUA at different concentrations or for different times. In some cases, cells were pretreated for 2 h with an ER stress inhibitor (500 μM 4-phenylbutyrate [4-PBA], Sigma, St. Louis, MO, USA) or a ROS scavenger (1 mM *N*-acetyl-L-cysteine [NAC], Sigma) before treatment with ω-HUA. All control cells were incubated with only the same volume of vehicle (ethanol).

2.3. Cell cytotoxicity assay

Following ω-HUA treatment, cell viability was measured by a color reaction with WST-8. Mitochondrial dehydrogenase cleavage of WST-8 to formazan dye provided a measure of cell viability. After ω-HUA treatment for 30 h, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) colorimetric water-soluble tetrazolium dye (Dojindo Laboratory, Kumamoto, Japan) was added for a 2 h incubation, and absorbance at 450 nm was measured using an automated microplate reader (BioTek, Winooski, VT, USA).

2.4. Detection of apoptosis by flow assisted cell sorting (FACS) analysis

Apoptotic cells were detected using the Annexin V/propidium iodide (PI) reagent kit (BD Biosciences, San Jose, CA, USA). Cells were treated with the indicated concentrations of ω-HUA for 24 h. Cells were harvested and incubated in Annexin V/PI staining solution, and then apoptotic cells were detected by flow cytometry (BD Biosciences). The total population of apoptotic cells was obtained from Annexin V-positive and PI-negative (early apoptosis) and finally to Annexin V-positive and PI-positive (end stage apoptosis and death). The data analysis was done with Cell Quest software (BD Biosciences).

2.5. Lactate dehydrogenase (LDH) assay

Plasma membrane integrity was assessed by LDH leakage into the culture medium from cells using an LDH cytotoxicity assay kit (Cayman, Ann Arbor, MI, USA). ω-HUA-induced LDH leakage (in percent) was calculated as 'LDH activity in culture medium/total LDH activity' by measuring optical density at 490 nm.

2.6. ROS measurement

The 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) probe is oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by ROS [12]. ROS production was measured using OxiSelect™ ROS assay kit (Cell Biolabs Inc., San Diego, CA, USA) according to the manufacturer's guidelines. ROS were analyzed by measuring the oxidation of the non-fluorescent probe H2DCF-DA to the fluorescent metabolite DCF by mitochondrial ROS. The fluorescence intensity of DCF was measured at an excitation of 480 nm and emission of 530 nm, and the concentration of total ROS liberated by ω-HUA was calculated from a hydrogen peroxide standard curve. Quantification of fluorescence was performed using both a fluorescence plate reader and fluorescence microscopy. Fluorescence images were captured and analyzed with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) and relative ROS production was quantified using the Axiovert 200 M fluorescence microscope software.

2.7. Examination of morphology changes

Changes in the cellular morphology were photographed using an inverted microscope (IX71, Olympus, Tokyo, Japan) at 200× magnification.

2.8. siRNA transfection

siRNA oligonucleotides were synthesized by Genolution Pharmaceuticals (Seoul, Korea). The cells were transiently transfected with small interfering RNA against CHOP or irrelevant scrambled siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. At 30 h after transfection, cells were challenged with 500 μM ω-HUA. The CHOP siRNA sequence used was GTCCTGTCTTCAGATGAATT.

2.9. Western blotting

Immunoblotting was performed to detect ER stress-associated proteins and apoptotic molecules. After ω-HUA treatment, cells were harvested and lysed with RIPA buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin] on ice for 30 min. The supernatant protein concentration was quantified using the BioRad protein assay reagent (Hercules, CA, USA). Protein samples were resolved by 10–15% SDS–polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane (Bio-Rad). After blocking the membranes in 5% non-fat dry milk in PBS-T (0.1% Tween 20), the membranes were probed with antibodies against cleaved caspase-9, cleaved caspase-6, cleaved poly (ADP-ribose) polymerase (PARP), CHOP, phosphorylated eIF2α (Ser51), and tubulin (Cell Signaling Technology, Beverly, MA, USA). The membranes were washed with PBS-T and incubated with a secondary horseradish peroxidase-conjugated antibody (Cell Signaling Technology) for 90 min. The protein bands were

detected by Pierce ECL-Plus chemiluminescence (Thermo Scientific, Schwerte, Germany).

2.10. Statistical analysis

All data was analyzed by a Student's *t*-test using the EXCEL package (Microsoft, Redmond, WA, USA). Data are expressed as means ± standard error of three separate experiments. A $P < 0.01$ was considered significant.

3. Results

3.1. ω-HUA-induced NSCLC cell toxicity

To test the anti-proliferation effect of ω-HUA on NSCLC cells (H1299, HCC827, and A549), we used the WST-8 tetrazolium salt to monitor the rate of survival of NSCLC cells exposed to ω-HUA for 30 h. Incubating the cells with ω-HUA led to decreased cell viability in a dose-dependent manner (Fig. 1A). The measurement of cell viability was based on the reduction of WST-8 to water-soluble formazan. The loss of cell viability was linked with cytoskeletal changes in damaged cells (data not shown). Because H1299 cells were most sensitive to ω-HUA-induced toxicity compared with the HCC827 and A549 NSCLC cells (Fig. 1A), we used this cell line for further studies.

3.2. ω-HUA-stimulated apoptosis, caspase activation and PARP cleavage in NSCLC cells

To determine whether ω-HUA induces apoptosis in NSCLC cells, we examined cell membrane integrity and phosphatidylserine externalization of the cells using Annexin V/PI staining. After treatment with vehicle and 500 μM ω-HUA for 24 h, the populations of Annexin V-positive cells were 8.57% (4.91 + 3.66%) and 78.36% (12.27 + 66.09%), respectively (Fig. 1B). These results indicate that the fatty acid derivative ω-HUA induced apoptosis in H1299 cells. Similar results were obtained with the A549 and HCC827 NSCLC cell lines (data not shown).

As a next step, the expression of apoptosis-related protein was assessed after ω-HUA treatment for various times. Western blotting showed that ω-HUA significantly triggered cleavage of caspase-9, caspase-6, and PARP in H1229 cells (Fig. 1C), confirming the onset of apoptosis.

3.3. ω-HUA-induced LDH release in NSCLC cells

We also investigated whether ω-HUA induced LDH release by measuring LDH activity released from the cytosol of damaged cells into the culture medium. As shown in Fig. 1D, LDH released into the supernatant was significantly elevated after ω-HUA treatment in all NSCLC cells in a dose-dependent manner. These results show

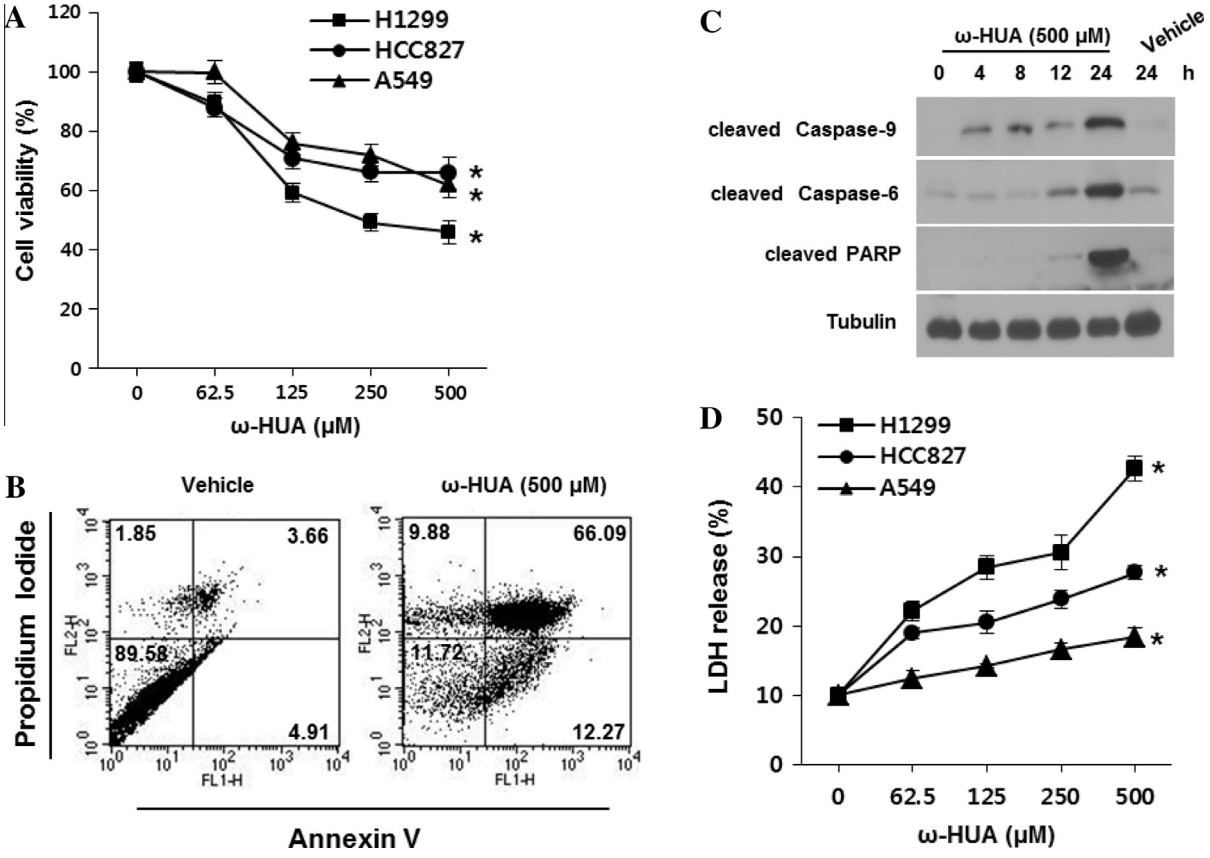

Fig. 1. Induction of apoptotic cell death and activation of the caspase cascade by ω-HUA in NSCLC cells. (A) The NSCLC H1299, HCC827 and A549 cell lines were challenged with the indicated concentrations of ω-HUA for 30 h. Cell viability was measured using a cell counting kit containing WST-8 (■ H1299, ● HCC827, ▲ A549). Results represent means ± standard error of three experiments (*$P < 0.01$ vs. vehicle control; Student's *t*-test). (B) H1299 cells were challenged with 500 μM ω-HUA for 24 h. Apoptotic cells were detected by Annexin V/PI staining. This figure is representative of three independent experiments. (C) H1299 cells were challenged with 500 μM ω-HUA or vehicle control for the indicated time periods. The levels of cleavage of caspase-9, caspase-6, and PARP were estimated by immunoblot analysis using specific antibodies. The blots shown are representative of three separate experiments. (D) The NSCLC H1299, HCC827, and A549 cell lines were challenged with the indicated concentrations of ω-HUA for 28 h. LDH release was determined by measuring the conversion of a tetrazolium salt to red-colored formazan. (■ H1299, ● HCC827, ▲ A549). Results represent means ± standard error of three experiments (*$P < 0.01$ vs. vehicle control; Student's *t*-test).

that ω-HUA-treated cells also underwent late apoptosis or postapoptotic necrosis as well as typical apoptosis.

3.4. ω-HUA-caused activation of ER stress signals

Some fatty acids such as polyunsaturated linoleic acid and saturated palmitic acid are known to induce ER stress [13]. To investigate whether ω-HUA treatment causes ER stress, ER-specific signals were measured. Western blot analysis revealed that the levels of phosphorylated eIF2α (Ser51) and CHOP increased in response to ω-HUA treatment compared to that of vehicle treatment (Fig. 2A), indicating that ER stress occurs in ω-HUA-treated H1299 cells. Similar results were obtained with the A549 and HCC827 NSCLC cell lines (data not shown).

3.5. Involvement of ER stress in ω-HUA treatment-induced apoptosis

CHOP, a C/EBP family transcription factor activated during ER stress, is a major component of the ER stress-mediated apoptosis pathway [1]. We evaluated the role of CHOP in ω-HUA-induced cell death and caspase activation to investigate whether ER stress is involved in ω-HUA-induced apoptosis. ω-HUA treatment significantly reduced cell viability, as expected (Fig. 2B). However, down-regulation of CHOP expression by CHOP siRNA significantly attenuated ω-HUA-induced cell death in H1299 cells (Fig. 2B). Moreover, CHOP siRNA, but not scrambled siRNA, significantly inhibited ω-HUA-induced caspase-9/-6 cascade and PARP cleavage as well as the induction of CHOP (Fig. 2C). We also found that 4-PBA, an ER stress inhibitor, efficiently attenuated ω-HUA-induced cell

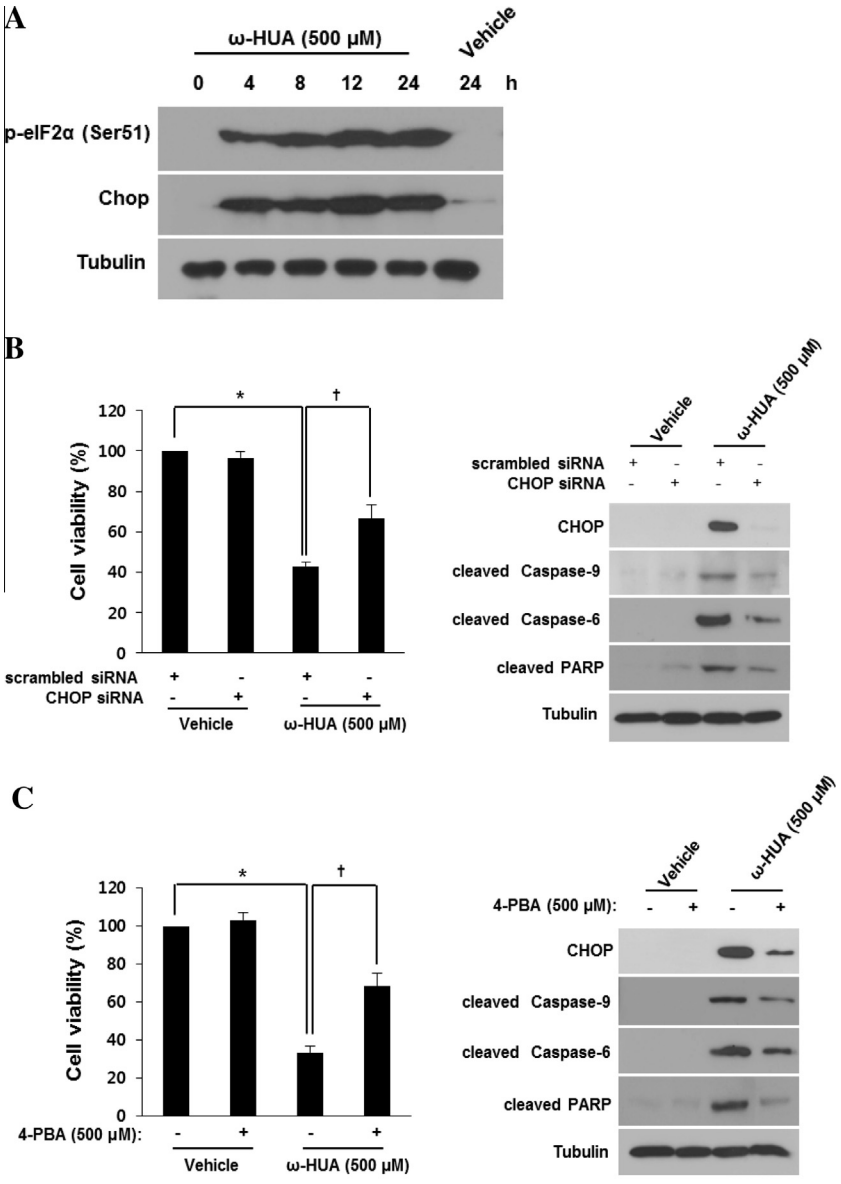

Fig. 2. Involvement of the ER stress pathway in ω-HUA treatment-induced apoptotic cell death and caspase-mediated PARP cleavage in H1299 cells. (A) Cells were challenged with 500 μM ω-HUA or vehicle control for the indicated time periods. The levels of phosphorylated eIF2α (Ser51) and CHOP were analyzed by Western blotting. Blots shown are representative of three independent experiments. (B) Cells were transfected with 100 nM CHOP-targeting siRNA or random siRNA control for 48 h and then treated with 500 μM ω-HUA for 30 h (for cell viability assay) or for 24 h (for protein assay). (*Left panel*) Cell viability was measured using WST-8. Data shown represent means ± standard error of three independent experiments (*$P < 0.01$ vs. vehicle control, †$P < 0.01$ vs. scrambled siRNA; Student's *t*-test). (*Right panel*) Cleavage of caspase-9, caspase-6, and PARP as well as CHOP expression were estimated by immunoblot analysis using specific antibodies. Blots shown are representative of three independent experiments. (C) Cells were pre-incubated with 500 μM 4-PBA for 2 h and then treated with 500 μM ω-HUA for 30 h (for cell viability assay) or for 24 h (for protein assay). (*Left panel*) Cell viability was measured using WST-8. Data shown represent means ± standard errors of three independent experiments (*$P < 0.01$ vs. vehicle control, †$P < 0.01$ vs. ω-HUA alone; Student's *t*-test). (*Right panel*) Cleavage of caspase-9, caspase-6, and PARP as well as CHOP expression were estimated by immunoblot analysis using specific antibodies. Blots shown are representative of three independent experiments.

death, caspase activation, and PARP cleavage as well as enhanced CHOP expression (Fig. 2D and E). ω-HUA-induced apoptosis, detected by Annexin V/PI staining, was also suppressed by CHOP siRNA and 4-PBA (data not shown). These results indicate that ER stress is involved in ω-HUA treatment-induced apoptotic cell death.

3.6. ω-HUA-induced intracellular ROS generation

Some fatty acids trigger the generation of ROS [4,5,12,14]. To investigate whether ω-HUA treatment generates ROS, we examined intracellular ROS production using a ROS-sensitive fluorescent probe, H2DCF-DA. DCF fluorescence intensity under ω-HUA treatment measured with fluorescence plate reader (Fig. 3A) and fluorescence microscopy (Fig. 3B) increased in a dose-dependent manner compared to that in the vehicle control H1299 cells. In addition, pretreatment for 2 h with the antioxidant NAC significantly reduced the levels of intracellular ROS, compared to cells treated with ω-HUA alone (Fig. 3C). Similar results were also obtained in HCC827 and A549 cells (data not shown).

3.7. Effects of ROS generation on ω-HUA-induced apoptotic cell death

H1299 cells were treated with ω-HUA in presence or absence of NAC to investigate whether ω-HUA-induced ROS is required to induce apoptotic cell death. A phase-contrast microscope was used to visualize live samples. The vehicle control H1299 cells appeared healthy and normal. After exposure to ω-HUA, the cells exhibited damaged morphology, which was attenuated efficiently by NAC pretreatment (Fig. 4A, upper panel).

To further evaluate the involvement of ROS in ω-HUA-induced apoptotic cell death, we pretreated with NAC and examined its effect on ω-HUA-induced apoptotic cell death by detecting WST-8 reduction, phosphatidylserine externalization on the plasma membrane, and caspase-mediated PARP cleavage. We observed that NAC significantly suppressed ω-HUA-induced apoptotic cell death and caspase-mediated PARP cleavage in H1299 cells (Fig. 4A, lower panel and 4B and C). Similar results were also obtained in HCC827 and A549 cells (data not shown). These results demonstrate that ROS generation is crucial for ω-HUA-triggered caspase-mediated apoptotic cell death.

3.8. Effects of ROS generation on ω-HUA-induced ER stress

Oxidative stress can be closely associated with ER stress and induce dysfunction of the ER [2,5,15]. We further investigated the role of ROS production in ER stress in ω-HUA-treated H1299 cells. ω-HUA upregulated the ER stress markers CHOP and phosphorylated eIF2α (Ser51), and these effects were inhibited efficiently by pretreatment with NAC (Fig. 4D). These results suggest that ROS production is the cause of ω-HUA-mediated ER stress in NSCLC cells.

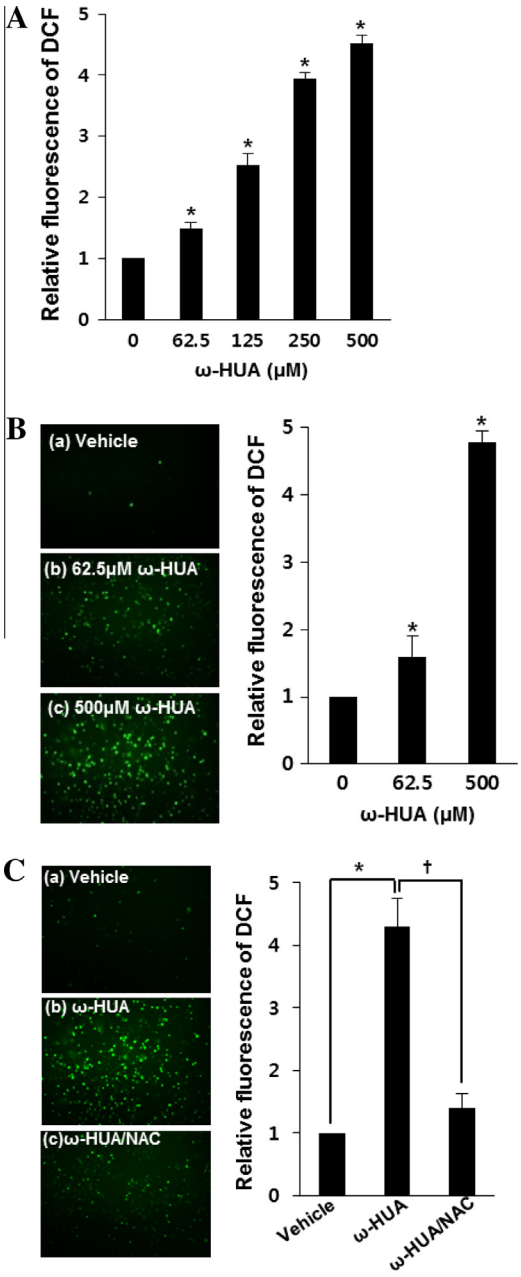

Fig. 3. Effects of ω-HUA treatment on ROS generation in absence or presence of the antioxidant NAC. H1299 cells were challenged with the indicated concentrations of ω-HUA for 90 min in absence or presence of a pretreatment (2 h) with 1 mM NAC. Cells were loaded with H2DCF-DA for 45 min at 37 °C and measured using a fluorescence plate reader (A) and fluorescence microscopy (B and C). Data shown represent means ± standard errors of three independent experiments ($^{*}P < 0.01$ vs. vehicle control, $^{\dagger}P < 0.01$ vs. ω-HUA alone; Student's *t*-test).

4. Discussion

Fatty acids not only can be linked to the risk of cancer but can also aid cancer prevention and treatment. Some fatty acids attenuate growth and induce apoptosis in a variety of human cancer cell lines [3–5,8,9,13,14,16–18]. Fatty acid-induced apoptosis may be correlated with increased activity of caspases [4,7,16,17]. All fatty acids are chains of carbon atoms with hydrogen atoms attached to the carbon atoms. Saturated fatty acids have all single bonds between the carbon atoms, whereas unsaturated fatty acids have one or more double bonds between carbon atoms. Compared to saturated fatty acids, a number of unsaturated fatty acids exhibit anticancer activity. In addition, contrary to other fatty acids, the biological functions (such as anti-cancer activity) of hydroxyl fatty acids acquired from bioconversion are not well known. Our group produced a hydroxyl mono unsaturated fatty acid derivative called ω-HUA through a multi-step biocatalysis in an *E. coli* system [11]. Here, we elucidated the possible role of ω-HUA in cancer cell apoptosis using human cell lines of NSCLC, which is the most common type of lung cancer. The results obtained by cell viability assay, Annexin V/PI staining, Western blotting for cleavage of caspase-9, caspase-6 and PARP demonstrated that ω-HUA induced caspase-mediated apoptotic cell death.

The ER is a major site of protein folding in the cell and is very sensitive to stress that inhibits protein folding. ER stress, which

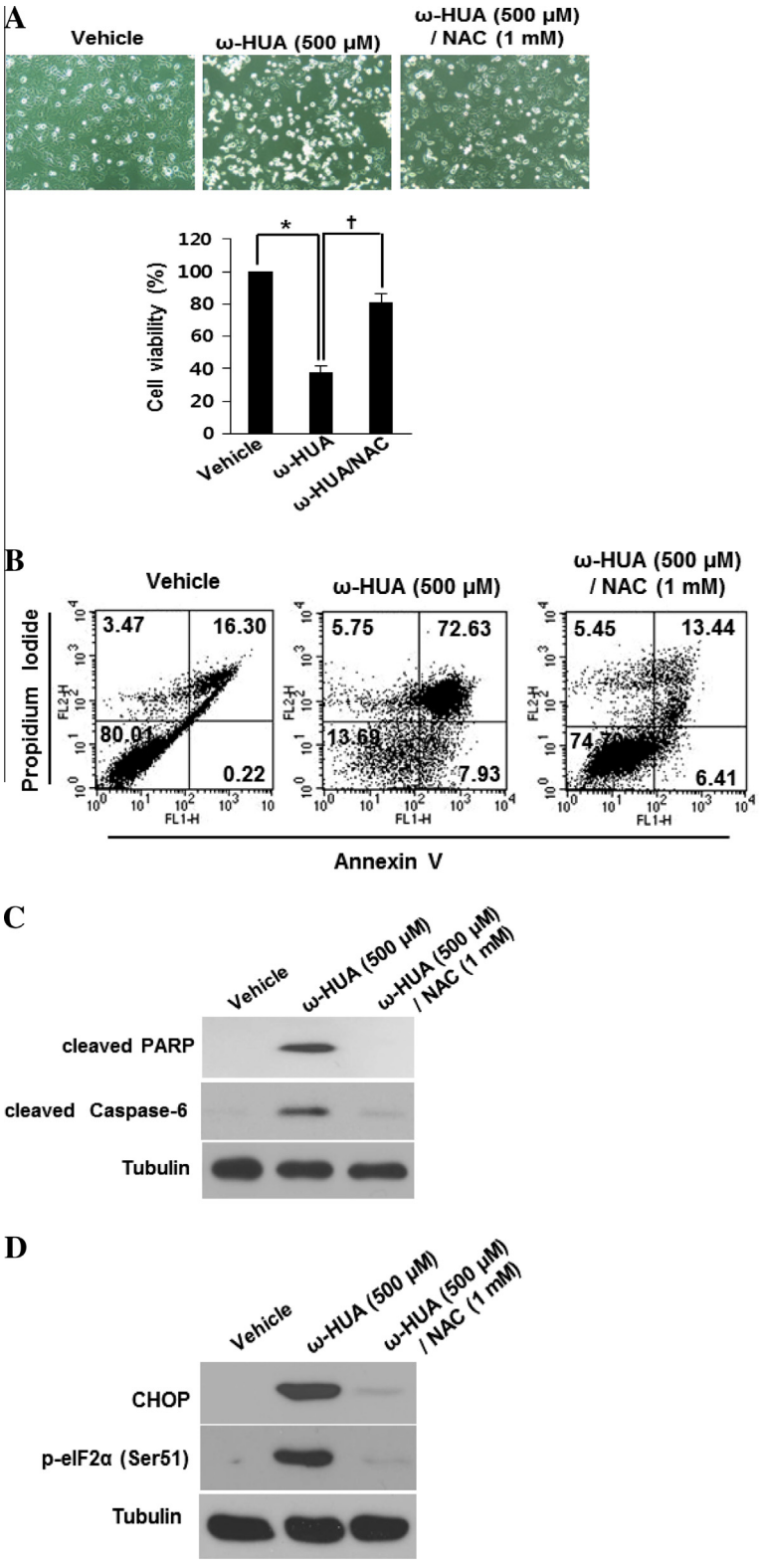

Fig. 4. Effect of a ROS scavenger on ω-HUA-induced caspase-mediated apoptotic cell death and ER stress. H1299 cells were challenged with 500 μM ω-HUA for 30 h (for morphological change observation and cytotoxicity assay) or 24 h (for apoptosis assay and protein assay) in absence or presence of pretreatment (2 h) with 1 mM NAC. (A) (*Upper panel*) Morphological change was visualized by phase-contrast microscopy. This figure is representative of three independent experiments. (*Lower panel*) Cell viability was detected using a cell counting kit containing WST-8. Data shown represent means ± standard errors of three independent experiments (*$P < 0.01$ vs. vehicle control, † $P < 0.01$ vs. ω-HUA alone; Student's *t*-test). (B) Apoptotic cells were detected by Annexin V/PI staining. This figure is representative of three independent experiments. (C and D) Cleavage of caspase-6 and PARP (C) as well as expression of CHOP and phosphorylated eIF2α (Ser51) (D) were estimated by immunoblot analysis using specific antibodies. Blots shown are representative of three independent experiments.

includes conditions interfering with the ER function, activates the unfolded protein response signals to alleviate this stress and restore ER homeostasis, promoting cell survival and adaptation. However, the failure to resolve ER stress drives cells to undergo apoptosis. Targeting the ER stress response can be a useful anti-cancer strategy. The induction of ER stress by unsaturated fatty acid in cancer cells has also been documented [5,13,18]. The results of our study indicated that ω-HUA triggered ER stress-inducible cellular signaling, e.g., phosphorylation of eIF2α (Ser51) and expression of CHOP. We also examined the contribution of ER stress to ω-HUA treatment-induced apoptosis by introducing CHOP siRNA and the ER stress inhibitor 4-PBA to cells. Cell viability and Western blotting to analyze key apoptosis markers indicated that CHOP siRNA and 4-PBA, but not control siRNA or the vehicle control, reduced ω-HUA treatment-induced cell death and caspase activation. Both CHOP siRNA and 4-PBA also significantly attenuated ω-HUA treatment-induced apoptosis detected by Annexin V/PI staining (data not shown). These findings suggest that ω-HUA treatment-induced apoptosis and caspase activation in human NSCLC cells involves the ER stress pathway.

ROS not only play a role in redox regulation during normal physiological functions but are highly reactive molecules that have the potential to cause cellular damage. Both saturated and unsaturated fatty acids may trigger generation of intracellular ROS [4,5,12,14]. Other studies have emphasized the role of ROS in caspase activation and ER stress during apoptosis [2,4,5,15]. In this study, we showed that ROS are generated during ω-HUA-induced apoptosis in human NSCLC cells, and that the oxidant scavenger NAC suppressed ω-HUA-induced ROS generation, cytotoxicity, and caspase activation. Next, we determined whether stimulating ER stress depends on the production of ROS in ω-HUA-treated cells. Quenching ROS with NAC prevented the expression of phosphorylated eIF2α and CHOP, indicating that ROS are required for ω-HUA-induced ER stress as well as caspase-mediated apoptosis in human NSCLC cells.

Overall, our results suggest that ω-HUA induces apoptotic cell death via activation of the caspase cascade accompanying increased ER stress and that ER stress is involved in ω-HUA-induced apoptosis and caspase activation. Moreover, our data provide a critical role for ROS in the activation of ER stress, caspase-mediated PARP cleavage, and subsequent apoptotic cell death. Taken together, these results suggest that ω-HUA, an unsaturated fatty acid derivative, may be a promising candidate for cancer treatment of NSCLC cells. Additional studies are underway to measure the effects of ω-HUA in other types of cancer.

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References

[1] R. Sano, J.C. Reed, ER stress-induced cell death mechanisms, Biochim. Biophys. Acta 2013 (1833) 3460–3470.
[2] J.D. Malhotra, R.J. Kaufman, Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?, Antioxid Redox Signal. 9 (2007) 2277–2293.
[3] D. Rovito, C. Giordano, D. Vizza, P. Plastina, I. Barone, I. Casaburi, M. Lanzino, F. De Amicis, D. Sisci, L. Mauro, S. Aquila, S. Catalano, D. Bonofiglio, S. Andò, Omega-3 PUFA ethanolamides DHEA and EPEA induce autophagy through PPARγ activation in MCF-7 breast cancer cells, J. Cell. Physiol. 228 (2013) 1314–1322.
[4] M. Fukui, K.S. Kang, K. Okada, B.T. Zhu, EPA, an omega-3 fatty acid, induces apoptosis in human pancreatic cancer cells: role of ROS accumulation, caspase-8 activation, and autophagy induction, J. Cell. Biochem. 114 (2013) 192–203.
[5] A.S. Pierre, M. Minville-Walz, C. Fèvre, A. Hichami, J. Gresti, L. Pichon, S. Bellenger, J. Bellenger, F. Ghiringhelli, M. Narce, M. Rialland, Trans-10, cis-12 conjugated linoleic acid induced cell death in human colon cancer cells through reactive oxygen species-mediated ER stress, Biochim. Biophys. Acta 2013 (1831) 759–768.
[6] K. Eitel, H. Staiger, M.D. Brendel, D. Brandhorst, R.G. Bretzel, H.U. Häring, M. Kellerer, Different role of saturated and unsaturated fatty acids in beta-cell apoptosis, Biochem. Biophys. Res. Commun. 299 (2002) 853–856.
[7] Y. Wei, D. Wang, F. Topczewski, M.J. Pagliassotti, Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells, Am. J. Physiol. Endocrinol. Metab. 291 (2006) E275–E281.
[8] J. Cao, D.L. Dai, L. Yao, H.H. Yu, B. Ning, Q. Zhang, J. Chen, W.H. Cheng, W. Shen, Z.X. Yang, Saturated fatty acid induction of endoplasmic reticulum stress and apoptosis in human liver cells via the PERK/ATF4/CHOP signaling pathway, Mol. Cell. Biochem. 364 (2012) 115–129.
[9] C. Parolin, N. Calonghi, E. Presta, C. Boga, P. Caruana, M. Naldi, V. Andrisano, L. Masotti, G. Sartor, Mechanism and stereoselectivity of HDAC I inhibition by (R)-9-hydroxystearic acid in colon cancer, Biochim. Biophys. Acta 2012 (1821) 1334–1340.
[10] Y. Suzuki, Structure of a new antitungal C11-hydroxy fatty acid isolated from leaves of wild rice (*Oryza officinalis*), Biosci. Biotechnol. Biochem. 59 (1995) 2049–2051.
[11] J.-W. Song, E.-Y. Jeon, D.-H. Song, H.-Y. Jang, U.T. Bornscheuer, D.K. Oh, J.-B. Park, Multistep enzymatic synthesis of long-chain α,ω-dicarboxylic and ω-hydroxycarboxylic acids from renewable fatty acids and plant oils, Angew. Chem. Int. Ed. Engl. 52 (2013) 2534–2537.
[12] M.F. Cury-Boaventura, R. Curi, Regulation of reactive oxygen species (ROS) production by C18 fatty acids in Jurkat and Raji cells, Clin. Sci. 108 (2005) 245–253.
[13] Y. Zhang, R. Xue, Z. Zhang, X. Yang, H. Shi, Palmitic and linoleic acids induce ER stress and apoptosis in hepatoma cells, Lipids Health Dis. 11 (2012) 1–8.
[14] E.J. Park, A.Y. Lee, S. Park, J.H. Kim, M.H. Cho, Multiple pathways are involved in palmitic acid-induced toxicity, Food Chem. Toxicol. 67 (2014) 26–34.
[15] S. Hanada, M. Harada, H. Kumemura, M.B. Omary, H. Koga, T. Kawaguchi, E. Taniguchi, T. Yoshida, T. Hisamoto, C. Yanagimoto, M. Maeyama, T. Ueno, M. Sata, Oxidative stress induces the endoplasmic reticulum stress and facilitates inclusion formation in cultured cells, J. Hepatol. 47 (2007) 93–102.
[16] H.Y. Xu, Z.W. Chen, H. Li, L. Zhou, F. Liu, Y.Y. Lv, J.C. Liu, 12-Deoxyphorbol 13-palmitate mediated cell growth inhibition, G2-M cell cycle arrest and apoptosis in BGC823 cells, Eur. J. Pharmacol. 700 (2013) 13–22.
[17] J. Gasmi, J.T. Sanderson, Jacaric acid and its octadecatrienoic acid geoisomers induce apoptosis selectively in cancerous human prostate cells: a mechanistic and 3-D structure–activity study, Phytomedicine 20 (2013) 734–742.
[18] C.H. Jakobsen, G.L. Størvold, H. Bremseth, T. Follestad, K. Sand, M. Mack, K.S. Olsen, A.G. Lundemo, J.G. Iversen, H.E. Krokan, S.A. Schønberg, DHA induces ER stress and growth arrest in human colon cancer cells: associations with cholesterol and calcium homeostasis, J. Lipid Res. 49 (2008) 2089–2100.