

# Dihydroartemisinin induces endoplasmic reticulum stress-mediated apoptosis in HepG2 human hepatoma cells

Xiaoling Gao<sup>1</sup>, Ziguo Luo<sup>1</sup>, Tingxiu Xiang<sup>2</sup>, Kejian Wang<sup>3</sup>, Jian Li<sup>1</sup>, and Pilong Wang<sup>4</sup>

<sup>1</sup>Institute of Life Science, Chongqing Medical University, Chongqing; <sup>2</sup>Molecular Oncology and Epigenetics Laboratory, First Affiliated Hospital of Chongqing Medical University, Chongqing; <sup>3</sup>Department of Anatomy, Chongqing Medical University, Chongqing; <sup>4</sup>Department of Gastroenterology, First Affiliated Hospital of Chongqing Medical University, Chongqing, PR China

## ABSTRACT

**Aims and background.** Previous studies showed that dihydroartemisinin (DHA) possessed antitumor activity in many human tumor cells through the induction of apoptosis. The aim of this study was to investigate the effects of DHA on apoptosis in the human hepatocellular carcinoma cell line HepG2 and the possible molecular mechanisms involved.

**Methods.** The inhibitory effect of DHA on HepG2 cells was measured by MTT assay. The percentage of apoptotic cells was detected by flow cytometry with double staining of fluorescein isothiocyanate-annexin V/propidium iodide. The intracellular production of reactive oxygen species (ROS) and intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) were detected by fluorescence spectrophotometry. Protein expression of GADD153, Bcl-2 and Bax in HepG2 cells was examined by Western blot and immunocytochemistry.

**Results.** DHA significantly inhibited proliferation of HepG2 cells in a dose- and time-dependent manner. The apoptosis rates in HepG2 cells treated with 0, 50, 100 and 200 mol/L DHA for 24 hours were 2.53 ± 0.88%, 24.85 ± 3.63%, 35.27 ± 5.92% and 48.53 ± 7.76%, respectively. Compared with the control group, DHA significantly increased ROS generation and [Ca<sup>2+</sup>]<sub>i</sub> level (*P* < 0.05), with the generation of ROS preceding the increase in [Ca<sup>2+</sup>]<sub>i</sub>. An increase in GADD153 and Bax expression and a decrease in Bcl-2 were observed in DHA-treated cells. Pretreatment with the antioxidant N-acetylcysteine could attenuate the effects of DHA in the experiments.

**Conclusion.** DHA could inhibit proliferation and induce apoptosis in HepG2 cell lines through increasing the intracellular production of ROS and [Ca<sup>2+</sup>]<sub>i</sub>. Endoplasmic reticulum stress-induced apoptosis may contribute to this effect by regulating the expression of GADD153, proapoptotic Bax, and antiapoptotic Bcl-2.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer-related mortality after lung and stomach cancer<sup>1</sup>. It can be treated with curative therapies including liver resection and liver transplant when detected early. However, most patients are diagnosed at an advanced stage, which is no longer amenable to curative therapies, and have to be treated with palliative treatments such as systemic chemotherapy, chemoembolization or hormonal therapy, all of which have severe side effects<sup>2</sup>. To date no proven or standard systemic therapy for advanced HCC has been defined. Like in other cancers, dysregulation of the balance between proliferation and cell death is a protumorigenic principle in human hepatocarcinogenesis<sup>3</sup>. Promoting apoptosis has become an important strategy in developing cancer chemopreventive drugs<sup>4</sup>.

**Key words:** dihydroartemisinin, apoptosis, endoplasmic reticulum stress, reactive oxygen species, calcium, GADD153, Bcl-2, Bax.

## Abbreviations

A: absorbance  
ATF6: activation of transcription factor 6  
[Ca<sup>2+</sup>]<sub>i</sub>: intracellular Ca<sup>2+</sup> concentration, DCFH: 2',7'-dichlorofluorescein diacetate  
DHA: dihydroartemisinin  
DMSO: dimethyl sulfoxide  
ER: endoplasmic reticulum  
FACS: fluorescence-activated cell sorter  
FCM: flow cytometry  
FITC: fluorescein isothiocyanate  
GADD153: growth-arrest-and-DNA-damage-inducible gene 153  
HCC: hepatocellular carcinoma  
IR: cell proliferation inhibition rate  
IRE1: inositol-requiring transmembrane kinase and endonuclease 1  
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
NAC: N-acetylcysteine  
PERK: protein kinase-like ER kinase  
PI: propidium iodide  
ROS: reactive oxygen species  
SERCA: sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase  
UPR: unfolded protein response

*Correspondence to:* Ziguo Luo, Associate Professor, Institute of Life Science, Chongqing Medical University, Chongqing 400016, China.  
Tel +86-023-68485838;  
fax +86-023-68741033;  
e-mail luoliangwen@yahoo.com.cn

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It was widely reported that bioactive components obtained from herbal plants have high potential in preventing and controlling carcinogenesis<sup>5,6</sup>. Dihydroartemisinin (DHA) is a derivative of artemisinin, which is isolated from the leaves of the herb *Artemisia annua* L. It is a sesquiterpene lactone containing an unusual endoperoxide trioxane moiety and has been used widely and safely to treat malaria thanks to its ability to generate organic free radicals through cleavage of the endoperoxide bridge<sup>7,8</sup> and its extremely potent inhibition of pfATP6, the plasmodium orthologue of mammalian sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPases (SERCA)<sup>9</sup>. In addition to its impressive parasitocidal properties, DHA has been reported to exert antitumor activity in many human tumor cells *in vitro* and *in vivo* through a mechanism which is thought to be the induction of the mitochondrial apoptotic pathway<sup>10-14</sup>. However, little attention has been paid to the other pathways of apoptosis. DHA has a similar structure to thapsigargin, which is also a sesquiterpene lactone and acts as an endoplasmic reticulum (ER) stress inducer via specific SERCA inhibition<sup>15-17</sup>. This similarity sparked our interest in further investigating the molecular mechanisms to determine whether the ER-stress-mediated pathway participates in apoptosis induced by DHA in human hepatoma HepG2 cells.

All eukaryotes respond to ER stress through a set of pathways known as the unfolded protein response (UPR), which elicits paradoxical outputs, inducing cytoprotective and destructive functions that promote apoptosis<sup>18,19</sup>. However, excessive or prolonged UPR results in apoptotic cell death<sup>17,20</sup>. The transcription factor growth-arrest-and-DNA-damage-inducible gene 153/CCAAT/enhancer-binding protein homologous protein (GADD153/CHOP), which is regarded as a marker of ER stress<sup>21</sup>, has been suggested to play an important role in ER-stress-induced apoptosis<sup>20</sup>.

The purpose of this study was to elucidate the molecular mechanism of the DHA-induced apoptotic pathway in HepG2 cells. We investigated the effect of DHA on the growth of human HepG2 cells and the underlying intracellular signal transduction pathways involved in regulating apoptosis. We found that DHA-induced apoptosis in HepG2 cells is accompanied by elevation of intracellular reactive oxygen species (ROS) and calcium concentration, and modulation of GADD153, Bax and Bcl-2 expression. DHA may induce apoptosis via the ER-stress-mediated apoptotic pathway.

## Materials and methods

### Materials

DHA was obtained from Holley Pharmaceuticals Co., Ltd. (Chongqing, China). Stock solution of 0.2 mol/L DHA was prepared in dimethyl sulfoxide (DMSO) and

diluted with complete RPMI 1640 medium before experiments. The final concentration of DMSO was less than 0.01%. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). The annexin V-FITC apoptosis detection kit was from KeyGen Biotech. Co., Ltd (Nanjing, China). N-acetylcysteine (NAC), 2',7'-dichlorofluorescein diacetate (DCFH), Fluo-3/AM, cell lysis buffer for Western blot and IP, the DAB horseradish peroxidase color development kit and mouse monoclonal anti- $\beta$ -actin antibody were obtained from Beyotime Company (Jiangsu, China). Rabbit polyclonal anti-Bcl-2, anti-Bax and anti-GADD153 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### Cells and cell culture

HepG2 human hepatoma cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated neonatal bovine serum, 100 U/mL penicillin G, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air.

### MTT assay

Cell viability was measured by the MTT assay as previously described<sup>22</sup> with some modifications. HepG2 cells were seeded in 96-well plates at a density of 5000 cells/well. After overnight incubation, cells were pretreated with or without NAC (5 mmol/L) for 0.5 hour, and subsequently incubated with increasing concentrations (0, 12.5, 25, 50, 100 and 200  $\mu$ mol/L) of DHA for 24 and 48 hours. Non-treated cells were used as control. Each treatment condition was tested in 3 replicate wells. Then 20  $\mu$ L of MTT (5 mg/mL) was added to each well and further incubated for 4 hours at 37 °C. Supernatants were gently aspirated and the precipitated formazan crystals were dissolved by adding 100  $\mu$ L/well of DMSO. The absorbance (A) was read at 540 nm using a microplate reader (Tecan, Grödig, Austria) after agitating the plates for 5 minutes. Each condition was tested in triplicate. The cell viability was calculated based on the following formula<sup>23</sup>: relative cell proliferation inhibition rate (IR) = (1 - average A<sub>540</sub> of the experimental group/average A<sub>540</sub> of the control group)  $\times$  100%.

### Annexin V-FITC and propidium iodide (PI) double staining

An annexin V-FITC apoptosis detection kit (Keygen, China) was used according to the manufacturer's protocol. HepG2 cells were seeded in 6-well plates at a density of approximately  $2 \times 10^5$  cells/well. After overnight incubation, cells were pretreated with or without NAC (5 mmol/L) for 0.5 hour, followed by cotreatment with

DHA at final concentrations of 0, 50, 100 and 200  $\mu\text{mol/L}$ , respectively, for 24 hours, then harvested, rinsed twice with PBS, collected by centrifugation at 2000 r/min for 5 minutes, and resuspended at a density of  $1 \times 10^6$  cells/mL in 500  $\mu\text{L}$  binding buffer and stained simultaneously with 5  $\mu\text{L}$  FITC-labeled annexin V and 5  $\mu\text{L}$  PI for 15 minutes at room temperature in darkness. Samples were scanned with a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson, USA), and the data were analyzed with CellQuest software (Becton Dickinson). Plots in the annexin V-positive/PI-negative quadrant were counted as apoptotic cells<sup>24</sup>. Each experiment was performed in triplicate.

#### *Measurement of intracellular ROS*

The fluorescence probe DCFH-DA (Beyotime) was used for assessment of ROS production in HepG2 cells<sup>25</sup>. Briefly, cells were seeded ( $1 \times 10^5$  cells/mL) in 25-cm<sup>2</sup> culture flasks overnight. After treatment as described in the flow cytometry (FCM) assay for 6 and 24 hours, cells were harvested, washed twice with PBS, resuspended in serum-free RPMI 1640 medium containing 10  $\mu\text{mol/L}$  of DCFH-DA at a density of  $2 \times 10^6$  cells/mL, and incubated for 20 minutes at 37 °C in darkness. After rinsing with serum-free RPMI 1640 medium thrice, the dichlorofluorescein (DCF) fluorescence of the cell suspensions was read at 488 nm for excitation and 525 nm for emission with a fluorescence spectrophotometer (Shimadzu RF-540, Japan).

#### *Measurement of the intracellular Ca<sup>2+</sup> concentration*

The intracellular Ca<sup>2+</sup> concentration,  $[\text{Ca}^{2+}]_i$ , was measured by the fluorescent probe Fluo-3/AM according to the method of Namba<sup>24</sup> and Mima<sup>26</sup>, with some modifications. After DHA was treated with or without NAC as described in the part on measurement of ROS, cells were harvested and washed twice with PBS, then suspended in serum-free RPMI 1640 medium and loaded with 5  $\mu\text{mol/L}$  Fluo-3/AM (Beyotime) for 45 minutes at 37 °C in darkness, with occasional gentle agitation of the cell suspension. Washed again, each sample was resuspended in serum-free medium, transferred to a quartz cuvette ( $5 \times 10^6$  cells/mL) and measured with a fluorescence spectrophotometer (Shimadzu RF-540, Japan) at 488 nm excitation and 525 nm emission. The intracellular Ca<sup>2+</sup> concentration was calculated according to the formula,  $[\text{Ca}^{2+}]_i = \text{Kd} (F - F_{\text{min}}) / (F_{\text{max}} - F)$ , where Kd is the apparent dissociation constant (400 nmol/L) of the fluorescent dye-Ca<sup>2+</sup> complex. The maximal Fluo-3 fluorescence intensity (F<sub>max</sub>) was obtained by adding 0.1% Triton X-100 and 1 mmol/L CaCl<sub>2</sub>, and the minimal fluorescence (F<sub>min</sub>) was determined by quenching Fluo-3 fluorescence with the addition of 5 mmol/L EGTA. F is the fluorescence measured without the addition of Triton-X-100 or EGTA.

#### *Western blot analysis*

HepG2 cells were seeded in 6-well plates at a density of  $2 \times 10^5$  cells/well, cultured until they became confluent, and then treated as described in the FCM assay for 6 and 24 hours. Cells were lysed using cell lysis buffer containing 1 mol/L phenylmethanesulfonyl fluoride (Beyotime). The lysates were collected by scraping from the plates and then centrifuged at  $12,000 \times g$  at 4 °C for 5 minutes. Western blot was performed as previously described<sup>12</sup> with some modifications. Briefly, proteins (20  $\mu\text{g}$ ) were loaded on a 12% SDS-polyacrylamide gel for electrophoresis, then transferred to a polyvinylidene fluoride membrane. Membranes were blocked with blocking solution (5% skimmed milk in Tris-buffered solution containing 0.1% Tween-20) at 4 °C overnight, followed by incubation with anti-GADD153, anti-Bcl-2 and anti-Bax antibodies (1:500 dilution) at 4 °C overnight.  $\beta$ -actin (1:400 dilution) was used as a loading control. Detection was performed using a DAB horseradish peroxidase color development kit (Beyotime) according to the manufacturer's instructions. For semi-quantification of immunoblots, the integrated optical density (IOD) of bands was semiquantified by the CM-2000B Biomedicine Image Analysis System (Beihang, China). The values of the different protein bands were normalized to those of the loading control.

#### *Immunocytochemistry*

Immunostaining was performed as previously described<sup>27</sup>. HepG2 cells were seeded on axenic glass coverslips in 24-well culture plates at a density of  $2.5 \times 10^4$  cells/well overnight, pretreated with 5 mmol/L NAC for 0.5 hours then cotreated with 100  $\mu\text{mol/L}$  DHA or treated with 100  $\mu\text{mol/L}$  DHA alone. After 24 hours of treatment, cells were fixed in 95% ethyl alcohol for 20 minutes on ice, and then blocked with 0.3% H<sub>2</sub>O<sub>2</sub> and 5% bovine serum albumin. The primary rabbit polyclonal anti-GADD153 antibody (1:100 dilution), anti-Bcl-2 antibody (1:50 dilution) and anti-Bax antibody (1:50 dilution) were applied to cells at 4 °C overnight, followed by incubation with biotinylated secondary antibody (Boster, China) at 37 °C for 0.5 hours, horseradish peroxidase-streptavidin biotin-peroxidase complex (Boster) at 37 °C for another 0.5 hours, and DAB staining. In order to model negative controls, the primary antibodies were substituted with PBS. The reaction products were observed with a microscope (Olympus BX51, Japan). The IOD of the positive brown particles was determined semiquantitatively by examining 5 fields randomly at  $\times 400$  magnification in each sample and analyzed by the CM-2000B Biomedicine Image Analysis System.

#### *Statistical analysis*

All data were presented as means  $\pm$  SD. Statistics were calculated with the SAS 9.12 package. Significance for

comparison of samples was determined by analysis of variance (ANOVA), *F* test and Student-Newman-Keuls (SNK) test. *P* values <0.05 were considered statistically significant throughout the study.

## Results

### Effects of DHA on proliferation of HepG2 cells

DHA was tested for its ability to inhibit HepG2 cell proliferation by MTT assay after 24 or 48 hours' exposure. As shown in Table 1, DHA at a range of concentrations from 12.5 to 200  $\mu\text{mol/L}$  could reproducibly suppress the proliferation of HepG2 cells in a dose- and time-dependent manner ( $P < 0.05$ ). However, the proliferation inhibition of cells treated with different concentrations of DHA combined with 5 mmol/L NAC for 24 and 48 hours was noticeably lower than that of cells exposed to the corresponding concentrations of DHA alone and the difference was statistically significant ( $P < 0.05$ ). NAC or DMSO itself had no effect on cell viability (data not shown). These findings indicated that DHA had a strong toxic effect on HepG2 cells, whereas NAC could attenuate the effect. To further investigate the cytotoxic mechanisms, more experiments were performed.

### Effects of DHA on apoptosis induction in HepG2 cells

Because a decrease in cell proliferation may result from the induction of apoptosis, we investigated whether DHA-induced growth inhibition of HepG2 cells was due to an increase in apoptosis. The percentage of apoptotic cells was determined by FITC-labeled annexin V and PI double-staining FCM. Figure 1 shows the percentages of apoptotic HepG2 cells treated with 50, 100 and 200  $\mu\text{mol/L}$  DHA for 24 hours, which were  $24.85 \pm 3.63\%$ ,  $35.27 \pm 5.92\%$  and  $48.53 \pm 7.76\%$ , respectively, significantly higher than in the control group,  $2.53 \pm 0.88\%$  ( $P < 0.01$ ). However, when cells were cotreated with DHA and NAC, the percentages of DHA-induced apoptotic cells were markedly lower ( $P < 0.05$ ). This agreed with the result of the MTT assay and therefore suggested that NAC, the ROS scavenger, may give

HepG2 cells significant protection from DHA-induced apoptosis, and that ROS may play a role in this process. In the NAC-alone group, only  $2.93 \pm 0.72\%$  of cells stained positive for annexin V, which was statistically nonsignificant when compared to the control group.

### Effects of DHA on ROS production in HepG2 cells

The membrane-permeable dye DCFH-DA enters cells and produces a fluorescent signal after intracellular oxidation. Oxidant stress is a well-known inducer of apoptosis in several cell types<sup>28</sup>. Previous studies indicated that some artemisinins triggered the generation of ROS which were known to elicit apoptosis<sup>14,29</sup>. To test the contribution of ROS to the apoptosis induction of HepG2 by DHA, we measured the ROS levels in HepG2 cells 6 and 24 hours after DHA and DHA+NAC treatment by a fluorescence spectrophotometer. DHA concentrations of 50, 100 and 200  $\mu\text{mol/L}$  were found to have significantly increased ROS generation in a dose- and time-dependent manner ( $P < 0.05$ ), with peak points of  $80.24 \pm 7.51$  for 6 hours and  $86.03 \pm 8.58$  for 24 hours in the highest concentration group, compared with only  $12.07 \pm 4.21$  for 6 hours and  $13.34 \pm 3.92$  for 24 hours in the control group. In the presence of NAC, however, the ROS generation by DHA was dramatically reduced ( $P < 0.05$ ) (Figure 2). This, combined with the result of the MTT and FCM assays, suggested that ROS is probably a key mediator in the induction of HepG2 cell death by DHA and that NAC by itself has no effect on the intracellular level of ROS.

### Effects of DHA on intracellular $\text{Ca}^{2+}$ concentration in HepG2 cells

DHA was reported to increase the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in lung cancer PC-14 cells and  $[\text{Ca}^{2+}]_i$  was known to be an important intracellular signal during the apoptotic pathway<sup>30</sup>. We therefore investigated whether DHA evoked an elevation of  $[\text{Ca}^{2+}]_i$  in HepG2 cells and also examined the relationship between ROS and  $[\text{Ca}^{2+}]_i$ . As demonstrated in Figure 3, treatment of HepG2 cells with 50, 100 and 200  $\mu\text{mol/L}$  DHA caused an increase in  $[\text{Ca}^{2+}]_i$  levels in a dose-dependent manner at 24 hours ( $P < 0.05$ ), with a peak point of  $498.79 \pm 109.86$  nmol/L in the highest concentration group, whereas the

**Table 1 - Effects of DHA and DHA + NAC on proliferation of HepG2 cells by MTT reduction activity (inhibitory rate, %)**

Concentration groups ( $\mu\text{mol/L}$ )	24 hours		48 hours	
	DHA	DHA + NAC <sup>#</sup>	DHA	DHA + NAC <sup>#</sup>
12.5	$11.79 \pm 3.62$	$3.98 \pm 1.18^*$	$14.96 \pm 5.31$	$9.55 \pm 2.28^*$
25	$16.84 \pm 6.18$	$9.20 \pm 2.40^*$	$25.88 \pm 9.38$	$17.05 \pm 6.55^*$
50	$43.35 \pm 10.51$	$21.49 \pm 9.41^*$	$60.12 \pm 11.05$	$36.22 \pm 9.52^*$
100	$65.51 \pm 11.24$	$30.59 \pm 8.71^*$	$72.89 \pm 13.11$	$48.48 \pm 10.23^*$
200	$79.25 \pm 16.83$	$56.12 \pm 10.65^*$	$83.71 \pm 17.64$	$64.17 \pm 13.48^*$

Values represent the means  $\pm$  SD ( $n = 9$ ). \* $P < 0.05$  vs corresponding DHA concentration group.

<sup>#</sup>HepG2 cells were pretreated with 5 mmol/L NAC for 0.5 h, then cotreated with different concentrations of DHA and 5 mmol/L NAC.

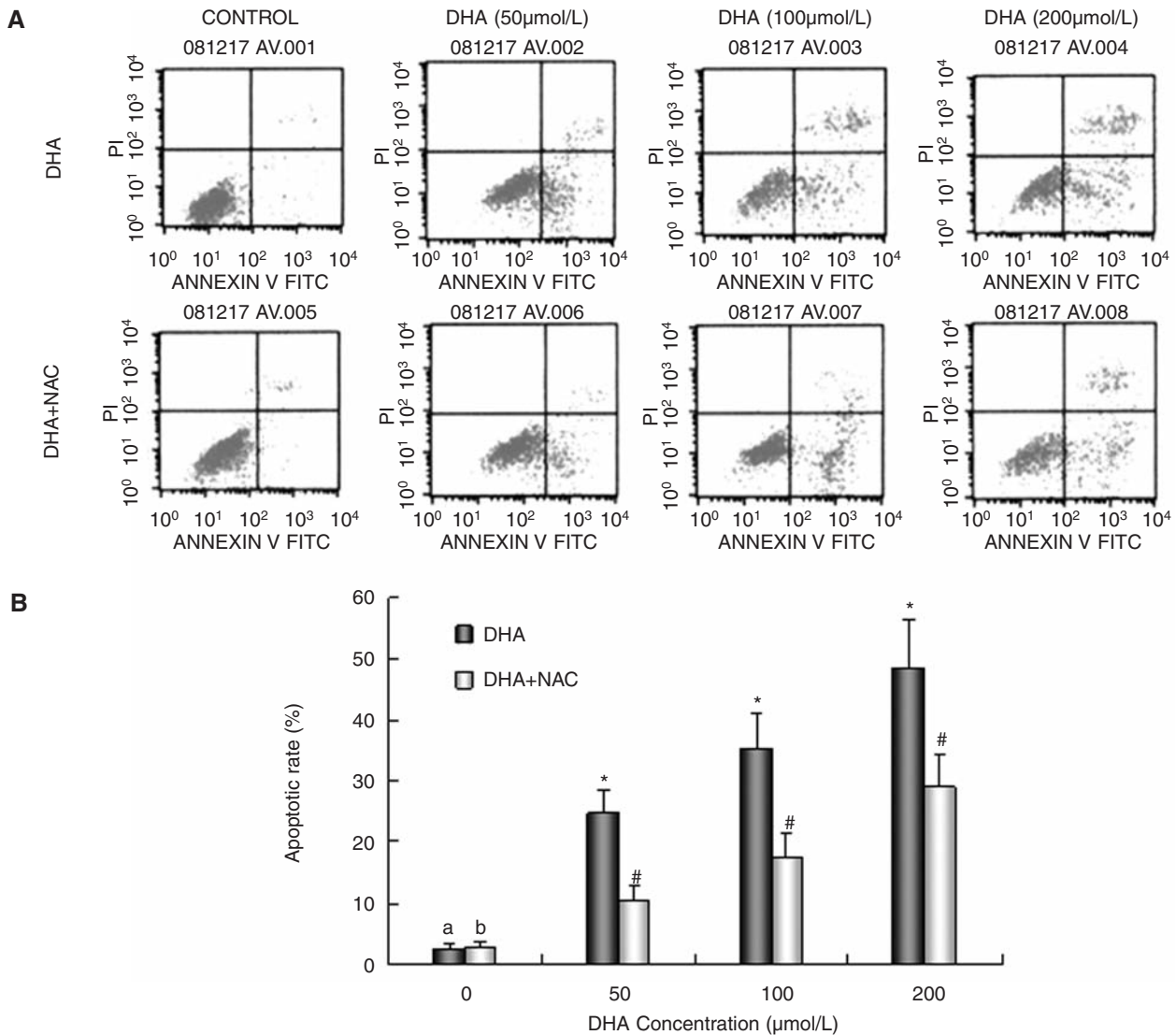


Figure 1 - Effects of DHA and DHA+NAC on apoptotic rate of HepG2 cells after 24 hours.

A) The percentage of apoptotic cells was determined by FITC-labeled annexin V and PI double-staining flow cytometry. B) a, control group; b, NAC-alone group. Values represent the means  $\pm$  SD ( $n = 3$ ). \* $P < 0.01$  vs control group, # $P < 0.05$  vs corresponding DHA concentration group.

control group peaked only at  $83.69 \pm 38.24$  nmol/L. When the same experiment was performed for the 6-hour treatment, no significant changes in  $[Ca^{2+}]_i$  levels were observed ( $P > 0.05$ ). This suggests that the ROS are probably generated prior to the increase in  $[Ca^{2+}]_i$ . As we expected, the addition of NAC (5 mmol/L) statistically attenuated the  $[Ca^{2+}]_i$  levels induced by DHA ( $P < 0.05$ ). However, NAC by itself did not show this effect at the concentration levels used in this experiment.

#### Regulation of the protein expression of GADD153 in HepG2 cells

It was reported that ROS and increasing intracellular  $Ca^{2+}$  can elicit ER-stress-induced apoptosis<sup>25</sup>. In this study, we found that ROS played an important role in

DHA-induced apoptosis, and  $[Ca^{2+}]_i$  was involved in it. Hence, Western blot analysis and immunocytochemistry were used to determine whether DHA can regulate the expression of ER-stress-induced apoptotic pathway related protein, GADD153, and whether free radical scavengers could attenuate this effect. It can be seen from Figure 4 that GADD153 protein was constitutively slightly expressed in control cells. After cell exposure to 100  $\mu$ mol/L DHA for 6 and 24 hours, DHA increased GADD153 expression in a time-dependent manner compared with the expression in the control group ( $P < 0.05$ ). In order to determine whether ROS were involved in the upregulation of GADD153 by DHA, NAC was employed in this experiment and the results showed that the effect of GADD153 upregulation was suppressed by the presence of NAC (Figure 4).

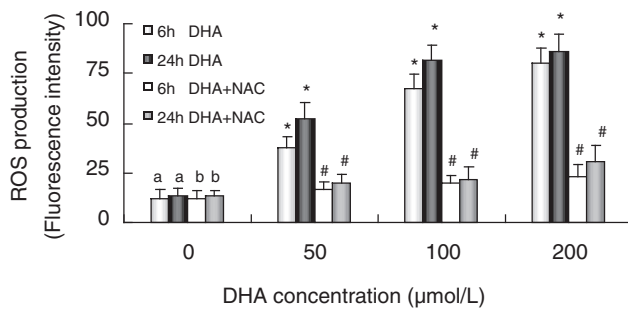


Figure 2 - Effects of DHA and DHA+NAC on ROS production of HepG2 cells by DCFH-DA (fluorescence intensity). a, control group; b, NAC-alone group. Values represent the means  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  vs control group; # $P < 0.05$  vs corresponding DHA concentration and exposure time group.

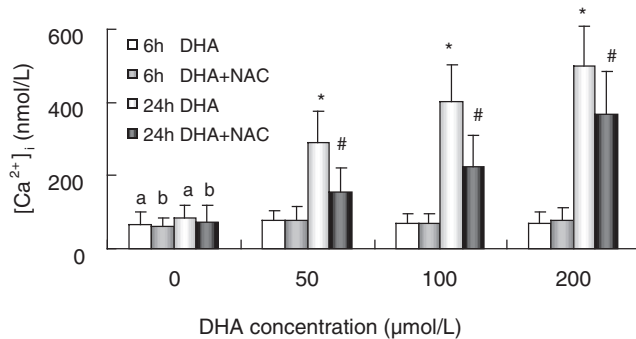


Figure 3 - Effects of DHA and DHA+NAC on intracellular  $\text{Ca}^{2+}$  concentration of HepG2 cells by Fluo-3/AM. a, control group; b, NAC-alone group. Values represent the means  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  vs control group; # $P < 0.05$  vs corresponding DHA concentration and exposure time group.

Immunochemical staining for GADD153 was mainly located in the cell nucleus. The positive brown particles were increased in cell nuclei when treated with DHA 100  $\mu\text{mol/L}$  for 24 hours compared with control cells, which showed very weak immunoreactivity, and NAC could attenuate the induction of GADD153 expression (Figure 5). Analysis of the staining intensity showed significant differences when the DHA group was compared with the control group ( $P < 0.05$ ) and DHA+NAC group ( $P < 0.05$ ).

#### Regulation of the protein expression of Bax and Bcl-2 in HepG2 cells

To investigate the mechanism underlying the apoptosis induced by DHA, we tested the effect of DHA on the expression levels of Bcl-2 and Bax, the 2 downstream targets of GADD153 and important regulators of apoptotic signaling pathways. As shown in Figure 4, Western blot analysis revealed that, compared with the control group, the protein expression of Bax in cells treated with 100  $\mu\text{mol/L}$  DHA for 24 hours was upregulated ( $P < 0.05$ )

while that of Bcl-2 was downregulated ( $P < 0.05$ ). Compared with DHA alone, cotreatment with NAC could prevent the increase in Bax expression and the decrease in Bcl-2 expression ( $P < 0.05$ ).

Immunocytochemistry analysis of Bcl-2 and Bax showed strongly positively stained cytoplasm and cell membranes for Bcl-2 and weak staining for Bax in the control groups. However, when cells were exposed to DHA 100  $\mu\text{mol/L}$  for 24 hours, the staining intensities were weakened for Bcl-2 and strengthened for Bax ( $P < 0.05$ ). NAC was found to counteract these effects (Figure 5).

## Discussion

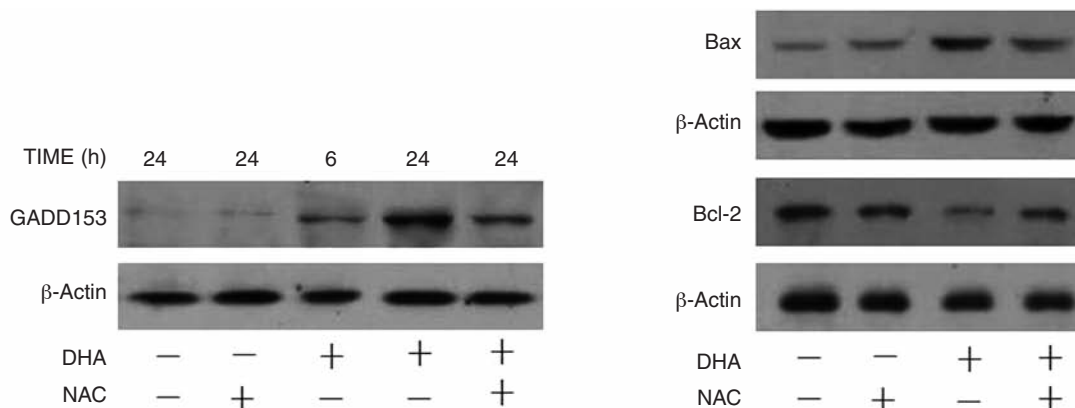
Apoptosis is a genetically regulated, programmed form of cell death that plays an important role in a variety of biological events, including surveillance against tumors<sup>31,32</sup>. Apoptosis can occur through 3 principal routes: death receptor-mediated, mitochondrial-mediated, and ER-stress-induced pathways<sup>33</sup>. It is now widely recognized that evasion of apoptosis is a cytopathological hallmark of most cancers. Induction of apoptosis has also been increasingly recognized as a strategy for identifying chemotherapeutic agents<sup>34</sup>.

In the present study, DHA caused proliferation inhibition of HepG2 cells in a dose- and time-dependent manner. FCM analysis revealed that DHA increased apoptosis in HepG2 cells in a dose-dependent manner. The cytotoxicity and apoptosis induced by DHA were partially reversed by NAC, a ROS scavenger. These findings suggest that DHA-induced apoptosis contributes to the growth inhibition of HepG2 cells, and that intracellular ROS generation induced by DHA may act as an important trigger.

Previous studies demonstrated that DHA harbored an endoperoxide bridge whose cleavage by an iron (II) species abundant in cancer cells resulted in the generation of ROS<sup>14,22</sup> and an increase in  $[\text{Ca}^{2+}]_i$  levels<sup>30</sup>, both of which could trigger ER stress. In light of the fact that DHA has a similar structure to thapsigargin, which is considered an ER-stress inducer, we investigated whether the ER-stress-induced pathway was involved in apoptosis induction by DHA.

ER is a key site for lipid and protein biosynthesis, calcium storage and signaling. It is highly sensitive to accumulation of unfolded or misfolded proteins, perturbation in calcium homeostasis and redox status, which has been referred to as ER stress<sup>35-38</sup>. Our study showed that DHA significantly increased intracellular ROS generation and  $[\text{Ca}^{2+}]_i$  levels, and that the effect could be attenuated by the addition of NAC. Interestingly, we detected a marked increase in ROS at 6 hours but no significant changes in  $[\text{Ca}^{2+}]_i$  levels at the same treatment time. This seems to suggest that ROS are generated prior to the increase in  $[\text{Ca}^{2+}]_i$  and ROS are probably a mediator of calcium increase.

## A



## B

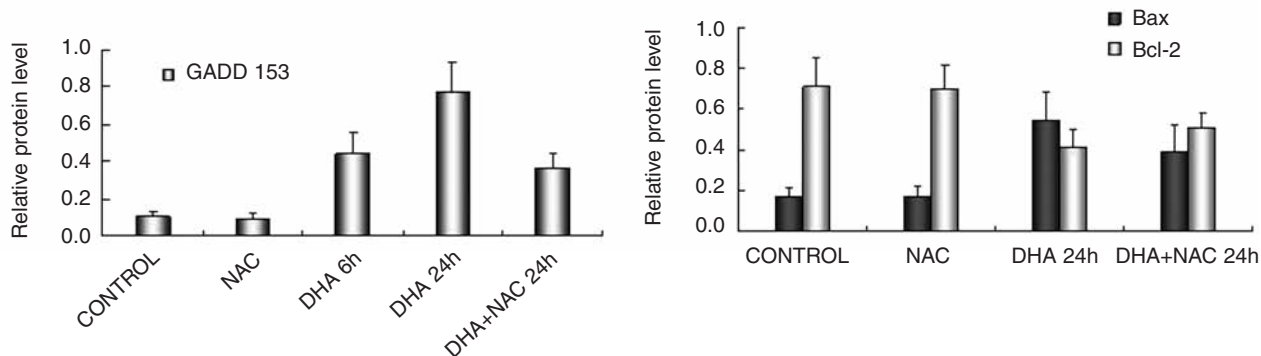


Figure 4 - Effects of DHA and DHA+NAC on protein expression of GADD153, Bcl-2 and Bax in HepG2 cells determined by Western blot analysis. A) GADD153, Bcl-2 and Bax mobility spectrum. The blot is representative of 3 independent experiments. B) Graph represents the changes in relative protein expression of GADD153, Bcl-2 and Bax based on densitometry. Normalized yield for Bcl-2, Bax and GADD153 fragments relative to  $\beta$ -actin. a, control group; b, NAC-alone group. The data represent the means  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  vs control group; # $P < 0.05$  vs corresponding DHA exposure time group.

The maintenance of intracellular  $\text{Ca}^{2+}$  homeostasis is crucial for cell survival, and its disruption may be involved in many pathological disorders, including ER stress<sup>24</sup>. The resting intra-ER  $\text{Ca}^{2+}$  concentration (400  $\mu\text{mol/L}$ ) is 3 to 4 orders of magnitude higher than cytosolic  $\text{Ca}^{2+}$  (0.1  $\mu\text{mol/L}$ ). This gradient is generated by the SERCA proteins, which pump  $\text{Ca}^{2+}$  into the ER, and by inositol (1,4,5)-trisphosphate and ryanodine receptors that release  $\text{Ca}^{2+}$  from the ER<sup>20,38</sup>. ROS may cause depletion of the calcium store in the ER via inhibition of  $\text{Ca}^{2+}$ -ATPase, generate oxidatively modified, abnormal proteins, and accumulate unfolded proteins through ROS-induced functional perturbation of ER foldases and/or chaperones, all of which can trigger ER stress<sup>39</sup>.

In higher eukaryotes, ER stress elicits UPR through 3 ER-resident transmembrane proteins: IRE1 (inositol-requiring transmembrane kinase and endonuclease 1), PERK (protein kinase-like ER kinase) and ATF6 (activation of transcription factor 6)<sup>19,20,40</sup>. The initial combined activation of IRE1, PERK and ATF6 produces cytoprotective outputs. However, when the ER stress is ex-

tensive or sustained, PERK, ATF6 and IRE1 activate proapoptotic molecules such as GADD153/CHOP<sup>18,41-43</sup> and murine caspase-12/human caspase-4<sup>20,43</sup> to initiate the apoptotic response.

Strong induction of GADD153 is regarded as a marker of ER stress<sup>21</sup>. Our data showed that the proapoptotic transcription factor GADD153 was increased in HepG2 cells that were treated with DHA at 6 and 24 hours, indicating a shift from the cytoprotective UPR to the initiation of apoptotic cell death. Although the precise mechanism by which GADD153 induces apoptosis remains inadequately understood, apoptosis-related molecules such as those of the Bcl-2 family were reported to be its target<sup>41</sup>. Thus, we tested the Bcl-2 and Bax expression regulated by DHA in our experiments, and found that DHA increased proapoptotic Bax expression and suppressed antiapoptotic Bcl-2 expression. As we expected, the presence of NAC could attenuate the effects of DHA on the expression of GADD153, Bcl-2 and Bax. This suggests that ROS exert their function through the signal transduction pathway.

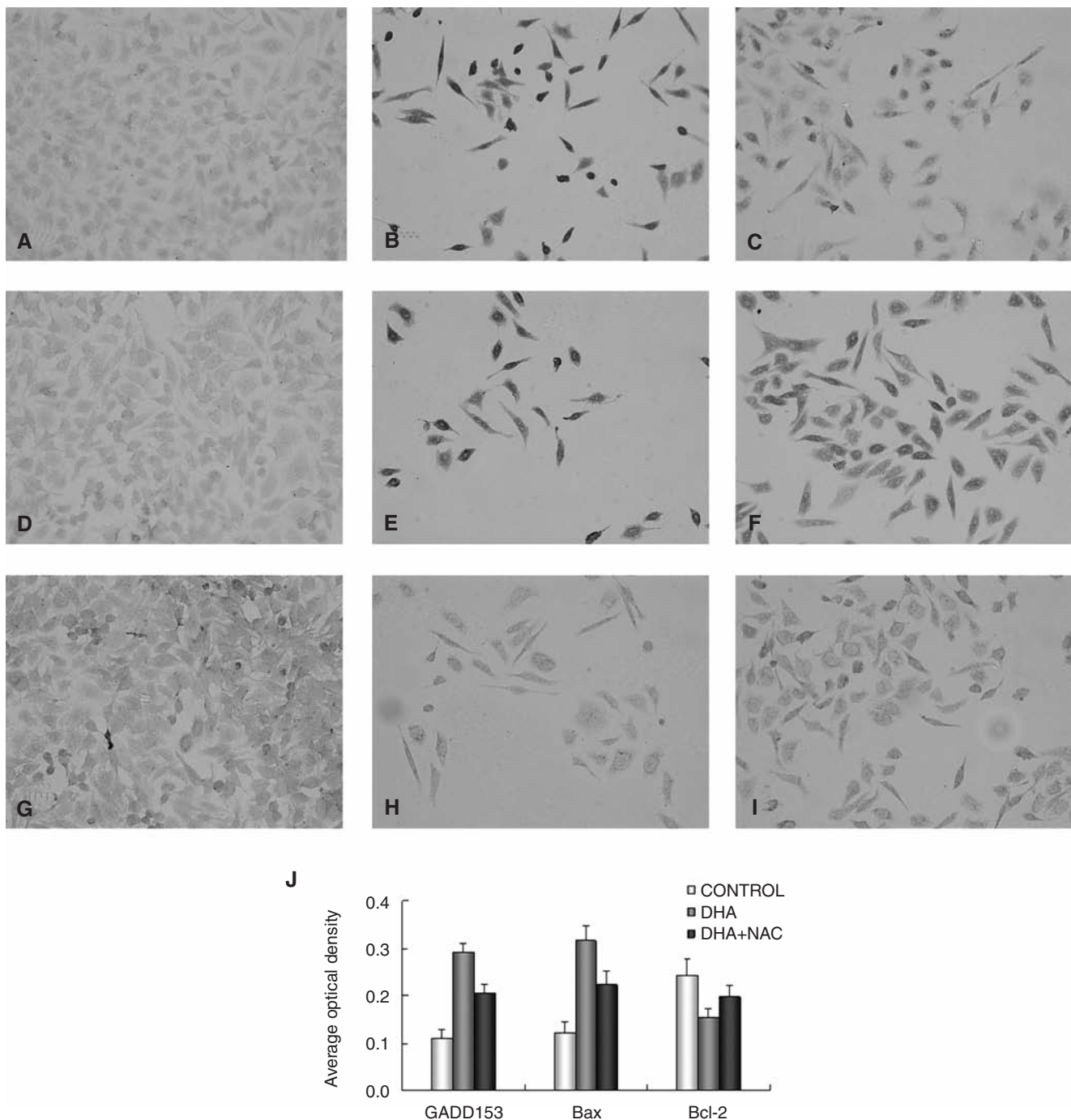


Figure 5 - Effects of DHA and DHA+NAC on protein expression of GADD153, Bax and Bcl-2 in HepG2 cells by immunocytochemistry ( $\times 400$ ). A, B, C: Expression of GADD153 in control, DHA and DHA+NAC group, respectively; D, E, F: Expression of Bax in control, DHA and DHA+NAC group, respectively; G, H, I: Expression of Bcl-2 in control, DHA and DHA+NAC group, respectively. J: Integrated optical density of the positive brown particles semiquantified by examining 5 fields randomly at  $\times 400$  magnification in each sample. Values represent the means  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  vs control group, # $P < 0.05$  vs DHA-treated group.

Previous studies suggested that members of the Bcl-2 family, usually considered as regulators of mitochondrial-mediated cell death, are also involved in the regulation of ER-stress-mediated apoptosis<sup>38</sup>. The Bcl-2 family has been found to localize at the ER, besides in the

mitochondria and nucleus, where it modulates the permeability of the ER membrane to  $\text{Ca}^{2+}$ <sup>38</sup>. Antiapoptotic Bcl-2 physically associates with the inositol (1,4,5)-trisphosphate receptor and alters its ability to release  $\text{Ca}^{2+}$  from the ER. Conversely, proapoptotic Bax can reg-

ulate ER Ca<sup>2+</sup> dynamics and cause a loss of ER content<sup>44</sup>. This forms a positive feedback of Ca<sup>2+</sup>-GADD153-Bcl-2 family-Ca<sup>2+</sup> to elicit ER-stress-induced apoptosis.

In summary, DHA was found to have strong ability to inhibit the proliferation of HepG2 cells and induce the apoptosis of these cells. Since DHA treatment elicited ER-stress-associated phenotypes in HepG2 cells, which included ROS generation, abnormal Ca<sup>2+</sup> leakage, and modulation of GADD153 and its 2 downstream target genes, Bcl-2 and Bax, the ER-stress-induced pathway might be involved in DHA-induced apoptosis. Furthermore, our results suggest that ROS might act as a key initiator and mediator of signal transduction. Future studies on other ER-stress-related genes, such as caspase-12/caspase-4, and animal models may be helpful in improving our understanding of DHA-induced ER-stress-mediated apoptosis.

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