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Cytotoxic effect of oxaloacetate on HepG2-human hepatic carcinoma cells via apoptosis and ROS accumulation

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Oxaloacetate (OA) is one of the intermediates of the Krebs cycle. In addition to its role in energy production, OA may have other effects on the cell. We report here that OA could have a cell type dependent cytotoxic effect on the human hepatic carcinoma cell line HepG2 through induction of apoptosis and reactive oxygen species (ROS) accumulation. In our study, OA decreased the viability and colony formation of HepG2 cells and induced cell death. Caspase-3 activity was increased, the pro-apoptotic protein Bax was up-regulated, and the anti-apoptotic protein Bcl-2 was down-regulated in OA-treated HepG2 cells indicating that apoptosis through the intrinsic pathway was involved in the cell death. The ROS level in OA-treated HepG2 cells was increased. The anti-oxidant N-acetylcysteine (NAC) and glutathione (GSH) prevented the OA-induced decrease in cell but did not alter the enhanced apoptotic Bax/Bcl-2 mRNA ratio. These results suggest that the OA-induced apoptosis of HepG2 cell is not driven by oxidative damage and at least two distinct mechanisms, one mediated by ROS and one involving apoptosis, result in the cytotoxic effects of OA on HepG2 cells. These studies expand the biological functional repertoire of OA and provide a mechanism by which hepatocellular carcinoma may be targeted by OA.

Key words: oxaloacetate, HepG2 cells, apoptosis, reactive oxygen species

Oxaloacetate (OA) is an intermediate of the Krebs cycle and participates in the energy production via a reaction with acetyl-CoA [1]. During this metabolic process, OA is exhausted and recovered by metabolism of glutamine. This process is more active in rapidly growing cells, such as tumor cells, in which nearly 90% of OA can be recovered by metabolism of glutamine, promoting mitochondrial oxidative phosphorylation and increasing the stores of cellular energy [2, 3] . In addition to its role in the Krebs cycle as a substrate, OA may have other biological effects. OA can promote proliferation in cultured cells [4] and alleviate glutamine-depletion mediated apoptosis [5, 6]. The mechanism of OA-induced cell proliferation is poorly understood and believed to be unrelated to its role in the Krebs cycle [4]. Our studies showed that OA induced cellular death in a cell type-dependent manner. Excess treatment with OA induced cell death in the human hepatic carcinoma cell line HepG2 but not in human cervical carcinoma cell line HeLa. Interestingly, citrate, another intermediate of the Krebs cycle, induces apoptosis [7-9]. This phenomenon has not been previously reported for OA to the best of our knowledge. In the present study, we hypothesized that the cytotoxic effect of OA on HepG2 cells may be related to induction of apoptosis and ROS accumulation.

Hepatocellular carcinoma (HCC) is one of the most globally prevalent malignancies and a frequent cause of cancer-related death [10, 11]. HCC is characterized by elevated glycolysis, gluconeogenesis, β-oxidation with reduced activity of the tricarboxylic acid cycle and Δ-12 desaturase and increased levels of antioxidants, together with decreased inflammatory-related polyunsaturated fatty acids and phospholipase A2 [12]. Currently, the major clinical treatment for HCC is surgical removal, but high recurrence rates limit long-term patient survival [13]. There is an urgent need for more effective HCC treatment, and studies on the metabolic mechanisms of cancer may provide insight into novel therapeutic strategies [14]. The discovery of citrate's cytotoxic effects on gastric cancer cells suggests potential treatment options for cancer [8, 15]. In the present study, we found that OA selectively induced death in the human hepatic carcinoma cell line HepG2 through apoptosis and

ROS accumulation, which suggests future treatment options for hepatocellular carcinoma.

Materials and methods

Reagents. OA was purchased from Sigma-Aldrich (St Louis, MO, USA). The primers used for RT-PCR were synthesized by Sangon (Shanghai, China).

Cell culture. HepG2 cells and HeLa cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibico, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA), 1% penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin) and 50 mM HEPES (Beyotime, China). The cells were maintained in a 5% CO2 humidified atmosphere at 37 °C.

Cell viability assay. Cell viability was assessed using MTT assays. Briefly, cells were grown in a final volume of 100 μl DMEM medium containing 10% FBS per well in 96-well plates at a density of 7×10^4 cells/ml. After 24 hours, the cells were treated with 50, 75 and 100 mM of OA for 24 hours or treated with 75 or 150 mM OA plus different concentrations of antioxidants for 24 hours. MTT (3- [4,5-dimethylthiazol-2-yl] -2,5 diphenyltetrazolium bromide)(Beyotime, China) was added to each well at a final concentration of 0.5 mg/ml and incubated for 4 hours in a moist chamber at 37 °C. The medium was then removed and 0.1 ml DMSO was added. The absorbance (A) of the formazan product was measured at 490 nm with a microtiter plate reader (Bio-Tek, Winooski, VT, USA).

Morphology of HeLa and HepG2 cells. HeLa and HepG2 cells were seeded in 96-well culture plates. After 24 hours, OA was added to the wells at concentrations of 0 or 75 mM in DMEM medium supplied with 10% FBS for 24 hours. The morphological changes in the cells were observed under an inverted optical microscope (Nikon, Japan).

Colony formation assay. HepG2 cells were plated in 6-well plates at 200 cells per well in 2 ml of DMEM for 24 hours. OA was added to a final concentration of 75 mM. Then the media was not changed throughout the course of the experiment. After 7-10 days, colonies were fixed in methanol and stained with 0.1% crystal violet. Dye was extracted with 10% acetic acid and the relative absorbance was determined at 595 nm with a spectrophotometer [16].

Flow cytometry analysis of cell death. The induction of cell death by OA was evaluated by double staining with propidium iodide (PI) and annexin V-FITC. After treatment with 75 mM OA for 24 hours, cells were harvested, washed twice with PBS, and assayed for cell death with a PI and annexin V-FITC assay kit (Beyotime, China). Briefly, 10^5 cells were resuspended in a binding buffer, stained with 5 μl of annexin V-FITC for 10 minutes, and then stained with 10μl of PI for another 10 minutes. The cells were immediately analyzed with a flow cytometer (FACScan; BD Biosciences, Milano, Italy).

Caspase-3 activity assay. In vitro caspase-3 protease activity was measured using a caspase-3 assay kit (Beyotime, China). According to the manufacturer's instruction, HepG2 cells were treated with OA at a concentration of 75 mM for 1 or 3 hours. The cells were then lysed and prepared to detect the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate Ac-DEVD-pNA. The absorbance was measured at wavelength of 405 nm with a microtiter plate reader.

RNA isolation and RT-PCR assay. To determin the Bcl-2 and Bax mRNA levels in HepG2 cells, RT-PCR was performed. Total RNA of the cells was isolated from HepG2 cell using RNA plus (TaKaRa). Then, 5 μg of RNA, 1 μl of Anchored Oligo(dT)18, 10 μl of 2×TS Reaction Mix and 1 μl of TransScript RT/RI Enzyme Mix (Transgene) were mixed and the final volume was brought up to 20 μl with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 °C for 30 minutes and 85°C for 5 minutes. PCR amplification of DNA was performed in a reaction volume of 20 μl containing 1 μl of template cDNA, 1 μl of each set of primers at a concentration of 10 pm, 10 μl of the 2×EasyTaq PCR SuperMix and 7μl of ddH_2O. The primers used in the study included forward (5-CGACGACTTCTCCCGCCGCTACCGC-3) and reverse (5-CCGCATGCTGGGGCCGTACAGTTCC-3) primers for human Bcl-2, forward (5-GTGCACCAAGGTGCCGGAAC-3) and reverse (5-TCAGCCCATCTTCTTCCAGA-3) primers for human Bcl-2, and forward (5-GAAGGTGAAGGTCGGAGTC-3) and reverse (5-GAAGATGGTGATGGGATTTC-3) primers for human GAPDH an internal control for the PCR. The expected size of the PCR products was 318 bp for Bcl-2, 205 bp for Bax, and 226 bp for GAPDH. PCR was carried out using a GeneAmp 9600 PCR system (Perkin-Elmer, Norwalk, CT, U.S.A.) under the following conditions: initial denaturation at 94 °C for 5 minutes followed by 36 amplification cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 60 second, and a final extension step at 72 °C for 10 minutes. The mRNA levels the RT-PCR product for each of the mRNA species were calculated densitometrically using Molecular Analyst TM version 1.4.1 (Bio-Rad, Hercules, CA, U.S.A.).

Detection of ROS accumulation. To detect ROS accumulation, we used a Reactive Oxygen Assay Kit from Beyotime, China. Briefly, HepG2 cells were treated with 75 mM OA for 16 hours, and the cells were collected and exposed to serum-free medium containing 10 μM DCFH-DA. After 20 minutes of incubation in the dark, cells were washed with DMEM three times, and fluorescence intensity was measured by fluorescence microscopy and flow cytometry with excitation and emission wavelengths of 488 and 525 nm, respectively.

Statistical analysis. All experimental data are expressed as the mean ± SD from at least three independent experiments. Single comparisons between groups were performed using paired, two-tailed Student's t-test, and statistical significance was set at $P<0.01$.

Results

Effects of OA on viability, morphology and colony formation of HepG2 and HeLa cells. HepG2 cell viability was reduced by OA treatment in a dose-dependent manner. The IC50 value of OA for inhibition of HepG2 cell viability was 61.47±0.73 mM. At concentrations of 50, 75 and 100 mM, OA reduced the viability of HepG2 cells to 78.77±0.169%, 36.1±0.085%, and 8.0±0.003% of the control value, respectively. However, the same concentrations of OA had no significant effect on HeLa cell vialbility (Figure 1A). We also performed a dose-response experiment to access the cytotoxic effects of OA on a normal hepatic cell line, LO-2, using MTT assays. The decrease in LO-2 cell viability was observed at 24 hours after the cells were treated with 75 mM OA, and the slope change of the inhibition curve of LO-2 cells by OA was not significant compared with that of HepG2 cells by OA. The IC50 value of OA for the inhibition of the normal hepatic cell line LO-2 cell was 90.28±0.23 mM. Under an optical microscope, HepG2 cells showed significant differences in morphology and overall count after treatment with 75 mM OA vs. the control for 24 hours. HeLa cells showed no changes under the same conditions (Figure 1B). Colony formation was also significantly reduced by treating the cells with OA, and the absorbance of HepG2 cells treated with 75mM OA was 44.1% of the control value (Figure 1C). There was no change in 75 mM OA-treated HeLa cells vs. the control (data not shown).

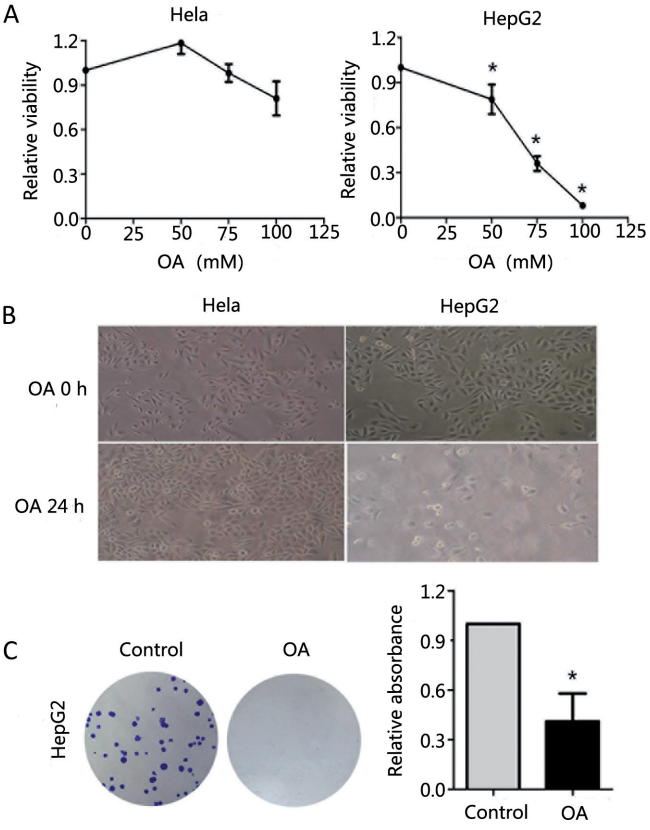

Figure 1. Effects of OA on the viability of HeLa and HepG2 cells. (A) HeLa and HepG2 cells were treated with different concentrations of OA for 24 hours. Viability of the cells was measured using an MTT assay. The experiments were repeated at least three times. The results are presented as the mean±SD. *Stands for p<0.01 as compared with OA-untreated group. (B) Morphology of HeLa and HepG2 cells treated with OA. HeLa and HepG2 cells were treated with 75 mM OA for 0 and 24 hours. Morphological changes of the cells were observed under an inverted microscope. (C) Colony formation assay. HepG2 cells were seeded in a six-well plate at a density of 200 cells per well for 24 hours and then treated with 0 (cont) or 75 mM OA for 7-10 days. Cell colonies were fixed in methanol and stained with 0.1% crystal. Dye in the colonies was extracted with 10% acetic acid, and the relative absorbance was determined at 595 nm by a spectrophotometer. The experiments were repeated at least three times. The results are presented as the mean±SD. *Stands for p<0.01 as compared with OA-untreated control (cont).

Effects of OA on cell death, Bax/Bcl-2 mRNA expression and caspase 3 activity of HepG2 cells. After treatment with 75 mM of OA for 24 hours, HepG2 cells became apoptotic. The distribution of apoptotic HepG2 cells was determined by flow cytometry, and the results showed that the numbers of early and late-apoptotic HepG2 cells were 21.63±2.64% of the total cells, which was significantly higher than that of non-OA treated control cells at 6.17±1.47% (Figure 2A and B). RT-PCR analysis indicated that the expression of Bax mRNA was increased and the expression of Bcl-2 mRNA was decreased in the HepG2 cells treated with 75 mM OA in a time-dependent manner. After HepG2 cells were treated with 75 mM OA for 1 hour or 3 hours, the ratio of Bax/Bcl-2 mRNA, a biomarker of apoptosis, was 1.3- or 2.5–fold that of the non-OA treated control cells respectively(Figure 2C and D). Caspase-3 activity, another biomarker of apoptosis, was significantly increased after HepG2 cells were treated with 75 mM of OA for 4 hours and was 1.2-fold of non-OA-treated control HepG2 cells (Figure 2E).

Effect of OA on ROS accumulation in HepG2 cells. ROS accumulation in HepG2 cells was increased by treatment with OA and alleviated by the antioxidant NAC. After HepG2 cells were treated with 75 mM OA for 12 hours, cellular fluorescence intensity, a measure of ROS accumulation, was 1.9-fold that of the non-OA-treated control HepG2 cells. The increase in the fluorescence intensity treated by OA was returned to approximately 90% of the control value by simultaneous treatment with NAC (Figure 3A and B).

Effects of antioxidants on viability and the Bax/Bcl-2 mRNA ratio of HepG2 cells treated with OA. The antioxidants NAC and GSH rescued the viability of HepG2 cells treated with OA. After HepG2 cells were treated with 75 mM (or 150 mM) OA and 5, 10 or 20 mM NAC for 12 hours, the ratio of absorbance, an index of viability measured by MTT assays, in these treated cells to the HepG2 cells treated with 75 mM (or 150 mM) OA only was 1.09±0.006 (1.20±0.068), 1.21±0.011 (1.69±0.23), and 1.30±0.001 (2.57±0.71), respectively (Figure 4A and 4B). For GSH, the ratio was 1.08±0.003 (1.21±0.017), 1.24±0.008 (1.6±0.043), and 1.40±0.024 (2.25±0.008), respectively (Figure 4C and 4D). Based on

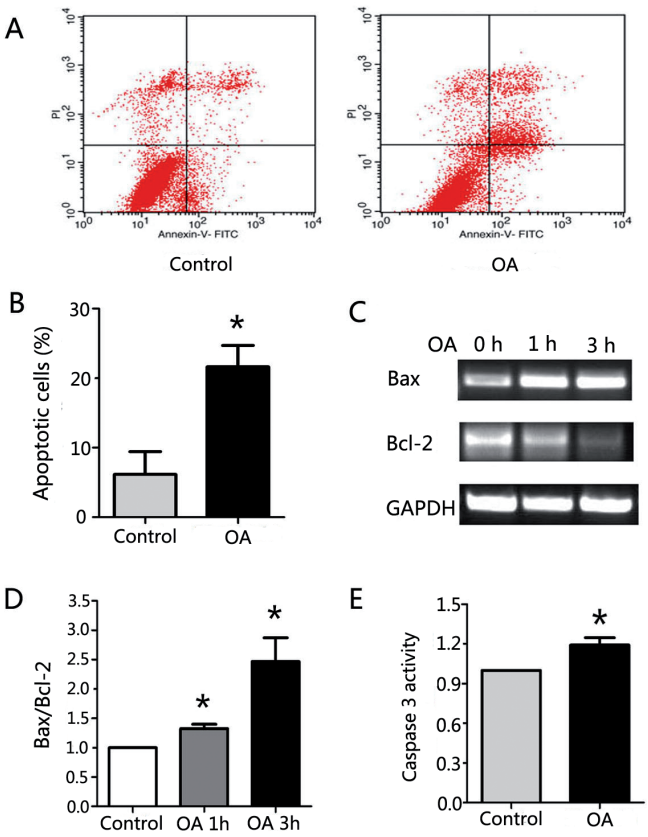

Figure 2. Flow cytometric analysis of cell death. (A) HepG2 cells were treated with 0 (control) or 75 mM OA for 24 hours and then harvested and double stained with Annexin V-FITC and PI. The cells were sorted by a flow cytometer. (B) The apoptotic cells represent early and late apoptotic cells (bottom right and up right). Data are expressed as the mean ± SD of three separate experiments. * Stands for p<0.01 as compared with OA-untreated control. (C) Apoptosis of HepG2 cells induced by OA. The mRNA levels of Bax, Bcl-2 and GAPDH in HepG2 cells were determined by RT-PCR. The mRNA levels of GAPDH were used as a loading control. (D) The levels of Bcl-2 and Bax mRNA were quantitatively analyzed with an image analyzer and expressed as the ratio of Bax/Bcl-2. *Stands for p<0.01 as compared with OA-untreated control group. (E) Caspase-3 activity assay. Caspase-3 activity was assayed after HepG2 cells were treated with 0 (control) or 75 mM OA for 4 hours. The experiment was repeated for three times. Data are presented as mean ± SD. *Stands for p<0.01 as compared with OA-untreated control group.

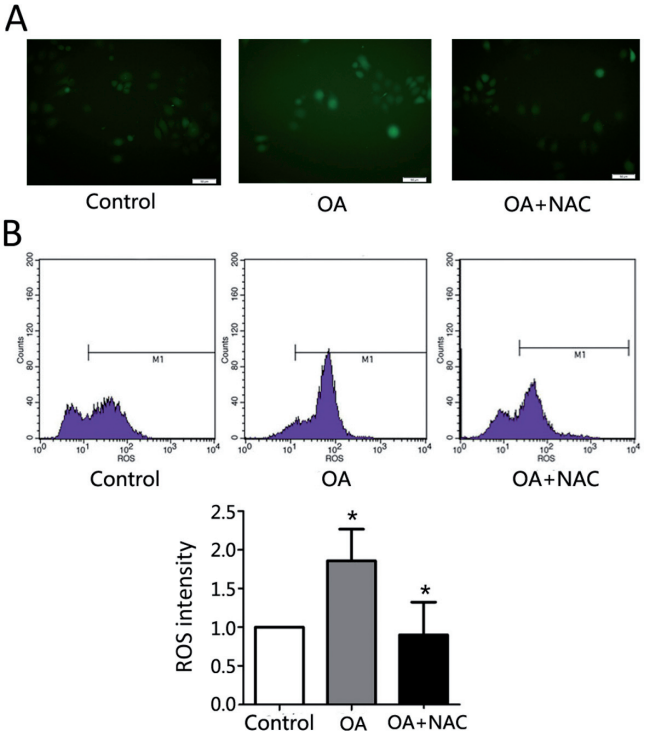

Figure 3. Accumulation of reactive oxidative species (ROS) induced by OA. (A) HepG2 cells were treated with HepG2 cells were treated with 0 mM, 75 mM OA, or 75 mM OA plus 20mM anti-oxidant NAC for 16 hours and incubated with DCFH-DA probe for 20 minutes. The intensity of green fluorescence reflecting the level of ROS was observed with a fluorescence microscope. (B) HepG2 cells were treated with 0 mM, 75 mM OA, or 75 mM OA plus 20mM NAC for 16 hours. After incubating with DCFH-DA probe for 20 minutes, the intensity of the green fluorescence in the cells was quantitatively analyzed by flow cytometry. The experiment was repeated for at least three times. Data are presented as mean ± SD, *Stands for p<0.01 as compared with untreated control group.

the results above, the restorative effects of NAC and GSH on the viability of HepG2 cells treated with OA was better for 150 mM OA treated cells than those treated with 75 mM OA. OA also increased the ratio of Bax/Bcl-2 mRNA, which could not be alleviated by NAC or GSH. The increase in the viability of HepG2 cells treated with OA by NAC or GSH could not be direct actions of on HepG2 cells, as NAC or GSH did not increase the viability of HepG2 cells with no OA. At concentrations of 5, 10 and 20 mM of NAC or GSH, the viability of HepG2 cells was 86.5%, 88.3% and 84.0% for GSH and 94.6%, 100% and 84% for NAC of the control value, respectively. After HepG2 cells were treated with 75 mM OA or 75 mM OA plus 20mM of the anti-oxidants NAC or GSH for 3 hours, mRNA expression of Bax or Bcl-2 in these cells was assayed by RT-PCR. The ratio of Bax/Bcl-2 mRNA was 2.49±0.082, 2.44±0.054, and 2.5±0.054-fold that of non-OA-treated HepG2 cells in HepG2 cells treated with OA, OA plus NAC or OA plus GSH, respectively. There were no significant changes of the ratio of Bax/Bcl-2 mRNA between these treated cells (Figure 4F).

Discussion

OA is a well-known intermediate of the Krebs cycle. In addition to its role in cellular metabolism, OA has been reported to be able to promote cell proliferation through an obscure mechanism [3]. We hypothesized that OA may have other direct biological effects on the cells via a mechanism unrelated to its metabolic roles. Surprisingly, our study showed that OA exhibited a cytotoxic effect on HepG2 cells. There were three key findings in the present study: 1) OA decreased

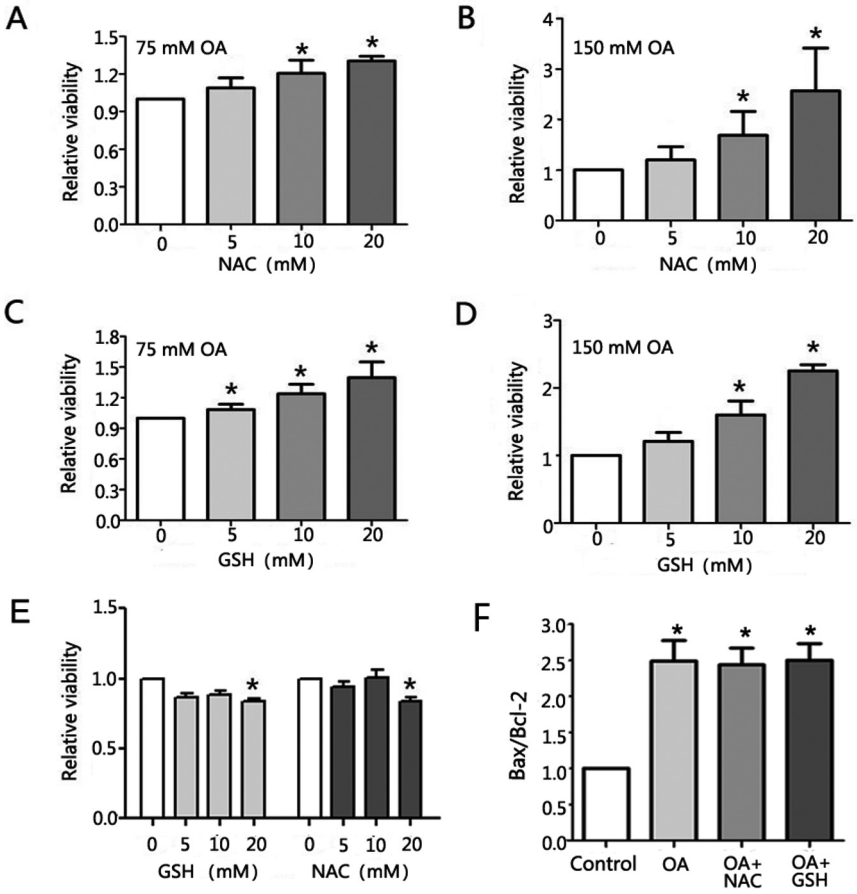

Figure 4. Effects of anti-oxidants on HepG2 cells treated with OA. HepG2 cells were treated with 75 mM OA and different concentrations of NAC (A) or GSH (C), or treated with 150 mM OA and different concentrations of NAC (B) or GSH (D) for 12 hours. The viability of HepG2 cells was measured with a MTT assay. (E) HepG2 cells were treated with different concentrations of GSH or NAC for 12 hours. The viability of HepG2 cells was measured with MTT assays. (F) HepG2 cells were treated with 75 mM OA or 75 mM OA plus 20 mM anti-oxidants NAC or GSH for 3 hours. The mRNA levels of Bax and Bcl-2 were measured using the RT-PCR assay. The experiments were repeated at least three times. The results are presented as the mean ± SD. * Stands for p<0.01 as compared with untreated control group.

the viability and numbers of HepG2 cells and this effect was cell type dependent as HeLa cells had no such response; 2) OA induced apoptosis of HepG2 cells through the intrinsic pathway of apoptosis; 3) the cytotoxic effect of OA on HepG2 cells was mediated not only via apoptosis, but through ROS accumulation as well.

The differential effects of OA on the viability of HepG2 and HeLa cells may reflect different cellular responses to adjacent bioactive substances as a result of varying genetic backgrounds. Cells expressing higher levels of oncogene c-Myc were shown to be highly vulnerable to apoptotic injures [17, 18]. Therefore, we examined and compared the expression of metabolism-regulating oncogenes including c-Myc, c-Jun and c-Fos and k-Ras in HepG2 and HeLa cells [19-21]. We found that expressions of these genes in both HepG2 and HeLa cell lines were decreased except for c-Myc in HepG2 cells, which was significantly elevated (data not shown). The differential expression levels of c-Myc may explain the different responses of HepG2 and HeLa cells to OA. The increase in the ratio of Bax/Bcl-2 mRNA expression and caspase 3 activities in HepG2 cells in the present study suggested that apoptotic injury may be responsible for the cytotoxic effects of OA on HepG2 cells. The increase in the ratio of Bax/Bcl-2 mRNA expression in the OA-treated HepG2 cells indicates that intrinsic apoptotic pathway may mediate the cytotoxic effects of OA on HepG2 cells [22-24].

ROS accumulation also contribute to the selective cytotoxic effect of OA on HepG2 cells. Tumor cells with high c-Myc expression have been reported to be metabolically active. These cells have a relatively high ROS content and are sensitive to apoptosis [25-27]. Apoptosis and ROS both contribute to cell death but have a contradictory relationship: ROS can either elicit cellular apoptosis through the intrinsic pathway or inhibit it [28], while our results showed that OA-induced ROS accumulation in HepG2 cells did not affect OA-induced apoptosis. Although the antioxidants NAC or GSH could prevent

the OA-induced decrease in viability of HepG2 cells, neither NAC nor GSH could alleviate the OA-induced increase in the Bax/Bcl-2 mRNA ratio of the cell. Lower levels of ROS have been reported to induce apoptosis, while higher levels of ROS induce a necrosis [29, 30]. The ROS levels in the OA-treated HepG2 cells in our study were relatively high, which can induce non-apoptotic cell death. Glutamate consumption may be responsible for the ROS accumulation in HepG2 cells induced by OA. OA is a catalytic substrate of aspartate-aminotransferase through which OA consumes a large amount glutamate, which reacts with alpha ketoglutaric acid to generate aspartic acid [31]. Glutamate is involved in glutathione synthesis, which can increase the intracellular antioxidant capacity[32, 33]. Glutamate consumption by OA in HepG2 cells may result in a decrease in glutathione content, which promotes accumulation of ROS inside the cell.

The cytotoxic effects of OA on HepG2 cells may be due to the amount of OA we used, which was greater than the requirement of the cells for OA. In the present study, 75 mM OA caused a cytotoxic effect on HepG2 cells, while 20-30 mM OA increased cell proliferation (data not shown). The latter finding is consistent with a published report in which 30 mM OA promoted DNA synthesis in cultured primary hepatocytes [4]. Therefore, for a given cell, OA hemostasis maintains normal function. Our study also demonstrated that NAC and GSH prevented the decrease in viability of HepG2 cells induced by OA but could not reverse the enhanced apoptotic Bax/Bcl-2 mRNA ratio, which suggests that at least two distinct mechanisms, one mediated by ROS and one involving apoptosis, lead to the cytotoxic effect of OA on HepG2 cells. These studies expand the biological functional repertoire for OA and provide a mechanism by which hepatocellular carcinoma may be targeted by OA.

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