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Reactive oxygen species accumulation contributes to gambogic acid-induced apoptosis in human hepatoma SMMC-7721 cells

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ABSTRACT

It is reported that gambogic acid (GA), the main active compound of gamboge which is a dry resin extracted from *Garcinia hanburyi* tree, has potent antitumor activity both *in vivo* and *in vitro*. Activation of mitochondrial apoptotic pathway in cancer cells is one effective therapy for cancer treatment. In the present study, we focus on the effect of GA on induction of reactive oxygen species (ROS) accumulation and triggering the mitochondrial signaling pathway in human hepatoma SMMC-7721 cells. The results indicated that GA induced ROS accumulation and collapse of mitochondrial membrane potential in SMMC-7721 cells in a concentration-dependent manner and subsequently induced that release of Cytochrome *c* and apoptosis-inducing factor from mitochondria to cytosol, which inhibited ATP generation and induced apoptosis in the cells. Moreover, GA elevated the phosphorylation of c-Jun-N-terminal protein kinase (JNK) and p38, which was the downstream effect of ROS accumulation. Furthermore, N-acetylcysteine, a ROS production inhibitor, partly reversed the activation of JNK and p38 and the induction of apoptosis in GA-treated cells. Collectively, our study demonstrated that accumulation of ROS played an important role in GA-induced mitochondrial signaling pathway, which provided further theoretical support for the application of GA as a promising anticancer agent.

1. Introduction

Gambogic acid (GA, $C_{38}H_{44}O_8$) is a major active ingredient of gamboges, a brownish to orange resin extracted from *Garcinia hanburyi* tree in Southeast Asia (Guo et al., 2004). Recent studies have demonstrated that GA exerts potent anticancer activity both *in vitro* and *in vivo* (Guo et al., 2006; Liu et al., 2005; Lu et al., 2007; Wu et al., 2004; Zhang et al., 2004). It has been previously demonstrated that GA activates cell apoptosis through transferrin receptor and nuclear factor-κB signaling pathway (Kasibhatla et al., 2005). It also has been reported its elevation of the phosphorylation levels of JNK-1 and p38 in MCF-7 cells (Chen et al., 2008) and upregulation of death inducer-obliterator 1 in Jurkat T cells (Wang et al., 2008). In addition, the chronic toxicity study revealed that the targets of GA in rats are the kidney and liver (Qi et al., 2008). We have reported that GA induces human hepatoma SMMC-7721 cells apoptosis involved in downregulation of Bcl-2 protein expression and activation of caspase-3 (Yang et al., 2007). These evidences implied that mitochondrial pathway might play an important role by which GA exerts the anticancer activity.

Mitochondria play a pivotal role during the process of cell apoptosis which involves in a variety of key invents, including loss of mitochondrial membrane potential (MMP), mitochondrial swelling and release of apoptotic proteins (Preston et al., 2001). Loss of MMP is a crucial step, which subsequently triggers the release of apoptotic factors, such as Cytochrome *c* (Cyt *c*) and apoptosis-inducing factor (AIF), from the mitochondrial intermembrane space into cytosol where these factors induce the propagation of the apoptotic cascade and execution of cell death (Kroemer et al., 2007). Mitochondria also play a central role in cellular energy metabolism (Kroemer et al., 2007). The release of Cyt *c* interrupts the electron flow between respiratory chain complexes III and IV, together with the uncoupling of the respiratory chain due to the oxidation of NAD(P)H, thus inhibits the ATP generation and accelerates cell death (Cadenas and Davies, 2000; Raha and Robinson, 2000).

The structure of GA (Fig. 1) shows the presence of an α,β-unsatured ketone which plays an important role in GA cytotoxicity (Kasibhatla et al., 2005). As α,β-unsatured ketone groups are

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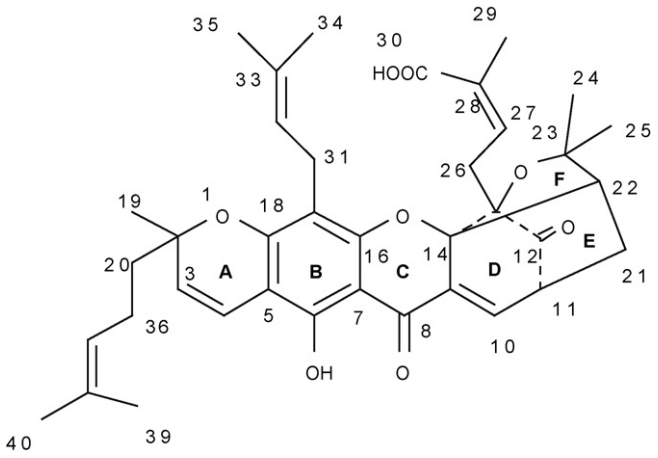

Fig. 1. Chemical structure of GA.

present in some drugs that are demonstrated to induce apoptosis through the generation of ROS (Chen et al., 2005; Kondo et al., 2002), we presume that the cytotoxic effect of GA might be mediated through the generation of ROS, which is known to affect MMP, therefore triggers the mitochondria-associated cell apoptosis (Park et al., 2005). Both the mitochondrial structural integrity and function are subject to a preferential redox balance. When the balance disrupted, excess ROS production and/or antioxidant depletion, then oxidative stress occurs (Garcia-Ruiz and Fernandez-Checa, 2006). In tumor cells, the oxidative stress could be alleviated by several antioxidants, such as reduced glutathione hormone (GSH) and superoxide dismutase. GSH depletion-mediated cell death is also induced by a ROS accumulation in mitochondria, which is an early signal of mitochondria-dependent ROS induced cell apoptosis (Domenicotti et al., 2003).

It has been demonstrated that Mitogen-activated protein kinase (MAPK) signal pathways, which contains extracellular signal-regulated kinases (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) pathway, are responsible for ROS mediated cell apoptosis (Liu and Huang, 2006). Therefore, we speculated that the apoptosis induced by GA was through the ROS-dependent MAPK-mediated mitochondrial apoptotic pathway.

In the present study, we demonstrate that GA induces a mitochondrial apoptosis characterized by the loss of MMP which is induced by the accumulation of ROS. Cyt *c* and AIF have released from mitochondria to cytosol in the GA treatment cells. Thus, ATP generation was disturbed. Moreover, GA could elevate the phosphorylation levels of JNK, p38 and increase the levels of downstream apoptotic proteins, such as caspase-9 and PARP, which have been activated. Antioxidant NAC, a ROS production inhibitor, partially reversed the GA-induced activation of JNK and p38 and apoptosis. Collectively, our results demonstrate that accumulation of ROS plays an important role in GA-induced mitochondrial signaling pathway.

2. Materials and methods

2.1. Medicine and reagents

GA was isolated from gamboge resin of *G. hanburyi*, with the purity of 99% as determined by HPLC according to previous study (Zhang et al., 2004). It was dissolved to a concentration of 0.01 M in 100% DMSO as a stock solution and stored at −20 °C. The final DMSO concentration did not exceed 0.1% DMSO throughout the study. N-acetylcysteine (NAC) was purchased from Sigma (St. Louis, MO) and dissolved in PBS to the concentration of 500 mM, then adjusted the pH value to 7.0 with 1 M NaOH as stock solution. Glucose was purchased from Sinopharm Chemical Reagent Co. Ltd. (Nanjing, Jiangsu, China). Antibodies of JNK, p-JNK, p38, p-p38, ERK1/2, p-ERK1/2, Histones, GAPDH, COX IV and β-actin were obtained from Santa Cruz Biotechnology (California). Anti-AIF and anti-Cyt *c* antibodies were purchased from Cell Signaling Technology (Beverly, MA) and Biosource (Camarillo, CA), respectively. IRDye™800 conjugated second antibodies were obtained from Rockland Inc. (Bedford, PA, USA.).

2.2. Cells culture

Human hepatoma SMMC-7721 cell line was offered by KeyGen Biology Technology Company (Nanjing, Jiangsu, China). Cells cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (Sijiqing, Hangzhou, Zhejiang, China), 100 U/ml penicillin, and 100 U/ml streptomycin, and maintained in a humidified atmosphere of 95% air + 5% CO_2 at 37 °C.

2.3. Subcellular fractionation preparation

Mitochondrial and cytosolic fractions of cells were prepared using a mitochondrial/cytosol fractionation kit purchased from Biovision Inc. (Moutain View, CA). Briefly, cells treated with different concentrations of GA (1.5, 3 and 6 μM) for 10 h and then harvested with cold phosphate-buffered saline (PBS). Afterward the cells were lysed in 400 μl extraction buffer containing dithiothreitol and protease inhibitor cocktail on ice for 30 min with homogenizing operation using a Kontes Dounce tissue grinder (Fisher, CA). After 10 min centrifugation (700 × *g*), the supernatant was centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant (cytosolic fraction) was collected, and the pellets were resuspended in the mitochondrial extraction buffer (mitochondrial fraction). Cell nuclear and cytoplasmic fractions were prepared using a nuclear/cytosol fractionation kit of Biovision Inc. (Moutain View, CA) according to the manufature's direction. Protein concentrations of the fractions were measured with the Bradford protein assay reagent (Pierce, WI).

2.4. Mitochondrial membrane potential determination

Quantitative changes of MMP at the early stage of the cell apoptosis was measured using JC-1 probe (Molecular Probes Inc., Eugene, OR). JC-1 exists as a monomer with an emission at 530 nm (green fluorescence) at low concentrations but forms J-aggregates with an emission at 590 nm (red fluorescence) at high concentrations. Mitochondria with intact membrane potential concentrate JC-1 into aggregates, whereas de-energized mitochondria cannot concentrate JC-1. Therefore, the changes of fluorescence of JC-1 can be considered as an indicator of the relative mitochondrial membrane polarization state.

Cells were incubated for 10 h in the presence or absence (control) of GA with rotenone treatment as a positive control. All floating and attached cells were harvested, fixed with ice-cold 4% paraform for 20 min and washed with ice-cold PBS. Then the cells were permeabilized with 0.3% Triton X-100, washed with ice-cold PBS, stained with JC-1 (5 μM) and observed under a fluorescence microscope (Olympus IX51, Japan) with a peak excitation wavelength of 340 nm. For flow cytometry detection, cells were harvested and resuspended in RPMI-1640 medium at a density of 0.5×10^6 cells/ml, then incubated with 5 μM JC-1 at 37 °C for 20 min. Relative fluorescence intensities were monitored using the flow cytometry (FACSCalibur, Becton Dickinson), and analyzed by the software Modfit and CellQuest (BD Biosciences, Franklin Lakes, NJ).

2.5. Measurement of intracellular ROS level

For detecting the accumulation of intracellular ROS in SMMC-7721 cells. Reactive oxygen species assay kit purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China) was used according to the method described previously (Lluis et al., 2007). Briefly, at the end of each treatment, 5×10^4 cells were collected and resuspended in 100 μM dihydrodichlorofluorescein diacetate (H_2DCFDA) with serum-free medium. Intracellular H_2DCFDA was deesterified to dichlorodihydrofluorescein which is oxidized by ROS to produce the fluorescent compound dichlorofluorescein. After a 30-min incubation at 37 °C, the fluorescence intensity was measured using the fluorescence plate reader (BD Falcon, CA) at Ex./Em. = 488/525 nm.

2.6. Measurement of intracellular GSH level

Intracellular GSH level was determined using the ApoSENSOR glutathione detection kit (BioVision, CA) according to the manufacturer's instructions. Briefly, after the treatment of different concentrations of GA (1.5, 3 and 6 μM) for 10 h, cells were harvested and centrifuged at 700 × *g* for 5 min, then lysed in 100 μl ice-cold lysis buffer on ice for 10 min and centrifuged at 12,000 × *g* for 10 min at 4 °C afterward. The supernatant was subjected to the test by the glutathione detection kit. The fluorescence value was measured in a fluorometer or fluorescence plate reader (BD Falcon, CA) at Ex./Em. = 380/460 nm.

2.7. Western blot analysis

Cells were collected after treatment, then they were lysed in lysis buffer (100 mM Tris–HCl, pH 6.8, 4% (m/v) sodium dodecylsulfonate(SDS), 20% (v/v) glycerol, 200 mM β-mercaptoethanol, 1 mM phenylmethyl sulfonylfluoride, and 1 g/ml aprotinin) for 1 h on the ice. The lysates were clarified by centrifugation at 4 °C

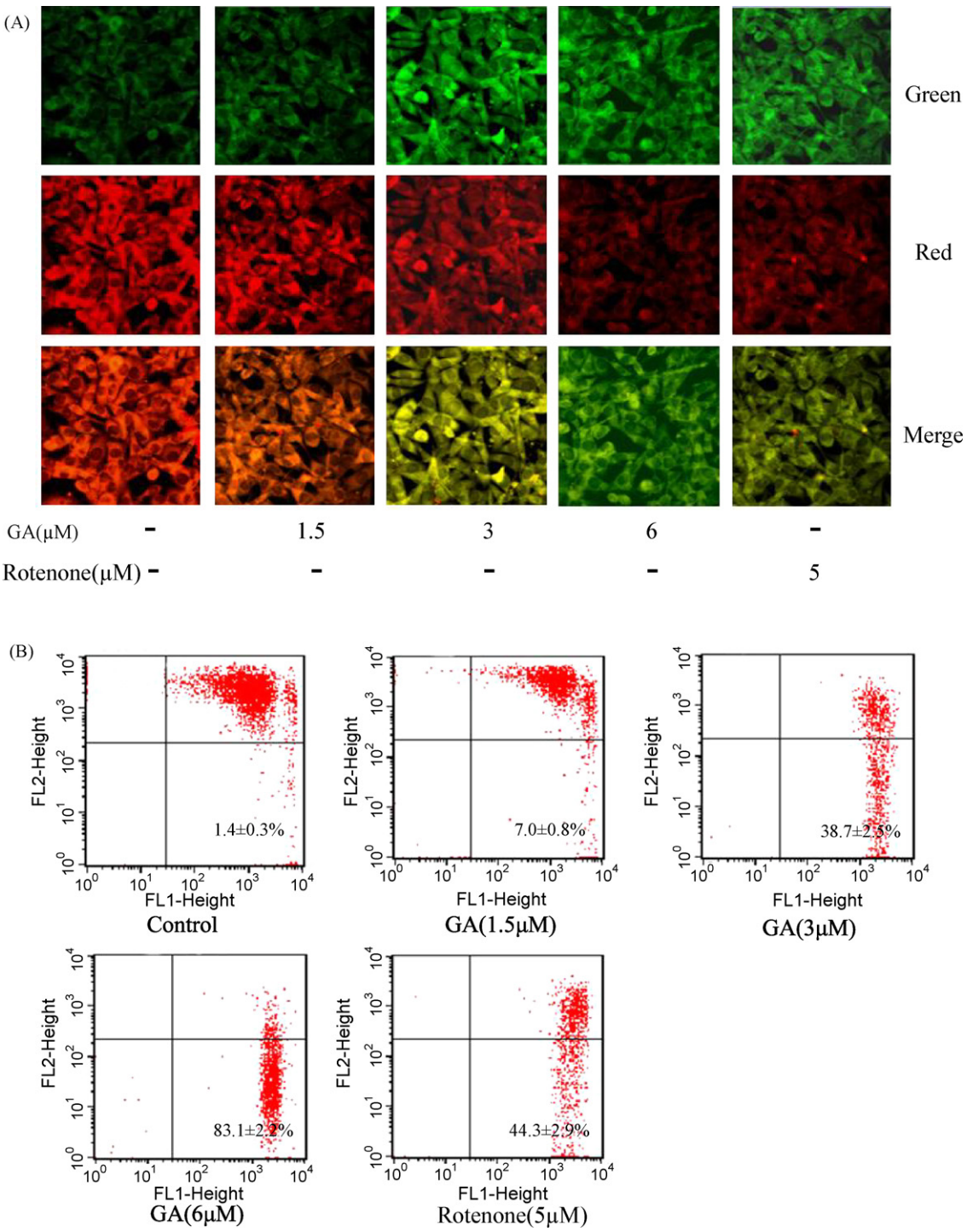

Fig. 2. The mitochondrial membrane potential decreased in GA-treated SMMC-7721 cells. (A) Cells were exposed to GA (1.5, 3 and 6 μM) or rotenone (5 μM) for 10 h, stained with JC-1 and visualized by confocal microscopy. (B) Uptake of JC-1 analyzed by flow cytometry. Cells with high and low red fluorescence (MMP) are found in the upper and lower right quadrant, respectively. The percentage in histogram of each profile represents the percentage of total cells with low fluorescence intensity. (For interpretation of the references to color, the reader is referred to the web version of the article.)

for 15 min at 12,000 rpm. The total protein concentration in the supernatants was detected using BCA assay with Varioskan multimode microplate spectrophotometer (Thermo, MA). The cytosolic, nuclear and mitochondrial fraction were prepared as described above. SDS-PAGE was carried out using 8–15% gradient or standard polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes, which were saturated with 10% milk in PBS/Triton X-100 (0.5%) and incubated with primary antibodies in 50 mM Tris–HCl buffer (pH 8.5, 500 mM NaCl, 0.1% Tween-20 and 0.1% bovine serum albumin) overnight at 4 °C. The membranes were washed three times with Tris-buffered saline containing Tween-20 buffer and incubated with the IRDye™ 800 conjugated anti-mouse and/or anti-rabbit secondary antibody for 1 h, followed by washing four times with PBS. Detection was performed with Odyssey Infrared Imaging System (LI-COR Inc., NE).

2.8. Measurement of ATP production

Intracellular ATP level was measured using the ApoSENSOR cell viability assay kit (BioVision, CA) according to the manufacturer's instructions. Briefly, cells were treated with GA (1.5, 3 and 6 μM), rotenone (5 μM) and glucose (1 mM) for 10 h, then incubated with 100 μl Nuclear Releasing Reagent for 5 min at 37 °C with gentle shaking, followed by further incubation with 1 μl ATP Monitoring Enzyme. Detection

was performed using the luminometer Orion II (Berthold DS, Bleichstr, Pforzheim, Germany).

2.9. Annexin V/PI double staining assay

Apoptotic cells were quantified using an Annexin V-FITC/PI kit (BioVision, CA, USA) and detected by flow cytometry (FACSCalibur, Becton Dickinson), and analyzed by the software Modfit and CellQuest (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, cells were pretreated 1 mM NAC for 1 h, then treated with 6 μM GA or/and 1 mM NAC for 10 h and washed with PBS. Then the cells were collected, resuspended in binding buffer (pH 7.5, 10 mM HEPES, 2.5 mM $CaCl_2$ and 140 mM NaCl), and incubated with Annexin V-FITC and PI for 10 min in the dark, then flow cytometric analysis were preformed. Cells in early stage of apoptosis were Annexin V positive; whereas Annexin V and PI positive cells were considered in the late stage of apoptosis.

2.10. Statistical analysis

All results shown represent means ± SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical differences were evaluated using the Student's *t*-test and considered significant at the $^{*}P < 0.05$ or $^{**}P < 0.01$ level. All the figures shown in this article were obtained from at least three independent experiments.

3. Results

3.1. GA decreases MMP in SMMC-7721 cells

The fate of cells succumbing to the intrinsic pathway of apoptosis is sealed by MMP. Loss of MMP is an important event during the mitochondrial pathway of apoptosis (Galluzzi and Kroemer, 2007), so we firstly investigated whether GA could induce the loss of MMP in SMMC-7721 cells. As shown in Fig. 2A, SMMC-7721 cells treated with GA for 10 h exhibited an increased green fluorescence signal and a decreased red fluorescence signal in a concentration-dependent manner. The percentage of fluoresced green increased to 6.5%, 38.73% and 85.19% in cells treated with GA at 1.5, 3 and 6 μM, respectively (Fig. 2B). These results demonstrated that GA diminished MMP in SMMC-7721 cells in a concentration-dependent manner.

3.2. GA increases the level of ROS in SMMC-7721 cells

Since a loss of MMP is associated with the generation of ROS (Chauhan et al., 2003), we detected the level of ROS in SMMC-7721 cells treated with various concentrations of GA for 10 h with the cellular oxidation of (H_2DCFDA), a probe that is oxidized to green fluorescent DCF by various peroxide-like ROS and nitric-oxide-derived reactive intermediates. As shown in Fig. 3A, the level of ROS in cells treated with GA was increased in a concentration-dependent manner with 1.8-fold increase for 6 μM GA treatment compared with that of vehicle-treated cells which is stronger than Rotenone (1.6-fold). These data demonstrated that GA significantly increased ROS production in SMMC-7721 cells, which may promote mitochondrial dysfunction and trigger mitochondria-mediated apoptosis. Excessively produced ROS could disturb the homeostasis between GSH and ROS. Then we tested the effect of GA on the intracellular GSH level in SMMC-7721 cells. As shown in Fig. 3B, 1.5, 3 and 6 μM GA treatment decreased the level of GSH by 17.05%, 32.75% and 72.59%, respectively compared with that of vehicle-treated cells.

3.3. GA induces pro-apoptotic proteins release from mitochondria to cytosol and triggers downstream cascade reactions

ROS generation induces apoptosis through the release of pro-apoptotic proteins such as Cyt *c* and AIF from mitochondria to the cytosol (Loeffler and Kroemer, 2000). In the present study, cells treated with different concentrations of GA for 10 h, the amount of Cyt *c* and AIF significantly decreased in mitochondria while

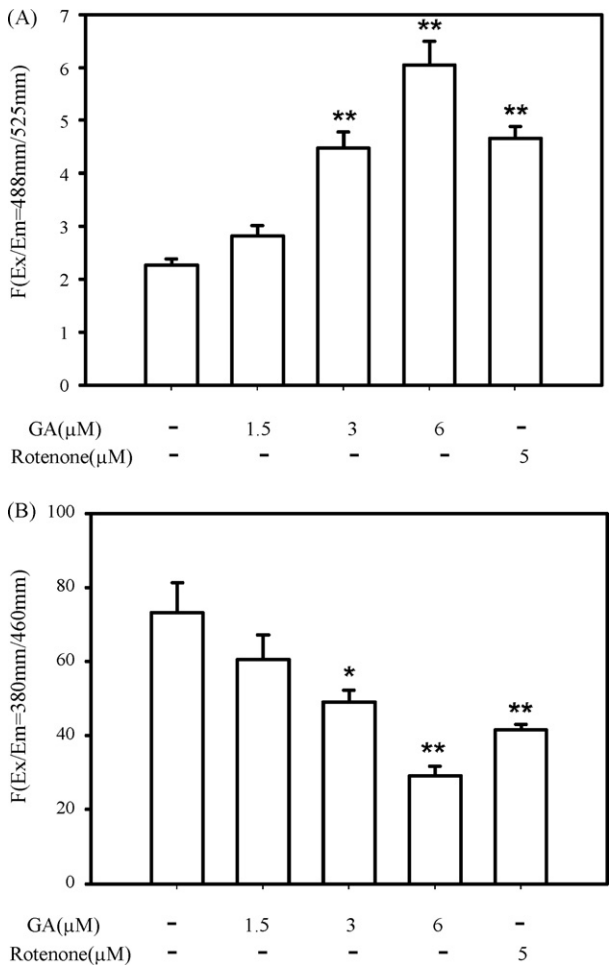

Fig. 3. GA increased ROS and decreased GSH levels in SMMC-7721 cells. Cells were treated with GA (1.5, 3 and 6 μM) or rotenone (5 μM) for 10 h, and then the intracellular levels of ROS (A) and GSH (B) were detected. Intracellular levels of ROS and GSH markedly increased and decreased in GA-treated cells, respectively, compared with that of vehicle control. Each sample was duplicated, and the figure is representative of three independent assays. Values are means ± S.D. for at least three independent experiments performed in triplicate ($^{*}P < 0.05$ and $^{**}P < 0.01$ compared with vehicle control).

increased in cytosol (Fig. 4). Cyt *c* releasing from mitochondria can activate caspase-9, which in turn activates executioner caspase-3 via cleavage induction. PARP, one important substrate of caspase-3 will be cleaved (Gambi et al., 2008), The results showed that expression of procaspase-9 was decreased and PARP was cleaved in GA-treated cells compared with control (Fig. 4B). AIF translocated from cytosol to nucleus, where it interacts with DNA, activates cyclophilin A (latent DNase), and to participate in chromatinolysis (Loeffler and Kroemer, 2000). Thus, we detected whether GA-induced relocation provokes this nuclear hallmark in SMMC-7721 cells. As shown in Fig. 4C, a portion of AIF was found in the nucleus while no AIF was detected in untreated cells.

3.4. GA decreases the intracellular ATP level in SMMC-7721 cells

To test whether the dysfunction of mitochondrial energy production occurred in GA-treated cells, we investigated the changes of intracellular ATP level in the GA-treated cells with rotenone (5 μM) and glucose (1 mM) as a positive and negative control. As shown in Fig. 5, after 10 h treatment with various concentrations of GA, the ATP level decreased in a concentration-dependent manner. The ATP level in cells treated with 1.5 μM GA was similar to

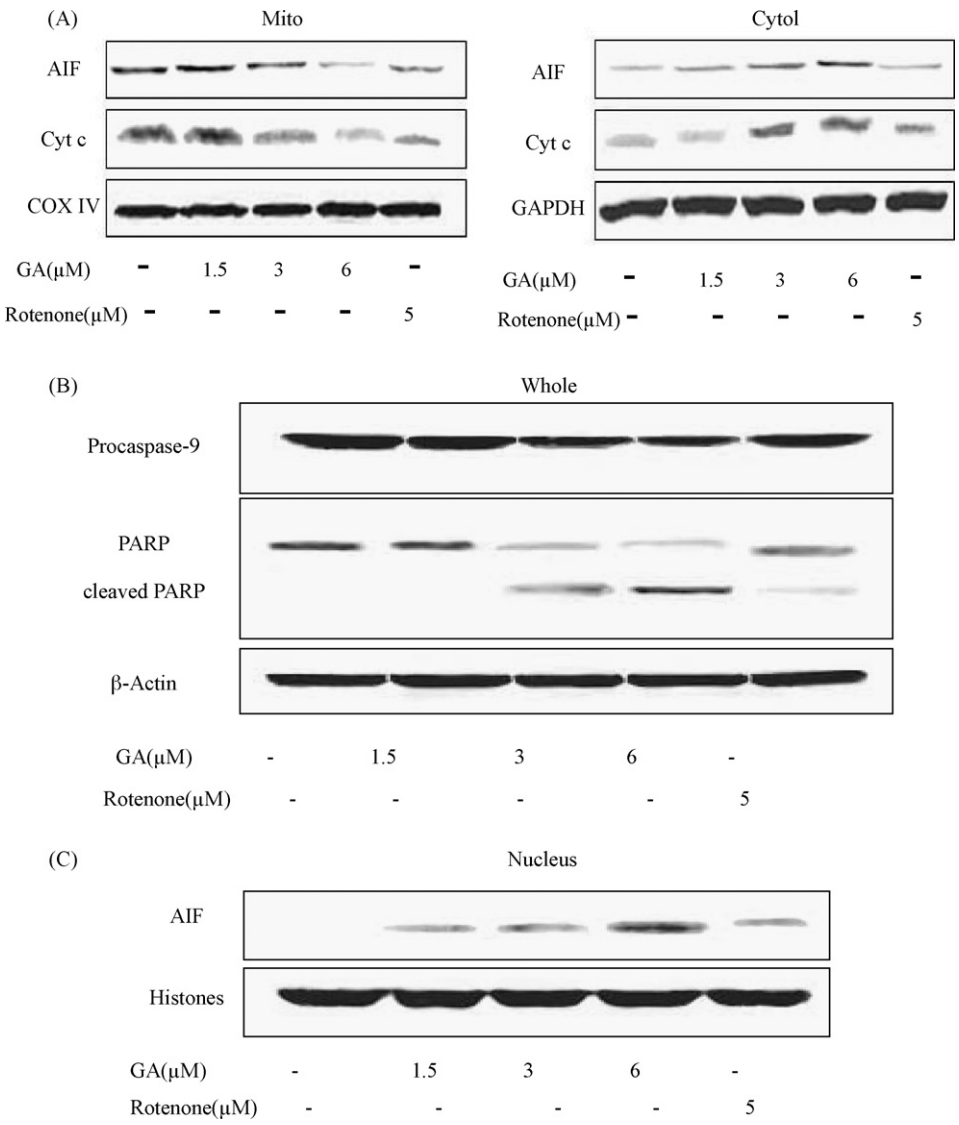

Fig. 4. Effects of GA on apoptosis-related proteins in SMMC-7721 cells. (A) Effect of GA on the subcellular redistributions of Cyt *c* and AIF in SMMC-7721 cells. Mitochondrial and cytosolic fractions were isolated as described in Section 2, and subjected to Western blot analysis. (B) Western blotting analysis of PARP, Procaspase-9 of SMMC-7721 cells. (C) Effect of GA on AIF translocation in SMMC-7721 cells. Nuclei fractions were isolated as described in Section 2, and subjected to Western blot analysis. Cells were treated with GA (1.5, 3 and 6 μM) or rotenone (5 μM) for 10 h, respectively. All Western blots were representatives of three independent experiments.

that of vehicle-treated cells, whereas those in cells treated with GA 3 and 6 μM decreased to 60.3% and 33.8% of that of untreated cells, respectively, which was more significant than the effect of rotenone.

3.5. Antioxidant NAC partly reverses GA-mediated ROS generation in SMMC-7721 cells

To illustrate the role of ROS in GA induced apoptosis and related signaling pathway, SMMC-7721 cells were treated with GA in the presence or absence of antioxidant, NAC. As shown in Fig. 6, NAC essentially abrogated GA-mediated generation of ROS (Fig. 6A) and apoptosis (Fig. 6B). For it has been reported that ROS could activate MAPK pathway and induce cell apoptosis (Boutros et al., 2008), here we also investigated the role of ROS in MAPK pathway activation in cells treated with GA. Cells were exposed to 6 μM GA for 10 h, the levels of phosphorylated JNK and p38 was increased, whereas the level of phosphorylated ERK remained unchanged (Fig. 6C). Moreover, the activation of JNK and p38 in cells treated with GA could be partly abolished by NAC (Fig. 6C).

4. Discussion

We have previously demonstrated that GA induces apoptosis through a caspase-dependent way in several human cancer cell lines (Guo et al., 2006, 2004; Liu et al., 2005; Wu et al., 2004; Zhao et al., 2004). However, the molecular mechanism of apoptosis and the precise characters of the mitochondriotoxicity of this agent have not been well elucidated. Loss of MMP is a key event of mitochondria-dependent apoptosis. In the present study, the results showed the significant decrease of MMP in SMMC-7721 cells treated with GA (Fig. 2). This indicated that GA-induced apoptosis possibly occurs via a mitochondrial pathway.

ROS are known to induce the collapse of MMP, therefore trigger a series of mitochondria-associated events including apoptosis (Park et al., 2005). Structure–activity relationship analysis showed that the tricyclic ring and α,β-unsaturated ketone present in the structure of GA, which are relevant for its cytotoxicity (Kasibhatla et al., 2005). Therefore, the presence and putative activity of α,β-unsaturated ketone suggested that GA can induce the generation of ROS, which can be signaling messenger that promote apop-

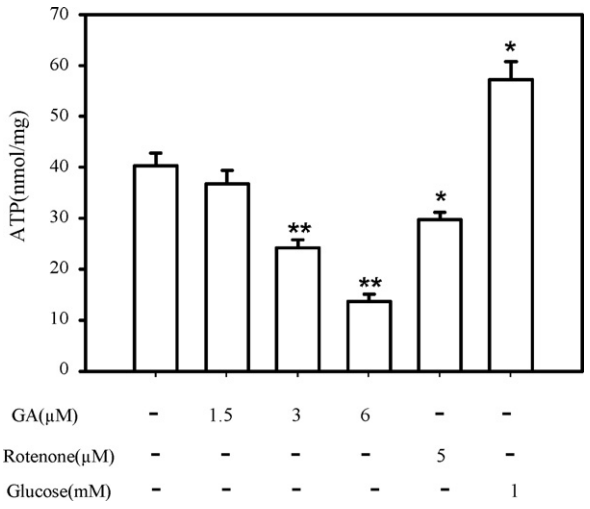

Fig. 5. Inhibitory effects of GA on intracellular ATP level in SMMC-7721 cells. Cells were treated with GA (1.5, 3 and 6 μM), rotenone (5 μM) or glucose (1 mM) for 10 h and then the intracellular ATP was detected. Values are means ± S.D. for at least three independent experiments performed in triplicate ($^{*}P < 0.05$ and $^{**}P < 0.01$ compared with vehicle control).

tosis (Zhang and Chen, 2004). We found that GA increased the level of ROS in cancer cells, which was coincident with the data published recently (Ortiz-Sanchez et al., 2009). Loss of MMP also induces apoptosis by causing the release of pro-apoptotic factors, such as Cyt *c* and AIF from the mitochondrial inner space to cytosol (Nonn et al., 2003). Cyt *c* in cytosol exerts its apoptogenic effects through participating in the activation of capase-9, which in turn activated executioner caspase-3 (Green, 2005). As one of the identified substrates of caspases-3, PARP is involved in the repair of DNA damage induced by certain anticancer agents and or radiation. During apoptosis, caspase-3 cleave PARP into two fragments, p89 and p24, thus suppressing PARP activity (Gambi et al., 2008). AIF is expelled from mitochondria and translocate to the nucleus after some apoptotic stimuli, which contribute to DNA and nuclear fragmentation, thereby functioning via a caspase-independent pathway (Modjtahedi et al., 2006). As demonstrated in the present study, Cyt *c* released from mitochondria to cytosol, caspase-9 was activated, and PARP was cleaved in GA-treated SMMC-7721 cells (Fig. 4A and B). Our result also indicated that AIF released from mitochodria to cytosol and translocated to nucleus (Fig. 4A and C). Moreover, the mitochondrion is at the core of cellular energy metabolism, whose oxidative phosphorylation is the major ATP synthetic pathway. During this process, mitochondrial

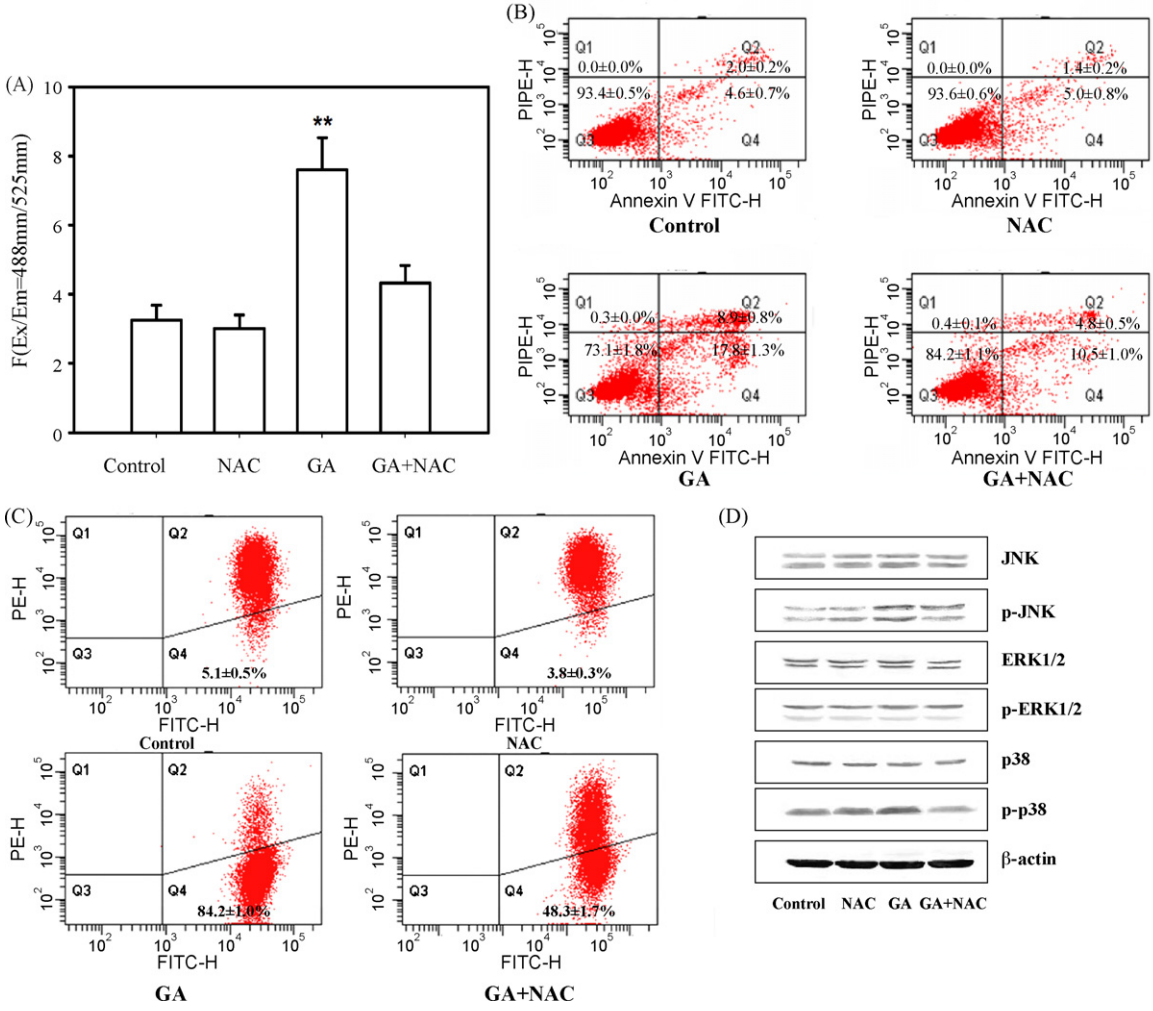

Fig. 6. GA-induced apoptosis in SMMC-7721 cells can be partly reversed by NAC. (A) Cells were pretreated with 1 mM NAC for 1 h, then treated with/without 6 μM GA for 10 h, then ROS level was detected. Values are means ± S.D. for at least three independent experiments performed in triplicate ($^{*}P < 0.01$ compared with vehicle control). (B) Cells treated as in (A) were incubated with Annexin V and PI for 15 min at 37 °C and then subjected to flow cytometry analysis. The figure is representative of three independent experiments yielding similar results. (C) Cells treated as in (A) and whole cell extracts were collected in lysis buffer, applied to each lane and subjected to electrophoresis by 12% SDS-PAGE. p-JNK, p-ERK1/2, p-p38, JNK, ERK1/2 and p38 were identified by Western blot analysis as described in Section 2. Blots shown were representatives of three observations.

complexes I–IV participated in the respiratory chain and establish a H^+ gradient across the inner mitochondrial membrane, and then the electrochemical energy of this gradient can be used to drive ATP synthesis by complex V (ATP synthase) (Loeffler and Kroemer, 2000). MMP diminishment in GA-treated cells indicated that the H^+ gradient disturbed, with consequent loss of Cyt *c* interrupts electron flow between respiratory chain complexes III and IV, thereby causing loss of ATP (Fig. 5). All these changes have been described to occur during apoptosis and should be intrinsically lethal.

ROS could be scavenged by the redox-related enzymes, such as GSH, catalase, superoxide dismutase, and thioredoxin which can protect cells against ROS-induced toxicity (Lluis et al., 2007). Low GSH level is sometimes associated with mitochondrial dysfunction and induction of apoptosis, thereby decreasing the chemoresistance of tumors (Ramos et al., 2006). Our results also showed that GA dramatically stimulated GSH depletion (Fig. 3B). In addition, GA treatment with NAC, an antioxidant protect the cells from oxidative stress, significantly decreased the amount of GA-induced ROS generation (Fig. 6A). And the apoptosis induced by GA was also partially blocked by NAC in SMMC-7721 cells (Fig. 6B). Moreover, ROS signaling appears to be triggered by the activation of the mitochondrial-dependent cell death pathway through activation of the MAPK pathways, with subsequent loss of MMP and cell death (Kim et al., 2005; Kuo et al., 2007; Zhang and Chen, 2004). Our previous study have shown that GA can elevate the phosphorylation of JNK and p38 in MCF-7 cells (Chen et al., 2008), therefore we attempted to investigate whether the MAPK pathways were involved in GA-treated SMMC-7721 cells. The results indicated that GA is an activator of MAPK including ERKs, JNK, and p38 kinase in SMMC-7721 cells (Fig. 6C). We further observed that GA-induced elevation of p38 and JNK phosphorylation could also be partially reversed by NAC (Fig. 6C). These data suggested that ROS might act as an upstream signal that triggers p38 and JNK activation in SMMC-7721 cells.

In summary, our data indicated that GA can induce ROS production, lead to loss of MMP, decrease the levels of ATP and GSH, activate stress-responsive p38 and JNK pathway in SMMC-7721 cells. GA-induced apoptosis may act, at least partially, though a ROS-related mitochondrial pathway in SMMC-7721 cells. Future investigation should be undertaken to address the detailed molecular mechanism of GA interacting with mitochondria and stimulating ROS generation.

Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2009.03.010.

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