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Anti-oxidative and Anti-inflammatory Activities of Three Triterpenoid Acids in Human Bronchial Epithelial BEAS-2B Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMT designed this study. Authors MCY and ZHW performed experiments and wrote this manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Protective effects of three triterpenoid acids, asiatic acid (AA), glycyrrhizic acid (GA) or oleanolic acid (OA), for BEAS-2B cells, human bronchial epithelial cells, against hydrogen peroxide (H_2O_2) induced injury were examined. Cells were pre-treated with AA, GA or OA at 4 or 8 μmol/l for 48 hr at 37°C, and followed by 100 μM H_2O_2 treatment for 4 hr at 37°C. Cell viability, mitochondrial membrane potential, oxidative, apoptotic and inflammatory factors were measured. Western blot analysis was used to determine protein expression. Results showed that H_2O_2 treatment reduced both Na^+-K^+-ATPase activity and mitochondrial membrane potential of BEAS-2B cells. Pre-treatments from triterpenoid acids maintained Na^+-K^+-ATPase activity and mitochondrial membrane potential. H_2O_2 stimulated the formation of reactive oxygen species, interleukin-6, tumor necrosis factor-α and prostaglandin E_2 in BEAS-2B cells. Three triterpenoid acids pre-treatments lowered

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the levels of these factors. H_2O_2 enhanced the protein expression of $p47^{phox}$, $gp91^{phox}$, nuclear factor-κB (NF-κB) and p-p38. AA, GA or OA pre-treatments suppressed the expression of $p47^{phox}$, NF-κB p65 and p-p38; but only at 8 μmol/l decreased $gp91^{phox}$ expression. These findings suggest that these triterpenoid acids are potent protective agents for bronchial epithelial cells to mitigate oxidative and inflammatory injury.

Keywords: Triterpenoid acids; bronchial epithelial cells; ROS; NF-κB; Na^+-K^+-ATPase activity.

1. INTRODUCTION

Environmental contamination such as air pollution causes impairment in human respiratory system. Bronchial epithelial cells act as barriers for preventing the invasion of toxic materials including particulate matter, cigarette or heavy metals, which consequently protects lung and benefits the normal functions of respiratory system [1,2].

Oxidative stress due to the massive production of reactive oxygen species (ROS) is an important pathophysiological contributor toward the development and progression of airway disorders including lung cancer and asthma [3,4]. NADPH oxidase is an enzyme complex consisted of several subunits like $p47^{phox}$ and $gp91^{phox}$. The activation of NADPH oxidase is the major ROS source in bronchial epithelial cells, and it is involved in oxidation induced bronchial cell death [5,6]. It is known that ROS could activate nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) pathways in airway epithelium, which in turn stimulates the release of inflammatory mediators such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 [7,8]. In addition, increased cyclooxygenase-2 (COX-2) activity and prostaglandin E_2 (PGE_2) generation in bronchial epithelium also enhance respiratory inflammatory stress [9,10]. Therefore, any agent with the capability to decrease ROS and PGE_2 generation, and suppress the protein expression of NADPH oxidase, NF-κB and/or p38MAPK may be able to protect airway epithelial cells against oxidative and inflammatory injury.

Asiatic acid (AA), glycyrrhizic acid (GA), and oleanolic acid (OA) are pentacyclic triterpenoid acids naturally present in some edible plant foods such as brown mustard (*Brassica juncea*), basil (*Ocimum basilicum*), hawthorn fruit (*Crataegi pinnatifidae* fructus) and licorice (*Glycyrrhiza glabra*) [11-13]. The study of Yin et al. [11] revealed that dietary intake of AA and OA in mice increased the bioavailability of these compounds in mice tissues like liver or heart. It is reported that these three pentacyclic triterpenoid acids possess anti-oxidative and anti-inflammatory activities [14,15]. Wächter et al. [16] indicated that OA could protect respiratory system through its anti-tubercular activity. Liu et al. [17] reported that ursolic acid, an OA isomer, at 3.2, 6.3, 12.5 and 25 μmol/l, protected human normal bronchial epithelial cells against cigarette smoke extract induced injury, and led to cell survival rate at 73-86%. These previous studies suggest that OA and other pentacyclic triterpenoid acids like AA and GA may benefit survival and functions of human bronchial epithelial cells.

BEAS-2B cells are human normal bronchial epithelial cells, and have been widely used in researches about bronchial disorders [1,18]. Hydrogen peroxide is an oxidant in cigarette smoke extract, could induce apoptotic and oxidative damage in bronchial epithelial cells [19]. Thus, BEAS-2B cells were used in our present study to examine the protective effects of AA, GA and OA against hydrogen peroxide caused oxidative, apoptotic and inflammatory injury. The influence of these triterpenoid acids upon the activity and/or protein expression of COX-2, NADPH oxidase, NF-κB and p38MAPK was evaluated.

2. MATERIALS AND METHODS

2.1 Materials

AA, GA, OA (95%) and Rhodamine123 (Rh123) were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). BEAS-2B cell line was bought from American Type Culture Collection (Rockville, MD, USA). Medium, plates and antibiotics used for cell culture were purchased from Difco Laboratory (Detroit, MI, USA).

2.2 Experimental Design and Cell Culture

Cells were treated by each compound for 24, 36 or 48 hr. After washed twice by phosphate buffer

saline (PBS), the concentration of each compound in collected PBS was determined according to the HPLC method described by Yin et al. [11]. The compound retained in cells was defined as incorporated. Our preliminary experiments revealed that 24, 36 and 48 hr incubation resulted in 47.8, 70.5 and 95.9% incorporation of test compound into BEAS-2B cells. Thus, 48 hr incubation was applied for our present study. On the other hands, the improvement upon cell survival of test compounds at 1, 2, 4 and 8 μmol/l against H_2O_2 was also evaluated in preliminary experiments, and results showed that these agents at 1 or 2 μmol/l did not significantly increase cell survival. Consequently, these compounds at 4 and 8 μmol/l were used for present study. Three triterpenoid acids were dissolved in dimethyl sulfoxide (DMSO) and further diluted with bronchial epithelial growth medium (BEGM). The final DMSO concentration in cell culture was less than 0.5%, and did not affect any measurements. BEAS-2B cells were cultured in BEGM containing 10% fetal calf serum, L-glutamine, 100 units/ml streptomycin and 100 units/ml penicillin (pH 7.4) under CO_2 and air at 5% and 95%, respectively, at 37°C. BEAS-2B cells were sub-cultured every week and culture medium was changed every 3 days. Cell number at 10^5/ml was adjusted by PBS for experiments and analyses. After aspiration, BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l at 37°C for 48 hr, and washed twice by PBS. Then, BEAS-2B cells were treated with 100 μM H_2O_2 at 37°C for 4 hr. Control groups had neither test compound nor H_2O_2 treatment.

2.3 Cell Viability Determination

Cell viabiliaty was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT at 0.25 mg/ml was added into medium, and incubated at 37°C for 3 hr. MTT formazan product was then quantified by a Bio-Rad microplate reader (Hercules, CA, USA) to monitor the change of absorbance at 570 nm, with 630 nm as a reference wavelength. Cell viability was presented as a percentage of control groups.

2.4 Assay of DNA Fragmentation

DNA fragmentation was measured by a cell death detection kit obtained from Roche Molecular Biochemicals (Mannheim, Germany). In brief, cells were mixed with 5 ml lysis buffer containing HEPES (10 mM, pH 7.9), PMSF (1 mM), DTT (1 mM), KCl (10 mM), $MgCl_2$ (1.5 mM), EDTA (0.1 mM) and Nonidet P-40 (0.6%) for 30 min. After centrifugation at 200 xg for 10 min, 20 μl supernatant was added on the plate pre-coated by streptavidin. Then, 80 μl immunoreagent was added and incubated at 25°C for 2 hr. After washing twice with PBS and adding substrate solution, the mixture was further incubated for 15 min. The change in absorbance at 405 nm, with 490 nm as a reference wavelength, was recorded by a microplate reader. DNA fragmentation, expressed as enrichment factor, was measured by the equation: absorbance of sample ÷ absorbance of control.

2.5 Mitochondrial Membrane Potential (MMP) Measurement

MMP was determined by a Beckman-FC500 flow cytometry (Beckman Coulter, Fullerton, CA, USA) and a fluorescent dye, Rh123. After centrifugation at 1200 xg for 5 min, cells were collected, re-suspended in BEGM medium and mixed with Rh123 at 100 μg/l. After 45 min incubation at 37°C, cells were collected and washed twice by PBS. Fluorescence intensity was measured by a flow cytometry, and mean fluorescence intensity (MFI) was recorded.

2.6 Isolation of Mitochondrial Fractions

After treated by the lysis buffer containing HEPES (10 mM, pH 7.9), PMSF (1 mM), DTT (1 mM), KCl (10 mM), $MgCl_2$ (1.5 mM), EDTA (0.1 mM) and Nonidet P-40 (0.6%) for 30 min, lysed cells were centrifuged at 200 xg, 4°C for 10 min. The supernatant was collected and centrifuged again at 4°C, 10,000 xg for 20 min to obtain mitochondrial pellet. These pellets were re-suspended in PBS, and protein concentration was determined by a kit (Pierce Biotechnology Inc., Rockford, IL, USA).

2.7 Determination of Na^+-K^+-ATPase Activity

Na^+-K^+-ATPase activity was determined by measuring the released inorganic phosphate (Pi) from ATP. The mitochondrial fraction of cells was mixed with Tris-HCl buffer (pH 7.4, 30 mM), and followed by adding 2 mM ATP to initiate this assay. After incubating at 37°C for 15 min, reaction was terminated by adding trichloroacetic acid (15%). The change in absorbance of 640 nm was monitored for Pi level. Activity was shown as a percentage of control groups.

2.8 Assay of Caspase Activity

Caspase-3 or -8 activity was assayed by fluorometric assay kits (Upstate, Lake Placid, NY, USA) according to the instruction of manufacturer. The inter-assay and intra-assay CV values were 4.4-6.3% and 4.5-5.1%, respectively. Cells were lysed by lysis buffer. Cell lysate at 50 μl was mixed with 25 ml reaction buffer and 5 ml fluorogenic specific substrates for either caspase-3 or -8. After incubating at 37°C for 1 hr, fluorescence was measured by a fluorophotometer (Hitachi F-4500, Tokyo, Japan), in which excitation and emission wavelengths were set at 400 nm and 505 nm, respectively. Activity was expressed as a percentage of control groups.

2.9 Measurement for ROS, 8-Hydroxydeoxyguanosine (8-OHdG) and Glutathione (GSH) Levels

After washing and re-suspending in medium, cells were reacted with $DCFH_2$-DA at 50 μmol/l for 30 min to measure ROS level. Cell were washed and re-suspended by PBS, and centrifuged at 4°C, 412 xg for 10 min. Then, Triton X-100 at 1% was added to dissolve the collected cells. The fluorescence change was recorded by a fluorescence microplate reader. Both excitation and emission wavelengths were set at 485 nm and 530 nm. Result was expressed as relative fluorescence unit (RFU)/mg protein. A DNA Extractor WB kit (Wako Pure Chemical Industries Ltd., Tokyo, Japan) was used to extract DNA fraction of cells. This fraction at 1 mg/ml was dissolved in distilled water. 8-OHdG is an indicator for DNA oxidative injury. An ELISA assay kit for 8-OHdG analysis was purchased from OXIS Health Products Inc. (Portland, OR, USA), and the detection limit was 10 pg/mg protein. A colorimetric GSH kit (OxisResearch, Portland, OR, USA) was used to determine GSH concentration (ng/mg protein).

2.10 Quantification for TNF-α, IL-6, PGE_2 and COX-2

Cells were homogenized and centrifuged, supernatants were collected and used for determining TNF-α or IL-6 level (pg/mg protein) by cytoscreen immuno-assay kits (BioSource International, Camarillo, CA, USA). PGE_2 level (pg/mg protein) and COX-2 activity were assayed by commercial kits purchased from Cayman Chemical Co. (Ann Arbor, MI, USA).

2.11 Western Blot Analyses

After washing by PBS, cells were scraped and suspended in lysis buffer. The protein concentration was determined by a commercial kit purchased from Pierce Biotechnology Inc. (Rockford, IL, USA), in which bovine serum albumin was used as a standard. Protein sample at 40 μg was applied for gel electrophoresis containing SDS-polyacrylamide at 10%, and followed by transferring to a Millipore nitrocellulose membrane (Bedford, MA, USA) for 1 hr. After treated with a solution containing 5% non-fat milk for another 1 hr for blocking non-specific antibody binding, nitrocellulose membrane was further reacted with monoclonal antibodies (Boehringer-Mannheim, Indianapolis, IN, USA) against Bcl-2, Bax, $p47^{phox}$, $gp91^{phox}$, NF-κB p50, NF-κB p65, p38 or p-p38 (1:1000) at 4°C overnight. Sample was further reacted with horseradish peroxidase conjugated antibody for 3.5 hr at 25°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the loading control, and the produced bands were processed by an image analyzer (Tokyo, Japan). Data were quantified against GAPDH.

2.12 Statistical Analysis

Three different preparations were used to examine the effects of treatments (n=3), and duplicates were performed for each preparation. Statistical analysis was processed by one-way ANOVA. Post-hoc comparison was processed by Student's *t*-test. Difference was $p<0.05$ level was considered to be significant.

3. RESULTS

3.1 Effects of AA, GA or OA upon Cell Viability

AA, GA or OA at test doses alone did not affect BEAS-2B cells viability (Fig. 1, $p>0.05$). H_2O_2 treatment reduced viability and increased DNA fragmentation of BEAS-2B cells (Fig. 2, $p<0.05$). However, AA, GA or OA pre-treatments raised cell survival and decreased DNA fragmentation in BEAS-2B cells ($p<0.05$).

3.2 Effects of AA, GA or OA upon Mitochondrial Stability

H_2O_2 decreased Na^+-K^+-ATPase activity and MMP in BEAS-2B cells (Table 1, $p<0.05$). AA, GA or OA pre-treatments reserved Na^+-K^+-ATPase activity and MMP ($p<0.05$). H_2O_2 treatment also increased the activity of caspase-

3 and caspase-8 in BEAS-2B cells (Fig. 3, p<0.05). AA, GA or OA pre-treatment lowered caspase-3 activity; and only at 8 μmol/l reduced caspase-8 activity (p<0.05).

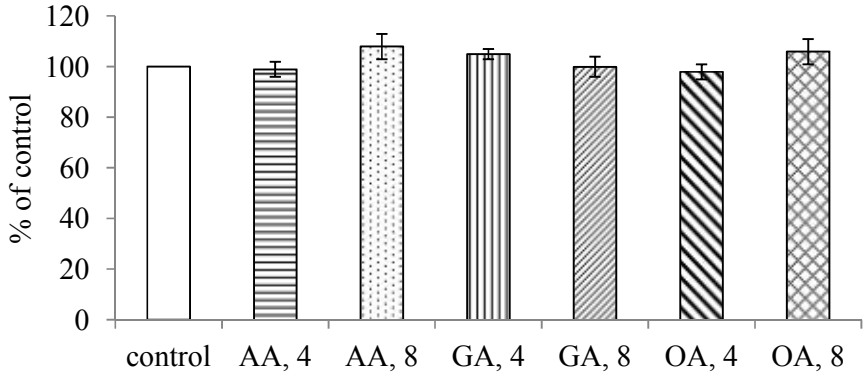


Fig. 1. Effects of AA, GA and OA upon the growth of BEAS-2B cells, without H_2O_2 treatment. BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l for 48 hr

Control groups were cells without test compound. Data are mean±SD (n=3)

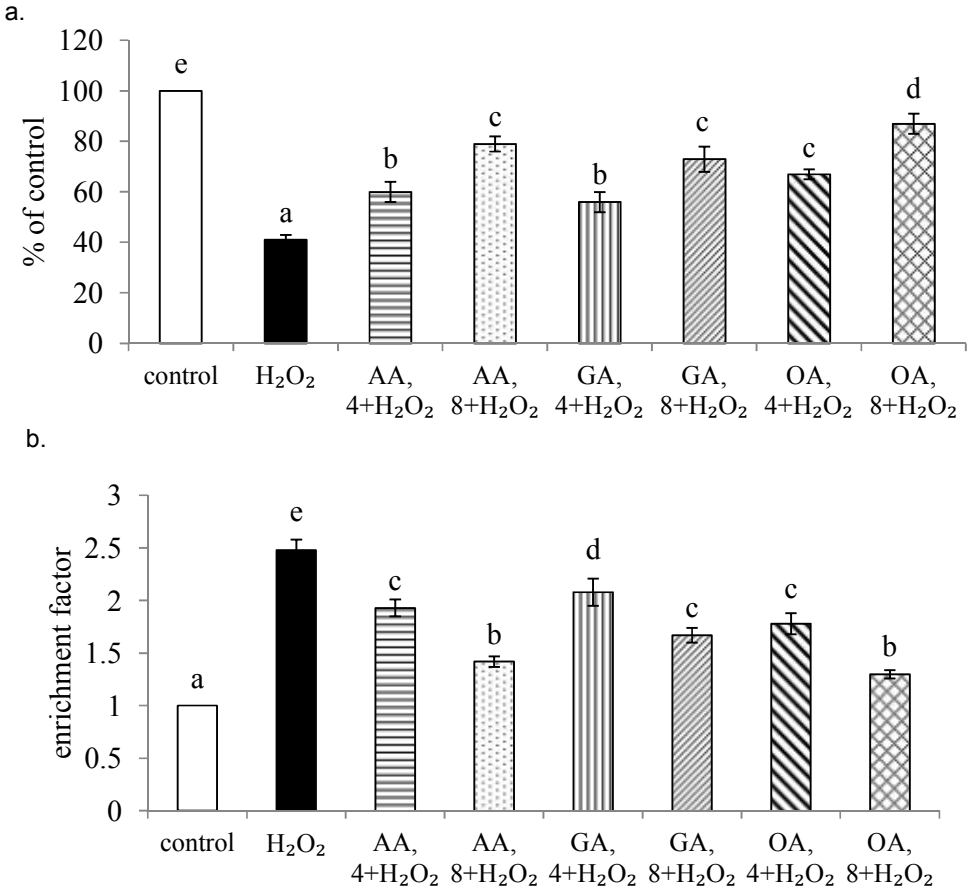


Fig. 2. Effects of AA, GA and OA upon viability (a) and DNA fragmentation, expressed as an enrichment factor (b) in BEAS-2B cells. BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l for 48 hr, and H_2O_2 at 100 μM was added to induce cell injury

Control groups were cells contained neither test compound nor H_2O_2 treatment. Data are mean±SD (n=3).
$^{a-e}$values among bars without a common letter differ, p<0.05

3.3 Effects of AA, GA or OA upon Bcl-2 and Bax Expression

H_2O_2 treatment down-regulated Bcl-2 expression and up-regulated Bax expression in BEAS-2B cells (Fig. 4, p<0.05). AA, GA or OA pre-treatments increased Bcl-2 expression (p<0.05); but did not alter Bax expression (p>0.05). The pre-treatments of AA, GA, or OA also raised the ratio of Bcl-2/Bax (p<0.05).

Table 1. Effects of AA, GA or OA upon mitochondrial membrane potential, determined as MFI, and Na^+-K^+-ATPase activity in BEAS-2B cells. BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l for 48 hr, and H_2O_2 at 100 μM was added to induce cell injury

	MFI	Na^+-K^+-ATPase activity
Control	100^e	100^e
H_2O_2	30±4^a	43±2^a
AA, 4+ H_2O_2	63±2^c	63±3^c
AA, 8+ H_2O_2	77±4^d	80±4^d
GA, 4+ H_2O_2	45±3^b	52±4^b
GA, 8+ H_2O_2	67±5^c	67±2^c
OA, 4+ H_2O_2	65±2^c	69±5^c
OA, 8+ H_2O_2	81±3^d	83±3^d

Control groups were cells contained neither test compound nor H_2O_2 treatment. Data are mean±SD (n=3). $^{a-e}$values in a column without a common letter differ, p<0.05

3.4 Effects of AA, GA or OA upon Oxidative and Inflammatory Stress

H_2O_2 treatment enhanced ROS and 8-OHdG production, and lowered GSH level in BEAS-2B cells (Table 2, p<0.05). AA, GA or OA pre-treatments decreased ROS and 8-OHdG levels; and increased GSH content (p<0.05). H_2O_2 increased TNF-α, IL-6 and PGE_2 levels (Table 3, p<0.05). AA, GA or OA pre-treatments reduced TNF-α, IL-6 and PGE_2 levels, and COX-2 activity (p<0.05).

Table 2. Effects of AA, GA or OA upon level of ROS (RFU/mg protein), 8-OHdG (pg/mg protein) and GSH (ng/mg protein) in BEAS-2B cells. BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l for 48 hr, and H_2O_2 at 100 μM was added to induce cell injury

	ROS	8-OHdG	GSH
Control	0.11±0.02^a	21±4^a	114±8^e
H_2O_2	2.21±0.19^f	188±10^e	49±3^a
AA, 4+ H_2O_2	1.39±0.08^d	143±7^d	64±2^b
AA, 8+ H_2O_2	0.67±0.05^b	80±5^b	85±7^d
GA, 4+ H_2O_2	1.62±0.15^e	152±11^d	58±4^b
GA, 8+ H_2O_2	1.04±0.1^c	109±9^c	78±5^c
OA, 4+ H_2O_2	1.3±0.12^d	122±12^c	73±3^c
OA, 8+ H_2O_2	0.72±0.06^b	77±4^b	89±4^d

Control groups were cells contained neither test compound nor H_2O_2 treatment. Data are mean±SD (n=3). $^{a-f}$values in a column without a common letter differ, p<0.05

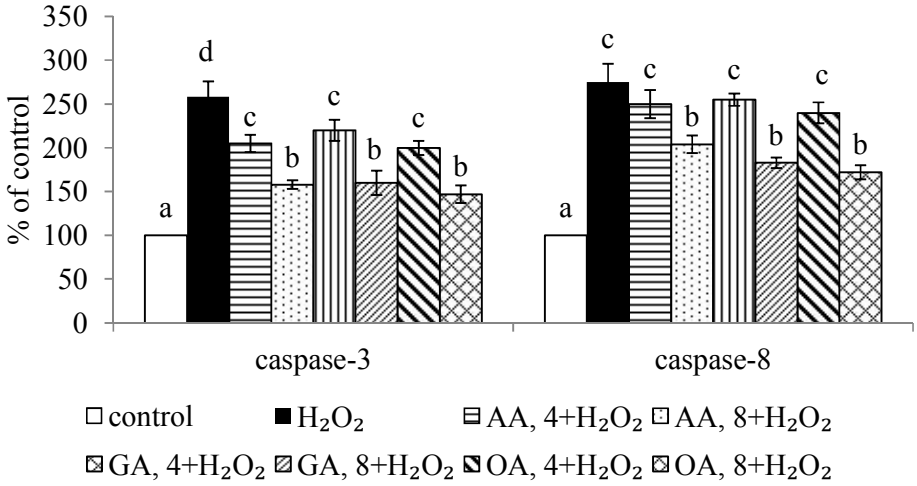
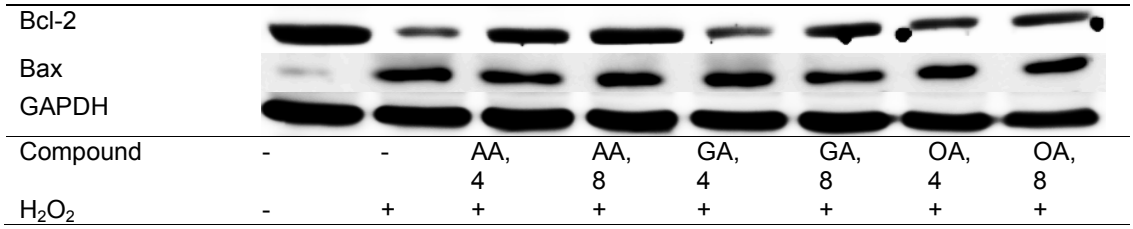


Fig. 3. Effects of AA, GA and OA upon the activity of caspase-3 and caspase-8 in BEAS-2B cells. BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l for 48 hr, and H_2O_2 at 100 μM was added to induce cell injury

Control groups were cells contained neither test compound nor H_2O_2 treatment. Data are mean±SD (n=3). $^{a-d}$values among bars without a common letter differ, p<0.05



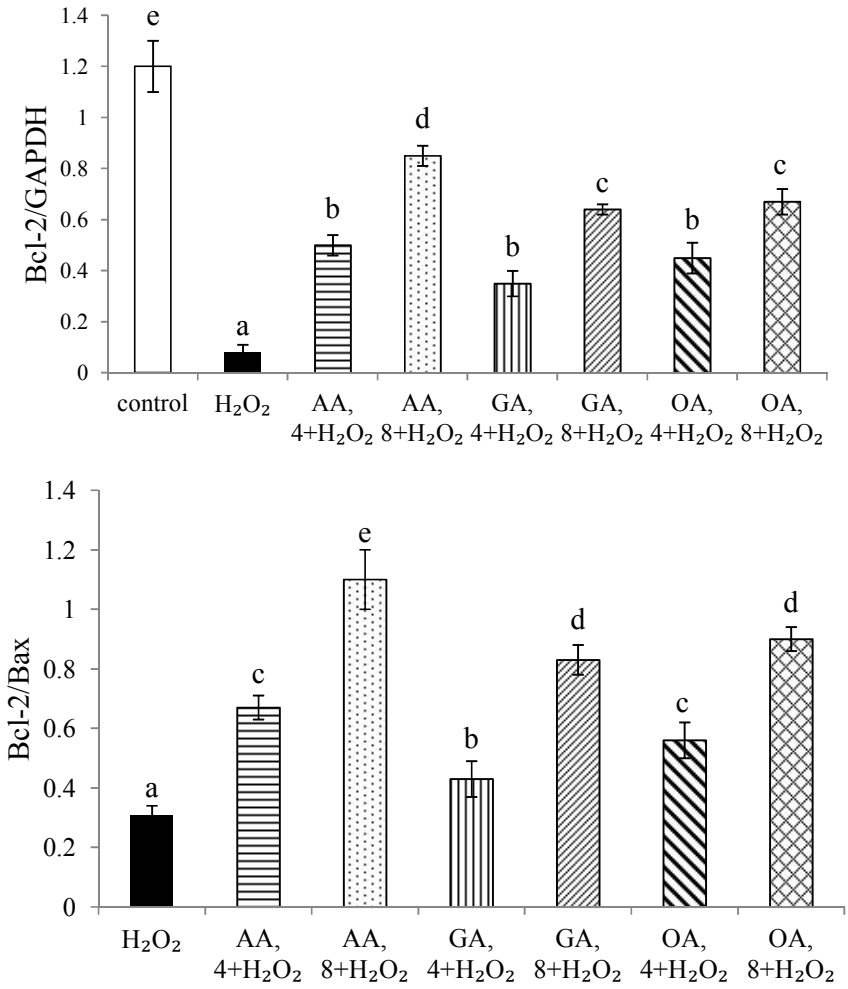


Fig. 4. Effects of AA, GA or OA upon protein expression of Bcl-2 and Bax, and ratio of Bcl-2/Bax in BEAS-2B cells. BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l for 48 hr, and H_2O_2 at 100 μM was added to induce cell injury

Control groups were cells contained neither test compound nor H_2O_2 treatment. Data are mean±SD (n=3). $^{a-e}$values among bars without a common letter differ, p<0.05

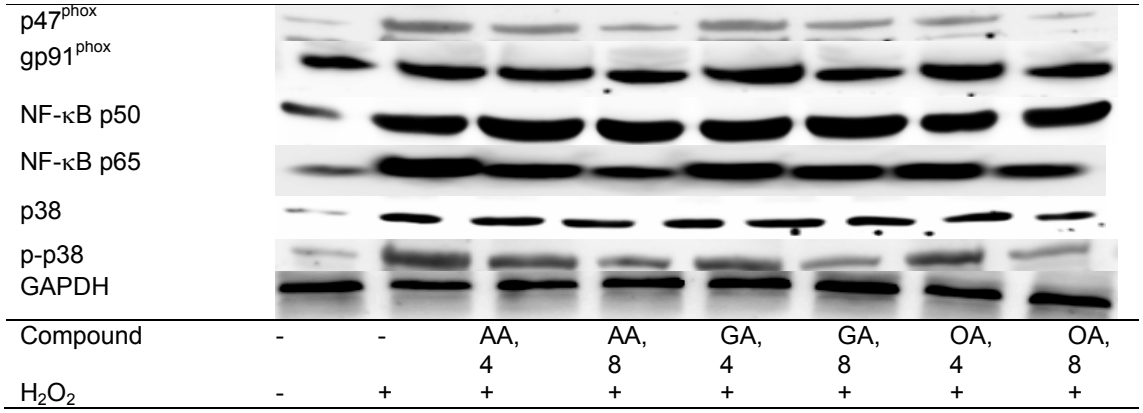
3.5 Effects of AA, GA or OA upon NADPH Oxidase, NF-κB and p38 Expression

H_2O_2 challenge increased protein expression of $p47^{phox}$, $gp91^{phox}$, NF-κB p50, NF-κB p65, p38 and p-p38 of BEAS-2B cells (Fig. 5, $p<0.05$). AA, GA or OA pre-treatments suppressed the expression of $p47^{phox}$, NF-κB p65 and p-p38 ($p<0.05$); but these compounds only at 8 μmol/l decreased $gp91^{phox}$ expression ($p<0.05$). Three test agents failed to change NF-κB p50 expression ($p>0.05$).

Table 3. Effects of AA, GA or OA upon level (pg/mg protein) of IL-6, TNF-α, and PGE_2, and COX-2 activity (U/mg protein) in BEAS-2B cells. BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l for 48 hr, and H_2O_2 at 100 μM was added to induce cell injury

	IL-6	TNF-α	PGE_2	COX-2
Control	10 ± 4^{a}	16 ± 2^{a}	23 ± 5^{a}	0.18 ± 0.04^{a}
H_2O_2	144 ± 12^{d}	171 ± 15^{e}	180 ± 13^{e}	1.76 ± 0.19^{d}
AA, 4+ H_2O_2	91 ± 5^{c}	113 ± 9^{d}	131 ± 10^{d}	1.25 ± 0.08^{c}
AA, 8+ H_2O_2	62 ± 6^{b}	72 ± 5^{c}	74 ± 8^{b}	0.76 ± 0.05^{b}
GA, 4+ H_2O_2	86 ± 7^{c}	94 ± 8^{d}	102 ± 12^{c}	1.33 ± 0.12^{c}
GA, 8+ H_2O_2	60 ± 3^{b}	49 ± 2^{b}	67 ± 3^{b}	0.69 ± 0.07^{b}
OA, 4+ H_2O_2	82 ± 5^{c}	99 ± 10^{d}	90 ± 4^{c}	1.19 ± 0.1^{c}
OA, 8+ H_2O_2	57 ± 4^{b}	65 ± 3^{c}	61 ± 2^{b}	0.7 ± 0.09^{b}

Control groups were cells contained neither test compound nor H_2O_2 treatment. Data are mean±SD (n=3). $^{a-e}$values in a column without a common letter differ, p<0.05



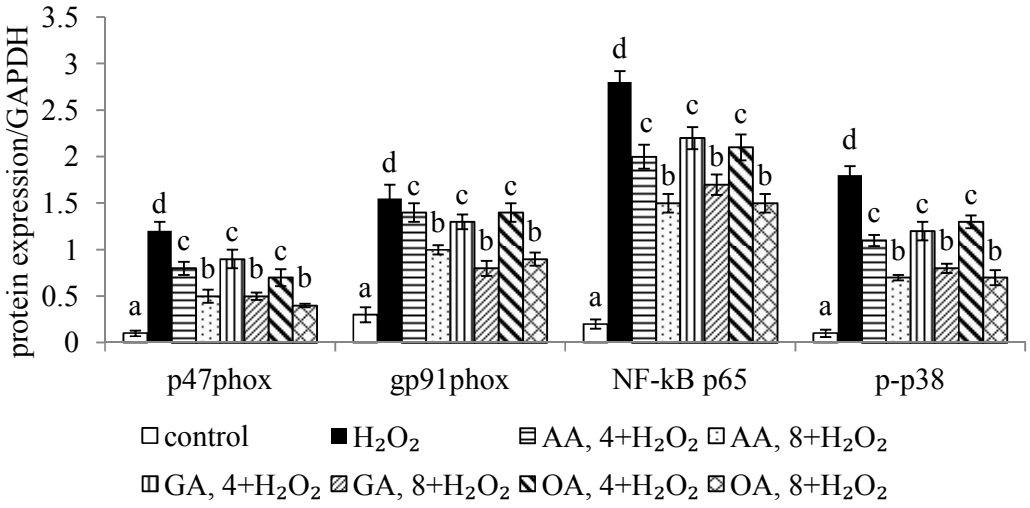


Fig. 5. Effects of AA, GA or OA upon protein expression of $p47^{phox}$, $gp91^{phox}$, NF-κB p50, NF-κB p65, p38 and p-p38 in BEAS-2B cells. BEAS-2B cells were pre-treated by AA, GA or OA at 4 or 8 μmol/l for 48 hr, and H_2O_2 at 100 μM was added to induce cell injury

Control groups were cells contained neither test compound nor H_2O_2 treatment. Data are mean±SD (n=3). $^{a-d}$values among bars without a common letter differ, p<0.05

4. DISCUSSION

Hydrogen peroxide could be released from cigarette smoking or particulate matter into airway epithelial cells, which in turn induce apoptotic, oxidative and/or inflammatory injury in those cells [20,21]. In our current study, pre-treatments from AA, GA and OA, three triterpenoid acids, effectively protected human bronchial epithelial BEAS-2B cells against hydrogen peroxide caused apoptotic, oxidative and inflammatory injury, which consequently enhanced cell survival. Furthermore, we found that these agents mediated protein expression of NADPH oxidase, NF-κB and p38MAPK in BEAS-2B cells. In addition, our data revealed that these triterpenoid acids treatments alone did not alter the survival and growth of BEAS-2B cells. These findings suggest that these test triterpenoid acids seems safe, and may favor the stability and functions of respiratory system.

Na^+-K^+-ATPase plays an important role in maintaining ion homeostasis of mitochondrial membrane. Abnormality of this enzyme impairs ion homeostasis and leads to mitochondrial malfunctions [18]. Thus, the observed decrease in Na^+-K^+-ATPase activity and mitochondrial membrane potential in BEAS-2B cells due to hydrogen peroxide exposure indicated that mitochondrial structure and functions of those cells were impaired, which subsequently caused apoptotic insult. However, the pre-treatments from three triterpenoid acids substantially increased both Na^+-K^+-ATPase activity and mitochondrial membrane potential in BEAS-2B cells. These results implied that these agents could stabilize mitochondrial structure for those cells. In addition, we found that these triterpenoid acids ameliorated hydrogen peroxide induced DNA fragmentation, and lowered the generation of 8-OHdG, an indicator of DNA oxidative stress. These findings implied that these agents might be able to penetrate into these bronchial epithelial cells, where they protected DNA, and attenuated apoptotic stress induced by hydrogen peroxide. It is reported that loss in mitochondria membrane potential stimulates the activation of caspase-3 and caspase-8 [22]. These two caspases are apoptotic executors because they could cause nuclear protein cleavage and/or cell morphological changes [23]. The increased caspase-3 and caspase-8 activities in BEAS-2B cells due to hydrogen peroxide exposure as we observed might be partially resulted from mitochondrial membrane potential change. Thus, it was reasonable to observe the lower caspases activities since three triterpenoid acids already increased mitochondrial membrane potential of those cells. In addition, three triterpenoid acids increased the expression of Bcl-2, an anti-apoptotic factor, and also raised the ratio of Bcl-2/Bax in hydrogen peroxide treated BEAS-2B cells. These data agreed that these test agents alleviated hydrogen peroxide-induced apoptotic stress in BEAS-2B cells.

Hydrogen peroxide promoted oxidative and inflammatory stress in BEAS-2B cells, which was evidenced by the massive production of ROS, 8-OHdG, IL-6, TNF-α and PGE_2. It is reported that these test agents possess anti-oxidative and anti-inflammatory activities [12,24]. Thus, the observed lower level of oxidative and inflammatory factors in triterpenoid acid-treated BEAS-2B cells seems reasonable. COX-2 is responsible for PGE_2 synthesis, and PGE_2 is involved in the progression of cigarette smoking associated lung cancer [25]. Our data revealed that three triterpenoid acids decreased COX-2 activity in hydrogen peroxide treated BEAS-2B cells, which contributed to lower PGE_2 generation, and mitigated inflammatory injury in those cells.

NADPH oxidase complex is a crucial regulator responsible for ROS formation within the development and progression of respiratory disorders [26,27]. We found that three test triterpenoid acids down-regulated the expression of $p47^{phox}$ and $gp91^{phox}$, two components of NADPH oxidase complex, which in turn diminished ROS production in hydrogen peroxide treated BEAS-2B cells. Obviously, these triterpenoid acids could execute their anti-oxidative action in BEAS-2B cells via mediating NADPH oxidase. In addition, ROS could activate NF-κB and p38MAPK signaling pathways, which further facilitate the generation of inflammatory factors [28,29]. In this current study, triterpenoid acids pre-treatments at test doses suppressed protein expression of NF-κB p65 and p-p38. Apparently, the suppression of test triterpenoid acids upon NF-κB p65 and p-p38 could be partially attributed to these agents already reducing ROS production. The other possibility is that these triterpenoid acids were able to directly limit NF-κB p65 expression and p38 phosphorylation. Consequently, the lower levels of inflammatory cytokines including IL-6 and TNF-α in triterpenoid acids treated BEAS-2B cells could be explained since the up-stream pathways have been declined. These findings suggest that these triterpenoid acids could

protect BEAS-2B cells against hydrogen peroxide induced inflammatory stress through restricting NF-κB and p38MAPK pathways.

Three triterpenoid acids are present in many herbs, vegetables and fruits. Human could obtain these compounds through consuming some edible plant foods. Thus, these agents should be safe for human application. Our previous animal study reported that dietary AA and OA intake increased their bioavailability in organs of mice [11]. The bioavailable triterpenoid acids definitely favor their application. Although using these agents for protecting respiratory system seems feasible, further *in vivo* study is necessary in order to prove the safety of these agents for airway disorders prevention or attenuation.

5. CONCLUSION

Asiatic acid, glycyrrhizic acid or oleanolic acid protected human bronchial BEAS-2B cells to counteract hydrogen peroxide induced apoptotic, oxidative and inflammatory stress via increasing mitochondrial stability, decreasing ROS, TNF-α and PGE_2 production, and suppressing protein expression of NAPDH oxidase, NF-κB p65 and p-p38. These findings support that these triterpenoid acids could provide benefits for bronchial epithelial cells and respiratory system.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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