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Research Article

Effect of *Hibiscus* anthocyanins-rich extract induces apoptosis of proliferating smooth muscle cell via activation of P38 MAPK and p53 pathway

Chia-Wen Lo, Hui-Pei Huang, Hui-Mei Lin, Cheng-Ting Chien and Chau-Jong Wang

Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan

Hibiscus sabdariffa L. (*Malvaceae*), an attractive plant believed to be native to Africa, is cultivated in Sudan and in eastern Taiwan. It has been reported to contain a number of protocatechuic acid and anthocyanins. *In vitro* experimental studies have shown that anthocyanins administration of the extract produces anti-inflammation and chemoprevention effects. In spite of the wide use of *Hibiscus sabdariffa* L. in folk medicine for treating various diseases, our previous study indicated a potency of *Hibiscus sabdariffa* extract (HSE) in anti-atherosclerosis. The mechanisms of anthocyanins administration of the extract produce from *Hibiscus sabdariffa* L. to attenuate atherosclerosis were not clarified. In this study, we found that *Hibiscus* anthocyanins (HAs) could inhibit the serum-stimulated proliferation of smooth muscle cell (SMC) and result in cell apoptosis. The HAs inducing cell apoptosis was dose dependent. We further used SB203580 (p38 inhibitor) to block cellular apoptosis and evaluate its effect on the HAs-inducing SMC death via some apoptosis criteria including DNA fragmentation and flow cytometry. We suggested that the mechanisms of the inhibitory effect of HAs on atherosclerosis could be via inhibiting the proliferation of SMC. HAs induces apoptosis via (i) activating p38 MAP kinase that subsequently phosphorylates target protein c-Jun and transduces the signal to further activate the apoptotic protein cascades that contain Fas-mediated signaling (Fas/caspase-8 signaling module) and (ii) activating p53 and inducing bax expression. As an outcome of the events, cytochrome c releases from the mitochondria, leading to cell apoptosis. In these experiments, HAs showed strong potential to induce SMC cell apoptosis via p38 and p53 pathway. In consequence, the rate of atherosclerotic formation is slowed down, and the progress is suppressed.

Keywords: Apoptosis / Atherosclerosis / *Hibiscus* anthocyanins / P38 phosphorylation / Rat aortic smooth muscle cells (A7r5)

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1 Introduction

Abnormal vascular smooth muscle cell (SMC) proliferation is thought to play an important role in the pathogenesis of both atherosclerosis and restenosis [1]. Proliferation of SMC in atherosclerotic lesions includes both their migration into the intima and excessive proliferation in neointima [2]. During the process of atherogenesis, many growth factors and cytokines can stimulate SMC proliferation [3]. Some natural foods have recently gained considerable attention as agents that can reduce the risk of atherosclerosis [4–6]. In previous reports, it has been shown that extracts from red wine such as polyphenols and epigallocatechin can prevent the progression of atherosclerosis because of their anti-oxidative and possible anti-proliferative effects [7, 8]. *Hibiscus sabdariffa* L. (*Malvaceae*) is a kind of soft drink material in local regions, and is used effectively in folk medicines against hypertension [9, 10], inflammation [11], and adipogenesis [12]. The sour tea (*H. sabdariffa*) is a popular soft drink in Taiwan.

Previously, we have demonstrated that HSE, *Hibiscus* anthocyanins (HAs), and protocatechuic acid exhibit remarkable anti-oxidative [13, 14] and anti-tumor activity [15, 16]. We also found that HSE can inhibit LDL oxidation [17] and reduce the atherosclerosis rate by preventing foam cell formation and inhibiting SMC proliferation [18]. HSE

Correspondence: Professor Dr. Chau-Jong Wang, Chung Shan Medical University, No.110, Sec. 1, Chien-Kuo N. Road Taichung, 402, Taiwan, R.O.C.
E-mail: wcj@csmu.edu.tw
Fax: +886- 4-2324-8167

Abbreviations: **HAs**, *Hibiscus* anthocyanins; **HSE**, *Hibiscus sabdariffa* extract; **SMC**, smooth muscle cell

contains polyphenolic acids, flavonoids, protocatechuic acid (PCA) and anthocyanins. Anthocyanins are water-soluble and exist among the most important groups of plant pigments. In our previous studies, HAs have been shown not only to inhibit LDL oxidation and oxLDL-mediated macrophage apoptosis, but also to induce HL-60 cells apoptosis [19]. In the light of these studies, HAs isolated from the dried flower of *H. sabdariffa* L. (Malvaceae) might also show anti-atherosclerosis effects, though the mechanism remains unclear. Therefore, in the present study, we investigated the influence of HAs on SMC proliferation and SMC apoptosis.

Recent studies have reported that curcumin [20] and green tea polyphenol [21] can inhibit the growth of aortic SMC through the induction of apoptosis. Besides, HAs mediated HL-60 apoptosis via the p38-FasL and Bid pathway [19]. The purpose of this investigation was to study the effect of HAs and its downstream signal. It has been suggested that HAs may trigger SMC apoptosis through activation of p38 MAPK signal and caspases. However, the exact role of HAs in anti-proliferation and apoptosis of SMC still remains to be investigated.

2 Materials and methods

2.1 Chemicals

Tris-HCl, EDTA, SDS, PMSF, BSA, protease inhibitor cocktail, Nonidet P-40, deoxycholic acid, SB203580 (4-[4-fluorophenyl]-2-[4-methylsul-finylphenyl]-5-[4-pyridyl]-1H-imidazole), PD098059 (2-[2-amino-3-methoxyphenyl]-4H-1-benzophyran-4-one), SP600125 (1,9-pyrazoloanthrone), and wortmannin were purchased from Sigma (St. Louis, MO). These inhibitors were stored in DMSO and added to the culture medium to a final concentration as described in the figure legends. However, the amount of DMSO in the cell culture medium did not exceeded 0.2% upon drug treatment. Protein assay kit was obtained from Bio-Rad Labs. (Hercules, CA). DMEM, fetal bovine serum, penicillin-streptomycin mixed antibiotics, L-Glutamine, Dulbecco's PBS, sodium pyruvate, and trypsin-EDTA, were purchased from Gibco/BRL (Gaithersburg, MD). RNase A, proteinase K, DNase, polyclonal antibody against a-tubulin, and actin were purchased from Sigma. Polyclonal antibody against phospho-p38 MAP kinase (Thr180/Tyr182), phosphor-p53 (Ser 392) and phospho-c-Jun (Ser-73) were purchased from Cell Signaling Technology (Beverly, MA). Antibody against FAS (FL-335), FAS-L(C-178), caspase-8 (H-134), caspase-3 (H-277), BID (C-20), Bcl-2 (N-19), Bax (P-16), and cytochrome c (A-8) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAb with reactivity to human cytochrome oxidase subunit IV was purchased from Molecular Probes (Eugene, OR). Anti-rabbit and anti-mouse secondary antibodies were purchased from Sigma. The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, UK).

2.2 Preparation of HAs

HAs had been prepared from the dried flower of *H. sabdariffa* L. (20 g) with methanol containing 1% HCl for 1 day at 4°C. The extract was filtered and then concentrated under reduced pressure at 25°C. The concentrated forms was collected, stood on an Amberlife Diaion HP-20 resin column for 24 h, and then cleaned with distilled water containing 0.1% HCl solution and eluted with methanol. The filtrate was collected and then lyophilized to obtain approximately 2 g of HAs and stored –20°C before use.

Total anthocyanins were determined using the Fuleki and Francis (1968) method [22]. Briefly, 100 μL of HAs (10 mg/mL) were diluted to 3 mL with the pH 1.0 and 4.5 buffers, respectively. The OD of the sample was measured at 535 nm, using distilled water as blank. The difference in OD was obtained by subtracting the total OD at pH 4.5 from that at pH 1.0. Both values were calculated from the OD readings using the appropriate dilution and calculation factors. For the standardization of HAs, delphinidine in HAs was determined by HPLC using a symmetry shield RP18 column (3.5 μm, 4.6 × 150 mm) and a UV/visible detector (monitored at 530 nm). The mobile phase consisted of H_2O and 10% formic acid/methanol (65/35, v/v). The sample (1 mg) was dissolved in 1 mL acidic methanol (HCl–CH_3OH = 1:1, v/v) and boiled at 95°C for 30 min, and 10 μl of it was injected into chromatography. The flow rate was set at 1 mL/min. The result was evaluated with the commercially available standard delphinidine.

Spectrophotometric analysis of HAs showed that the purity of HAs was approximately 85–95%. For HAs standardization, delphinidine contained in the HAs was determined using HPLC. The HPLC analysis confirmed that delphinidine is the major component in the *H. sabdariffa* L. anthocyanin, consisting of approximately 3–4% of HAs in each analysis [19].

2.3 Cell lines

A7r5, a rat embryonic aortic smooth muscle cell line, was grown in DMEM (supplemented with 10% fetal bovine serum (FBS), 1.0 mM sodium pyruvate, 4 mM L-glutamine,1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 100 units/mL penicillin, and 100 μg/mL streptomycin). Prior to the experiments, confluent SMC were starved by culturing for 48 h (after 24 h one medium change) in 0.5% FBS [20].

2.4 Assessment of cell viability

A7r5 cells (10^5 cells/mL) were treated with HAs. The culture was exposed to various HAs concentrations (0–6 mg/mL) for 24 h. Subsequently, the medium was removed and

replaced with 3-(4, 5-dimethylthiazol-2-xl)-2, 5-diphenyltetrazolium bromide (MTT, 0.1 mg/mL) for 4 h. The number of viable cells was directly proportional to the production of formazan, which was solubilized in isopropanol and measured spectrophotometrically at 563 nm.

2.5 DNA laddering

Cleavage of DNA into oligonucleosomal fragments, perceptible as a DNA ladder when electrophoresed on an agarose gel, is usually taken as biochemical hallmark of apoptosis. A7r5 (10^5 cells/mL) were plated and after 24 h were treated with 0–5 mg/mL HAs and treated with 5 mg/mL HAs for indicated times, and washed with PBS. The cell pellet was isolated by the DNeasy Tissue Kit (Qiagen, Germany) for efficient isolation of total cellular DNA. Then the DNA was electrophoresed on a 1.8% agarose gel, and stained ethidium bromide. The bands of DNA fragmentation were photographed under UV light.

2.6 Flow cytometric analysis

Cells were synchronized at the G_0 phase by serum starvation for 48 h [20], washed and incubated in fresh medium containing 10% v/v FBS to allow the progression through the cell cycle. At various time periods after relief of the quiescent state, cells were analyzed for cell cycle distribution by flow cytometry [23]. After various treatments, floating and adherent A7r5 cells were collected and pelleted at 1000 rpm for 5 min at 4°C Cells were fixed in ice-cold 70% ethanol. After incubation at –20°C for at least 24 h, and resuspended in 1 mL each RNase (50 μg/mL) and propidium iodide (50 μg/mL in 0.1% Triton X-100 in PBS). Cells were analyzed on a BD Biosciences FACScan and analyzed using CellQuest™ Pro software. Cell cycle distribution is presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence, and the extent of apoptosis was determined by counting cells of DNA content at the Sub-G1 peak.

2.7 Western blotting

Western blotting was performed according to a previously described method [24]. To analyze the expression of proteins, HAs (1–5 mg/mL) were added to the culture for 1 h or 6–24 h. Cells were washed in ice-cold PBS and lysed in buffer (50 mM Tris, PH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) plus protease inhibitor cocktail. Protein content in clarified lysates (0.5 μg purified protein) was mixed with an equal volume of electrophoresis sample buffer and then boiled for 10 min, followed by analysis using SDS-PAGE, Mini-PROTEAN II cell (Bio-Rad), and transfer of protein from the gel to nitrocellulose membranes using Mini Trans-Blot cell (Bio-Rad). Incubations with primary antibodies were performed overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detection via chemiluminescence.

2.8 Preparation for cytosolic and mitochondrial fraction

Subcellular fractionation was performed as follows and washed with PBS. The cell pellet was isolated by the Mitochondria Isolation Kit (Pierce, USA) for the separation of mitochondria from cytosolic components. Cytochrome c proteins in the two fractions were analyzed by Western blot.

3 Results

3.1 Effect of HAs on A7r5 cells

We first sought to determine whether HAs could inhibit the growth of A7r5 cells. Cells were plated in triplicate, at a density of 10^5 cells/mL and were allowed to recover for 4 h. Cultures were then treated with 0–6.0 mg/mL HAs for 24 h and cell viability was determined by MTT assay. As shown in Fig. 1, growth of A7r5 cells was inhibited in a dose-dependent fashion.

3.2 Induction of apoptosis by HAs

The below experiment evaluated the effects of HAs on the growth of A7r5 cells by underlying apoptosis. When cells undergo apoptosis, the degradation of DNA into a particular pattern of fragment is a characteristic feature. Agarose-gel electrophoresis of chromosomal DNA extracted from HAs-

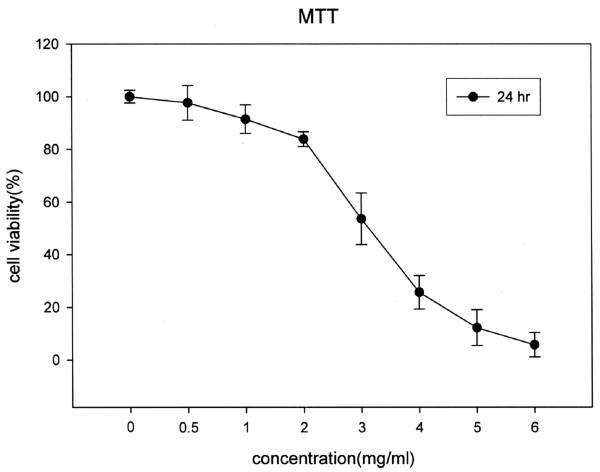

Figure 1. Dose response of the viability of A7r5 cells after treatment with HAs. After overnight attachment of cells, the culture was exposed to indicate concentration of HAs for 24 h (●). Then, the medium was removed and isopropanol was added to dissolve the formazan crystal for MTT assay. The viable cell number is directly proportional to the production of formazan ($n = 4$ in each group, mean ± SE).

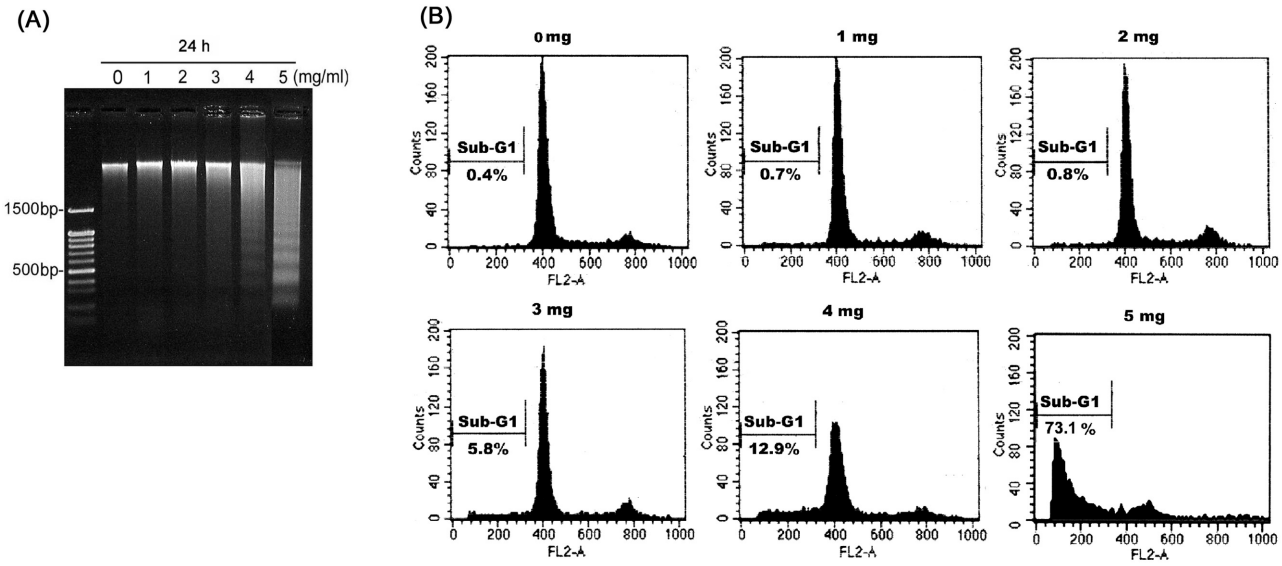

Figure 2. The apoptosis induced by HAs was characterized by flow cytometry and DNA laddering in A7r5 cells. Genomic DNA was isolated from A7r5 cells and treated with indicated concentration of HAs for 24 h. DNA fragmentation was evaluated by electrophoresis on agarose gel containing ethidium bromide which was photographed under UV light (A). Flow cytometric DNA fluorescence profiles of A7r5 cells. PI-stained DNA histograms of HAs-treated cells are shown. Cells were treated with 0–5 mg/mL HAs for 24 h (B).

treated cells revealed a DNA ladder consisting of multimers of approximately 180–200 bp. After 24 h exposure to HAs, the degradation of cellular DNA increased in the dose-dependent manner (Fig. 2A). To further examine the effect of HAs on apoptosis, we used flow cytometry to quantify the apoptotic status. Similarly as in DNA fragmentation assay, A7r5 cells exposed to higher concentrations of HAs for 24 h produced higher percentage of Sub-G1 phage (Fig. 2B). These data unambiguously demonstrate that HAs induced A7r5 cells apoptosis in a dose-dependent manner.

3.3 Effect of MAP kinase inhibitors on HAs-induced apoptosis

To determine which signaling pathway is involved in HAs-induced A7r5 apoptosis, we used MAP kinase and phosphatidyl inositol 3-kinase (PI3-kinase) inhibitors including PD98059 (an inhibitor of MAP kinase kinase), SB203580 (an inhibitor of p38 MAPK), SP600125 (an inhibitor of c-Jun N-terminal kinase) or wortmannin (an inhibitor of phosphatidylinositide-3-kinase) to treat A7r5 cells. By FACScan analysis, only treatment with SB203580 repressed significantly the HAs-induced apoptosis of A7r5 cells (Fig. 3). Therefore, we proposed that p38 pathway was involved HAs-induced apoptosis.

3.4 HAs-induced apoptosis in A7r5 cells involves p53, MAP kinase family proteins, and apoptosis-related proteins

P53 and p38 kinase activation is known to be associated with the stress-induced apoptosis process, such as chemotherapeutic drugs, UV irradiation, and Fas [25, 26]. To examine the level of p53, MAP kinase family proteins and bcl-2 family proteins we used Western blot analysis. Incubation of A7r5 cells with HAs led to a dose-dependent phosphorylation of p53, p38 and c-Jun (Fig. 4A). Accordingly, we suspected that p38 could regulate FasL transcription via c-Jun. Therefore, we measured the protein levels of FasL. The data showed increased FasL and phosphorylation of c-Jun in A7r5 cells that were treated with HAs in a dose-dependent fashion (Fig. 4B). In addition, Bcl-2 family proteins have exhibited a complex network that regulates apoptosis in multiple biological systems. We examined the cellular levels of Bcl-2, Bax, and Bid in A7r5 cells treated with HAs for 6 h. The expression of the anti-apoptotic protein Bcl-2 showed no specific changes, but Bax and tBid (activated Bid), the proapoptotic Bcl-2 family proteins, revealed increased expression levels (Fig. 4B). These data suggested that the activation of p53, p38 and c-Jun kinases was a primary effect during HAs-induced apoptosis in A7r5 cells. The further experiment showed that Fas death receptor activating caspase-8 and p53 regulating Bcl-2 family proteins eventually induced cytochrome c release.

3.5 Effect of HAs on caspase cleavage and mitochondrial cytochrome c releases

We wondered whether HAs could trigger caspases activation and mitochondrial cytochrome c release when A7r5 cells undergo apoptosis. The release of cytochrome c from the mitochondria is reported to be involved in the activation of the caspase cascade and triggering apoptosis. The level of cytochrome c in the cytosol was examined by Western

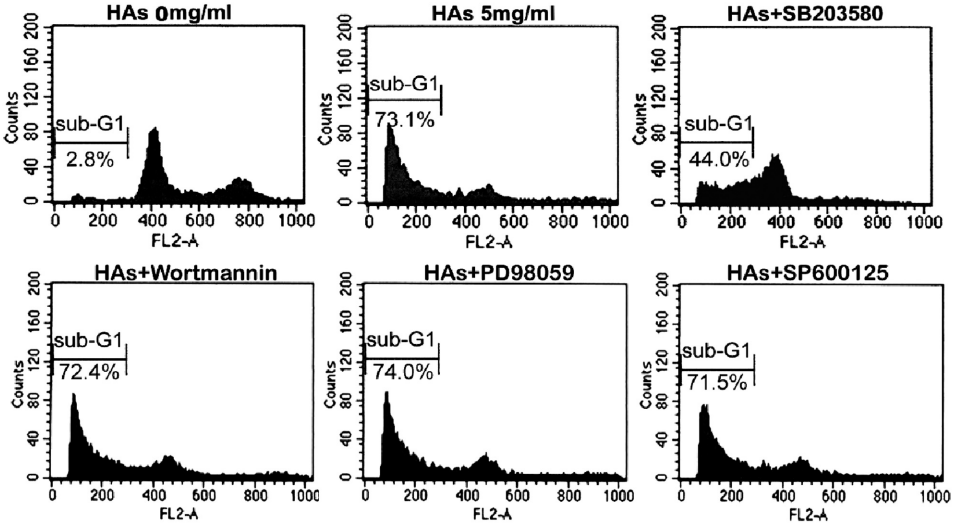

Figure 3. Effect of inhibitors blocking HAs-induced apoptosis in A7r5 cells. A7r5 cells (10^4 cells/dish) were preincubated for 1 h with various inhibitors, including SB203580 (10 μM), SP600125 (25 μM), PD98059 (25 μM), and wortmannin (100 nM), and then incubated 0–5 mg/mL HAs for 24 h. Quantitative analysis of apoptosis was determined by flow cytometry assay. Similar results were obtained in three independent experiments, a representative one is shown here.

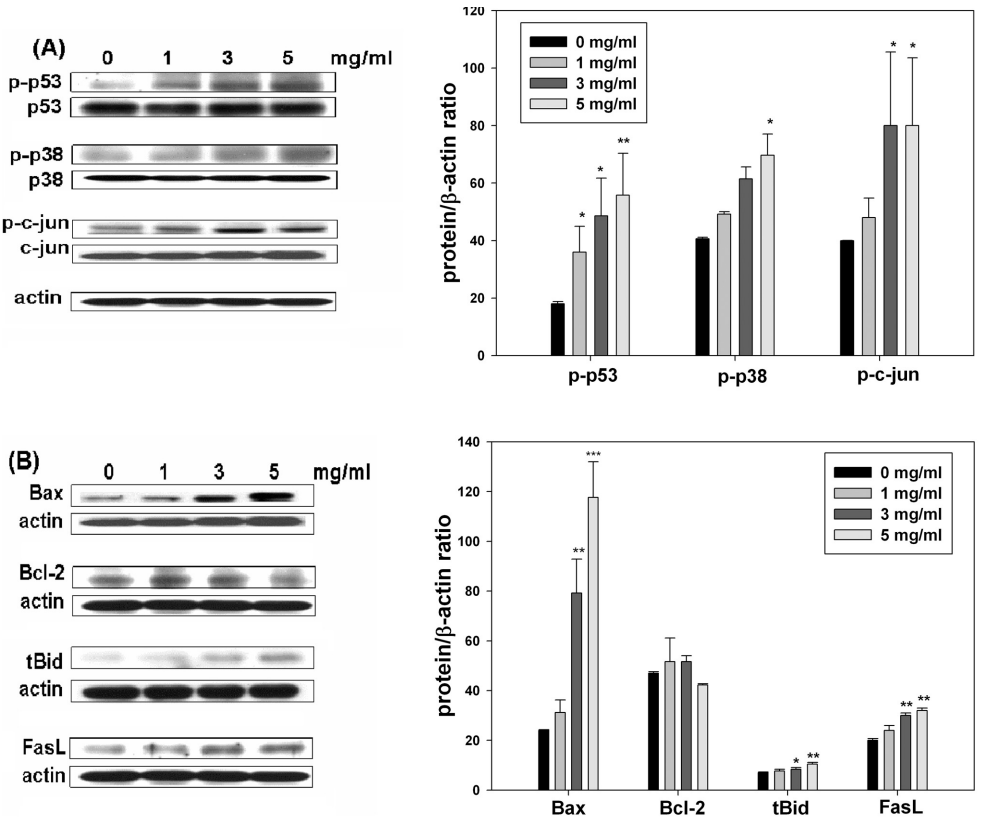

Figure 4. HAs activates MAP kinases in a dose-dependent manner. A7r5 cells were incubated with 0–5 mg/mL HAs for 1 h (A) or 6 h (B). The levels of upstream and downstream of c-Jun and Bcl-2 family protein expression were determined by Western blotting using specific antibodies. Actin was used as the loading control. The quantitative data were presented as means ± SD of three independent experiments. *p <0.05; **p <0.01, ***p <0.001, compared with the non-treated group.

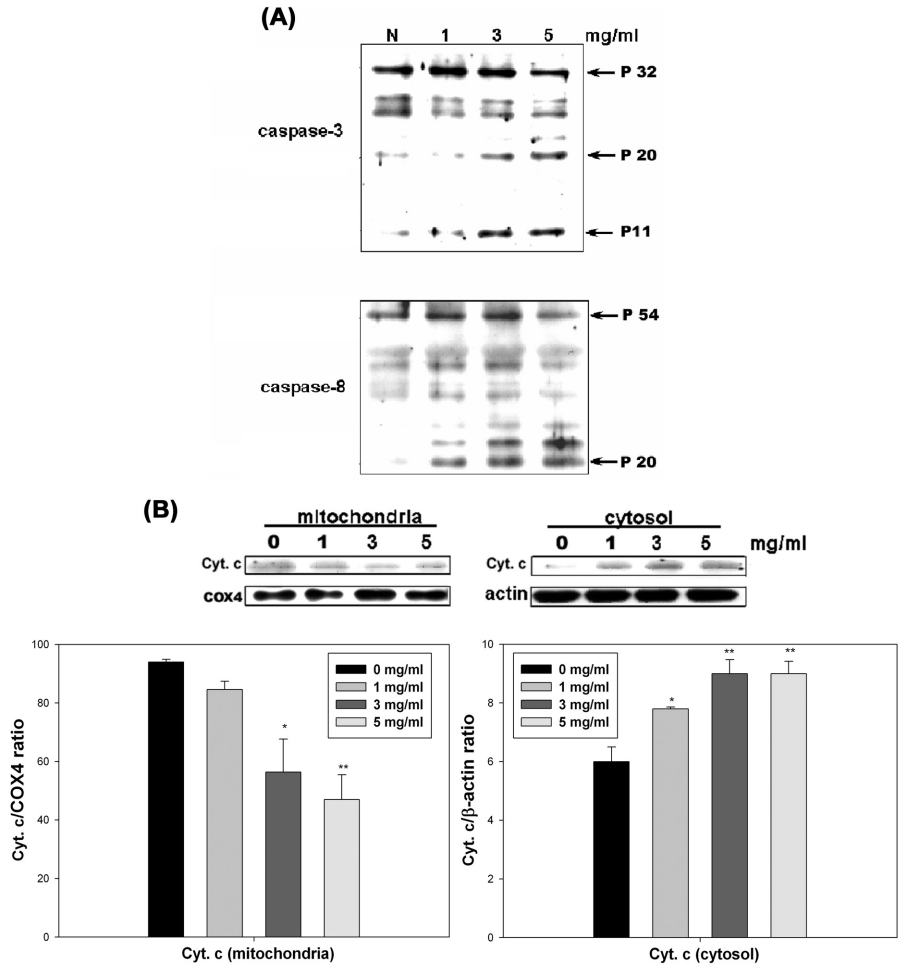

Figure 5. Effect of HAs-induced caspases activation and cytochrome c release. The levels of cleaved caspase-3/-8 and pro-caspase-3/-8 proteins in A7r5 cells with 0–5 mg/mL HAs for 6 h were analyzed using immunoblotting with anti-cleaved-caspase-3/-8 and pro-caspase-3/-8 antibody. Maker indicates cpspase-3 precursor (32-kDa) and cleavage fragment (20 and 11-kDa); caspase-8 precursor (54-kDa) and cleavage fragment (20-kDa) (A). A7r5 cells were treated with 0–5 mg/mL HAs for 6 h, and the expression of cytochrome c in the cytosol and the mitochondria was assayed by immunoblotting (B). Actin and COX4 were the loading controls. The quantitative data were presented as means ± SD of three independent experiments. *p <0.05; **p <0.01, compared with the non-treated group.

blot analysis. The results showed that compared to the control (no treatment with HAs), cells exposed to HAs had significantly increased cytosolic fraction of cytochrome c (Fig. 5B). Caspase-3 and caspase-8 are cytosolic proteins that exist normally as a higher molecular weight of inactive precursor. They are cleaved proteolytically into a low molecular weight when the cells undergo apoptosis [27]. To determine whether caspase-3 and caspase-8 are involved in this process, we performed Western blot analysis for cleavage status of caspase-3 and caspase-8. Our data confirmed that caspase-3 and caspase-8 were indeed activated and catalyzed into the active form in a dose-dependent manner in HAs-treated A7r5 cells (Fig. 5A). In contrast with HAs-treated A7r5 cells, the caspase-3 and caspase-8 were not cleaved in cells which were not treated with HAs. These data showed that caspase 3 and caspase-8 activation and mitochondrial cytochrome c release were involved in the HAs-induced apoptosis.

3.6 Effect of SB203580 on HAs-induced apoptosis

To confirm the signaling pathway involved in HAs-induced A7r5 apoptosis, we supposed that the inhibitor SB203580 might also suppress the downstream effectors of c-Jun, FasL, and cytochrome c, which are involved in the HAs-induced apoptosis. As shown in Fig. 6, A7r5 cells treated with HAs (5 mg/mL) alone showed significantly elevated levels of c-Jun, FasL, and cytochrome c (lane 2) compared with lane 1. The pretreatment of A7r5 cells with SB203580 repressed the expressions of the proteins induced by HAs

but no change in p53 was observed (lane 3). These results again emphasize that p38 activation is involved in the HAs-induced apoptosis pathway.

4 Concluding remarks

Anthocyanins are commonly present in human diet and are abundantly contained in aronia fruits, black currant, raspberry, grapes and apples. Because of their chemical structure, they are able to act as antioxidative, anti-inflammatory and cardioprotective agents [28]. Our laboratory has shown that HAs (which are extracted from the dried calyx of *H. sabdariffa*) possess antioxidant bioactivity both *in vivo* and *in vitro* [13, 14]. Previously, we have reported that HSE and its components (anthocyanins and protocatechuic acid) can inhibit LDL oxidation and thiobarbituric acid reactive substances (TBAR) formation *in vitro* [18, 29], and also inhibit progression of atherosclerosis in cholesterol-fed rabbits [17]. Many investigations highlight an additional role of polyphenolic acid, flavonoids, and anthocyanins that may act as antioxidants or via other mechanisms contributing to cardioprotective actions [30–32]. We have recently found that HSE inhibited LDL oxidation *in vitro*, decreased serum lipids in cholesterol and high fructose-fed rats [17, 18], and inhibited progression of atherosclerosis through anti-proliferation of SMC. Furthermore, we showed that HAs inhibited progression of atherosclerosis through anti-proliferation effects of SMC. This mechanism may contribute to the cardioprotective actions.

This study showed for the first time evidence that HAs repressed SMC proliferation and induced cell apoptosis. The effects of HAs in inhibiting cell growth and leading to apoptosis were observed by DNA laddering (Fig. 2) and flow cytometry (Fig. 3). Two major apoptosis pathways have been defined in mammalian cells, the Fas/TNF-R1 death receptor pathway and the mitochondria pathway. FasL-mediated apoptosis can usually activate caspase-8, leading to a series of downstream events, including activation of pro-caspase-3 and cleavage of Bid. Activated Bid is translocated to mitochondria and induces cytochrome c release [33]. HAs-treated cells revealed the expressions of FasL and tBid (Fig. 4), the cleavage of caspase-3 and caspase-8 proteins (Fig. 5A), and cytochrome c release from the mitochondria (Fig. 5B). The results suggest that HAs-induced SMC apoptosis is involved in FasL-mediated pathway. In addition, the phosphorylation of c-Jun through p38 signaling has been found to trigger apoptosis [34, 35]. In this study, increased phosphorylation of p38 and c-Jun was also observed in the HAs-treated A7r5 cells (Fig. 4). Furthermore, we found that the reaction of HAs-treated A7r5 cells was repressed by the co-treatment with SB203580 (p38 MAPK inhibitor), which was related to FasL expression (Fig. 6). These results confirmed that the HAs-mediated apoptosis is also induced through the p38 signaling

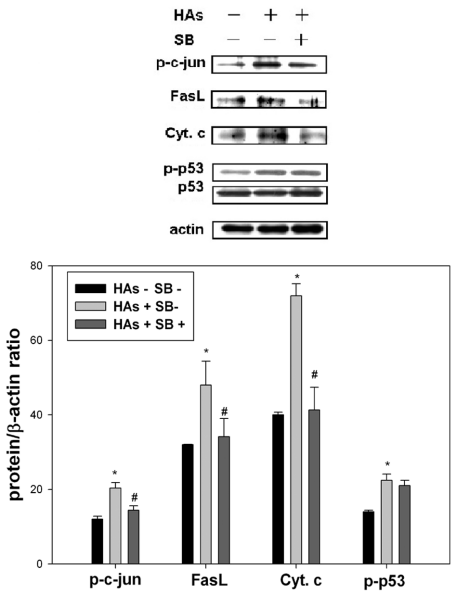

Figure 6. Effect of p38 MAPK inhibitor (SB203580) on HAs-induced apoptosis in A7r5 cells. A7r5 cells (10^4 cells/dish) were preincubated for 1 h with SB203580 (10 μM), and then incubated with 0–5 mg/mL HAs for 1 h (phospho-c-Jun and p-p53) or 6 h (FasL and cytochrome c). The levels of phospho-c-Jun, FasL, cytochrome c, and p-p53 protein expressions were analyzed by Western blotting. Actin was used as the loading control. The quantitative data were presented as means ± SD of three independent experiments. *p <0.05, compared with the non-treated group. #p <0.05, compared with the HAs-treated group.

pathway (Fig. 6). The tumor suppressor protein p53 can induce growth arrest and cell death via apoptosis in response to cellular stress [36]. The p53 protein is a potent regulator of apoptosis, most notably by mediating up-regulation and mitochondrial translocation of Bax [26]. Results also showed that HAs-induced apoptosis involved the up-regulation of p53 and Bax protein (Fig. 4), cleavage of Bid, and the release of cytochrome c. HAs-induced apoptosis was also accompanied by the activation of caspases-3 and -8.

The findings of this study are schematically presented in Fig. 7. This figure shows that HAs-induced apoptosis was involved in activation of p53 and p38 MAP kinase, subsequent phosphorylation of the target c-Jun and signal transduction, activation of apoptotic protein cascades that involve Fas-mediated signaling (Fas/caspase-8/tBid signaling module) and mediation of mitochondria dysfunction. These events resulted in the release of cytochrome c from the mitochondria into the cytosol, activated caspase-3, and eventually caused apoptosis. The above results imply that HAs may be helpful for the study and treatment of the pathological changes in atherosclerosis.

Some reports indicate that SMC apoptosis is involved in plaque rupture, vessel remodeling, coagulation, inflammation and calcification in advancement of atherosclerosis

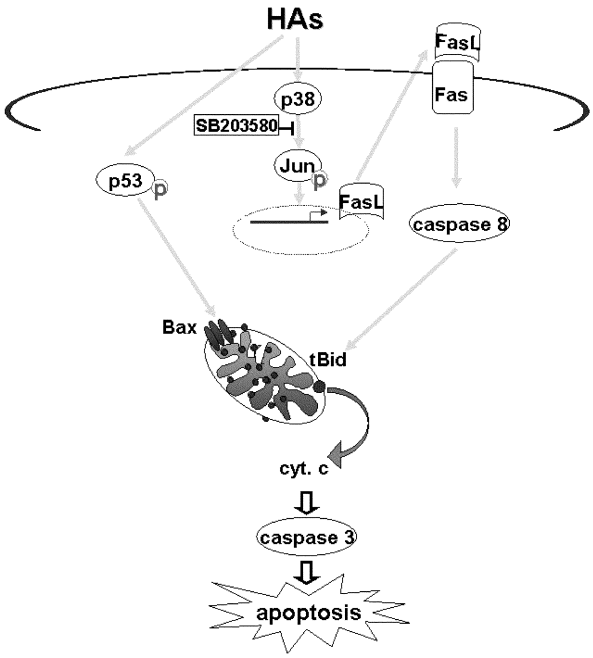

Figure 7. Model showing pathways that mediate HAs-induced apoptosis in A7r5 cells. HAs were been shown to be capable of inducing A7r5 cell apoptosis. P38 signaling activation involves mitochondrial membrane alterations resulting in the release of cytochrome c and caspases activation. See the text for discussion.

[37, 38]. In the lesion progressions of atherosclerosis, SMC migrate from the medial portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous plaque. In our study, we observed the effect of HAs on the proliferation of cultured rat SMC stimulated by fetal bovine serum (FBS) *in vitro*. HAs significantly inhibited the proliferation of rat vascular SMC induced by the FBS stimulant. HAs inhibiting serum-stimulated proliferation of SMC and resulting in cell apoptosis may be useful for the study and treatment of the lesion progressions of atherosclerosis. For treatment of atherosclerosis, the dose range of HAs, which can inhibit the uncontrolled response of cell division without an adverse effect on the stability of atherosclerotic plaque should be used.

In our previous study, we explored whether HAs could prevent or delay the development of leukemia in *in vitro* and *in vivo* tests. The results showed that HAs not only led to the death of human promyelocytic leukemia cells (HL-60 cells) but also inhibited N-Nitroso-N-Methylurea (NMU)-induced rat leukemia. In the research mentioned above, the effective dose of HAs was 3 mg/mL (treatment 0–4 mg/mL) in the cell model and 0.2% (treatment 0–0.2%) in the animal model, respectively. Based on the calculation of the weight of the edible dry food for an adult of 500 g/day, and an HAs content of 0.2%, the dosage recommendation for an adult is 1 g/day. In this study, we found that 0–5 mg/mL HAs inhibited significantly SMC proliferation in arteriosclerosis. In animal studies, feeding HSE (0.5 and 1% in the diet) to rabbits significantly reduced severe atherosclerosis in the aorta of the rabbits. The final extract (HSE) was composed of 2.5% anthocyanins. According to the results of this study, the recommended dosage of HAs for human consumption is 1 g/day as diet supplements due to their perceived potential health benefits. In Taiwan, HSE was also made for health foods, and available on the market. The net weight of HSE of a capsule is 500 mg. Each capsule contains 17.1–231 mg anthocyanins, 7.6–12.5 mg flavonoids, and 11.2–16.8 mg total polyphenols. HSE dosage recommendations for humans are 1.5 g three times per day. HAs are the major component of HSE. According to our results, HAs may play the most cardioprotective role in HSE.

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