Chrysin inhibits human airway smooth muscle cells proliferation through the extracellular signal-regulated kinase 1/2 signaling pathway

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Abstract. Asthma is a chronic airway inflammatory disease characterized by an increased mass of airway smooth muscle (ASM). Chrysin (5,7-dihydroxyflavone), a natural flavonoid, has been shown to exert multiple biological activities, including anti-inflammatory, anti-proliferative and anti-oxidant effects, as well as the potency to ameliorate asthma in animal models. The objective of the present study was to identify the underlying mechanism of the therapeutic effects of chrysin. The impact of chrysin on basal and platelet-derived growth factor (PDGF)-induced proliferation and apoptosis of human airway smooth muscle cells (HASMCs) was investigated. Furthermore, the activation of the extracellular signal-regulated protein kinase (ERK) signaling pathway was evaluated in HASMCs. The results revealed that chrysin significantly inhibited basal as well as PDGF-induced HASMC proliferation, most likely through the suppression of ERK1/2 phosphorylation. However, chrysin did not significantly reduce PDGF-induced apoptosis of HASMCs. The present study indicated that chrysin may be a promising medication for controlling airway remodeling and clinical manifestations of asthma.

Introduction

Asthma is a complex chronic airway inflammatory disease, which is characterized by reversible airflow obstruction, bronchial hyperresponsiveness, airway inflammation and airway remodeling. Among these, airway remodeling, comprising a range of structural alterations, including increased deposition of extracellular matrix proteins (ECMs), epithelial denudation with goblet cell metaplasia, angiogenesis and increased airway smooth muscle (ASM) mass, has been proposed to result in persistent airflow limitation and a decreased baseline lung function (1-3).

A number of studies have demonstrated that ASM may participate in multiple processes associated with asthma (4,5). ASM cells are considered to be the main cell type involved in bronchial hyperresponsiveness due to their contractibility. The ASM not only serves as the major target of inflammatory mediators in the asthmatic inflammatory process, but also exerts pro-inflammatory and immunomodulatory functions through expressing a host of cell adhesion molecules, responding and secreting a myriad of cytokines and growth factors and upregulating the expression of toll-like receptors in ASM cells (6). Furthermore, increases in the ASM mass have a key role in asthmatic bronchial remodeling, and hyperplasia and hypertrophy of airway smooth muscle cells are considered to be the primary cause of airway obstruction (1,4,5). The mechanisms accounting for ASM hyperplasia include increased proliferation, reduced apoptosis and enhanced migration of myofibroblasts within the ASM layer (4). Proliferation of ASM cells can be induced by a variety of mitogens, including growth factors, cytokines, inflammatory mediators and enzymes, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), endothelin-1 and tryptase (4,7). Inhibiting ASM-cell proliferation can be an effective approach for the treatment of asthma. However, ASM remodeling is insensitive to currently used asthma medications, which are usually effective in controlling acute asthma exacerbation and bronchial inflammation (8).

Flavonoids, commonly present in vegetables, nuts, fruits, beverages and herbal remedies, are health-promoting and disease-preventing dietary supplements (9). Chrysin (5,7-dihydroxyflavone) is a natural flavonoid, which is contained in medicinal herbs (10,11). Previous studies showed that chrysin exerts multiple biological activities, including anti-inflammatory, anti-proliferative and anti-oxidative effects (12-14). Chrysin is beneficial for asthma in numerous aspects (15,16); however, the target cells and the mechanisms involved have

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remained to be identified. The proliferation of ASM cells is involved in various aspects of the pathogenesis of asthma (8,17). The present study aimed to investigate whether chrysin affects basal and PDGF-induced proliferation of human ASM cells (HASMCs) as well as the possible underlying mechanisms.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from (Thermo Fisher Scientific, Waltham, MA, USA). Chrysin (purity, 97%) was obtained from Invitrogen-Gibco (Paisley, UK). Recombinant human PDGF-BB was purchased from PeproTech (Rocky Hill, NJ, USA). Cell Counting Kit-8 (CCK-8; cat. no. CK04) and Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (cat. no. KGA107) were purchased from Dojindo (Kumamoto, Japan) and KeyGen Biotech (Nanjing, China), respectively. The Total Protein Extraction kit (cat. no. KGP250) was purchased from KeyGen Biotech. Rabbit polyclonal antibody against p44/42 mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK)1/2 (cat no. 9102) and rabbit monoclonal antibody against phospho (p)-p44/42 MAPK (ERK1/2) (Thr202/Tyr204; cat no. 4370) were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Mouse monoclonal antibody against GAPDH (cat no. MB001) was purchased from Bioworld Technology (St. Louis Park, MN, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G (H&L) (cat no. BS13278) and HRP-conjugated goat anti-mouse IgG (H&L) (cat no. BS12478) were obtained from Bioworld Technology (St. Louis Park, MN, USA). The Bicinchoninic acid (BCA) Protein Assay kit (cat. no. P0012) and Enhanced Chemiluminescence (ECL) Detection kit (cat. no. P1007-1) were obtained from Beyotime Institute of Biotechnology (Nantong, China) and Jinan Ubio Biological Technology (Jinan, China), respectively.

Cell culture. HASMCs were purchased from Sciencell Research Laboratories (cat. no. 3400; Carlsbad, CA, USA). The cells were grown in DMEM medium containing 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen-Gibco), and incubated at 37˚C in a humidified 5% CO_2 atmosphere (18). Cells at passage 4-8 were used in all experiments.

Proliferation. HASMCs cultured in DMEM supplemented with penicillin, streptomycin and 10% FBS were seeded in 96-well plates at a density of 5,000 or 3,500 cells per well for treatments for 24 and 48 h, respectively. The cells were then serum-deprived in DMEM containing 0.5% FBS for 24 h when they were ~60-70% confluent. Cells were treated with chrysin (10, 20 or 40 μM) alone or pre-treated with chrysin for 30 min prior to stimulation with PDGF-BB (10 ng/ml). After 24 or 48 h, the Cell Counting kit-8 assay was used to assess the number of viable cells. After the medium was aspirated, CCK-8 solution (10 μl) diluted in serum-free DMEM (100 μl) was added to each well and the cells were further incubated at 37˚C in the presence of 5% CO_2 for 2 h. The absorbance of the wells was measured at 450 nm using the Bio-Rad iMark™ Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

Apoptosis assay. *An* Annexin V-FITC Apoptosis Detection kit was used to determine the percentage of apoptotic cells. Briefly, cells were seeded in six-well plates, cultured as described above, and growth-arrested in DMEM containing 0.5% FBS for 24 h when reaching ~70% confluence. The cells were then stimulated with PDGF-BB (10 ng/ml) with or without 30-min chrysin (20 μM) pre-treatment, or chrysin (20 μM) alone for 24 h. The treated cells were trypsinzed and washed with phosphate-buffered saline (PBS) twice, and subsequently incubated with Annexin V-FITC and propidium iodide (PI). Quantification of apoptosis and necrosis was performed using flow cytometry (BD FACSCanto™ II; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. HASMCs were seeded in six-well plates at a density of $2x10^5$ cells/well and cultured as described above and subsequently starved in DMEM containing 0.5% FBS overnight. The growth-arrested cells were stimulated with PDGF-BB (10 ng/ml) for 30 min with or without 30-min chrysin (20 μM) pre-treatment, or chrysin (20 μM) alone. After the treatments, cells were immediately washed with ice-cold PBS twice and lysed in lysis buffer containing 5 μl phosphatase inhibitors, 1 μl protease inhibitors and 5 μl 100 mM phenylmethanesulfonylfluoride in 1 ml buffer (Total Protein Extraction kit). The lysates were centrifuged at 14,000 xg for 15 min at 4˚C to obtain the total cell extracts. The BCA Protein Assay kit was used to determine the protein concentration. Equal amounts of protein extracts (20 μg) were subjected to 10% SDS-PAGEand transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked in a blocking buffer (5% non-fat milk, 20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20; Biosharp, Hefei, China) at room temperature for 2 h and then incubated with the primary antibodies, anti-GAPDH (1:5,000 dilution), anti-ERK1/2 (1:1,000 dilution) and anti-p-ERK1/2 (1:1,000 dilution) at 4˚C overnight. Subsequently, membranes were washed with 20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20 (TBST) three times, for 15 min each wash. Following this, the membranes were incubated for 1.5 h with the HRP-conjugated goat anti-rabbit IgG antibodies for ERK1/2 and p-ERK1/2, and the HRP-conjugated goat anti-mouse IgG antibody for GAPDH. The membranes were then washed with TBST 3 times, for 15 min each wash. Immunoreactive bands were detected using ECL reagents. The intensity of bands was quantified using the Bio-Rad Gel Doc/Chemi Doc Imaging System and Image Lab software, version 4.0 (Bio-Rad Laboratories). GAPDH was used as an internal control for protein loading.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Differences between groups were analyzed using the two-tailed Student's t-test with SPSS software, version 19 (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

Chrysin inhibits PDGF-induced HASMC proliferation. To clarify the mechanism of the beneficial effects of chrysin in

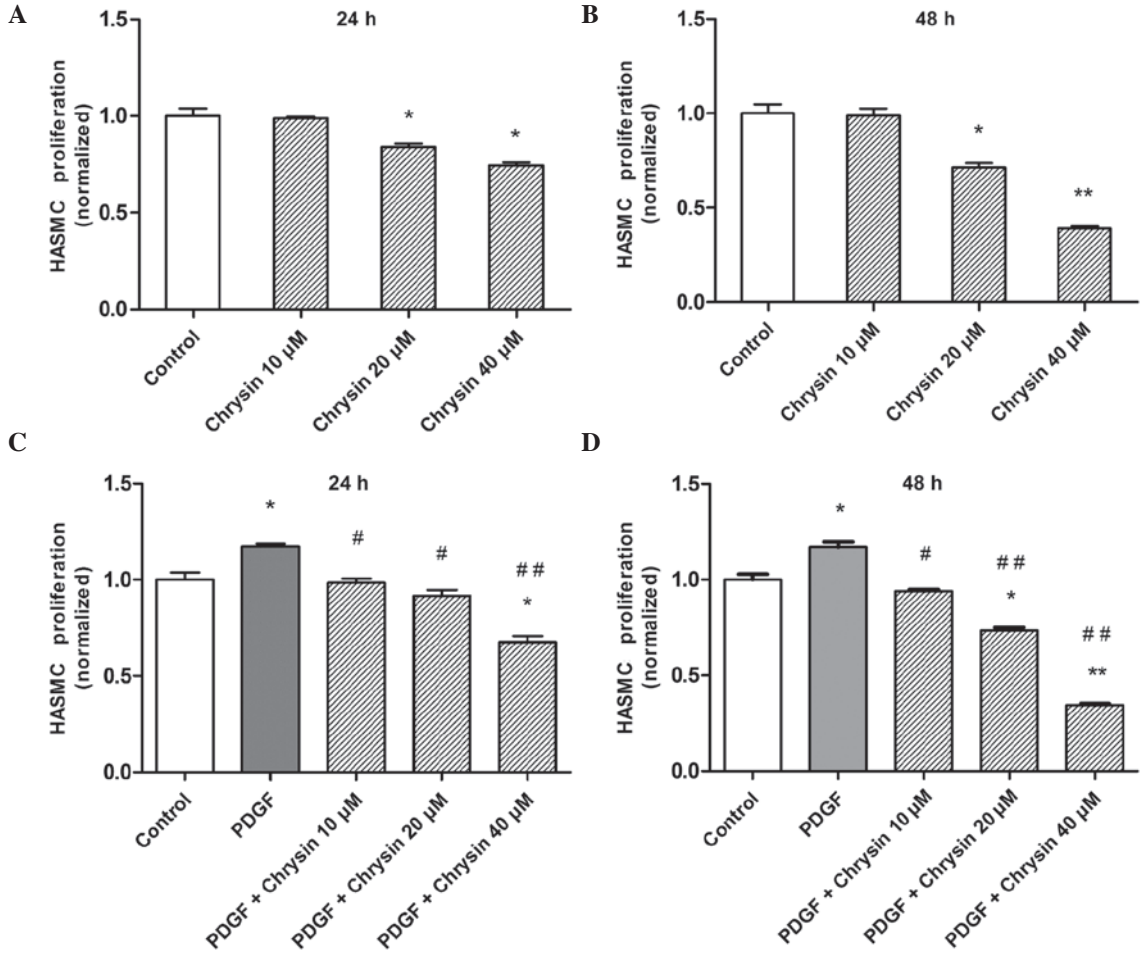

Figure 1. Chrysin inhibits basal as well as PDGF-stimulated proliferation of HASMCs. Growth-arrested cells were treated with (A and B) chrysin (10, 20 or 40 μM) alone or (C and D) pre-treated with chrysin for 30 min before stimulation with PDGF-BB (10 ng/ml). After 24 or 48 h, A Cell Counting Kit-8 assay was used to assess cell proliferation. Values are expressed as the mean ± standard error of the mean from three individual experiments. *P<0.05, **P<0.001 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.001 vs. PDGF-stimulated treatment group. PDGF, platelet-derived growth factor; HASMC, human airway smooth muscle cell.

patients with asthma, the present study investigated the role of chrysin on the basal as well as PDGF-induced proliferation in HASMCs. Chrysin inhibited the proliferation of HASMCs in a dose- and time-depended manner. Chrysin (20 or 40 μM for 24 or 48 h) significantly suppressed the proliferation of HASMCs (Fig. 1A and B). Subsequently, the present study examined whether chrysin was able to block PDGF-BB-induced HASMC proliferation. The results showed that PDGF-BB enhanced the proliferation, while chrysin significantly abrogated the PDGF-BB induced proliferation of HASMCs, resulting in a decreased number of viable cells compared with that in the control group at high chrysin concentrations (Fig. 1C and D).

Chrysin does not significantly affect the apoptotic rate of HASMCs. In order to investigate whether the anti-proliferative effects of chrysin were attributable to the induction of apoptosis, the present study next examined the effects of chrysin on HASMC apoptosis. An Annexin V-FITC apoptosis assay was used to determine the percentage of apoptotic cells. The results showed that PDGF-BB had no effects on the apoptotic rate compared with that in the control group. Treatment with chrysin increased the apoptotic rate of the HASMCs, but not significantly. However, chrysin had no effect on the apoptotic rate of cells treated with PDGF-BB (Fig. 2).

Chrysin abrogates PDGF-induced ERK1/2 phosphorylation. It has been established that ERK1/2 is activated during PDGF-stimulated cell proliferation (19). Therefore, the present study explored whether chrysin inhibited the proliferation of HASMCs by reducing the phosphorylation of ERK1/2. As shown in Fig. 3, PDGF-BB significantly increased the phosphorylation of ERK1/2 compared with that in the control group, which was significantly abrogated by chrysin. However, chrysin treatment alone did not affect the phoshorylation levels of ERK1/2. In addition, neither PDGF-BB nor chrysin had any effect on total ERK1/2 expression.

Discussion

The present study investigated the effects of chrysin on HASMCs. The results showed that chrysin inhibited basal and PDGF-induced proliferation and slightly, but not significantly, enhanced apoptosis. This effect was associated with decreased phosphorylation of ERK1/2.

Asthma is a complex chronic airway inflammatory disease, which is accompanied with oxidative stress. Increased production of reactive oxygen species has been identified to cause airway inflammation, bronchial hyperreactivity, increased vascular permeability, tissue injury and airway

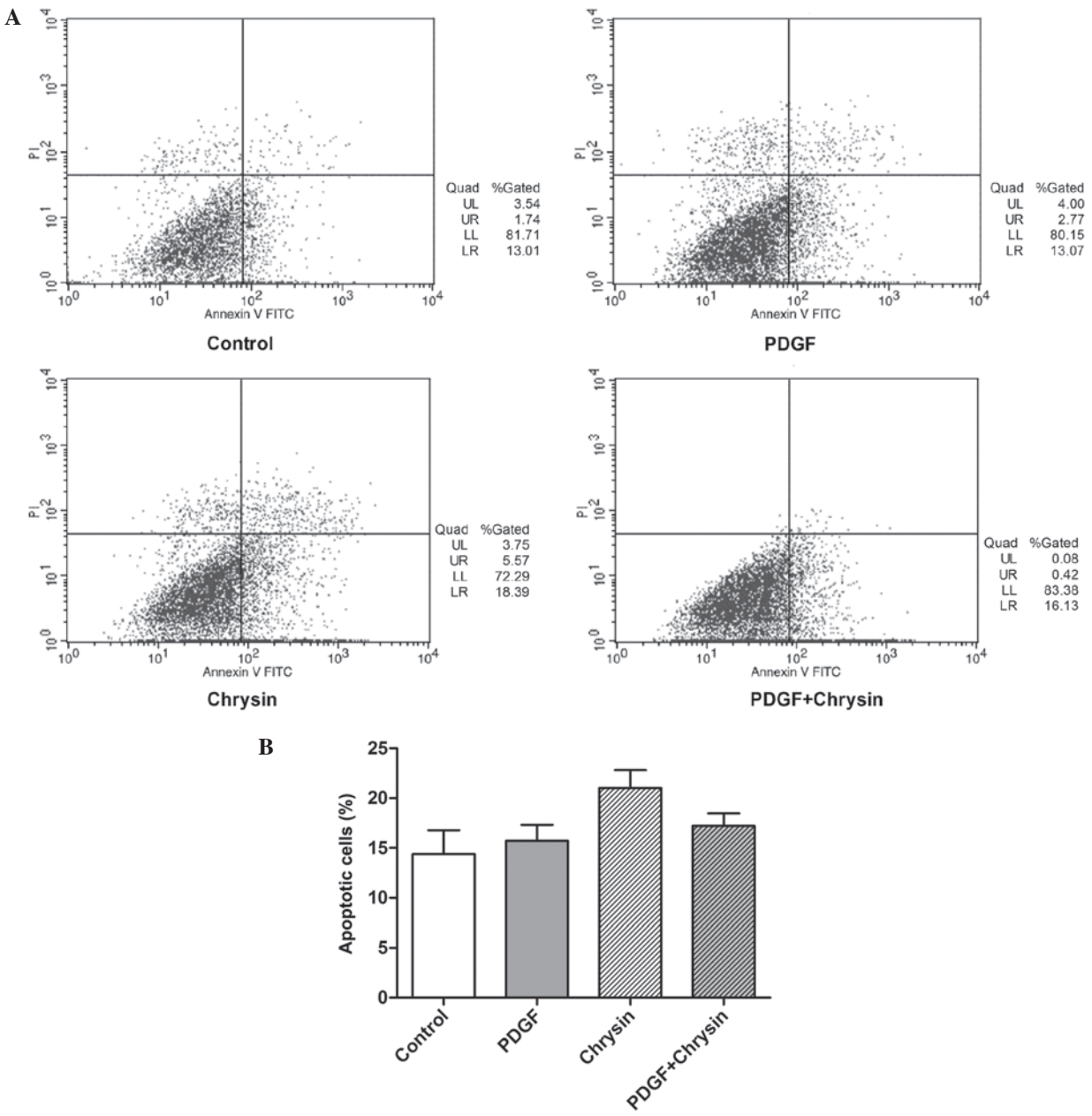

Figure 2. Chrysin does not significantly inhibit apoptosis of human airway smooth muscle cells. (A) Annexin V-FITC apoptosis detection kit was used to determine the percentage of apoptotic cells. Growth-arrested cells were stimulated with PDGF-BB (10 ng/ml) with or without 30-min chrysin (20 μM) pre-treatment, or chrysin (20 μM) alone for 24 h. (B) Bar graph representing the percent of apoptosis cells by densitometric analysis. There is no significant difference between groups. Values are expressed as the mean ± standard error of the mean from three individual experiments. PDGF, platelet-derived growth factor; FITC, fluorescein isothiocyanate; PI, propidium iodide; UL, upper left; LR, lower right; Quad, quadrant.

remodeling (1,20,21). Previous studies have demonstrated that chrysin has an anti-oxidant function (12,13). Chrysin may therefore be a suitable medication for treating asthma. A previous study reported that chrysin inhibits mast cell-derived allergic inflammatory reactions *in vivo* and *in vitro* by blocking histamine release and pro-inflammatory cytokine expression (16). Du *et al* (15) and Lee *et al* (22) have demonstrated that chrysin exerts anti-asthmatic effects in animal models. However, the target cells and mechanisms involved in chrysin-modulated amelioration of asthma have remained elusive. Studies have confirmed that chrysin exerts anti-proliferative effects on numerous cancer cell lines, including A549 and PC-3 (23,24), and also significantly suppresses the proliferation of human umbilical vein endothelial cells in a concentration-dependent manner (25). Since ASM have a crucial role in airway remodeling of asthma, primarily due to their increased mass, the present study investigated whether chrysin had an effect on the proliferation of HASMCs. The results revealed that chrysin inhibited HASMCs proliferation in a dose- and time-depended manner. PDGF-BB is a mitogen, which was extensively proved to potently induce the proliferation of ASM cells *in vitro* as well as *in vivo* (26-28). Furthermore, it has been reported that chrysin can inhibit PDGF-induced proliferation in rat vascular smooth muscle cell (19). In line with this finding, the results of the present study also indicated that chrysin suppressed the proliferation of HASMCs induced by PDGF-BB. This capacity

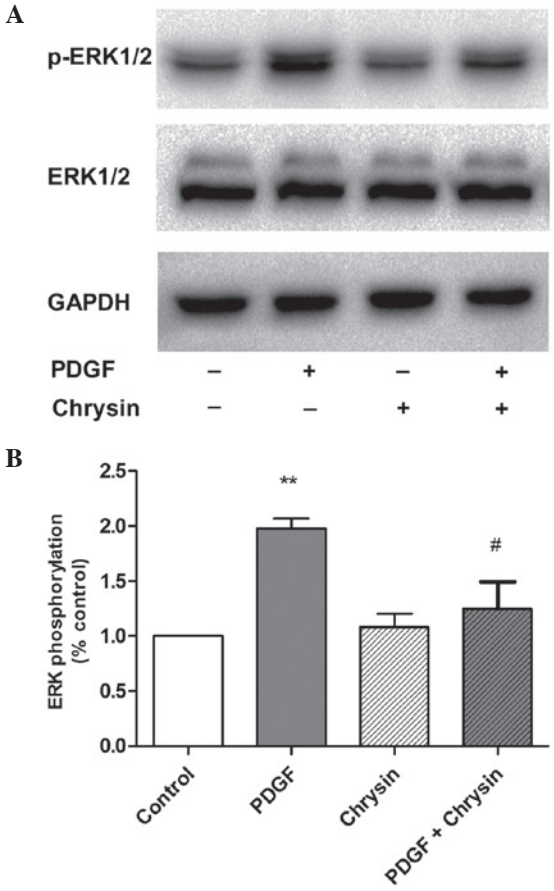

Figure 3. Chrysin inhibits the effect of PDGF-BB on the activation of ERK1/2. (A) HASMCs were stimulated with PDGF-BB (10 ng/ml) for 30 min with or without pre-treatment with chrysin (20 μM) for 30 min. (B) Bar graph representing the phosphorylation levels obtained by densitometric analysis. Values are expressed as the mean ± standard error of the mean from three individual experiments. **P<0.001 vs. control group; $^{\#}$P<0.05 vs. PDGF-stimulated treatment group. PDGF, platelet-derived growth factor; HASMC, human airway smooth muscle cell; p-ERK, phosphorylated extracellular signal-regulated kinase.

may be of potential clinical value in the treatment of airway remodeling.

The increased ASM mass in patients with asthma is associated with reduced apoptosis (4). Apoptosis has been observed to be responsible for the growth inhibition by chrysin in A549 and HepG2 cells (23,29). The present study explored whether chrysin inhibited the growth of ASM through inducing apoptosis. The results showed that chrysin induced apoptosis of HASMCs, although without statistical significance. This finding implied that the anti-proliferative effects of chrysin were not primarily the promotion of apoptosis.

ERK is required for HASMC proliferation and the phosphorylation of ERK1/2 is enhanced during PDGF-induced ASM-cell proliferation (30-32). Furthermore, chrysin has been confirmed to restore PDGF-induced ERK1/2 phosphorylation in rat vascular smooth muscle cell (19). To further elucidate the molecular mechanisms involved in the anti-proliferative effects of chrysin, the present study assessed whether chrysin inhibited the proliferation of HASMCs through the ERK pathway. The results revealed that chrysin reduced PDGF-induced ERK1/2 phosphorylation in HASMCs, indicating that chrysin may inhibit PDGF-induced proliferation through the ERK signaling pathway. However, chrysin alone had no effect on the basal phosphorylation of ERK1/2 and total ERK1/2. This finding indicated that other pathways contribute to the anti-proliferative effects of chrysin. Phosphoinositol-3-kinase (P13K), p38-MAPK, c-Jun N-terminal kinase, phospholipase C, protein kinase C, tyrosine kinases and CCAAT/enhancer binding protein-a are also involved in ASM-cell proliferation (7,33). Chrysin has been proved to induce growth inhibition and reduce the phosphorylation of Akt in A549 cells (23). Decreased proliferation induced by chrysin was observed in human and murine melanoma cells, which is associated with the activation of p38-MAPK (34). Therefore, the abovementioned signaling molecules may be additional targets of chrysin in ASM cells.

The PDGF - PDGF receptor (PDGFR) axis is involved in ASM-cell proliferation (35,36). It has remained elusive whether chrysin affects the interaction between PDGF and PDGFR or the downstream signaling of this axis to inhibit proliferation. A complex signaling network consisting of numerous signaling molecules participates in the proliferative process (33). Hence, it is possible that chrysin has a role in other pathways. In addition, asthma remodeling is associated with ASM migration (4). Previous studies suggested that chrysin inhibited the migration of cancer cells, endothelial cells and even vascular smooth muscle cells (19,37,38), indicating that chrysin may also affect ASM-cell migration. Since numerous other cell types, including inflammatory cells, participate in the pathology of asthma, ASM may not be the sole target cell type of chrysin in ameliorating asthma. Therefore, additional studies are required to gain deeper insight into the mechanisms of action of chrysin and provide data supporting its clinical application.

In conclusion, the present study demonstrated that chrysin inhibited PDGF-induced proliferation of HASMCs through reducing the phosphorylation of ERK1/2, suggesting that chrysin mat be a promising medication for controlling airway remodeling and clinical manifestations of asthma.

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