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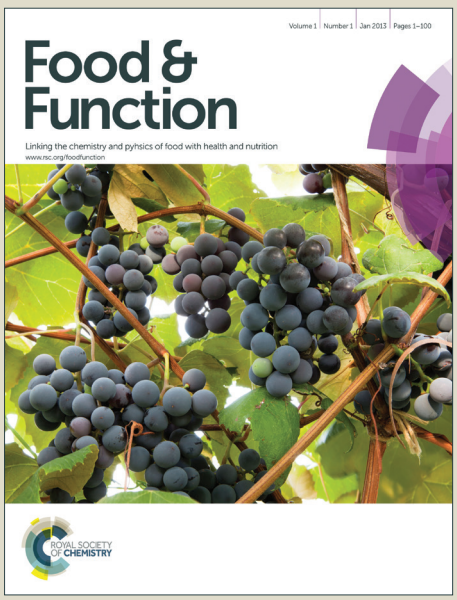

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Stigmasterol protects against Ang II-induced proliferation of A7r5 aortic smooth muscle cell-line

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Abstract:

Excessive proliferation of vascular smooth muscle cells is a crucial event in the pathogenesis of several cardiovascular diseases, including atherosclerosis and restenosis. In this study, we reported that stigmasterol was effective in inhibiting vascular cell proliferation exemplified using A7r5 cells stimulated by Ang II. Mechanism analysis showed that this inhibiting effect was mainly occurred by the arrest of cell-cycle and promotion of apoptosis. In addition, stigmasterol inhibition effects were detected to associate with decreased ROS production, enhanced SOD and CAT activity, decreased abundance of cyclin A, CDK2, PCNA, bax and bcl-2, and increased levels of p53 protein. Our study provided implications for the development of therapeutic strategies to protect against certain cardiovascular pathologies, such as atherosclerosis and restenosis.

Keywords：Stigmasterol, proliferation, vascular smooth muscle cells, A7r5 cells

1. Introduction

Vascular smooth muscle cells (VSMC), a major cellular component in the vascular wall, play a crucial role in the local regulation of vascular tone. Excessive proliferation of VSMCs has been reported to contribute to the initiation and progression of atherosclerosis and restenosis after percutaneous coronary intervention. Therefore, a novel agent that can inhibit VSMCs proliferation is highly desired for preventing atherosclerosis and restenosis.[1-3]

The excessive proliferation of VSMCs can be induced by various cytokines and growth factors, such as angiotensin II (Ang II), insulin, platelet-derived growth factor.[4] As a significant peptide of the rennin-angiotensin system, Ang II was reported to induce VSMCs proliferation through the regulation of oxidative stress and cell-cycle progression.[5] Recently, it was substantiated *in vitro* and *in vivo* that Ang II-mediated cardiomyocytes proliferation have associated with the P53-dependent apoptotic signaling pathway.[6-8]

Phytosterols are well known chemical in plants with structural similarity to cholesterol. Previous studies had reported that phytosterols could diminish cholesterol, modulate immune systems, reduce risks of coronary and cardiovascular diseases, and protect against cancers. As a member of phytosterol family, stigmasterol exists in plant spreads and oils such as peanut, sunflower, or corn oils. The chemical structure of stigmasterol was displayed in Fig. 1A. It has been reported that stigmasterol could lower the plasma and hepatic cholesterol levels in rats[9] and decrease hepatic lipid peroxidation in mice,[10] suggesting that an antiatherogenic potential for stigmasterol. Additional studies showed that stigmasterol exerted anti-proliferative effects and apoptosis induction in several cell lines, such as MCF-7 cells[11] and HepG2 cells. [12] However, the effect of stigmasterol on the proliferation in VSMCs is remains largely unknown. In the current study, we used rat vascular smooth muscle cell line A7r5 as a model cell line to study the effect of stigmasterol on the proliferation of Ang II-induced vascular smooth muscle cells. The cell proliferation rates, apoptosis rates, cell cycle progression in Ang II-induced A7r5 cells with or

without stigmasterol treatment were studied and compared. The oxidant/antioxidant levels and the expression of some cell cycle-related protein (Cyclin A, cyclin-dependent kinase 2, proliferating cell nuclear antigen) and apoptotic protein (p53, bax and bcl-2) were also analyzed.

1. Materials and methods

2.1 Chemicals

Stigmasterol was purchased from Chengdu Must Bio-technology Co. Ltd (Chengdu, China). Ang II was obtained from Alexis Corporation (Lausen, Switzerland). Propidium iodide (PI) and 3-(4, 4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were bought from Gibco BRL (NY, USA). All antibodies used for Western blot analysis were obtained from Boster Biotechnology Co. Ltd (Wuhan, China). Kits for analyzing reactive oxygen species (ROS), superoxide dismutase (SOD) and catalase (CAT) were purchased from Beyotime Institute of Biotechnology (Nantong, China) .

2.2 Cell culture

Rat embryonic thoracic aorto smooth muscle-derived A7r5 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM with high glucose supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin at 37°C with a humidified atmosphere of 5% CO_2.

2.3 Cell treatment

After the attainment of confluence (70-80%), A7r5 cells were counted and seeded into culture plates at a different densities for 24 h. Cells were then rinsed with phosphate-buffered saline (PBS) and cultured in medium containing 2% FBS, with or without Ang II (1.0 M) and stigmasterol at the required concentrations for 24 h. The concentration of Ang II used to stimulate cells proliferation was selected according to the literature.[13]

2.4 Cytotoxity and cell proliferation assay

Cytotoxity and cell proliferation were determined by the MTT method. Cells at a density of 3×10^3 cells/well were subjected to the same treatments as above, followed by addition of MTT at a final concentration of 0.5 mg/mL for 4 h at 37°C. The medium was then removed and the end product (formazan) was dissolved with 150 μL dimethyl sulfoxide (DMSO) to each well. After agitation of the plates for 15 min, the absorbance was measured at a wavelength of 570 nm with an automated microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Experiments were performed in triplicate.

2.5 Cell cycle regulation

Cell cycle regulation was determined by flow cytometry. Briefly, cells were seeded into 24-well plates at a density of 5×10^5 cells per well and treated as described above. After the treatment, cells were harvested, and fixed with ice-cold 70% ethanol overnight. The fixed cells were washed with cold PBS twice, and then incubated with PI solution (50 μg/mL PI in sample buffer containing 0.1 mg/mL of Rnase) in the dark for 10 min. Cell cycle stages were analyzed by flow cytometry (FACS Calibur cytometer BD Biosciences, USA). Experiments were performed in triplicate.

2.6 Cell apoptosis assay

Cell apoptosis was detected by flow cytometry. Cells at a density of 1×10^6 cells/well were seeded into 6-well culture plates. After different treatment as described above, cells were harvested and stained with Annexin V and PI for 15 min at room temperature in the dark. The percentages of positive cells were detected using flow cytometry (FACS Calibur cytometer BD Biosciences, USA). Experiments were performed in triplicate.

2.7 Oxidant/antioxidant levels determination

The production of ROS and the activity of SOD and CAT were tested to estimate oxidant/antioxidant levels. Cells at a density of 1×10^6 cells/well were seeded into

6-well culture plates, and treated as described above. Intracellular ROS production was assessed using 2',7'-dichlorofluorescein diacetate according to the reference. [14] The fluorescence intensity was determined with a fluorescence microscope (Leica, Germany) at excitation of 485 nm and emission of 524 nm. The activities of SOD and CAT were measured by commercial kits (Beyotime Institute of Biotechnology, Nantong, China). The protein concentration of the samples was measured by Bradford method (Beyotime Institute of Biotechnology, Nantong, China). ROS levels were expressed as the percentage of fluorescent positive cells to the total number of cells, SOD and CAT activities were expressed as U/mg protein. Experiments were performed in triplicate.

2.8 Western blot analysis

Western blot analysis was used to measure the protein levels of Cyclin A, cyclin-dependent kinase 2(CDK2), proliferating cell nuclear antigen (PCNA), p53, bax and bcl-2. Briefly, Cells at a density of 1×10^6 cells/well were seeded into 6-well culture plates. After being treated as described above, cells were lysed in the lysis buffer (50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% NP-40, 0.5 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM NaF, 0.1 mM Na_3VO_4 and 1 mM DTT). Then proteins lysed from cells were separated with SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% BSA for 2 h prior to incubation with the indicated primary antibody at 4 ºC overnight. After washing, the membrane was incubated with respective secondary antibody. Immunoblots were visualized with an ECL chemiluminescent assay kit (Amersham Biosciences, Buckinghamshire, UK). The Western blot bands were quantified by Quantity One (Bio-Rad,). Rat β-actin was used as an internal control to correct for differences in quantity between protein samples, and the expression levels of various proteins of interest were calculated as a ratio to β-actin protein. Experiments were replicated four times.

1.9 Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). Data were

analyzed by one-way analysis of variance followed by multi-range tests using the statistical software, SPSS 11.5 for Windows (SPSS, Chicago, IL, USA). A significant level of $P < 0.05$ was used for all comparisons.

3. Results

3.1 Effect of stigmasterol on cytotoxity in A7r5 cells in the absence of Ang II

The A7r5 cells were treated with various concentration of stigmasterol (0, 5, 10, 20, 40, 80 μg/mL) for 24 h and 48 h respectively, and the cytotoxity assay was analyzed by MTT assay. Considering the stigmasterol at concentration of 5, 10, and 20 μg/mL was not found to be statistically significant cytotoxity in A7r5 cells for 24 h (Fig.1B), the stigmasterol contration range of 5-20 μg/mL and incubation time of 24 were selected for the following experiments.

3.2 Effect of stigmasterol on cells proliferation induced by Ang II

The A7r5 cells were treated with 1μM Ang II and various concentration of stigmasterol (0, 5, 10, 20 μg/mL) for 24 h, and the cell proliferation was analyzed by MTT assay. The results showed that 20 μg/mL stigmasterol did not stimulate the excessive proliferation of A7r5 cells, however, 1 μM Ang II significantly promoted the proliferation of A7r5 cells from 100% to 131% (Fig.1C). After stigmasterol treatment, Ang II-induced cell proliferation was inhibited in a concentration-dependent manner.

3.3 Effect of stigmasterol on cell cycle progress induced by Ang II

In order to better understand the effect of stigmasterol on inhibition of cell proliferation, cell cycle progress was analyzed by flow cytometry. As shown in Fig.1D, only treatment with stimasterol did not alter the cell cycle progress of A7r5 cells. After being stimulated with Ang II, the proportions of A7r5 cells in the S phase increased from (13.02 ± 1.04)% to (41.15 ± 2.43)% ($P < 0.001$), with a concomitant decline in the proportions of A7r5 cells in the G_0/G_1 phase from (76.93 ± 5.69)% to (45.30 ± 5.25)% ($P < 0.001$) . However, pretreatment of Ang II-induced A7r5 cells with 5, 10, 20 μg/mL stigmasterol, the proportions of cells in the S phase decreased to (34.54 ± 2.36)% ($P < 0.01$), (25.23 ± 2.58)% ($P < 0.001$) and (18.23 ± 2.31)% ($P <$

0.001), while the proportions of cells in the G_0/G_1 phase increased to (50.07 ± 5.09)% ($P > 0.05$), (59.39 ± 7.06)% ($P < 0.01$) and (71.67 ± 3.46)% ($P < 0.001$) compared with Ang II treatment alone, respectively. Neither Ang II nor stigmasterol influenced the proportions of A7r5 cells in the G_2/M phase.

3.4 Effect of stigmasterol on cell apoptosis induced by Ang II

Apoptosis was determined by cytometry after staining with PI and PITC-labelled annexin □. The results (Fig. 2) showed that stigmasterol (20 μg/mL) did not reduce the apoptosis in A7r5 cells, and Ang II induced a substantially decrease in the number of apoptotic cells. Further, treatment with stigmasterol obviously reinforced this decrease trend induced by Ang II by showing even lower ratio of apoptotic A7r5 cells.

3.5 Effect of stigmasterol on oxidant/antioxidant levels induced by Ang II

ROS levels as well as the activities of SOD and CAT in the A7r5 cells were shown in Fig. 3. ROS levels were elevated in the Ang II-treated group compared with the untreated control group (~ 27% increase, $P < 0.001$). However, after treatment with stigmasterol in Ang II-induced cells for 24 h, the increase in cellular ROS levels was inhibited. Unlike the increasing trend of ROS, SOD and CAT activities in the Ang II group showed significant decrease for 38% and 29%, respectively. After the administration of stigmasterol, SOD and CAT activities restored to the normal levels with values close to the control group.

3.6 Effect of stigmasterol on Cyclin A1, CDK2 and PCNA induced by Ang II

To further substantiate the effect of stigmasterol on cell cycle, immunoblotting analysis was performed. As shown in Fig. 4, only stigmasterol (20 μg/mL) did not stimulate the expression of Cyclin A1, CDK2 and PCNA (all $P > 0.05$), and only Ang II (1 μM) significantly induced the expression of these proteins (all $P < 0.001$). Stigmasterol treatment in Ang II-induced A7r5 cells, on the other hand, inhibited Ang II-induced activation of these proteins. Nevertheless, the inhibition effect of stigmasterol on protein expression was largely related to the concentrations. As demonstrated in Fig.4, 5 μg/mL of stigmasterol showed partial inhibition. However, when the concentration of stigmasterol increased to 10 μg/mL, a strong inhibition effect was observed. With final concentration of 20 μg/mL of stigmasterol was applied, a complete inhibition effect was observed and no further protein expression could be

detected under the administration of this concentration (Fig. 4).

3.7 Effect of stigmasterol on p53, bcl-2 and bax induced by Ang II

The protein levels of p53, bax and bcl-2 were studied by western blot in order to understand the molecular mechanism of the effect of stigmasterol on cell apoptosis. The results were presented in Fig.5. It was found that Ang II markedly down-regulated the protein levels of p53 ($P < 0.01$) and bax ($P < 0.001$) and up-regulated the protein level of bcl-2 ($P < 0.001$). Treatment with stigmasterol for those Ang II-induced A7r5 cells significantly relieved the inhibition effect of Ang II by showing enhanced expression of p53 and bax and restored expression of bcl-2.

4. Discussion

The A7r5 clonal cell line, derived from embryonic rat thoracic aorta, retained many characteristics of vascular smooth muscle cells and was thought as a suitable model of VSMCs.[15] In the current study, we demonstrated that Ang II induced the cell proliferation and cell cycle arrest at S phase, and inhibited cell apoptosis in A7r5 cells, which was in line with other findings in vascular smooth muscle cells.[16-18] Stigmasterol, an important phytosterol present in plants, was reported to have various biological effects such as anti-stiffness, anti-cancer, anti-inflammatory, anti-oxidant and immune-modulating properties in previous studies.[10,19,20] In this study, we have provided the first evidence that treatment with stigmasterol significantly inhibited the excessive proliferation and cell cycle arrest, induced apoptosis, and counteract the detrimental effects induced by angiotensin II in A7r5 cell lines.

The proliferation of VSMCs is a crucial event in the pathogenesis of many vascular diseases, including atherosclerosis and restenosis.[21-24] In the eukaryotic cells, cell cycle is divided into four distinct phases: the G_0/G_1, S, G_2 and M phases. Under normal conditions, VSMCs retain in the quiescent G_0/G_1 phase, a non-proliferative state. However, when vessel injuries such as atherosclerosis and restenosis happen, VSMCs leave the G_0/G_1 phases and re-enter the cell cycle for proliferation (S phase) in response to various stimulatory cytokines and growth factors such as Ang II.[25,26] The present finding showed similar cell cycle progress in Ang II-treated A7r5 cells. The percentage of cells in the S phase increased after treatment with Ang-II.

Stigmasterol supplements significantly prevented Ang II-induced this increase, with a concomitant increase in the percentage of A7r5 cells in the G_0/G_1 phase. The stigmasterol effect on A7r5 cells determined in the current work was consistent with those previous studies using various cancer cells. It is also reported that stigmasterol inhibited the proliferation of hepatoma cells both *in vitro* and *in vivo*, which was related to the increase of cells in G_0/G_1 phase, decrease the cells in S or G_2/M phases, and induce cell apoptosis.[27] Other investigations reported that stigmasterol had potential anti-proliferative effects and apoptosis induction in liver hepatocellular carcinoma (HepG2) cells probably via reducing cell numbers in S phase and blocking the cell multiplication cycle.[12]

In S phase of cell cycle, the content of Cyclin A1/CDK2/PCNA complex is a key factor to ensure cells transit from S to G_2/M phase.[28] Cyclin A is one of the main S phase cyclins and regulates CDK2. In turn, CDK2 plays a critical role at the G_1/S transition. As an acidic nuclear protein, PCNA is closely related to the synthesis of DNA by promoting the ability of DNA polymerase. Blockade of the Cyclin A/CDK2/PCNA complex dramatically reduced aberrant vascular smooth muscle cell (VSMC) proliferation.[29,30] Here our findings using A7r5 smooth muscle cells were in agreement with previous studies using VMSCs,[31,32] showing that Ang II up-regulated the expression of Cyclin A1, CDK2 and PCNA. Therefore, for the first time that we demonstrated in this work stigmasterol could inhibit the upregulation of the expression of Cyclin A1, CDK2 and PCNA, which may further prevent excessive proliferation of A7r5 cells.

Besides the induction of reentry into the cell cycle, alteration of susceptibility to apoptosis in VSMCs also plays critical role in promoting cell proliferation.[33] Apoptosis, or programmed cell death, is a normal physiological process of cell death which designed to eliminate unwanted host cells. The p53, a major tumor suppressor in human cancers, orchestrates a broad array of cellular responses by regulating apoptosis, cell cycle arrest, senescence, DNA repair and genetic stability. Mounting evidence has demonstrated that p53 participates directly in the intrinsic apoptosis pathway by interaction with caspases and bax/bcl-2.[7,34] In our study, 1 μM Ang II

significantly induced apoptosis in A7r5 cells, primarily via down-regulation of p53 and bax expression along with up-regulation of bcl-2 expression. Similar results were reported in other studies. [6,13,35] Stigmasterol displayed potent apoptosis inductive effects by up-regulation of p53 and bax and down-regulating bcl-2 respectively in HepG2 cells[12] and U937 cell line, [36] which was consistent with our results found in Ang II-induced A7r5 cells.

Since VSMCs growth is redox-sensitive and oxidative stress stimulates the proliferation and migration of these cells, antioxidant treatment is considered as a potential therapy to reduce the risk of cardiovascular diseases. It is well known that Ang II stimulated the production of ROS in VSMCs and mediates oxidative stress during the development of the cardiovascular diseases.[37-39] In this work, similar results were detected by showing that stigmasterol could significantly inhibit the increment of ROS induced by Ang II in A7r5 cells. Based on the findings that SOD and CAT are important enzymes to defend against oxidative stress, we further investigated the influence of stigmasterol on the activities of SOD and CAT. Results showed that administration of stigmasterol increases the activities of these two enzymes in Ang II-treated A7r5 cells. These results clearly demonstrated an antioxidant role of stigmasterol in A7r5 cells, consistent with previous reports on various tissues and cell types.[10,40] Thus, the proliferative inhibitory effect and apoptosis inductive effect of stigmasterol is well proven to be related with its antioxidant activity.

5. Conclusion

This study reports for the first time that administration of stigmasterol to Ang II-induced A7r5 cells inhibited significantly cell proliferation and cell cycle arrest, and promoted cell apoptosis. The beneficial effects of stigmasterol may be associated with mediating oxidative stress, down-regulating Cyclin A1, CDK2, PCNA and bcl-2 and up-regulating p53 and bax expression. Our findings suggest that stigmasterol administration has promising potential as therapeutic strategy for preventing cardiovascular disease.

Conflict of interest statement

The authors declare here that they do not have any direct financial relation with the commercial identities mentioned in the paper that might lead to conflicts of interest. All research was done by the authors. There was no financial support except those mentioned in Acknowledgments.

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Fig. 1. Effects of stigmasterol on cytotoxity, proliferation and cell cycle distribution in A7r5 cells induced by Ang II. (A) Chemical structure of stigmasterol, (B) Cytotoxity was tested by MTT assay. A7r5 cells were incubated with or without stigmasterol (0 - 80 μg/ml) in the absence of Ang II (1 μM) for 24 h and 48 h. (C) Proliferation was measured by MTT assay. (D) Cell cycle distribution was assessed by cytometry assay. A7r5 cells were incubated with or without stigmasterol (0, 5, 10, 20 μg/ml) for 24 h, followed by stimulation with Ang II (1 μM). Data were expressed as means ± S.E.M. for three independent experiments with five duplicates. *$P<0.05$, ***$P<0.001$ represent significant difference when compared with control group (without treatment with stigmasterol or Ang II). $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ represent significant differences when compared with Ang II group (treatment with Ang II alone).

Fig. 2. Effect of stigmasterol on apoptosis in A7r5 cells induced by Ang II. (A) Apoptosis was determined by flow cytometry, (B) Cell apoptotisis rates were analyzed. After treatment stigmasterol (0 - 20 μg/ml) or/and Ang II (1 μM), cells were harvested and stained with 5μL Annexin V and 10 μL PI for 15 min at room temperature in the dark. Data were expressed as means ± S.E.M for three independent experiments with five duplicates. ***$P<0.001$ represent significant difference when compared with control group (without treatment with stigmasterol or Ang II). $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ represent significant differences when compared with Ang II group (treatment with Ang II alone).

Fig. 3. Effect of stigmasterol on oxidant/antioxidant levels in A7r5 cells induced by Ang II. (A) ROS levels were illustrated by fluorescence intensity (×200 magnification). (B) ROS levels were analyzed statistically. (C) SOD activities were analyzed statistically. (D) CAT activities were analyzed statistically. A7r5 cells were treated with stigmasterol (0 - 20 μg/ml) or/and Ang II (1 μM). Intracellular ROS production was assessed using 2', 7'-dichlorofluorescein diacetate. The activities of SOD and CAT were measured with Total Superoxide Dismutase Assay kit or Catalase Assay kit respectively. Fluorescence intensity was determined with excitation wavelength of 485 nm and emission wavelength of 524 nm. Data were expressed as means ± S.E.M for three independent experiments with five duplicates. ***$P<0.001$ represent significant difference when compared with control group (without treatment with stigmasterol or Ang II). $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ represent significant differences

when compared with Ang II group (treatment with Ang II alone).

Fig. 4. Effects of stigmasterol on the expressions of cyclin A1, CDK2 and PCNA in A7r5 cells induced by Ang II. (A) Representative gel images were illustrated by Western blot, (B) protein expression was quantified and analyzed statistically. After treatment stigmasterol (0 - 20 μg/ml) or/and Ang II (1 μM), cells were lysed and proteins lysed from cells were separated with SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Then the membranes were incubated with primary antibodies and respective secondary antibodies. β-actin expression was measured to confirm the equal amount of protein. Data were expressed as means ± S.E.M. for four independent experiments with four duplicates. ***$P<0.001$ represent significant difference when compared with control group (without treatment with stigmasterol or Ang II). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ represent significant differences when compared with Ang II group (treatment with Ang II alone).

Fig. 5. Effects of stigmasterol on the expressions of p53, bcl-2 and bax in A7r5 cells induced by Ang II. (A) Representative gel images were illustrated by Western blot, (B) protein expression was quantified and analyzed statistically. After treatment stigmasterol (0 - 20 μg/ml) or/and Ang II (1 μM) , cells were lysed and proteins lysed from cells were separated with SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Then the membranes were incubated with primary antibodies and respective secondary antibodies. β-actin expression was measured to confirm the equal amount of protein. Data were expressed as means ± S.E.M. for four independent experiments with four duplicates. ***$P<0.001$ represent significant difference when compared with control group (without treatment with stigmasterol or Ang II). $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ represent significant differences when compared with Ang II group (treatment with Ang II alone).

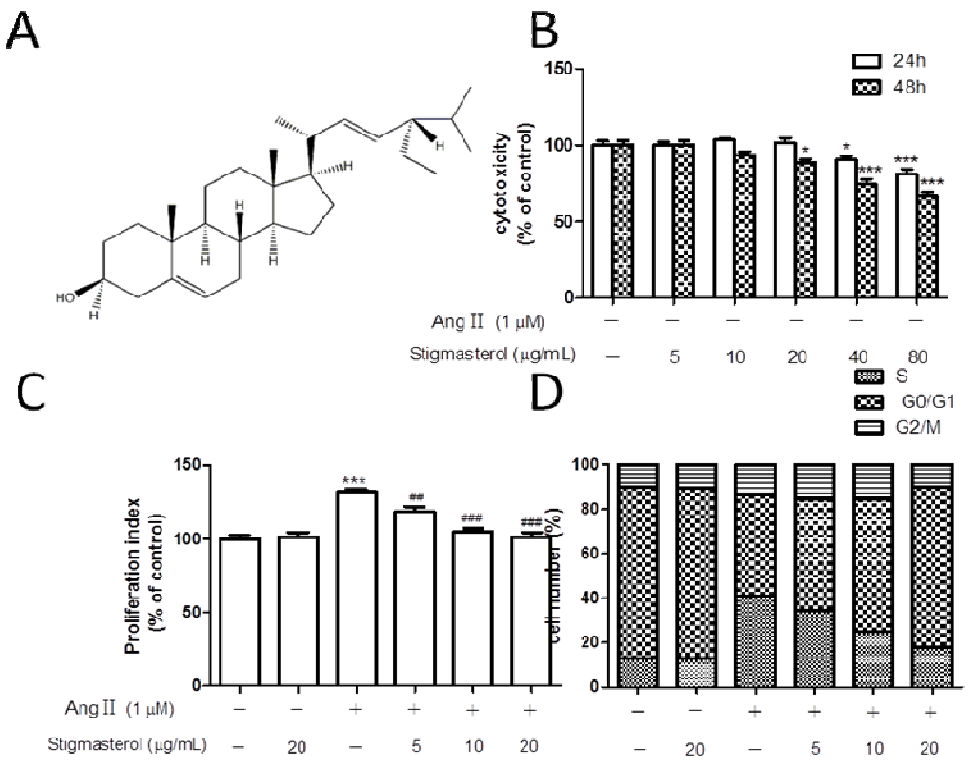


Fig. 1

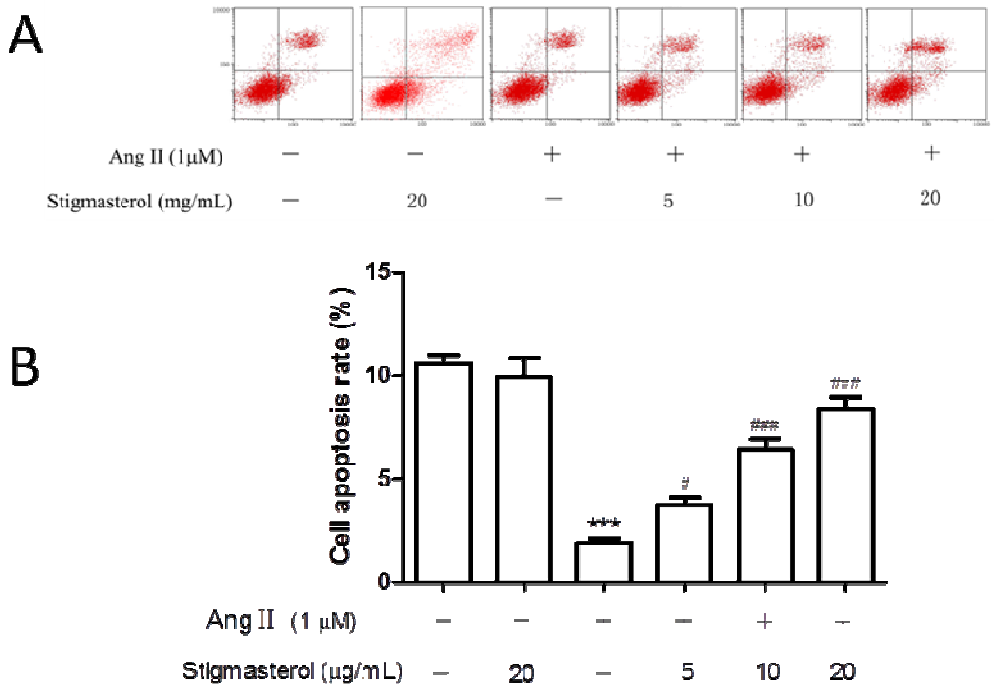


Fig. 2

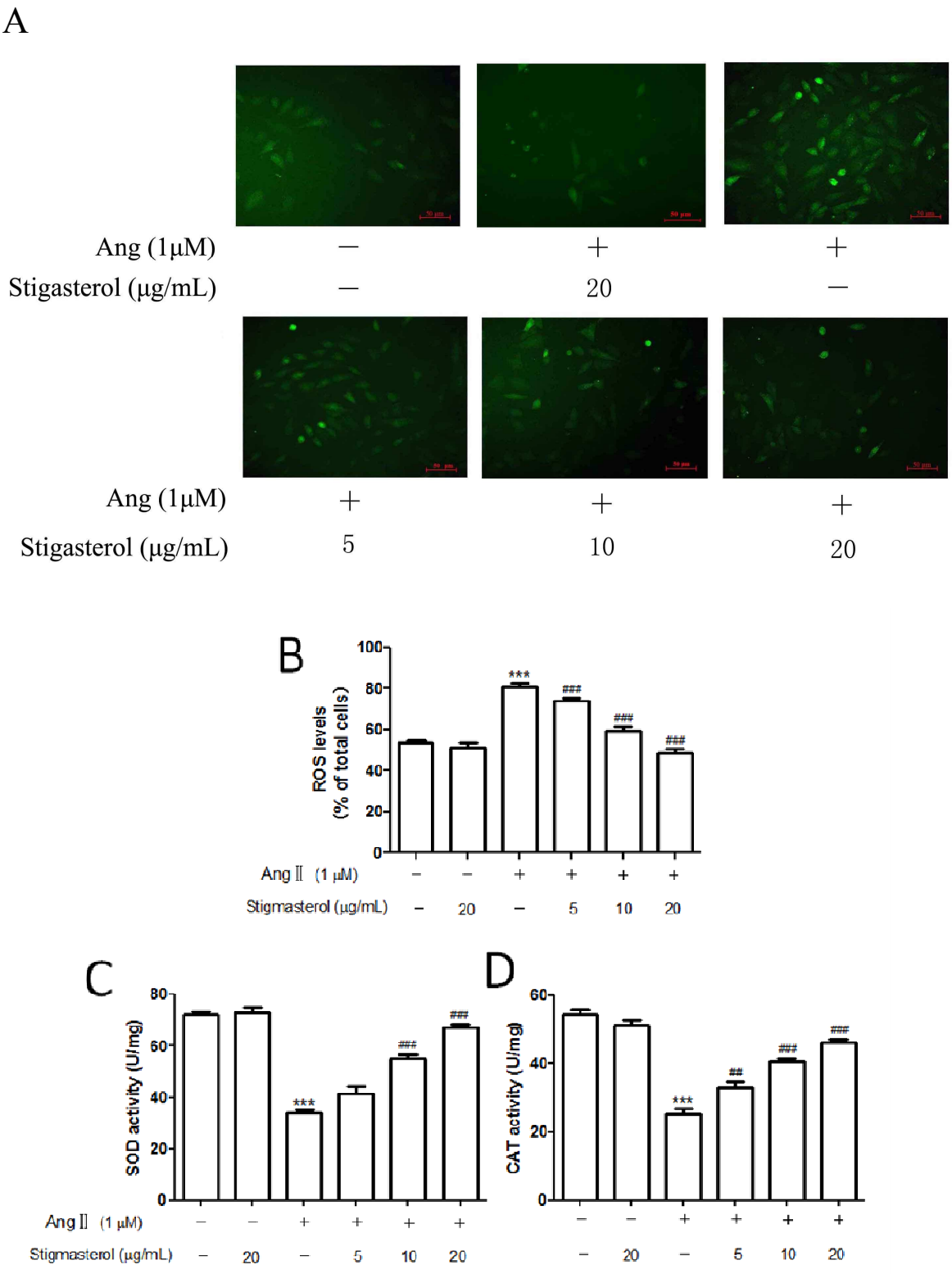
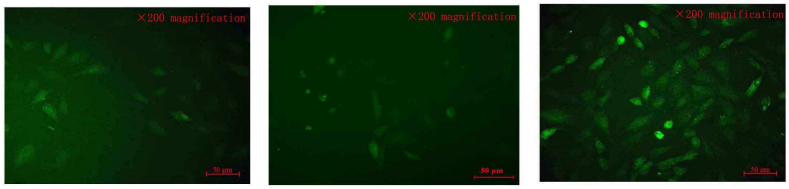
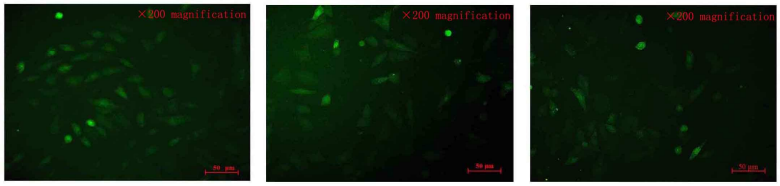


Fig. 3 (Fig. 3A is without magnification, and Fig. 3A with magnification is followed in the next page)



Ang (1μM)	−	+	+
Stigasterol (μg/mL)	−	20	−



Ang (1μM)	+	+	+
Stigasterol (μg/mL)	5	10	20

Fig3A with magnification

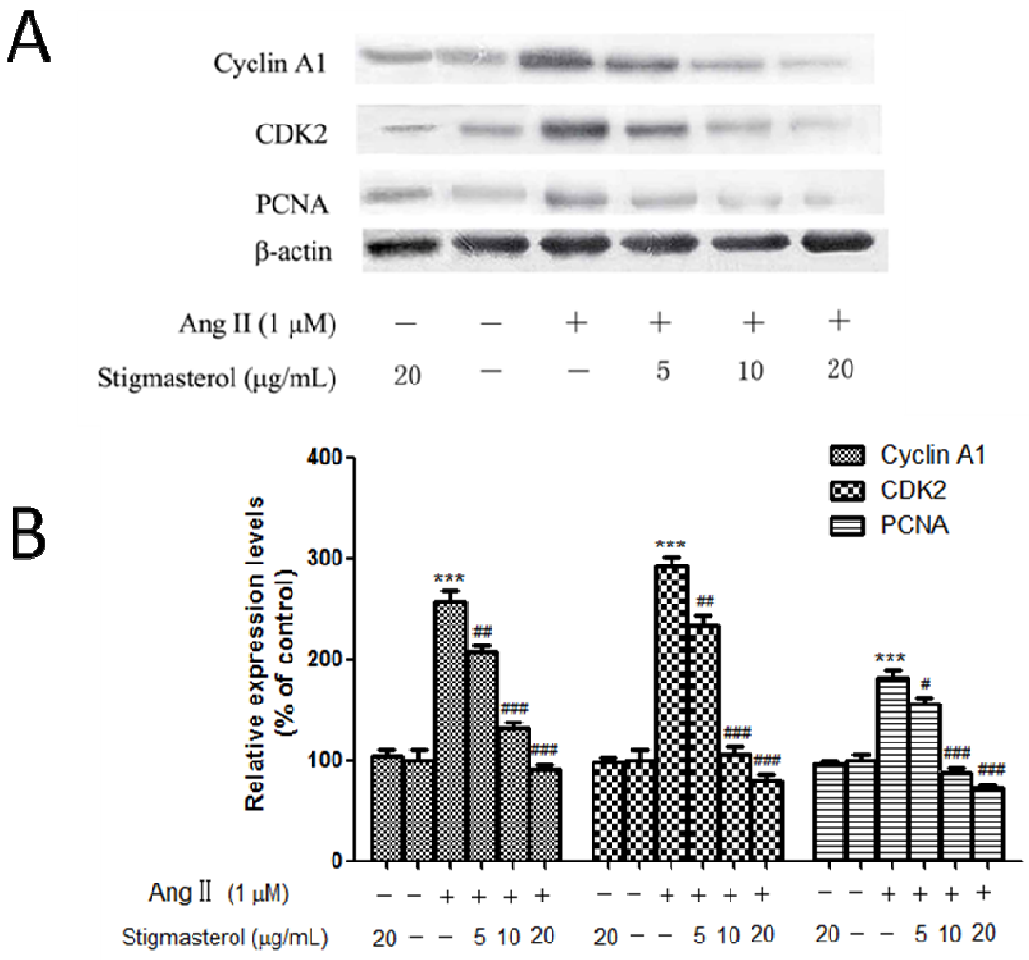


Fig. 4

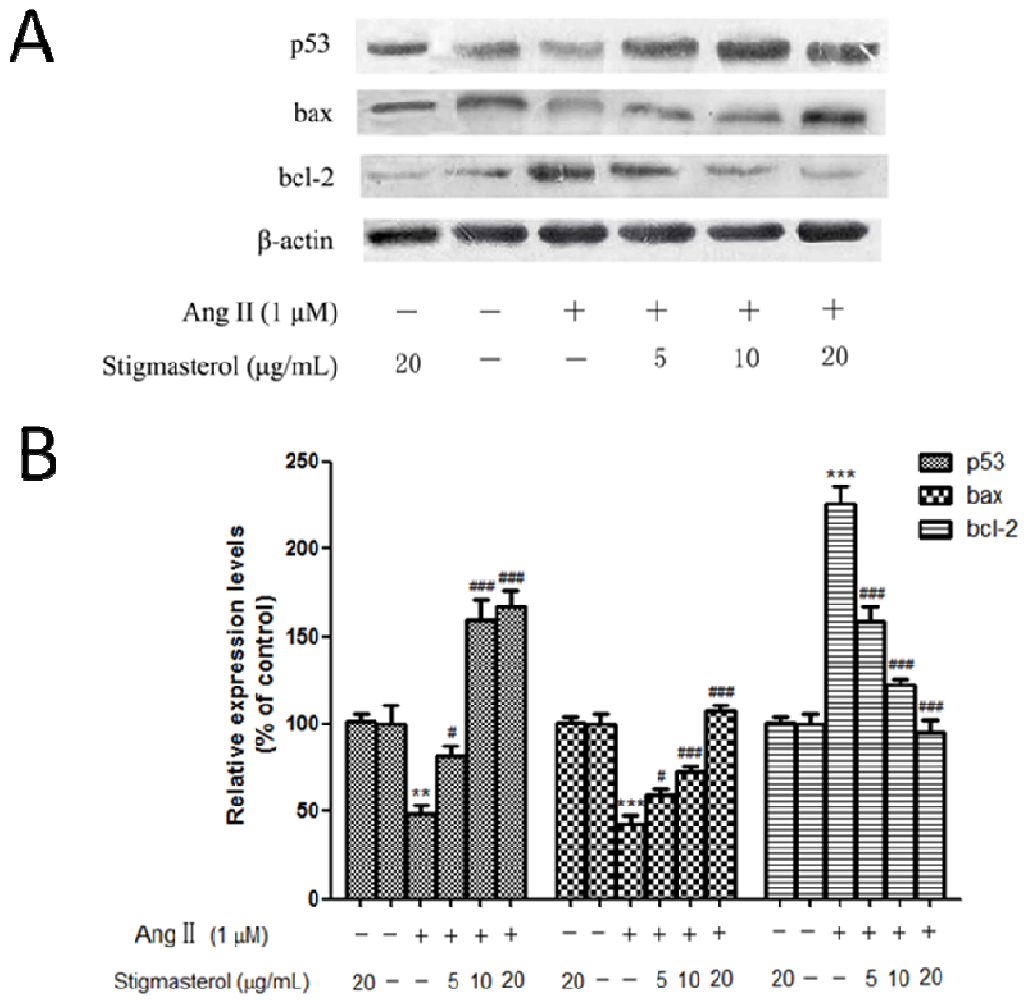


Fig. 5