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Inhibitory Effects of Saponins From *Anemarrhena asphodeloides* Bunge on the Growth of Vascular Smooth Muscle Cells[1]

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Objective To investigate the effects of saponins from *Anemarrhena asphodeloides* Bunge (SAaB) (Botanical Name: Anemarrhena Asphodeloidis Rhizoma) on the growth of vascular smooth muscle cells (VSMCs). **Methods** Cell proliferation was measured by a newly developed cell proliferation reagent, WST-1. Cell apoptosis was assayed by flow cytometry through detecting annexin V. Nitric oxide production was evaluated using confocal laser scanning microscopy with diaminofluorescein diacetate (DAF-2, DA). Cell aldose reductase (AR) activity, as well as the effect of Epalrestat and interleukin-1β were also explored. **Results** WST assay showed that cell proliferation induced by serum was significantly inhibited by SAaB ($P<0.01$). Flow cytometry analysis revealed that SAaB could enhance apoptotic rate of VSMCs ($P<0.01$). Nitric oxide production was significantly enhanced after administration of SAaB and interleukin-1β. Moreover, AR activity of VSMCs was also remarkably inhibited by both SAaB and Epalrestat ($P<0.01$). **Conclusion** SAaB can inhibit proliferation and enhance apoptosis of VSMCs. It may protect vascular cells by inhibiting VSMC proliferation and augmenting apoptotic rate of VSMCs via NO-dependent pathway.

Key words: *Anemarrhena asphodeloides* Bunge; Saponins; Vascular smooth muscle cells; Proliferation

INTRODUCTION

The proliferation of abnormal vascular smooth muscle cells plays a fundamental role in the pathogenesis of vascular complications, such as atherosclerosis, hypertension and restenosis[1-2]. Such proliferation is regulated by both autocrine and paracrine growth factors[3-6].

Medicinal herbs constitute indispensable components of the traditional medicine practiced worldwide due to their low cost, easy access and ancestral experience. *Anemarrhena asphodeloides* Bunge belongs to the family Liliaceae growing in China, Korea, and Japan. The *asphodeloides* rhizomes, having antipyretic, anti-inflammatory, sedative, diuretic, and anti-diabetic properties, are used in Chinese traditional medicine, and show anti-diabetic activity[7-8], platelet aggregation inhibitory activity[9-10], antifungal activity[11], anti-yeast activity[12], and inhibiting activity on cyclic AMP phosphodiesterase[13]. Chemical constituents from the rhizomes of *asphodeloides* that have been studied include steroidal saponins[14-16], xanthone C-glycosides[17], and norlignans[11]. Previous studies have demonstrated that inhibition of AR prevents the growth of VSMCs in culture as well as in balloon-injured arteries *in vivo*[18] and that *Anemarrhena asphodeloides* Bunge exhibits markedly inhibitory effect on the AR activity[19]. Whether proliferation of VSMCs is affected by SAaB remains unknown. Moreover, whether such effects are present in cell proliferation stimulated by the administration of foetal bovine serum (FBS) remains unknown, and the relationship between SAaB and the inhibitory effect of *Anemarrhena asphodeloides* Bunge on AR activity is unclear. In the present study, we therefore focused on the determination of the relationship between SAaB and proliferation of VSMCs induced by serum.

MATERALS AND METHODS

Preparation of Saponin Extract

Rhizomes of *Anemarrhena asphodeloides* Bunge (grown in Yi country, Hebei Province, China) were

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purchased from Shenyang Yaocai Co. (Shenyang, Liaoning Province, China). To obtain total saponins, four-fold 75% ethanol was added into 500 g Rhizoma *Anemarrhenae*, which was then extracted 3 times by heating under reflux, and each repetition lasted for forty minutes. When the filtrate solution was filtered and blended, it was condensed into 1000 mL, and shaken to be homogeneous, and put into the D101 large-aperture resin columns (glass column with internal diameter of 5 cm and column height of 30 cm, which contained 165 g resins with water inside). Then it was soaked for 15 minutes, and washed successively by 1000 mL water, 1000 mL 0.5% NaOH solution and 1000 mL 20% ethanol with a flow rate of 3 mL/min. After the elution by 60 mL 95% ethanol, the elution solution was collected and the ethanol was retrieved. The product after lyophilization was a slight yellow powder, weighing 16.5 g with a productive rate of 5.3%. The content of sapogenin in the final product was 26.7% and the crude saponin content was over 70%.

Materials

Sprague Dawley rats were purchased from Zhejiang Laboratory Animals Center and kept according to the Care Standard of Laboratory Animals (publication by the National Ministry of Health, 1998). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin and trypsin-EDTA solution were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone. NADPH, DL-glyceraldehyde, L-glutamine, mercaptoethanol and interleukin-1β were from Sigma Chemical Co. WST-1 was purchased from Dojindo Laboratories (Kumamoto, Japan). Annexin V kit (contained with fluorescein isothiocyanate-conjugated annexin V, propidium iodide and 4× binding buffer) was obtained from Caltag, USA. 4, 5-diaminofluorescein diacetate (DAF-2, DA) was from Calbiochem. AR inhibitor, Epalrestat, was a gift from Doctor Yi KONG. All other reagents and solvents used were of analytical grade.

Cell Culture

Primary cultures of vascular smooth muscle cells were obtained by the explant method from the media of thoracic aorta of 10-week-old male Sprague Dawley rats[20]. Briefly, aortas were stripped of adventitia, the endothelial cell layer of intima was scraped off, and the aorta was cut into small pieces. These pieces were placed in DMEM supplemented with heat-inactivated 20% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37℃. They exhibited a typical "hill and valley" growth pattern. Medium was replaced twice a week. When the cells became confluent, they were subcultured in the medium using 0.2% trypsin in 0.02% EDTA. Confluent cells at passage numbers 4-8 were used for experiments.

WST Cell Proliferation Assay

To analyze cell proliferation, a cell proliferation reagent WST-1 (4-[3(4–lodophenyl)-2-(4-nitrophenyl) -2 *H*-5-tetrazolio]-1, 3-benzen disulfonate) was used, and cell viability was assessed according to the manufacturer's instructions. This *in vitro* assay is based on the reduction of highly water-soluble tetrazolium salt WST-1. VSMCs were seeded onto 96-well tissue culture plates at a concentration of 1×10^4 cells/well in 100 μL DMEM containing 10% FBS. After the cells reached 70%-80% confluency, G_0/early G_1 synchronization was achieved by serum deprivation. The cells were then treated with 10% FBS containing DMEM in the absence or presence of various concentrations of SAaB for another two days. Control cells were cultured in DMEM containing 1% FBS or 10% FBS alone. After the treatment, 10 μL of WST-1 was added into each well, and the culture plates were incubated for 1.5 h at 37℃. Absorbency was measured at 450 nm with a reference wavelength at 690 nm by a microplate reader (BIO-RAD), and cell proliferation was presented as the relative cell viability in reference to the viability of control cells, which was set as 100%.

Cell Apoptosis Assay

Annexin V kit (contained with FITC-conjugated Annexin V, PI and 4×binding buffer; Caltag, USA) was used for two-color analysis on a flow cytometer (FACsort, Becton-Dickinson, USA) to evaluate apoptosis of VSMCs. Cells were seeded onto 24-well tissue culture plates at a concentration of 1×10^5 cells/well in 1 mL of 10% FBS containing DMEM and treated as described above. After the treatment, cells were washed twice with D-Hank's solution and harvested by trypsinization. Then cells were collected by centrifugation at 1200 rpm and resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL. Cells in binding buffer (100 μL) were transferred to a 5 mL culture tube, stained with 5 μL FITC-conjugated annexin V and 10 μL PI (50 μg/mL). After 15 minutes of incubation at 20-25℃ in the dark, cells in each tube were added 400 μL of 1× binding buffer and analyzed on the flow cytometer. Forward scatter (FSC) and side scatter (SSC) were collected in a linear mode, FL1 and FL2 were collected in a log mode. At least 10 000 cells were

collected for each sample, and the data were analyzed using the CELLQUEST^{TM} software (Becton-Dickinson, USA). This assay identifies normal cells as PI-negative and annexin V (FITC)-negative, apoptotic cells as PI-negative and annexin V (FITC)-positive and necrotic cells as PI-positive and annexin V (FITC)-positive[21].

Cell AR Activity Assay

VSMCs were seeded on 24-well plates at 2×10^4 cells/well in 500 μL of 10% FBS containing DMEM. After 24 hours, the culture medium was changed to a 0.1% FBS containing DMEM, and cells were cultured for an additional 24 h. The cells were then treated with 10% FBS containing DMEM in the absence or presence of SAaB for another two days. Control cells were cultured in 10% FBS containing DMEM alone. Cells were then washed with ice-cold PBS (pH 7.4) and harvested by scraping. The cell suspensions were homogenized with a glass Dounce homogenizer in 20 mmol/L sodium phosphate buffer (pH 7.0) containing 2 mmol/L dithiothreitol, 5 μmol/L leupeptin, 2 μmol/L pepstatin, and 20 μmol/L phenylmethylsulfonyl fluoride. After centrifugation of the homogenate at 2000 g for 10 min, the supernatant fraction was used for enzyme analysis. AR activity was measured by spectrophotometry with a decrease in the absorbance of NADPH at 340 nm using DL-glyceraldehyde as a substrate[22]. The assay mixture containing 0.7 mL of phosphate buffer (0.067 mol/L), 0.1 mL of NADPH (25×10^{-5} mol/L), 0.1 mL of cell supernatant, 0.1 mL of DL-glyceraldehyde (substrate) (5×10^{-4} mol/L) and 0.2 mol/L lithium sulfate to a final volume of 1 mL, was read against a reference cuvette containing all components but the substrate, DL-glyceraldehyde. The final pH of the reaction mixture was 6.2. The enzymatic reaction was started by the addition of the substrate, and the absorbance of NADPH at 340 nm was measured by spectrophotometry[23].

Nitric Oxide Measurements

NO generation in VSMCs was monitored by labellling with 4, 5-diaminofluorescein diacetate (DAF-2DA) that was de-esterified intracellularly to DAF-2. NO provided the third nitrogen to form a triazo ring from the two amino groups of the nonfluorescent DAF-2 and converted it to diaminotriazolofluorescein (DAF-2T) that could be monitored at 490 nm exication and 530 nm emission[24].

Determination of Cytotoxicity

To confirm whether the inhibitory effects of SAaB were due to toxicity or damage to the cells, trypan blue viability tests were carried out in cells treated in parallel with growth studies. There was no loss in viability of cells treated with SAaB and less than 3% of the cells took up the dye. Furthermore, no floating cells were observed on any particular day during the treatment. Thus, detachment and loss of cells did not account for the inhibition of cell proliferation[25].

Statistical Analysis

All data were statistically analyzed with Microsoft Excel for Windows using a Lenovo Pentium Ⅳ/2.0 GHz computer. The results were expressed as $\bar{x}\pm s$, and accompanied with the number of observations. Statistical analysis of the data was carried out with Student's *t*-test. Differences with a *P* value of less than 0.01 were considered statistically significant.

RESULTS

Effect of SAaB on Cell Proliferation

After treatment with SAaB or Epalrestat, cell viability was determined by WST assay. Viability of VSMCs cultured in 10% FBS containing DMEM in the absence or presence of SAaB was evaluated. 0.2 μg/mL of SAaB had no remarkable effect on viability of VSMCs; while 2 μg/mL, 20 μg/mL of SAaB, and 20 mmol/L Epalrestat reduced the cell viability significantly ($P<0.01$) (Fig. 1).

Effect of SAaB on Cell Apoptosis of VSMCs

To ascertain whether SAaB affected the apoptosis of VSMCs, the effect of SAaB on cell apoptosis was analyzed. The apoptotic rate of VSMCs in the 10% FBS treated group was significantly lower than that in the 1% FBS treated group (Figs. 2 and 3). The apoptotic rate of VSMCs in the 20 μg/mL SAaB treated group was significantly higher than that in the 10% FBS treated group, and in the IL-1β (10 ng/mL) treated group as well (Figs. 2 and 3). 0.2 μg/mL and 2 μg/mL SAaB had no significant effect on the apoptotic rate of cells cultured in 10% FBS containing DMEM (Figs. 2 and 3). These experiments indicated that 20 μg/mL SAaB and IL-1β (10 ng/mL) could enhance the apoptotic rate of VSMCs.

Effects of SAaB on Cell AR Activity

The effects of FBS, various concentrations of SAaB and Epalrestat on the level of AR activity in cultured VSMCs were assessed. AR activity of

VSMCs cultured in 10% FBS containing DMEM was remarkably increased compared with that of cells cultured in 1% FBS containing DMEM ($P<0.01$=, though no notable effect of 0.2 μg/mL SAaB on cell AR activity was detected. Two μg/mL, 20 μg/mL SAaB, and 20 mmol/L Epalrestat reduced the cell AR activity of VSMCs significantly (Fig. 4).

Effects of SAaB on Nitrite Production

The SAaB treated group exhibited a progressive increase in DAF-2 fluorescence intensity, so did the IL-1β (10 ng/mL) treated group, which indicated that SAaB and IL-1β could lead to increased NO generation in VSMCs compared with that in the control group ($P<0.01$) (Fig. 5).

DISCUSSION

The proliferation of vascular smooth muscle cells underlies the high propensity of diabetics for restenosis and may be an important factor in the increased incidence and severity of atherosclerosis due to diabetes[26]. Moreover, it is also the primary factor in reocclusion of arteries after angioplasty[27]. In the present study, we found that SAaB could reduce serum-induced VSMC proliferation, and act on cell viability, apoptosis, AR activity, and nitrite release. WST-1 is a newly developed cell proliferation reagent. WST assay is a colorimetric assay for the quantification of cell proliferation and cell viability based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells, and a non-radioactive alternative to the [^{3}H]-thymidine incorporation assay. The results of WST-1 assay showed that VSMCs cultured in 10% FBS containing DMEM had an elevated viability and proliferation rate compared with cells cultured in 1% FBS containing DMEM and this elevation was inhibited by SAaB (20 μg/mL) significantly (Fig. 1).

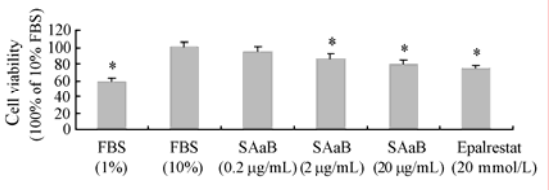

FIG. 1. Viability of VSMCs after treatment with SAaB or Epalrestat. *$P<0.01$ versus 10% FBS group.

As shown in Fig. 4, the results of AR activity assay showed that compared with VSMCs cultured in 1% FBS containing DMEM, VSMCs cultured in 10% FBS containing DMEM had an elevated AR activity, and this elevation was significantly inhibited by SAaB (20 μg/mL), suggesting that the inhibitory effect of SAaB on the proliferation of VSMCs is related to its effect on AR activity. We also observed that cell proliferation and AR activity were also remarkably inhibited by the AR inhibitor, Epalrestat, suggesting that inhibition of cell proliferation by SAaB is at least partly due to its AR inhibiting activity. Indeed, there is a growing body of evidences that acceleration of the polyol pathway is implicated in the pathogenesis of diabetic vascular complications[28-29]. The polyol pathway consists of two enzymes. One is aldose reductase which oxidizes sorbitol to fructose using NAD^+ as a cofactor, the other is sorbitol dehydrogenase (SDH) which reduces glucose to sorbitol with the aid of cofactor NADPH[30]. It has been reported that inhibition of AR prevents hyperproliferation and hypertrophy of VSMCs[31]. Previous studies have also demonstrated that inhibition of AR prevents VSMC growth in culture as well as in balloon-injured arteries *in vivo*[18], and inhibition of the AR by tolrestat and sorbinil diminishes DNA synthesis and VSMC proliferation in response to serum. In VSMCs, AR is a growth-responsive gene product and the inhibition of AR prevents VSMC growth and decreases intimal hyperplasia and restenosis. The involvement of AR in VSMC growth may be related to its ability to metabolize reactive end products of ROS signaling and to restore the cellular redox state altered by oxidative components of mitogenic signaling[32]. Based on these studies and the results presented in the present study, it can be suggested that the inhibitory effect of SAaB on the proliferation of VSMCs may be related to its effect on AR activity.

Apoptosis (programmed cell death) is a self destruction process during which multicellular organisms efficiently dispose of redundant, senescent, or injured cells. It is important in tissue remodelling as a counterbalance to cell proliferation[33]. The level of apoptotic cell death is also strongly related to the stage of development of atherosclerotic plaque, smooth muscle cells in adaptive intimal thickening, and fatty streaks show very little apoptosis[34-36]. As revealed by flow cytometry, the cell apoptosis rate of VSMCs was reduced by 10% FBS incubation and this effect was remarkably inhibited by SAaB (20 μg/mL), indicating that SAaB may interfere with the proliferation of VSMCs by augmenting cell apoptosis (Figs. 2 and 3). We also clearly demonstrated that SAaB could induce a progressive increase of nitrite release from VSMCs cultured in 10% FBS containig DMEM. A positive drug, IL-1β, had the similar effects on nitrite release and apoptotic rate of VSMCs with SAaB (Figs. 5, 2, and 3). These facts indicate that the inhibitory effect of SAaB on the proliferation

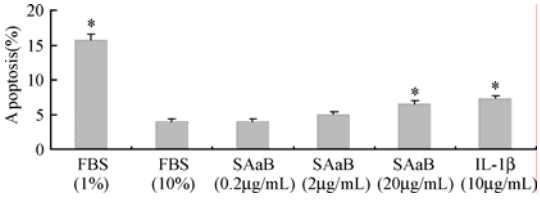

FIG. 2. Effect of SAaB and IL-1β on apoptosis of VSMCs cultured in 10% DMEM containing DMEM. $^{*}P<0.01$ versus 10% FBS treated group.

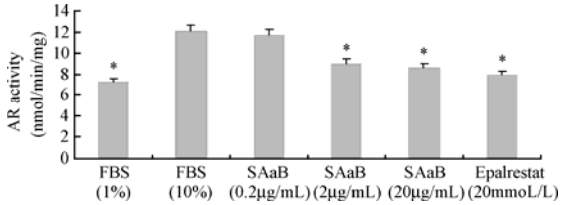

FIG. 4. Effect of SAaB and Epalrestat on the level of AR activity in cultured VSMCs. $^{*}P<0.01$ versus 10% FBS group.

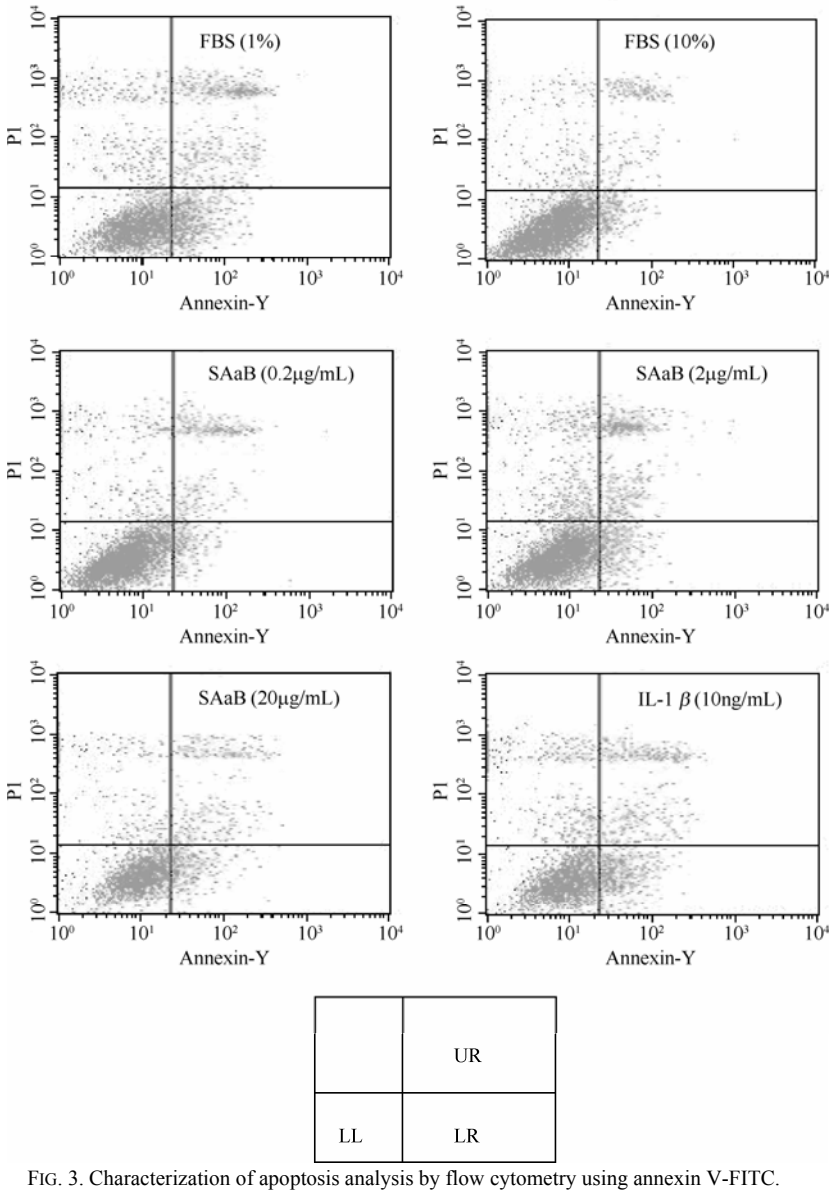

FIG. 3. Characterization of apoptosis analysis by flow cytometry using annexin V-FITC.

of VSMCs may be due to its activity of enhancing nitrite release and augmenting apoptosis via NO-dependent pathway. In fact, it is reported that NO can interfere directly with diverse cellular functions in some cases, thus suppressing cell proliferation in multiple ways. These effects include DNA replication[37],

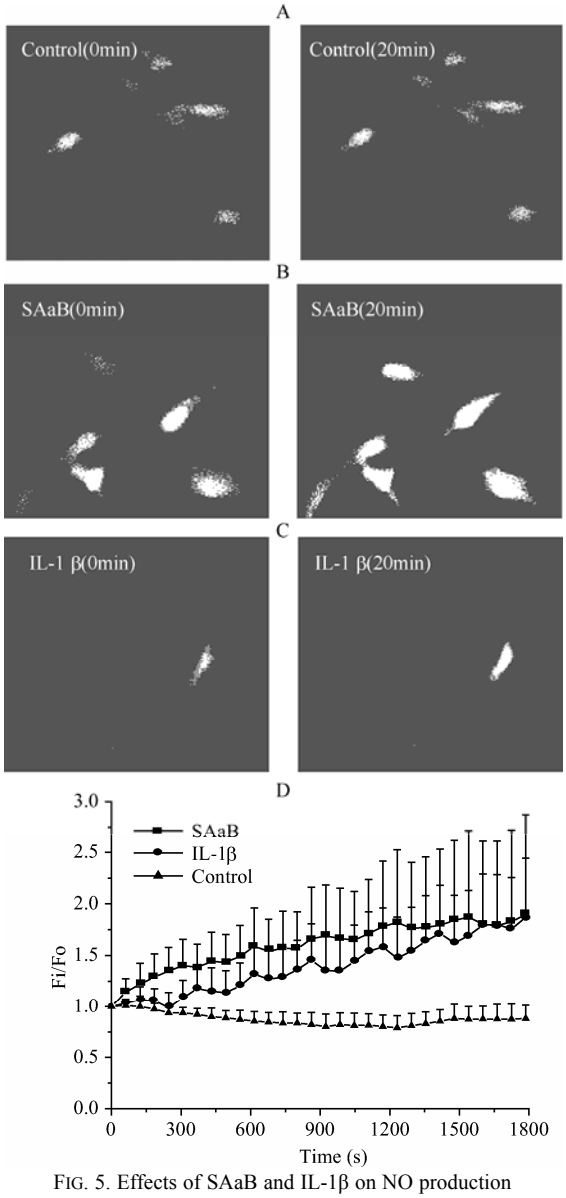

FIG. 5. Effects of SAaB and IL-1β on NO production by rat VSMCs. A-C: DAF-2 fluorescence change; D: statistical change of NO. Each data point represents $\bar{x} \pm s$ (n=18-20). $^{*}P<$ 0.01 compared with control group.

RNA, and protein synthesis[38], and modulation of cell cycle regulatory proteins[39-40]. NO possess different vasoprotective properties, including vasodilatation, inhibition of proliferation and migration of VSMCs. The ability of NO to initiate apoptosis also has been observed in various cells, including VSMCs[41-42]. Moreover, NO donors and eNOS gene transfer can inhibit VSMC proliferation *in vitro*[43]. Furthermore NO donors induce apoptosis in vascular VSMCs in a dose-dependent manner[42]. According to these studies and the results presented in our study, it can be suggested that the inhibitory effect of SAaB on the proliferation of VSMCs may be related to its ability to induce nitric oxide release.

In conclusion, SAaB can inhibit the proliferation of VSMCs stimulated by serum. The antiproliferation active effects of SAaB are, at least in part, derived from its AR inhibiting and nitric oxide release-inducing properties. SAaB may be a promising prophylactic and therapeutic agent for the treatment of vascular complications of diabetes mellitus, such as atherosclerotic plaque formation.

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