

Original Article

Globular adiponectin reduces vascular calcification via inhibition of ER-stress-mediated smooth muscle cell apoptosis

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Abstract: Objective: This study aims to explore the mechanism of globular adiponectin inhibiting vascular calcification. Methods: We established drug-induced rat vascular calcification model, globular adiponectin was given to observe the effect of globular Adiponectin on the degree of calcification. The markers of vascular calcification and apoptosis were also investigated. Meanwhile, the in vitro effect of globular Adiponectin on vascular calcification was also evaluated using primary cultured rat vascular smooth muscle cells. Results: We found that globular adiponectin could inhibit drug-induced rat vascular calcification significantly in vivo. The apoptosis of vascular smooth muscle cells was also reduced. The possible mechanism could be the down-regulation of endoplasmic reticulum stress by globular adiponectin. Experiments in primary cultured vascular smooth muscle cells also confirmed that globular adiponectin could reduce cell apoptosis to suppress vascular calcification via inhibition of endoplasmic reticulum stress. Conclusions: This study confirmed that globular adiponectin could suppress vascular calcification; one of the mechanisms could be inhibition of endoplasmic reticulum stress to reduce cell apoptosis. It could provide an effective method in the therapy of vascular calcification-associated diseases.

Keywords: Vascular calcification, globular adiponectin, cell apoptosis, endoplasmic reticulum stress

Introduction

During the development of vascular calcification (VC), the stiffness of the vascular wall increased in combination with reduced compliance. It often leads to multiple cardiovascular events such as myocardial ischemia, ventricular hypertrophy and heart failure, and is the common pathological and physiological basis of atherosclerosis, hypertension, cerebrovascular disease, diabetes, end-stage renal disease and other diseases [1, 2]. VC can induce the thrombosis and plaque rupture, and is one of the important factors of high incidence and mortality rate of cardiovascular and cerebrovascular diseases. Previous study found that 80% of vascular injury and 90% of coronary artery disease patients were complicated with VC [1]. Therefore, it has important scientific significance to investigate the detailed mechanism of VC and its therapy.

Recent study found that VC is an active and manageable biologic process similar to bone development; its main features include osteoblast-like phenotype conversion in vascular cells especially in vascular smooth muscle cells (VSMC) [2]. With the further development of VC, phenotypic characteristics of VSMC gradually reduce and begin to have the features and functions of osteoblasts. It can synthesize a variety of bone specific proteins with specific osteoblastic gene expression [3]. Proudfoot et al. found that VSMC showed the typical characteristics of cell apoptosis before calcification occurred [4]. Shroff et al. found that blood vessels after dialysis increased the activity of alkaline phosphatase (ALP) and strengthened the osteoblast-like phenotype transformation of VSMC [5]. After VSMC apoptosis, ALP activity obviously increased and the expression levels of BMP2, Runx2 and osterix increased, which suggested that apoptosis can promote the conversion of VSMC to osteoblast-like cells; inhibi-

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tion of apoptosis can relieve VSMC calcification [6]. So apoptosis of VSMC may be closely related to the VC.

Globular adiponectin (gAd) is a kind of specific bioactive polypeptide secreted by adipose cells, it has multiple physiological effects such as antidiabetes, anti-inflammatory and vaso-protective properties [7]. gAd can play the role of protection of vascular by inducing NO activation, inhibiting activation of vascular endothelial cells, inhibiting apoptosis induced by endoplasmic reticulum stress and promoting the repair of endothelial cells [8]. Luo et al. found that gAd can promote the expression of osteoprotegerin (OPG) and inhibit phenotype conversion of VSMC into osteoblast by combining with the globular adiponectin receptor 1 in the calcified VSMC [9]. Okamoto et al. found that the serum gAd levels were negatively correlated with the degree of coronary artery calcification [10]. These results suggested that gAd has the potential to improve vascular calcification, but its mechanism remains unclear. So in this study we explored the mechanism of gAd inhibiting vascular calcification, which could provide an effective method in the therapy of vascular calcification-associated diseases.

Materials and methods

Experimental animals

A total of 40 SPF grade 8-weeks healthy Sprague-Dawley (SD) male rats weighing 180 to 220 g were obtained from the experimental animal center of Shanxi Medical University. The rats were kept in clean and quiet environment with room temperature. They had free access to food and drinking water and were pre feeding for one week to adapt to the environment. They were randomly divided into 3 groups: control group (10 rats), vascular calcification group (10 rats) and treatment group (10 rats).

Rat model of vascular calcification was established with intramuscular injection of Vitamin D3 and intragastric administration of nicotine method [4]. Intramuscular injection of Vitamin D3 (300000U/kg) combined with intragastric administration of nicotine (25 mg/kg, dissolved with peanut oil) were performed at 8:00 am and intragastric administration of nicotine was performed at 18:00 pm again. Intramuscular injection of physiological saline combined with intragastric administration of peanut oil was

performed in control group. After the establishment of model, intravenous injection of 1 µg/kg globular Adiponectin was administrated in treatment group 1 times a day for 6 consecutive weeks and intravenous injection of 1 µg/kg physiological saline was administrated in control and model group.

Housing and procedures involving experimental animals were in accordance with the NIH Animal Care and Use Committee guidelines. All experimental procedures were approved by the Care of Experimental Animals Committee of Shanxi Medical University.

Von kossa staining

The thoracic aorta was stained using Von kossa staining method. The slice section of thoracic aorta was stained with 2% silver nitrate for 20-60 min and washed with water for 5 min. Then it was treated with 5% sodium thiosulfate for 3 min and washed with water for 5 min. It was stained with 1% toluidine blue for 2 min and washed with water for 5 min. Then it was sealed with neutral gum after routine dehydration.

Determination of thoracic aortic calcium content and the activity of ALP

About 1 cm of thoracic aorta was taken and rinsed, then baked to constant weight in the 60°C constant temperature box. 0.6 ml of 72% perchloric acid and 2.4 ml of 65% nitric acid were added and placed at room temperature for 12 hours. They were heated at 120°C until all liquid volatilized using the digestive tube, white crystal precipitated at the bottom of the tube. Water was added and set volume to 3 ml after cooling. Calcium content was determined using calcium assay kit according to the manual.

Tissue homogenate of thoracic aorta was prepared and centrifuged at 4°C with 8000 rpm for 10 min. The supernatant was used to determine the activity of ALP using ALP assay kit according to the manual.

Detection of the expression of OPG, Runx2 and apoptosis factors

Total RNA and proteins were extracted from thoracic aortic tissues after the intima and adventitia of thoracic aorta were stripped respectively.

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Total RNA was extracted using RNA extraction Kit according to the manufacturer's manual. RT-PCR was performed according to reference [11]. RNA was subjected to reverse transcription using reverse transcription kit and Real-time PCR was performed using Prime-Script™ RT Master Mix kit according to the manufacturer's manual. Primer sequences were as follows: OPG Forward: 5'-TGGAGTCG-AATTCTGCTTG-3', Reverse: 5'-TCAAGTGCTTGA-GGGCAAC-3'; Runx2 Forward: 5'-GAGCACAAC-CATGGCTGAGA-3', Reverse: 5'-TGGAGATGTTG-CTCTGTTTCG-3'; GRP78 Forward: 5'-CTGGGTAC-ATTTGATCTGACTGG-3', Reverse: 5'-GCATCCT-GGTGGCTTTCCAGCCATTC-3'; Caspase-12 Forward: 5'-TGAACGGGAAGCTCACTGG-3', Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'; CHOP Forward: 5'-AGCAGAGGTCAACAAGCACCT-3', Reverse: 5'-CTGCTCCTTCTCCTTCATGC-3'; β -actin Forward: 5'-GTC AGG TCA TCA CTA TCG GCA AT-3', Reverse: 5'-AGAGGTCTTACGGATGTCAA-CGT-3'. β -actin was used as an internal reference.

Total proteins were separated by SDS polyacrylamide gel electrophoresis. Then separated proteins were electro-transferred to the PVDF membrane. The membrane containing the proteins was used for western blotting with required antibodies. The protein bands were scanned and quantified as a ratio to β -actin.

Culture and identification of primary vascular smooth muscle cells of rats

The thoracic aorta was taken out using sterilized instruments from rats after they were anesthetized using 2% pentobarbital sodium, and then the thoracic aorta was placed into cold PBS buffer containing double antibiotics. The intima and adventitia of thoracic aorta were stripped carefully and rinsed, smooth muscle layer was cut into approximately 1 mm² tissue block. The tissue blocks were cultured in 25 cm² flask using high glucose DMEM medium containing 20% fetal bovine serum at 37°C with 5% CO₂. The purity of smooth muscle cells were identified using α -smooth muscle actin (α -SMA) antibody according to reference [11].

Establishment of a smooth muscle cell calcification model

The smooth muscle cell calcification model was established using 5-8 generation of primary

cultured smooth muscle cells according to reference [12]. Cells were seeded in 6-well plate and calcification medium containing 10 mmol/L β -glycerophosphate (β -GP) sodium was added into the wells to continue to cultivate for 14 days, the medium was changed every 2 days and different concentrations (0.5 μ g/ml, 1 μ g/ml and 2 μ g/ml) of gAd were added into them at the same time. 2 μ g/ml of gAd were added into the normal cells and normal cells without APN as control.

Determination of cellular calcium content and the activity of ALP and caspase-3

After culture for 14 days, the cellular calcium content was determined using calcium assay kit according to the manual, the activity of ALP was determined using ALP assay kit according to the manual and the activity of caspase-3 was determined using caspase-3 activity assay kit according to the manual.

Detection of the expression of calcification and apoptosis related proteins

The expression levels of OPG, Runx2, GRP78, caspase-12 and CHOP were detected using RT-PCR and Western blotting methods. The operational process was similar to above-mentioned.

Statistical analysis

The results were expressed as mean \pm SD. The SPSS software package 19.0 was used for the statistical tests. Variance analysis and t-test were used to compare among groups. $P < 0.05$ was considered statistically significant.

Results

Adiponectin improved vascular calcification in rats

Von kossa staining results showed that there were no calcareous sediments in rats' vascular smooth muscle of control group (**Figure 1A**) while there were lots of black granular calcium sediments in rats' vascular smooth muscle of model group (**Figure 1B**). There were fewer calcium sediments in rats' vascular smooth muscle of gAd treatment group than that of model group (**Figure 1C**).

Compared with control group, the calcium content of model group increased (75.41 ± 5.24

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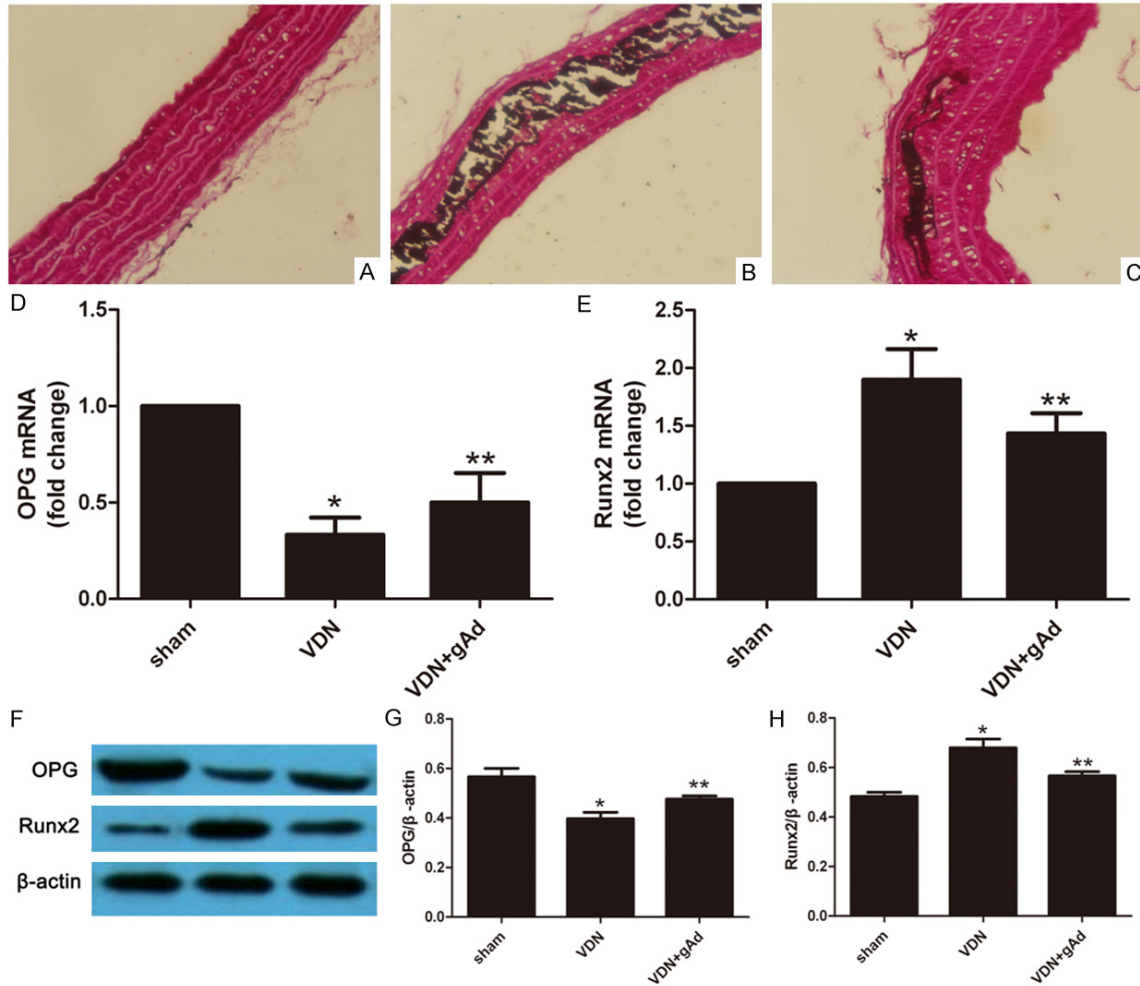


Figure 1. Adiponectin improved vascular calcification in rats. A: Von kossa staining results of control group; B: Von kossa staining results of vascular calcification model group; C: Von kossa staining results of gAd treatment group; D: OPG mRNA relative expression; E: Runx2 mRNA relative expression; F: Western blotting results; G: Relative OPG protein; H: Relative Runx2 protein. gAd: Globular Adiponectin; Sham: Control group; VDN: vitamin D3 nicotine.

Table 1. Determination of thoracic aortic calcium content and the activity of ALP

Group	Calcium content ($\mu\text{mol/g}$)	ALP activity (U/g)
Control	18.36 ± 1.27	18.63 ± 1.29
VC model	$75.41 \pm 5.24^*$	$98.63 \pm 6.92^*$
gAd	$56.43 \pm 5.62^{**}$	$72.52 \pm 4.91^{**}$

* $P < 0.05$ vs. model group; ** $P < 0.01$ vs. control group.

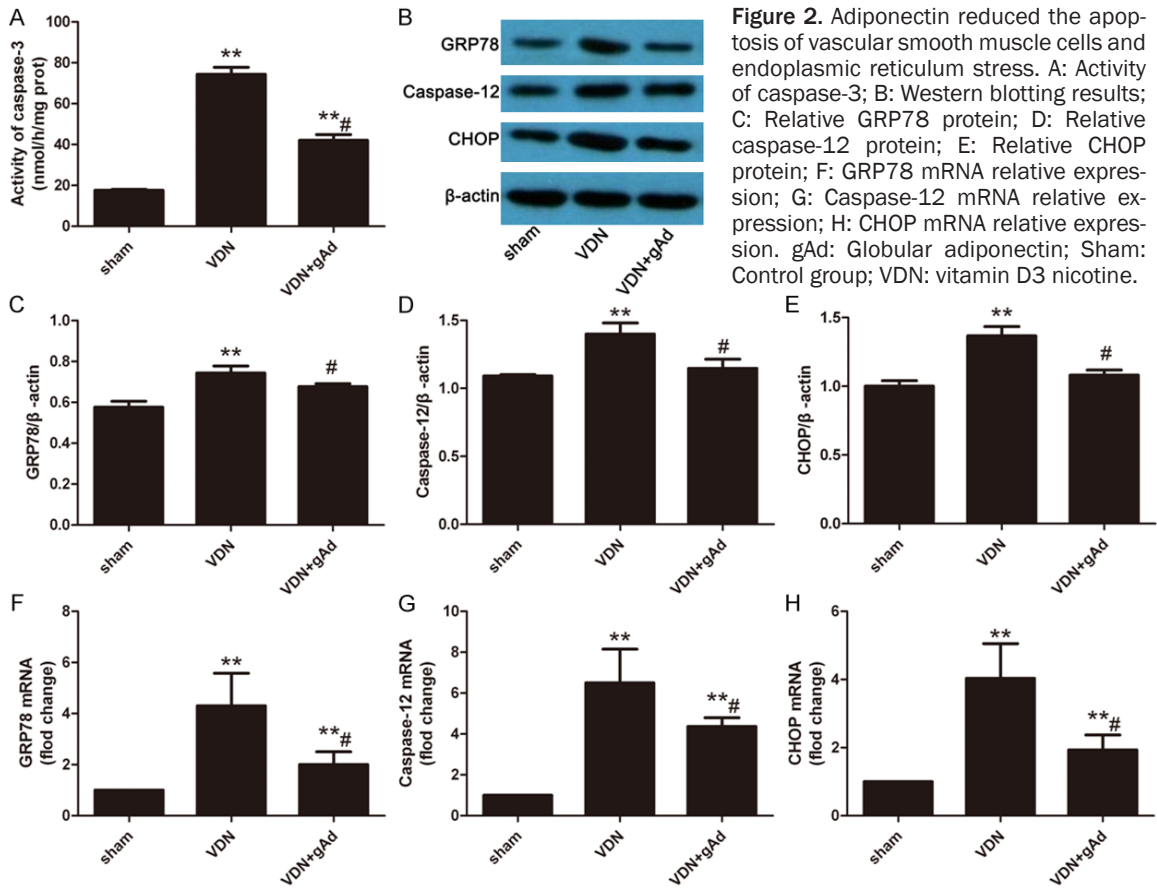
vs. $18.36 \pm 1.27 \mu\text{mol/g}$) while that of gAd treatment group decreased (56.43 ± 5.62 vs. $75.41 \pm 5.24 \mu\text{mol/g}$) significantly (Table 1). The activity of ALP in gAd treatment group was lower than that of model group significantly (72.52 ± 4.91 vs. 98.63 ± 6.92 U/g, $P < 0.05$, Table 1).

RT-PCR and western blotting results showed that the expression of OPG decreased and the expression of Runx2 increased significantly in model group. However, compared with model group, the expression of OPG and Runx2 reversed significantly in APN treatment group (Figure 1D-H).

Adiponectin reduced the apoptosis of vascular smooth muscle cells and endoplasmic reticulum stress

As shown in Figure 2, the activity of caspase-3 in gAd treatment group was lower than that of model group (Figure 2A), and the expression levels of GRP78, Caspase-12 and CHOP in APN treatment group was lower than that of model group (Figure 2B-H).

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Adiponectin improved the calcification of primary VSMC

As shown in **Figure 3**, we cultured VSMC of rats (**Figure 3A**) and got high purity of spindle shaped or polygonal VSMC cells through the passage purification method (**Figure 3B**) and its purity reached above 90% identified by α -SMA antibody (**Figure 3C**). Black calcium deposition and nodules appeared in cells after treated by β -GP which confirmed that the cell calcification model was established successfully (**Figure 3D**). Compared with control group, the calcium content and the activity of ALP increased in model group while gAd reversed it with dose dependent (**Table 2**). The expression levels of OPG and Runx2 increased in model group while gAd also reversed these with dose dependent (**Figure 3E-I**).

Adiponectin inhibited the apoptosis of VSMC

The activity of caspase-3 and flow cytometry analysis showed that apoptosis increased significantly in the process of VSMC calcification,

while gAd could inhibit the apoptosis of VSMC induced by β -GP (**Figure 4A-C**).

Adiponectin reduced the endoplasmic reticulum stress of VSMC induced by β -GP

Similar to the results in vivo, the endoplasmic reticulum stress of VSMC induced by β -GP in vitro was also activated. The expression of GPR78, caspase-12 and CHOP increased significantly. gAd could decrease the expression of GPR78, Caspase-12 and CHOP (**Figure 5A-G**). These results suggested that gAd could reduce the endoplasmic reticulum stress in the process of vascular calcification.

Discussion

VC could induce many dangerous complications, there is a lack of specific methods for the prevention and treatment of VC. Recent studies paid attention to the role of gAd in VC. It was confirmed that gAd could inhibit the conversion of VSMC to osteoblast-like cells, slow down the development process of cardiovascular tissue

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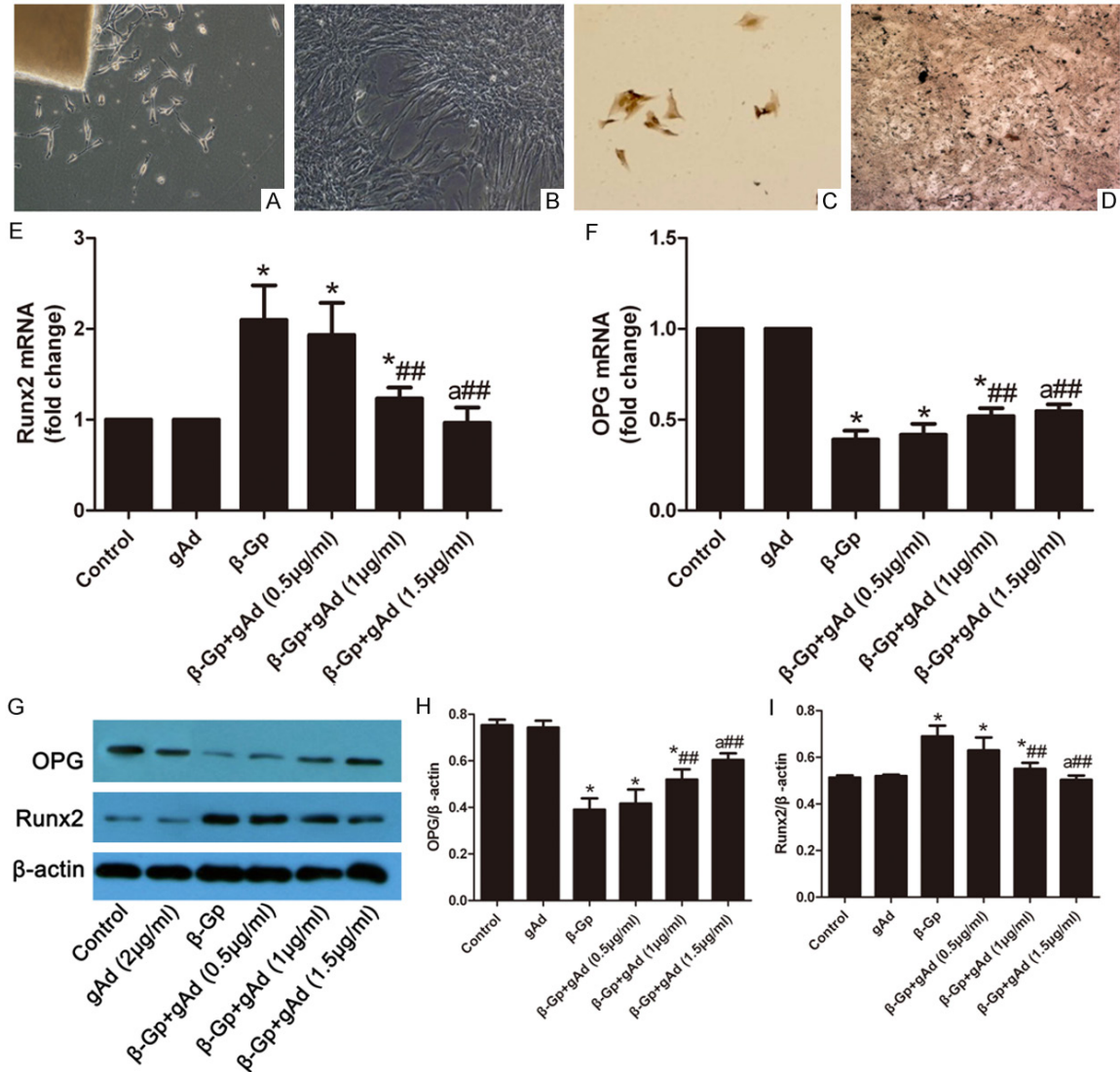


Figure 3. Adiponectin improved the calcification of primary VSMC. A: Primary VSMC of rats; B: High-purity VSMC passage cells; C: Identification by α -SMA antibody; D: Calcification model cells treated by β -GP; E: Runx2 mRNA relative expression; F: OPG mRNA relative expression; G: Western blotting results; H: Relative OPG protein; I: Relative Runx2 protein.

Table 2. Determination of calcium content and the activity of ALP in VSMC

Group	Calcium content (μ mol/ml)	ALP activity (U/ml)
Control	18.38 \pm 1.56	18.65 \pm 1.28
gAd	17.19 \pm 2.42	18.11 \pm 1.36
VC model	69.71 \pm 6.12*	92.70 \pm 7.17*
0.5 μ g/ml gAd + VC model	62.06 \pm 2.43*	82.56 \pm 8.11*
1 μ g/ml gAd + VC model	49.71 \pm 6.24*##	66.28 \pm 5.08*##
2 μ g/ml gAd + VC model	40.21 \pm 6.25*##	52.83 \pm 5.15*##

* P < 0.05 vs. control; ## P < 0.01 vs. model.

lesions [4, 5]. The anti-inflammatory effect of VC may also play an important role in delaying

vascular calcification process mediated by inflammatory mediators [14, 15]. The detailed mechanism of gAd improve vascular calcification is not yet clear. In this study we observed cell apoptosis and vascular calcification induced by gAd and found that gAd could significantly inhibit the apoptosis mediated by endoplasmic reticulum stress and improve the vascular calcification effectively.

Previous studies found that VSMC could present osteoblast-like phenotype conversion in vascular calcification process, and the phenotype characters of vascular smooth muscle cells in VSMC gradually reduced

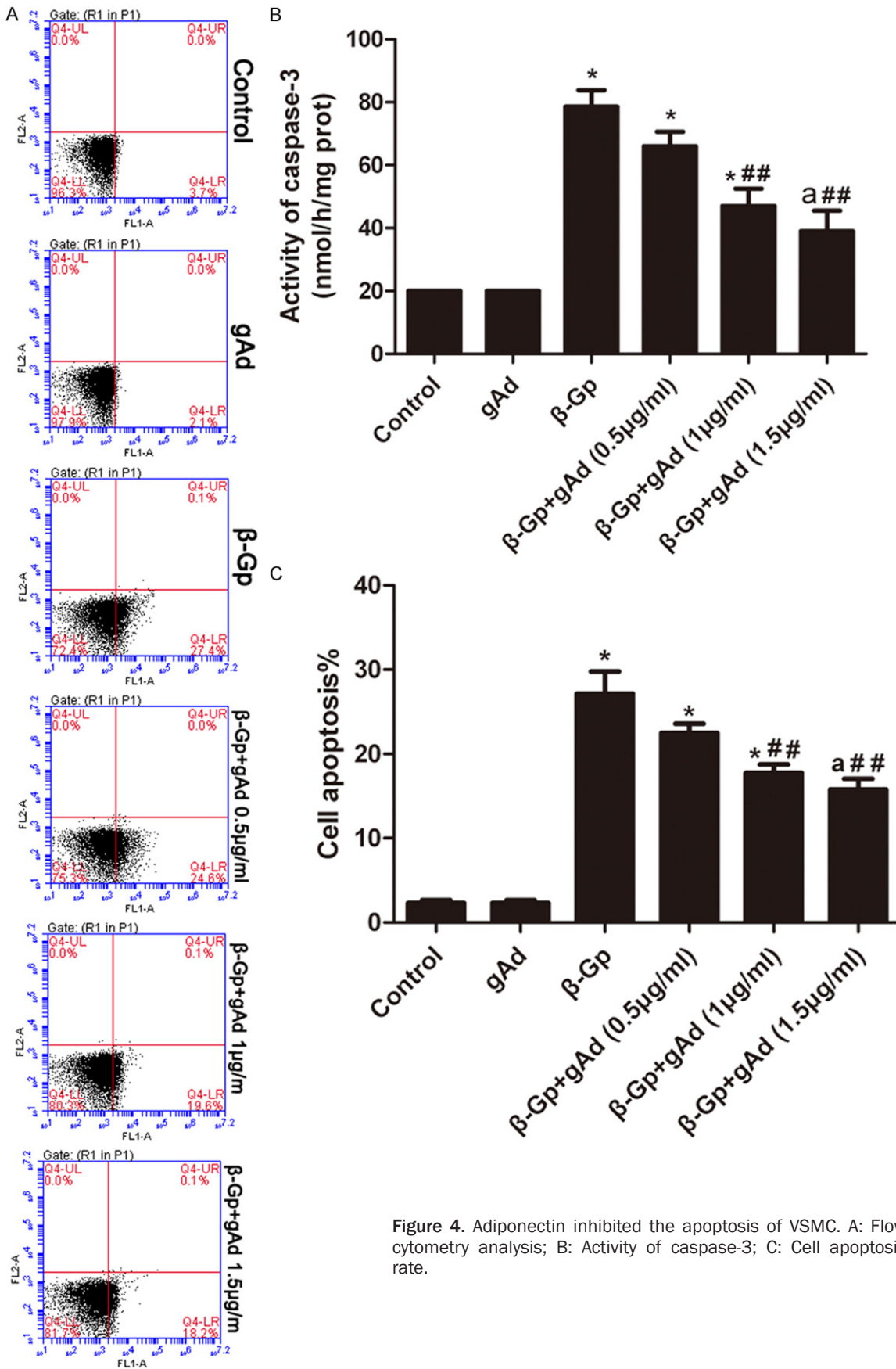
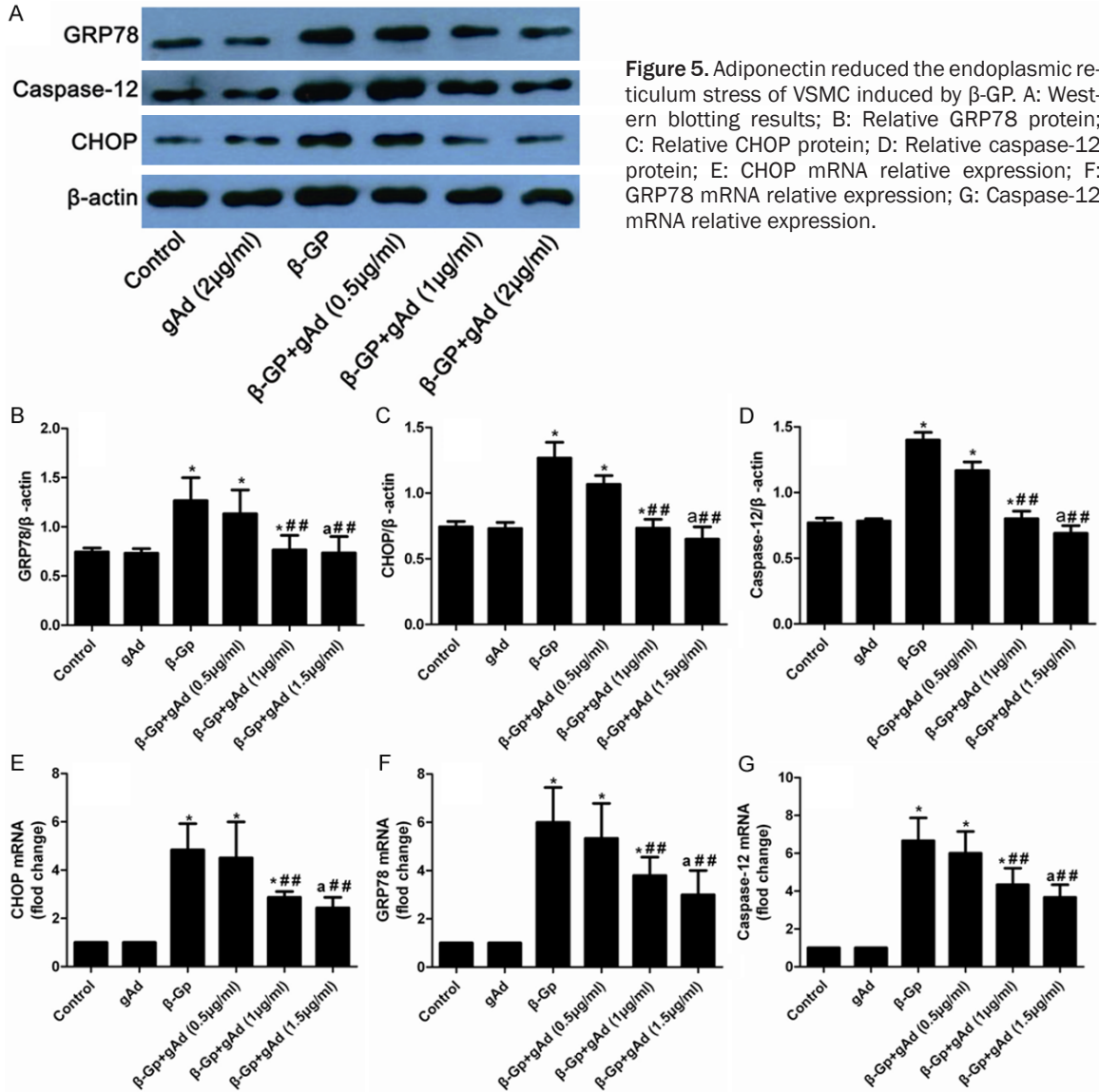


Figure 4. Adiponectin inhibited the apoptosis of VSMC. A: Flow cytometry analysis; B: Activity of caspase-3; C: Cell apoptosis rate.

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with the development process of calcification. VSMC apoptosis occurred in the process of VC, apoptotic body accumulated calcium and promoted bone differentiation, the activity of ALP increased and promote the expression of BMP2, Runx2 and Osterix gene, which accelerating the differentiation of VSMC cell to osteoblast like cells [6]. In some situations leading to the vessel wall damage such as hypertension and atherosclerosis VSMC apoptosis can be significantly observed, MGP and fetuin-A levels decreased and VC process significantly accelerated. Our study also confirmed that apoptosis appeared in VC models in vitro and in vivo. Chasseraud et al. found that induction of apoptosis in vitro significantly promoted calcification

in VSMC [16]. Therefore, inhibition of apoptosis may be able to improve the VC effectively.

In this study we found that the apoptosis of VSMC significantly reduced in vitro and in vivo using gAd interference, OPG levels increased and Runx2 levels decreased and the degree of calcification was also significantly reduced. So gAd interference may be an effective method for the prevention and treatment of VC. gAd can significantly inhibit the endoplasmic reticulum stress pathway of VSMC activated by drug. The endoplasmic reticulum is an important storage device of calcium ion in mammalian cells, and is the place for post-translational modification of protein and correct assembly and folding of

polypeptide chain [17]. Unfolded or misfolded proteins accumulated in endoplasmic reticulum activated endoplasmic reticulum stress under the condition of damaged homeostasis of endoplasmic reticulum caused by pathological factors to try to restore the endoplasmic reticulum homeostasis and function. Excessive endoplasmic reticulum stress could activate apoptosis process by activating CHOP and caspase-12 [18]. Endoplasmic reticulum stress may have an important connection with the activation of vascular smooth muscle and VC. Masashi et al. found that ATF4, the key transcription factor of endoplasmic reticulum stress, mediated the VC induced by stearic acid [19]. Increased stearic acid could induce over-expression of ATF4 by PERK-eIF2 α pathway and CHOP increased, which enhancing the endoplasmic reticulum stress and leading to osteoblastic differentiation and enhanced calcification of VSMC. Liberman et al. found that the expression of GRP78, phosphorylated IRE1 α XBP1 and Runx2 increased after human coronary artery smooth muscle cells were treated by BMP-2, resulting in the transformation of smooth muscle cells to the osteoblastic phenotype [20]. We also found that APN could reduce the expression of Runx2 and calcium deposition and mineralization in smooth muscle cells. All the results confirmed that endoplasmic reticulum stress can promote calcification of smooth muscle cells by increasing the expression of Runx2. Saito et al. confirmed that endoplasmic reticulum stress mediated by PERK-eIF2 α -ATF4 pathway played an important role in the transformation of smooth muscle cells into osteoblasts and osteoblastic differentiation [21]. In addition, the marker protein GRP78 of endoplasmic reticulum stress could cause formation of mineralized matrix by increasing the deposition of calcium and phosphorus [22].

In conclusion, this study demonstrated that gAd can significantly reduce the apoptosis of VSMC and VC in vitro and in vivo, its mechanism is probably associated with the inhibition of endoplasmic reticulum stress in VSMC cells, which slowing down the process of VC. This study provides a new theoretical foundation for the treatment of diseases associated with VC with gAd and its clinical application as drug candidate.

Disclosure of conflict of interest

None.

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