Adiponectin enhances biological functions of vascular endothelial progenitor cells through the mTOR-STAT3 signaling pathway

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Running title: Biological function of adiponectin

Abstract: Adiponectin (APN), an adipose tissue-excreted adipokine, plays protective roles in metabolic and cardiovascular diseases. In this study, the effects and mechanisms of APN on biological functions of rat vascular endothelial progenitor cells (VEPCs) were investigated *in vitro*. After administrating APN in rat VEPCs, the proliferation was measured by methyl thiazolyl tetrazolium (MTT) method, the apoptotic rate was test by Flow cytometry assay, mRNA expression of B-cell lymphoma-2 (Bcl-2) and vascular endothelial growth factor (VEGF) was determined by Real-time reverse transcriptase polymerase chain reaction (RT-PCR), and protein expression of mechanistic target of rapamycin (mTOR), signal transducer and activator of transcription 3 (STAT3) and phospho-STAT3 (pSTAT3) was analyzed by Western Blot. It was suggested that APN promoted the optical density (OD) value of VEPCs, enhanced mRNA expression of Bcl-2 and VEGF, and inhibited cell apoptotic rate. Furthermore, protein expression of pSTAT3 was also increased in the presence of APN. Moreover, APN changed-proliferation, apoptosis and VEGF expression of VEPCs were partially suppressed after blocking the mTOR-STAT3 signaling pathway by the mTOR inhibitor XL388. It was indicated that APN promoted biological functions of VEPCs through targeting the mTOR-STAT3 signaling pathway.

Key words: Adiponectin, VEPCs, proliferation, apoptosis, mTOR, STAT3

Abbreviation: Adiponectin, APN; vascular endothelial progenitor cells, VEPCs; methyl thiazolyl tetrazolium, MTT; B-cell lymphoma-2, Bcl-2; vascular endothelial growth factor, VEGF; real-time reverse transcriptase polymerase chain reaction, RT-PCR, mechanistic target of rapamycin, mTOR; signal transducer and activator of transcription 3, STAT3; phospho-STAT3, pSTAT3; optical density, OD; hemopoietic stem cell, HSC; bone marrow stromal cell, BMSC; nitric oxide, NO; vascular smooth muscle cell, VSMC; Fms-like tyrosine kinase 1, Flk-1; cluster of differentiation 31, CD31; Endothelial Cell Growth Medium 2, EGM-2; fetal bovine serum (FBS); propidium iodide (PI); standard deviation, SD; extracellular signal regulated kinase, Erk; endothelial nitric oxide synthase, eNOS; human brochial epithelial cells, HBECs; human hepatoma, HepG2; phosphoinositide 3 kinase, PI3K; human microvascular endothelial cell line, HMEC-1.

Introduction

Adiponectin (APN), a circulating adipokine, has been known for its potent insulin-sensitizing, anti-atherosclerotic, anti-inflammatory, and anti-diabetic properties (Shibata *et al*. 2005; Tajtakova *et al*. 2010; Villarreal-Molina and Antuna-Puente, 2012). *In vitro*, APN was previously shown to have the abilities to increase hemopoietic stem cell (HSC) proliferation (DiMascio *et al*. 2007) and to promote bone marrow stromal cell (BMSC) differentiation towards the osteoblastic lineage (Wu *et al*. 2014). Furthermore, APN could partly regulate the migration of angiogenic cells, cardiac fibroblasts, endothelial progenitor cells (EPCs) and muscle satellite cells (Chang *et al*. 2010; Shibata *et al*. 2008). Therefore, APN may play a crucial role in maintaining cell functions.

Especially, a variety of studies suggested that APN played a protective role in the cardiovascular system (Caselli *et al*. 2014). Plasma APN levels was believed to negatively correlate with cardiovascular disease, such as hypertension and metabolic disorders (Zhu *et al*. 2008). The reduction in plasma APN was responsible for hypertension-associated cardiovascular diseases (Kawai *et al*. 2013). The *In vitro* studies proved that APN could inhibit the activation of vascular endothelial cells by inducing nitric oxide (NO) activation, inhibiting apoptosis and promoting the repair of cells (Xu *et al*. 2010). Globular APN could resist vascular calcification via the inhibition of endoplasmic reticulum stress to reduce vascular smooth muscle cell (VSMC) apoptosis (Lu *et al*. 2015). Luo *et al*. (2009) found that globular APN could enhance mRNA expression of osteoprotegerin and suppress phenotype conversion of VSMCs into osteoblast by combining with the globular APN receptor 1 to slow down the development process of cardiovascular tissue lesions.

In 1997, EPCs was successfully isolated by Asahara *et al*. (1997). As the origins of vascular endothelial cells, EPCs played an important role in repairing the injured vascular endothelium, but their proliferative capacity was limited (Aragona *et al*. 2016; Matsuo *et al*. 2007), indicating that enhancing the proliferative ability of EPCs was a key step in improving the effects of EPC transplantation therapy. The findings in cultured EPCs have given us a clue that APN could promote cell number and stimulate the expression of the endothelial cell marker proteins Fms-like tyrosine kinase 1 (Flk-1) and cluster of differentiation 31 (CD31) (Shibata *et al*. 2008). However, these results only indicated that APN had the potential to improve vascular functions, but its mechanism remained unclear. Therefore, in this study, we explored the mechanism of APN in biological functions of EPCs from blood vessel, which could provide an effective method in the therapy of vascular associated diseases.

Materials and methods

Cell culture

The rat VEPCs purchased from American Type Culture Collection (ATCC) (BNCC338120, USA) were cultured in Endothelial Cell Growth Medium 2 (EGM-2) BulletKit (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), and antibiotic-antimycotic solution (Gibco®, Life Technologies, Thermo Fisher Scientific, California, USA). VEPCs were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Treatments

The experiments were randomly divided into the following groups: Control, APN treatment (1 μg/ml),

XL388 treatment, and APN plus XL388 treatment. XL388 (a specific mTOR inhibitor) was purchased from Santa Cruz Biotechnology (2145 Delaware Ave Santa Cruz, CA, USA), and APN was purchased from Genepharma (Shanghai, China).

MTT assay

VEPCs at a density of 1.0×10^4/ml were collected from the cultures and replated into a 96-well plate and underwent different treatments. Cell proliferation was determined using MTT method according to the previous study (Dong *et al*. 2016). A microplate reader was used to measure the absorbance at 450 nm (Bio-Rad, Hercules, CA, USA).

Flow cytometry

A total of 2.0×10^4 cells were seeded onto a 96-well plate and 24 h later treated with APN, XL388, and APN plus XL388 for indicated times, then cell apoptotic rate was identified by Flow cytometry according to the manufacturer instructions. Cells were collected after trypsinization, and then fixed in 70% ethanol. After ethanol was removed, the samples were stained with 50 μg/ml fluorescein isothiocyanate (FITC), Annexin V, and propidium iodide (PI) (BD Biosciences, San Jose, CA, USA), respectively. Cell apoptotic rate was evaluated using a FACScan flow cytometry apparatus (BD Biosciences, San Jose, CA, USA).

Western blot analysis

The examination of the protein expression levels of mTOR, STAT3 and pSTAT3 was performed separately using Western blot analysis. Total protein was extracted and the protein concentration was measured according to previously published article (Dong *et al*. 2016). β-actin was used as an internal control. All antibodies were

purchased from Santa Cruz Biotechnology, CA, USA. Band density was quantitated using Image J software.

Gene expression analysis

Expression of Bcl-2 and VEGF in VEPCs was determined at indicated times by RNA preparation and quantitative reverse transcription polymerase chain reaction (RT-PCR). Total cellular RNA was isolated from cells on 6-well plates using Trizol reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and RT-PCR was performed according to the reference (Dong *et al*. 2016). β-actin expression was used as an internal control. Specific primer sequences were synthesized in BIOSUNE Biological Technology Corp (Shanghai, China), and the sequences of the primers were as follows: β-actin, 5'-CACGATGGAGGGGCCGGACTCATC-3' (forward), 5'-TAAAGACCTCTATGCCAACACAGT-3' (reverse); Bcl-2, 5'-CTGGTGGACAACATCGCTCTG-3' (forward), 5'-GGTCTGCTGACCTCACTTGTG-3' (reverse); and VEGF, 5'-GGTGAGAGGTCTAGTTCCCGA-3' (forward), 5'-CCATGAACTTTCTGCTCTTC -3' (reverse).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Student's two-tailed t-test was used to determine the statistical differences between the treatment groups and the control groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

APN stimulated the proliferation of VEPCs

The cultured VEPCs were stimulated with APN at dose of 1 μg/ml for 12, 24, 48 and 72 h, and the viability

was assessed by MTT assay. The experiments revealed that there was no significance between the control group and the APN-treated group at 12 h ($P>0.05$). Moreover, after 24 h of culture, APN-stimulated VEPCs had a higher proliferation rate than the non-stimulated VEPCs ($P<0.05$) (Fig.1).

APN inhibited the apoptosis of VEPCs

APN at concentration of 1 μg/ml was added into VEPCs to evaluate cell apoptosis by Flow cytometry assay *in vitro*. The results of Flow cytometry assay demonstrated that the apoptotic rate of VEPCs was significantly decreased in the APN stimulated-group compared with the control group ($P<0.05$). The rate of apoptotic cells reached 4.06±0.58%, 3.24±0.72%, 2.75±0.39% and 3.37±0.98% at 12, 24, 48 and 72 h, respectively in APN stimulated-cells (Fig.2A). To reinforce these data, the expression of the apoptosis relative factor Bcl-2 was assayed employing RT-PCR in VEPCs. The results showed that, during 72 h of stimulation, the expression of Bcl-2 in VEPCs was higher in the presence of APN than that in VEPCs without APN stimulation, and there was an obvious significant difference between two groups following 24 h of stimulation ($P<0.05$) (Fig.2B).

APN enhanced the expression of VEGF in VEPCs

Using RT-PCR method, the influence of APN on the expression of VEGF in cultured VEPCs was determined at different periods of time. As shown in Fig.3, VEGF was present in the supernatants of cultured VEPCs at a very low level in the control group. Moreover, the expression level of VEGF in the supernatant of VEPCs was significantly enhanced in a time-dependent manner when treated with APN at concentration of 1 μg/ml ($P<0.05$).

APN activated the mTOR-STAT3 signaling pathway in VEPCs

To investigate whether APN regulated the mTOR-STAT3 signaling pathway in VEPCs, protein expression of mTOR, STAT3 and pSTAT3 was measured using Western blot analysis (Fig.4A). It was noted that, compared with the control group, there was a significant increase in pSTAT3 expression ($P<0.05$), but there were no changes in the bands of mTOR and STAT3 ($P>0.05$) in the APN-treated groups (Fig.4B).

APN regulated biological functions of VEPCs via the mTOR-STAT3 signaling pathway

To validate the role of the mTOR-STAT3 signaling pathway played in the proliferation, apoptosis and VEGF expression of VEPCs, after blocking the signal with inhibitor XL388, we further treated VEPCs with APN at concentration of 1 μg/ml for 48 h. As shown in Fig.5A, protein expression of pSTAT3 in VEPCs was evidently down-regulated following treatment with XL388, suggesting the good inhibitory efficiency of XL388 inhibitor (Fig.5A). After treated VEPCs with XL388 for 48 h, the proliferation of VEPCs was decreased markedly compared with the groups which was not treated with XL388 ($P<0.05$). Further, there was no difference between the XL388-treated group and the XL388 plus APN-treated group ($P>0.05$) (Fig.5B). Subsequently, it was observed that APN inhibited the apoptotic rate of VEPCs while inhibitor XL388 promoted it ($P<0.05$). There was also no difference in the apoptotic rate between the XL388-treated group and the XL388 plus APN-treated group ($P>0.05$) (Fig.5C). Additionally, the effect of APN on inducing VEGFs could be attenuated by XL388 the inhibitor of mTOR ($P<0.05$) (Fig.5D).

Discussion

APN, a peptide hormone secreted from the adipose tissue, regulates a number of metabolic processes as

energy metabolism, cell proliferation, differentiation and apoptosis (Caselli *et al*. 2014; Chang *et al*. 2010; Shibata *et al*. 2008). To date, the biological role and mechanism of APN in VEPCs have not been completely assessed. Here, we investigated the effects of APN on biological functions of VEPCs, showing that APN increased cell viability and VEGF expression while decreased the apoptosis of VEPCs through the activation of the mTOR-STAT3 signaling pathway.

APN has been reported to play important roles in protecting different kinds of cells from injuries and may have therapeutic implications in the treatment of diseases. For instance, administration of globular APN at 25 mM promoted EPC migration and tube formation, and dose-dependently upregulated phosphorylation of endothelial nitric oxide synthase (eNOS), Akt and augmented NO production (Huang *et al*. 2011). Globular APN promoted the proliferation, cell-cycle and wound repair of human brochial epithelial cells (HBECs) via the Ca(2+)/calmodulin signaling pathway, and inhibited the apoptosis of HBECs (Zhu *et al*. 2013). Besides, addition of globular APN increased quiescent porcine coronary artery VSMC proliferation, suggesting protection against atherosclerosis by the level of globular APN (Fuerst *et al*. 2012). APN was found to inhibit the growth of the murine endothelial cell line HECa 10 at concentrations from 10^{-5} to 10^{-12} M (Połowinczak-Przybyłek *et al*. 2009). In tumor cells, APN also exhibited potent anti-proliferative properties in human glioblastomas (Porcile *et al*. 2014), human hepatoma (HepG2) and breast cancer cells (Jia *et al*. 2016; Shrestha *et al*. 2014) via modulating cell cycle and apoptosis. In the present study, we also proved that APN promoted the OD values of VEPCs, indicating a proliferative response of VEPCs to APN. Additionally, compared to the control, APN significantly inhibited the apoptotic rate of VEPCs while promoted the expression

of the apoptosis relative factor Bcl-2. It was suggested that APN contributed to the increased proliferation and decreased apoptosis of VEPCs.

Vascular endothelial growth factor (VEGF) is a potent mitogen for vascular endothelial cells (Mitrou *et al*. 2014). Many studies have shown the participation of VEGF in atherosclerosis and angiogenesis in blood vessel (Greenberg and Jin, 2005, 2013). The correlation between APN and VEGF is still controversial. In a study, APN was shown to contribute to synovitis and joint destruction in rheumatoid arthritis by stimulating VEGF in fibroblast-like synoviocytes (Choi *et al*. 2009). Furthermore, APN promoted VEGF-A expression through the phosphoinositide 3 kinase (PI3K)/Akt and mTOR signaling pathways in human chondrosarcoma (Lee *et al*. 2015). On the other hand, over-expression of adiponectin decreased production of VEGF-A in prostate cancer cells (Gao *et al*. 2015). Srinivasan and Sulochana (2015) ever evaluated the correlation between VEGF and APN and suggested that human retinal pigment epithelial cell lines exposed to APN showed the decreased mRNA and protein expression of VEGF. However, our study demonstrated that APN stimulated VEPCs to secrete VEGF.

To further explore the possible mechanism of APN in VEPCs, we attempted to investigate the involvement of the mTOR-STAT3 signaling pathway. Recent studies have further proved that APN suppressed the proliferation and migration of VSMCs and induced VSMC apoptosis by inhibiting Ras/Raf or extracellular signal regulated kinase (Erk)1/2 signaling pathway (Zhang et al. 2015). Ding *et al*. (2011) found that APN promoted VSMC differentiation and preserved endothelial function via activating the Akt signaling. APN increased the proliferation of the human microvascular endothelial cell line (HMEC-1) through the activation of both PI3K/Akt and ERK/MAPK pathways (Alvarez *et al*. 2010). APN blocked interleukin-18-mediated

endothelial cell death via APPL1-dependent AMP-activated protein kinase (AMPK) activation and IKK/NF-kappaB/PTEN suppression (Chandrasekar et al. 2008). In contrast, APN inhibited leptin-induced proliferation of preneoplastic colon epithelial cells via inhibiting leptin-induced NF-kappaB-dependent autocrine IL-6 production and trans-IL-6 signaling (Fenton *et al*. 2008). In addition, APN inhibited IL-6-induced proliferation of MC-38 colon carcinoma cells by decreasing STAT-3 phosphorylation and activation (Fenton *et al*. 2010). In a new study, APN has ever prvoed to regulate EPC proliferation through the mTOR/STAT3 signal (Jiang *et al*. 2016). Our data also supported the findings of Jiang et al. and deeply discovered that APN could increase protein expression of pSTAT3 and promoted the proliferation and VEGF expression and inhibited the apoptosis in VEPCs through increasing the activity of the mTOR-STAT3 signaling pathway.

In conclusion, the novel data obtained from our experiments indicated that stimulation VEPCs with APN increased the proliferative capacity and VEGF expression of VEPCs through the activation of the mTOR-STAT3 signaling pathway. It was indicated that amplification of the proliferation potential of APN-stimulated VEPCs might be helpful in the therapeutic interventions for cardiovascular diseases.

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Conflict of interests

The authors declare that they have no conflict of interests.

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Figure legends

Fig 1. APN promoted the proliferation of VEPCs. After treated cells with 1μg/ml APN for 12, 24, 48 and 72 h, the number of viable VEPCs was determined by MTT assay. Three individual experiments were performed. Results were presented as mean ± SD. *P<0.05 was considered as significance compared with the control.

Fig 2. APN inhibited the apoptosis of VEPCs. After treated cells with 1μg/ml APN for 12, 24, 48 and 72 h, the apoptotic rate of VEPCs was determined by Flow Cytometry (A), and Bcl-2 expression was analyzed using RT-PCR method (B). Three individual experiments were performed. Results were presented as means ± SD. *P<0.05 was considered as significance compared with the control.

Fig 3. APN promoted the expression of VEGF. After treated cells with 1μg/ml APN for 12, 24, 48 or 72 h, mRNA expression of VEGF was determined by RT-PCR. Three individual experiments were performed. Results were presented as mean ± SD. *P<0.05 was considered as significance compared with the control.

Fig 4. The mTOR-STAT3 signaling pathway was activated by APN in VEPCs. After treated VEPCs with 1μg/ml APN for 48 h, protein expression of mTOR, STAT3 and pSTAT3 was determined by Western Blot analysis (A). Furthermore, band relative ratio was analyzed using Image J software. Three individual experiments were performed. Results were presented as means ± SD. *P<0.05 was considered as significance compared with the control.

Fig 5. The mTOR-STAT3 signaling pathway was involved in APN-induced cellular changes in VEPCs. Following the block of the mTOR-STAT3 signal using XL388, protein expression of pSTAT3 was detected by Western Blot analysis (A). After treated VEPCs with APN, XL388 or APN plus XL388, cell viability was

measured by MTT assay (B), the apoptotic rate was determined by Flow Cytometry (C) and mRNA expression of VEGF was evaluated by RT-PCR (D). Three individual experiments were performed. Results were presented as means ± SD. *P<0.05 was considered as significance compared with the control.

Figure 1

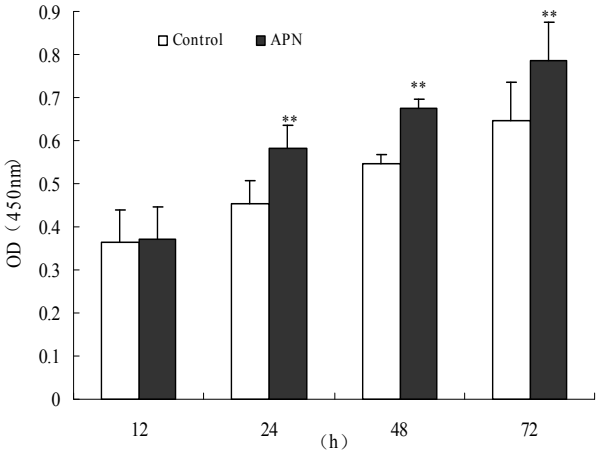
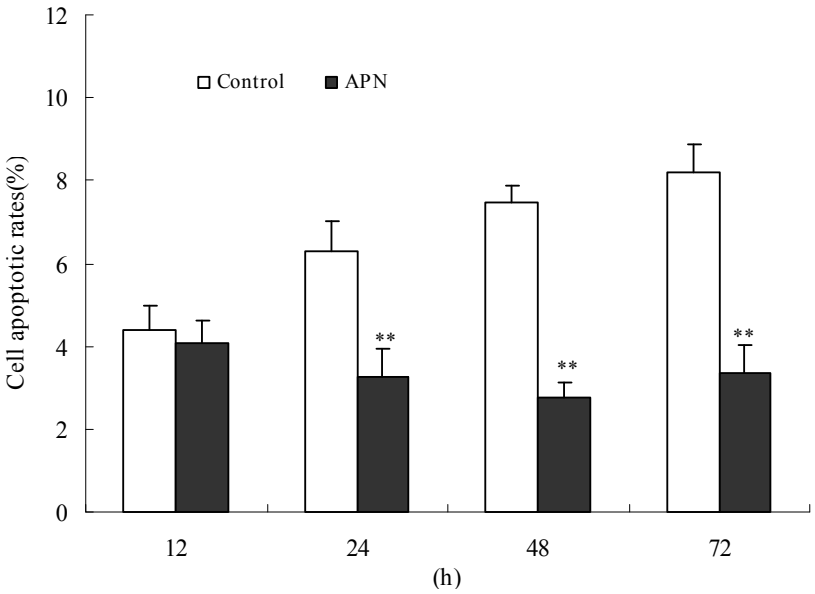


Figure 2

A


Control
APN
Cell apoptotic rates(%)
12
10
8
6
4
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**
**
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12
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48
72
(h)

B

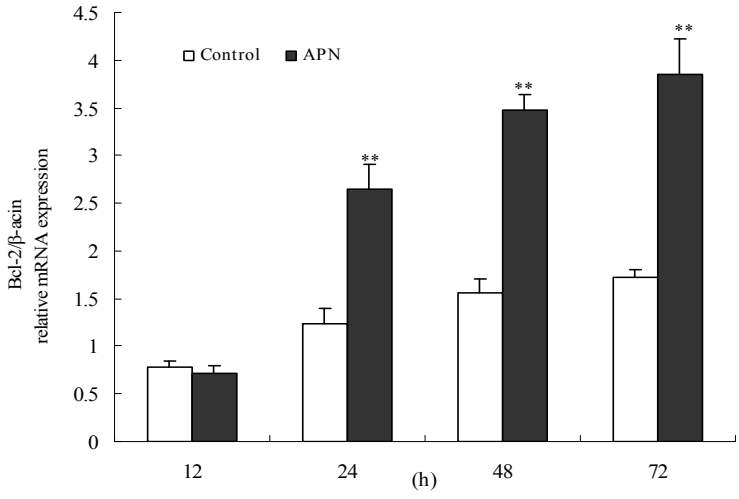

Control
APN
Bcl-2/β-acin
relative mRNA expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
**
**
12
24
48
72
(h)

Figure 3

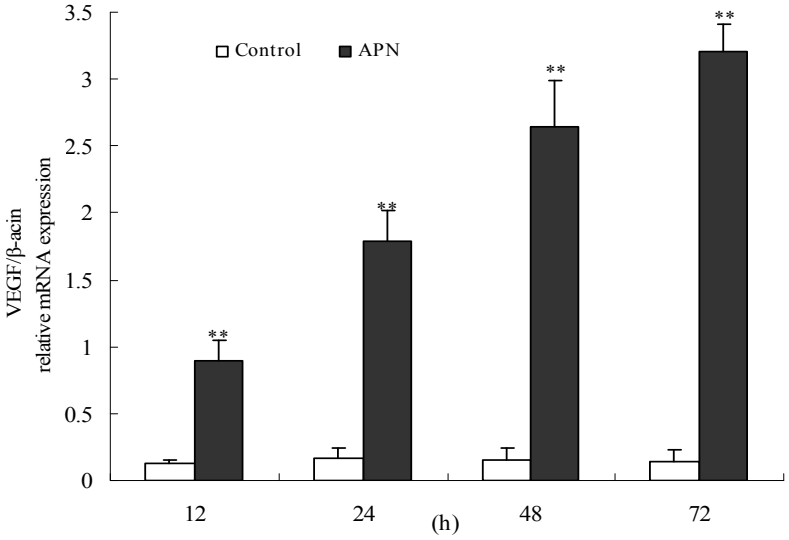
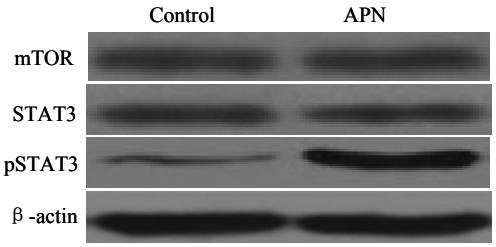

3.5
3
2.5
2
1.5
1
0.5
0
VEGF/β-acin
relative mRNA expression
Control
APN
**
**
**
**
12
24
48
72
(h)

Figure 4

A


Control
APN
mTOR
STAT3
pSTAT3
β -actin

B

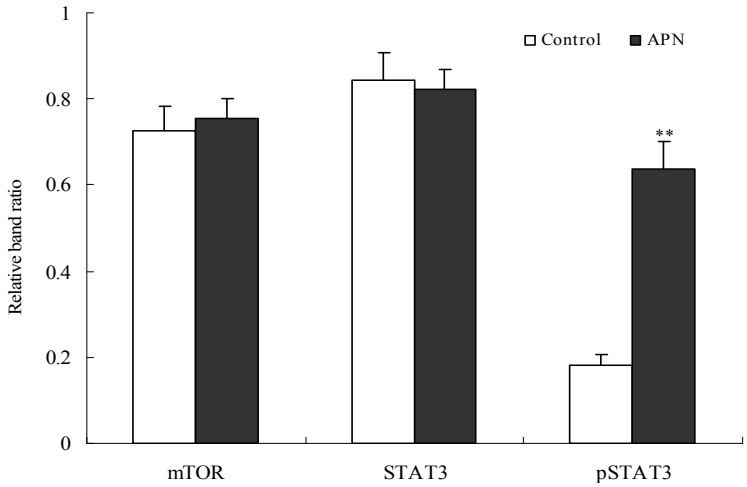
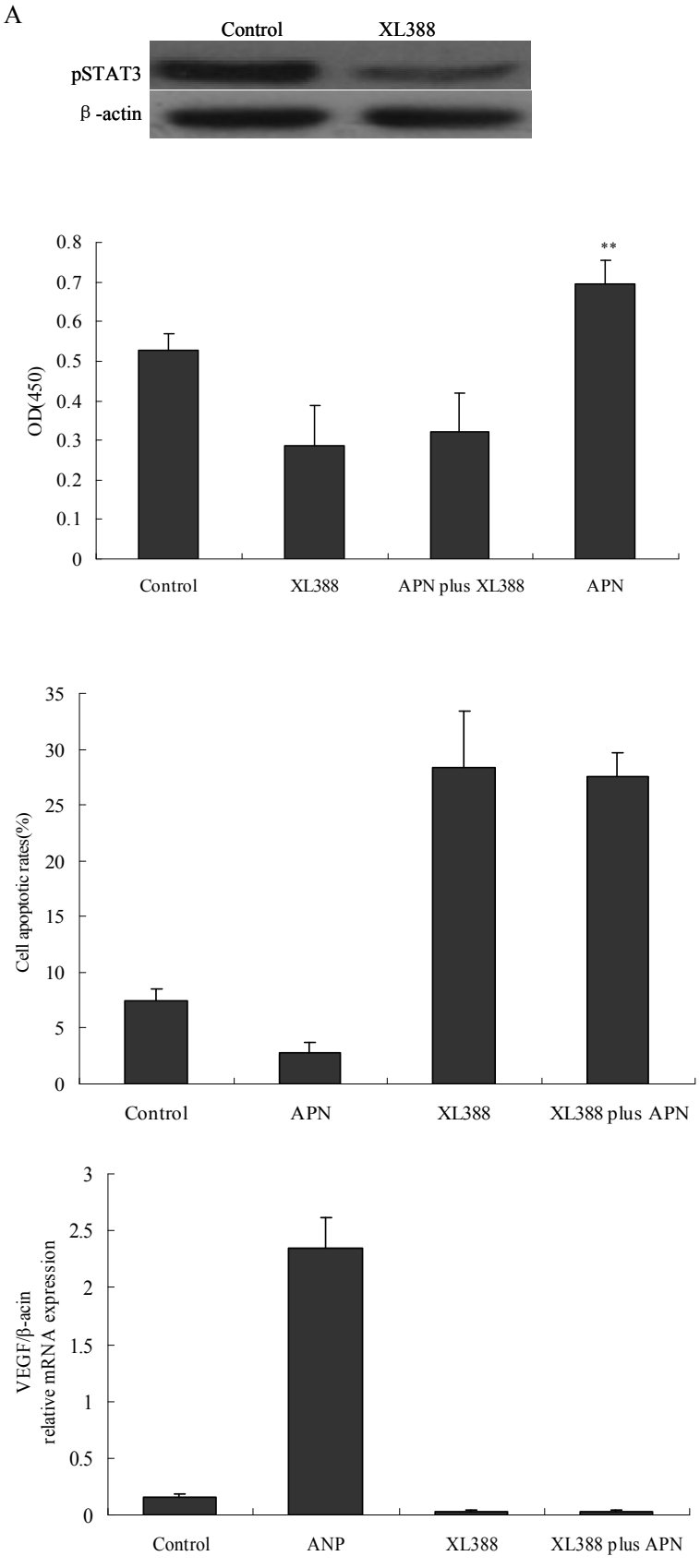

Relative band ratio
1
0.8
0.6
0.4
0.2
0
Control
APN
**
mTOR
STAT3
pSTAT3

Figure 5


A
Control
XL388
pSTAT3
β -actin
B
OD(450)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
Control
XL388
APN plus XL388
APN
C
Cell apoptotic rates(%)
35
30
25
20
15
10
5
0
Control
APN
XL388
XL388 plus APN
D
VEGF/β-acin
relative mRNA expression
3
2.5
2
1.5
1
0.5
0
Control
ANP
XL388
XL388 plus APN