European **Rev**iew for **Med**ical and **Pharmacol**ogical **Sci**ences 2017; 21: 5821-5826

Effect of β-arrestin on damage of human umbilical vein endothelial cell induced by angiotensin II

Z.-H. CAO, L. GAO, L. JIANG, P. ZHANG, H.-Y. NING, H. ZHANG

Departments of Cardiovascular Medicine, Tianjin Nankai Hospital, Tianjin, China

Zhenhua Cao and Lu Gao are Equal contributors

Abstract. – OBJECTIVE: β-arrestin (ARRB2) is a member of arrestin family and a negative regulatory protein of G-coupling receptor, which is closely associated with the pathogenesis of several autoimmune diseases. This study aimed to investigate the mechanism of the effect of ARRB2 on the damage of human umbilical vein endothelial cells (HUVECs), which is induced by angiotensin II (Ang II).

MATERIALS AND METHODS: ARRB2 at different concentration was used to interfere with the damage of HUVECs induced by Ang II or RNA interference technology to interfere with the expression of HUVECs followed by addition of Ang II to culture for 24 hours. Nitrate reduction method was used to measure the content of nitric oxide (NO) and radioimmunoassay was used to measure endothelin-1; Western blot assay was used to detect the expression of B-cell lymphoma-2 (Bcl-2), and flow cytometry was used to detect the intracellular level of reactive oxygen (ROS) and apoptosis of HUVECs.

RESULTS: Our study found that ARRB2 could significantly reduce the generation and release of ROS, endothelin-1 (ET-1), lactic dehydrogenase (LDH) of HUVECs induced by Ang II and promote the generation of NO, superoxide dismutase (SOD) and scavenging in a dose-dependent manner. On the contrary, when expression of ARRB2 was disturbed by siRNA, increased generation and release of ROS, ET-1, and LDH were observed with reduced generation of NO, SOD and scavenging. In addition, ARRB2 could reverse the apoptosis of HUVECs induced by Ang II and was related to upregulate the expression of Bax.

CONCLUSIONS: ARRB2 could protect the damage of HUVECs induced by Ang II and the mechanism was associated with upregulation of the expression of apoptosis and anti-apoptosis protein of Bcl-2.

Key Words

β-arrestin, Angiotensin II, G-protein coupling receptor, Human umbilical vein endothelial cells.

Introduction

β-arrestin (ARRB2) is widely expressed in the tissue of mammals[1] and is a member of arrestin family, which includes two subtypes, β-arrestin 1 (ARRB1) and β-arrestin 2 (ARRB2). ARRB2 could mediate G protein-coupled receptor and could be scaffolding protein involving in non-G protein signal of G protein-coupled receptor[2,3], which is closely associated to cell growth, immune function and pathogenesis of several diseases, such as depression, multiple sclerosis, asthma, type 2 diabetes[4-8], atherosclerosis and intimal hyperplasia[9] as well as inflammatory reaction caused by lipopolysaccharide and endotoxemia[10]. G protein-coupled receptor is involved in the regulation of downstream signal triggered by ARRB2, including angiotensin II receptor (AT2R). It is reported that ARRB2 may participant in the regulation of the vascular tension by angiotensin II (Ang II) in the process of pathology and multiple cell models dependent or independent apoptosis G protein-coupled receptor[11-14], participant in desensitizing reaction of G protein-coupled receptor, or participant in receptor endocytosis, degradation, intracellular signal transduction as scaffolding protein or adapter.

Some studies showed that there is ARRB2-specific binding domain in C-terminal of Ang II receptor[15]. ARRB2 is an intracellular regulatory protein which mainly regulates the signal transduction mediated by G protein-coupled receptor. After Ang II receptor is activated, ARRB2 exposes its specific binding domain and the effect of G protein-coupled receptor will be inhibited. During this process, ARRB2 as an inhibitor of signal pathway of G protein receptors plays an important role in uncoupling between Ang II receptor and G protein, and prevent

Corresponding Author: Hong Zhang, MD; e-mail: hongzhangqwe@163.com

the pathogenesis of several diseases[16,17]. Therefore, ARRB2 could regulate the effect of Ang II through G protein-coupled receptor. Through establishing the model of the damage of human umbilical vein endothelial cell which is induced by Ang II, this study aimed to investigate the protective effect and mechanism of β-arrestin on Ang II-induced damage of human umbilical vein endothelial cell.

Materials and Methods

Experimental Devices and Reagents

Experimental device: Western blot electrophoresis apparatus and qPCR device were both bought from Bio-Rad Co. Ltd. (Hercules, CA, USA). Cell culture plates were bought from Nest Company (San Diego, CA, USA). Flow cytometer were bought from BD Biosciences (San Jose, CA, USA). Reagents: HUVEC cells were derived from passage of our experimental room. Cytokines Ang II and ARRB2 were obtained from Peprotech Co. Ltd. (Rocky Hill, NJ, USA). The 1640 mediums were bought from Gibco (Rockville, MD, USA). Fetal calf serum (FCS) was bought from Hyclone (Logan, UT, USA). Bax and GAPDH antibody were anti-human polyclonal antibodies of mice, which were bought from Santa Cruz Biotech. (Santa Cruz, CA, USA). Anti-mice bi-antibodies of goat were bought from ZSGB Bio. Ltd. (Beijing, China). Si-RNA kit of ARRB2 was compounded by RiboBio Technol., Co. Ltd. (Guangzhou, China). ROS, ET-1, NO, LDH, SOD,O_2^- kit were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental Methods and Procedures

Cell culture: HUVEC cells were resuscitated in 1640 medium with 8% fetal calf serum (FCS) and cultured in an incubator with constant temperature of 37°C and 5% CO_2 saturated humidity. The conditions of cells were under observation every day. Cells were passaged every other day with fresh medium replaced.

Measurement of the Level of ROS, ET-1, NO, LDH, SOD, O2 $^-$

HUVEC cells were plated with a density of 100 thousands into each well. After cell attachment, 10^{-7} mol/L Ang II were added into each well. 48 h after cell culture, supernatant and cells were collected. Measurement of the level of ROS, ET-1, NO, LDH, SOD, O2$^-$ were performed according to the instruction of reagent kit

ARRB2 Transfection

Cells were plated after they were in exponential growth in big dish (6-well plate) with a density of 100 thousands in each well. After cell attachment, Ang II was added into the plates. Si-RNA kit compounded by Ruibo Company was used to interference the expression of ARRB2 of HUVECs. The efficacy of transfection was evaluated by fluorescence microscope and transfection rate was over 60%. 48 h after cell culture, supernatant and cells were collected and had related indicators measured. Primer sequence was shown in Table I.

Flow Cytometry Analysis of Cells Apoptosis

A total of 10^{-7} mol/L Ang II or Ang II (10^{-7} mol/L) + ARRB2 (10^{-6} mol/L) were added to HUVECs in exponential phase. They were co-incubated for 48 h and cells were collected for flow cytometry analysis. Apoptosis reagents kit was applied to detect cells apoptosis.

Western Blot Assay

Cells were plated after they were in exponential growth in big dish (6-well plate) with a density of 100 thousands in each well. After cell attachment, medium was replaced and drugs were added; then, transfection was performed. 48 h later, the total protein of cells was collected for protein analysis using an enhanced bicinchoninic acid (BCA) Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). After adjustment of internal reference, Western blot assay was performed.

Statistical Analysis

The SPSS 16.0 software (SPSS Inc., Chicago, IL ,USA) was introduced for statistical analysis. The measurement data was expressed as mean ± standard deviation (mean ± SD). Student's *t*-test was performed for the comparisons between groups. $p<0.05$ was considered as statistically significant.

Table I. siRNA primer of ARRB2 and GAPDH sequence.

Name	Primer sequence
ARRB2	5'-CGCTTCGCCAACTACAT-3' 5'-AGGGCATCCACTTCACAG-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC -3' 5'-TCCACCACCCTGTTGCTGTA -3'

Results

Effect of Cytokines ARRB2 on the Damage of HUVECs

Ang II of 10^{-7} mol/L was added to HUVECs in exponential phase. After co-incubation for 18 h, intracellular ROS, ET-1 and LDH were significantly increased compared to control group. However, NO, SOD and scavenging O_2^- were significantly reduced (p<0.05). In different concentration group of Ang II (10^{-7} mol/L+ARRB2), intracellular ROS, ET-1 and LDH were significantly lower than those in Ang II group, but NO, SOD and scavenging O_2^- were significantly higher. When the concentration of ARRB2 was at 10^{-7} mol/L-10^{-5} mol/L, generation of ROS, ET-1 and LDH in HUVECs were reduced, whereas, generation of NO, SOD and scavenging O_2^- was dose-dependently increased. With the increased concentration of ARRB2, intracellular ROS, ET-1 and LDH were gradually declined, and NO, SOD and scavenging O_2^- were significantly increased (p<0.05, Figure 1).

Effect of ARRB2 Gene Deletion on the Damage of HUVECs Induced by Ang II

Compared with control group, purely inference of ARRB2 could not change the level of ROS, ET-1, NO, LDH, SOD, scavenging O_2^-. Compared with Ang II group without interference, interference group displayed increased level of ROS, ET-1, LDH and reduced NO, SOD, scavenging O_2^-. In addition, there is significant difference in the change of LDH and NO level between these two groups (p<0.05, Figure 2).

Effect of ARRB2 on the Apoptosis of HUVECs Induced by Ang II

After Ang II (10^{-7} mol/L) was added, the apoptosis of HUVECs and the expression of Bax protein were significantly increased compared with control group. After both Ang II and ARRB2 were added, the apoptosis of HUVECs and the expression of B-cell lymphoma-2 (Bcl-2) protein were reduced significantly compared with addition of Ang II alone (Figure 3).

Discussion

β-arrestin is an important intracellular regulatory protein, which includes ARRB1 and ARRB2), and can regulate most signal transduction triggered by GRCR. After AT1R is activated, it

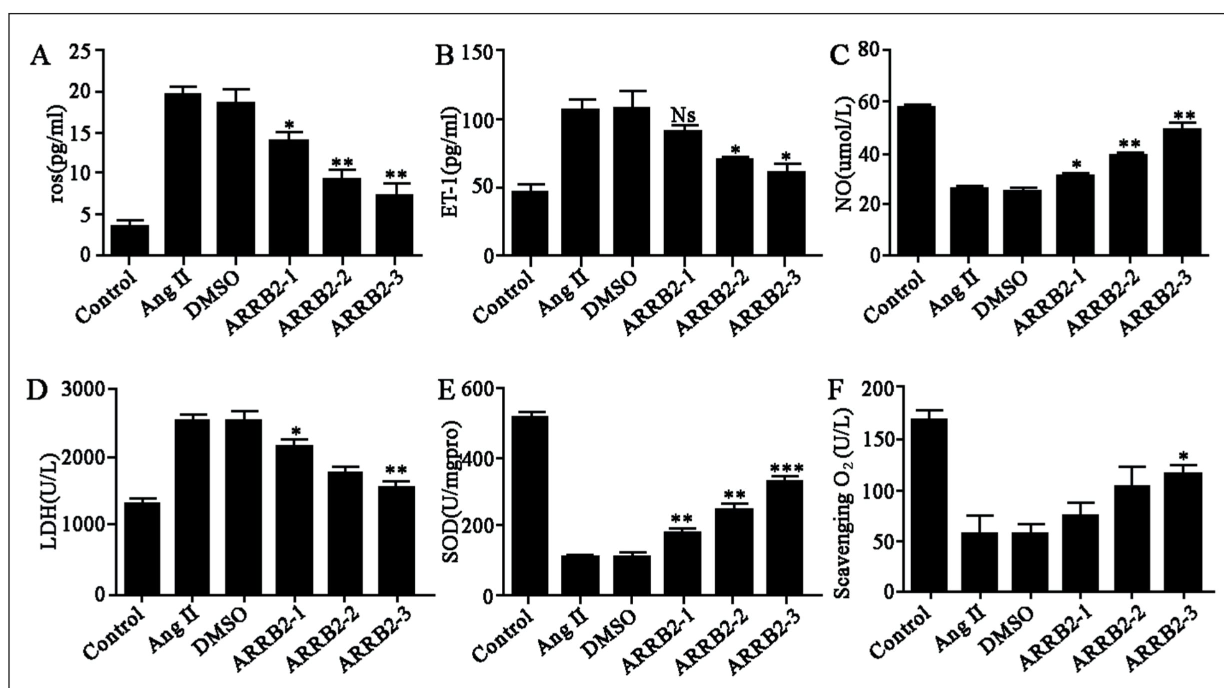

Figure 1. The effect of ARRB2 in different concentration on the release of ROS (A), ET-1 (B), NO (C), LDH (D), SOD (E), scavenging O_2^- (F) of the damage of human umbilical vein endothelial cell induced by Ang II (10^{-7} mol/L). ARRB2-1: 10^{-7} mol/L; ARRB2-2: 10^{-6} mol/L; ARRB2-3: 10^{-5} mol/L; DMSO: DMSO was diluted by PBS and the concentration of DMSO < 0.1%, n = 6. *p<0.05, **p<0.01, compared with Ang II.

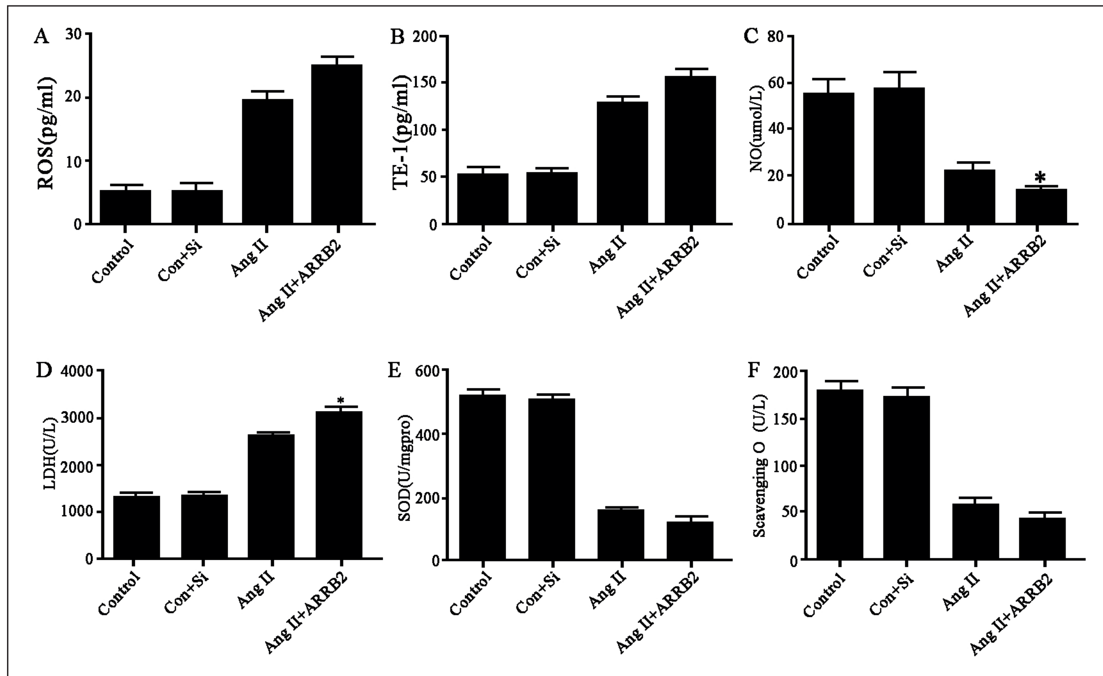

Figure 2. The effect of ARRB2 gene interferenced by siRNA on the damage (release of ROS (A), ET-1 (B), NO (C), LDH (D), SOD (E), scavenging O_2^- (F) of HUVECs induced by Ang II (10^{-7} mol/L). Con + si: HUVEC + 100 μm ARRB2 siRNA; Ang + ARRB2: HUVEC + Ang II + 100 μm ARRB2 siRNA. n = 6. *p<0.05, compared with Ang II group.

takes effect through recruiting β-arrestin to its specific binding domain. During this process, through promoting the couple of AT1R with G-protein, and combining clathrin, ARRB2 participants in endocytosis, desensitization and degradation of AT1R to influence the function of ANG 2 on endothelial cells. Some studies found that β-arrestin could affect the secretion of cytokines

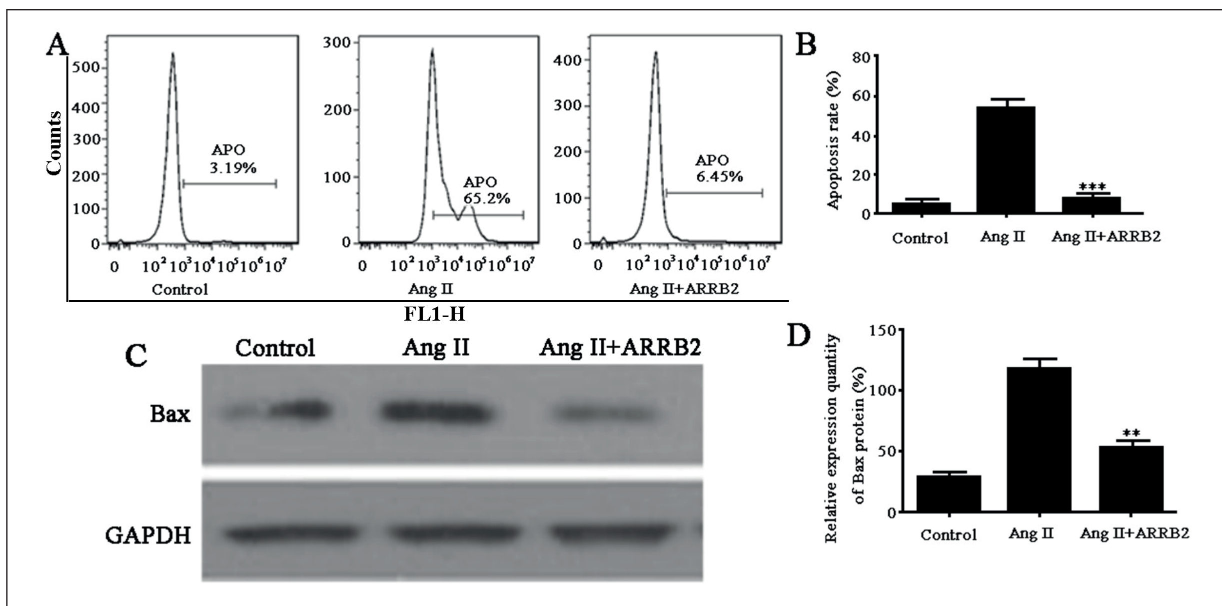

Figure 3. The effect of ARRB2 on the apoptosis of HUVECs induced by Ang II (10^{-7} mol/L). (A-B) The expression of Bax protein; (C-D) ARRB2: 10^{-6} mol/L, n = 6. *p<0.05, **p<0.01 vs. Ang II alone group.

from immune cells in mouse with acute asthma and involves in disease pathogenesis[18]. All these results confirm that β-arrestin participants in desensitization, internalization and degradation of β-AR. Moreover, some investigators suggested that signal transduction pathway of second activation, which relies on β-arrestin, provided the function of cardiac protection[19]. Therefore, β-arrestin plays non-substitutable importance role in maintaining the balance of human body function.

The various functions of β-arrestin are closely related to its self-structure. Meanwhile, β-arrestin contains specific sites, which have interaction with multiple signal factors. Once the configuration of β-arrestin is changed, many signal pathways are accordingly changed. Thus, it can regulate the functions of several cells, such as signal and transport related to cyto-membrane and cytoplasm. This study confirmed the change of expression quantity of β-arrestin on the expression of Bax. There is equilibrium between protein monomer and high-order structure of β-arrestin resulting in maintaining the homeostasis of human body and prevent the incidence of depression, multiple sclerosis, asthma, and type 2 diabetes. Thus, β-arrestin plays an important role in maintaining the body homeostasis and preventing the occurrence of diseases.

The results of this study showed that ARRB2 could inhibit the damage of human umbilical vein endothelial cell induced by Ang II. Ang II at 10^{-7} mol/L could cause apoptosis of HUVECs at 18 h. We found ROS, ET-1 and LDH were significantly increased, NO, SOD and scavenging O_2^- were significantly reduced after addition of Ang II at 10^{-7} mol/L, suggesting Ang II induces the damage of human umbilical vein endothelial cell possibly through ROS, ET-1, NO, LDH, SOD, scavenging O_2^-, consistent with previous reports[20,21]. The exact molecular mechanism may be the increased ROS generation and accelerated NO degradation leading to bioavailability of NO and finally inhibit the endothelial cell vitality and accelerate cell apoptosis[22,23]. After adding ARRB2 at different concentrations, levels of ROS, ET-1 and LDH were decreased and generation of NO, LDH, SOD, scavengingO_2^- was increased in a dose-dependent manner. At the same time, flow cytometry analysis of apoptosis showed that ARRB2 could significantly reduce the apoptosis of HUVECs induced by Ang II. Therefore, ARRB2 had inhibitory effects on the damage of HUVECs induced by Ang II. The secretion of Ang II may be increased in case of pathology. ARRB2 could reduce the generation of ROS and release of ET-1, promote the secretion of NO, or reduce eNOS uncoupling and accelerate generation of NO through inhibiting the generation of ROS resulting in amelioration of damage to body.

In this study we also found that the expression of Bax protein was significantly increased after addition of Ang II at 10^{-7} mol/L. This is because of initiation of apoptosis-promoting gene of Bcl-2 family. Bcl-2 family includes two types: one is anti-apoptosis gene, including Bcl-2, which is a negative regulator of cell death and prevents cells from apoptosis, which is stimulated by other factors. The other one is apoptosis-promoting gene, including Bax. The adding of ARRB2 could reduce the expression of Bax protein and inhibit the apoptosis of HUVECs. The expression of Bcl-2/Bax determines the initiation of apoptosis. The addition of Ang II could change the balance of the expression of Bcl-2/Bax, leading to initiation of cells apoptosis and ARRB2 could reverse the imbalance of the expression of Bcl-2/Bax. However, the exact mechanism by how ARRB2 regulates the balance of Bcl-2 and Bax remains unclear and requires further investigation.

Conclusions

We demonstrated that Ang II at 10^{-7} mol/L could promote the generation of ROS and LDH as well as secretion of ET-1, and inhibit the release of NO, SOD, scavenging O_2^- of HUVECs. We firstly found that ARRB2 *in vitro* could inhibit the generation of ROS and LDH as well as secretion of ET-1, and the release of NO, SOD, scavenging O_2^- of HUVECs. ARRB2 at 10^{-7} mol/L-10^{-5} mol/L could protect vascular endothelial function. The damage of HUVECs induced by Ang II was associated with the imbalanced expression of apoptosis-promoting and anti-apoptosis gene of Bcl-2 family. This study provides certain theoretic basis for the potential application of ARRB2 on the treatment of cardiovascular diseases.

Acknowledgments

This work was supported by Tianjin health and Family Planning Commission of Chinese Medicine combined with Western Medicine Scientific Research Task (2015031).

Conflict of Interest

The authors declare no conflict of interest.

References

1) Kingsmore SF, Peppel K, Suh D, Caron MG, Lefkowitz RJ, Seldin MF. Genetic mapping of the beta-arrestin 1 and 2 genes on mouse chromosomes 7 and 11 respectively. Mamm Genome 1995; 6: 306-307.
2) Li Y, Tian J, Ma XR, Li RT, Zhang SL, Wang PL, Yin XC, Bian JW, Lei JH, Liu HR. Increase in G protein-coupled receptor autoantibodies with decline of cardiac function in hypercholesterolemic rats. Eur Rev Med Pharmacol Sci 2017; 21: 1065-1073.
3) Shenoy SK, Lefkowitz RJ. Beta-arrestin-mediated receptor trafficking and signal transduction. Trends Pharmacol Sci 2011; 32: 521-533.
4) Mendez-David I, El-Ali Z, Hen R, Falissard B, Corruble E, Gardier AM, Kerdine-Romer S, David DJ. A method for biomarker measurements in peripheral blood mononuclear cells isolated from anxious and depressed mice: beta-arrestin 1 protein levels in depression and treatment. Front Pharmacol 2013; 4: 124.
5) Tsutsui S, Vergote D, Shariat N, Warren K, Ferguson SS, Power C. Glucocorticoids regulate innate immunity in a model of multiple sclerosis: reciprocal interactions between the A1 adenosine receptor and beta-arrestin-1 in monocytoid cells. FASEB J 2008; 22: 786-796.
6) Ko YC, Hsu WH, Chung JG, Dai MP, Ou CC, Wu WP. Proteomic analysis of CD4+ T-lymphocytes in patients with asthma between typical therapy (controlled) and no typical therapy (uncontrolled) level. Hum Exp Toxicol 2011; 30: 541-549.
7) Walker JK, Fong AM, Lawson BL, Savov JD, Patel DD, Schwartz DA, Lefkowitz RJ. Beta-arrestin-2 regulates the development of allergic asthma. J Clin Invest 2003; 112: 566-574.
8) Luan B, Zhao J, Wu H, Duan B, Shu G, Wang X, Li D, Jia W, Kang J, Pei G. Deficiency of a beta-arrestin-2 signal complex contributes to insulin resistance. Nature 2009; 457: 1146-1149.
9) Kim J, Zhang L, Peppel K, Wu JH, Zidar DA, Brian L, DeWire SM, Exum ST, Lefkowitz RJ, Freedman NJ. Beta-arrestins regulate atherosclerosis and neointimal hyperplasia by controlling smooth muscle cell proliferation and migration. Circ Res 2008; 103: 70-79.
10) Porter KJ, Gonipeta B, Parvataneni S, Appledorn DM, Patial S, Sharma D, Gangur V, Amalfitano A, Parameswaran N. Regulation of lipopolysaccharide-induced inflammatory response and endotoxemia by beta-arrestins. J Cell Physiol 2010; 225: 406-416.
11) Hennenberg M, Trebicka J, Kohistani AZ, Heller J, Sauerbruch T. Vascular hyporesponsiveness to angiotensin II in rats with CCl(4)-induced liver cirrhosis. Eur J Clin Invest 2009; 39: 906-913.
12) Yang X, Zhou G, Ren T, Li H, Zhang Y, Yin D, Qian H, Li Q. Beta-arrestin prevents cell apoptosis through pro-apoptotic ERK1/2 and p38 MAPKs and anti-apoptotic Akt pathways. Apoptosis 2012; 17: 1019-1026.
13) Zhang Z, Hao J, Zhao Z, Ben P, Fang F, Shi L, Gao Y, Liu J, Wen C, Luo L, Yin Z. Beta-arrestins facilitate ubiquitin-dependent degradation of apoptosis signal-regulating kinase 1 (ASK1) and attenuate H2O2-induced apoptosis. Cell Signal 2009; 21: 1195-1206.
14) Sun X, Zhang Y, Wang J, Wei L, Li H, Hanley G, Zhao M, Li Y, Yin D. Beta-arrestin 2 modulates resveratrol-induced apoptosis and regulation of Akt/GSK3ss pathways. Biochim Biophys Acta 2010; 1800: 912-918.
15) Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG. Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis*. J Biol Chem 2001; 276: 19452-19460.
16) Hunyady L, Catt KJ, Clark AJ, Gaborik Z. Mechanisms and functions of AT(1) angiotensin receptor internalization. Regul Pept 2000; 91: 29-44.
17) Thomas WG. Regulation of angiotensin II type 1 (AT1) receptor function. Regul Pept 1999; 79: 9-23.
18) DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK. Beta-arrestins and cell signaling. Annu Rev Physiol 2007; 69: 483-510.
19) Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. Beta-arrestin: a protein that regulates beta-adrenergic receptor function. Science 1990; 248: 1547-1550.
20) Wu M, Huang H, Zhou G, Deng Z, Wu X. [Expression changes of Ang-1, Ang-2, Tie-2 and VEGF in co-culturing of endothelial cells and astrocytes after irradiation by X-ray and their significances]. Zhonghua Yi Xue Za Zhi 2014; 94: 1815-1819.
21) Lee WS, Yang HY, Kao PF, Liu JC, Chen CH, Cheng TH, Chan P. Tetramethylpyrazine downregulates angiotensin II-induced endothelin-1 gene expression in vascular endothelial cells. Clin Exp Pharmacol Physiol 2005; 32: 845-850.
22) Desideri G, Bravi MC, Tucci M, Croce G, Marinucci MC, Santucci A, Alesse E, Ferri C. Angiotensin II inhibits endothelial cell motility through an AT1-dependent oxidant-sensitive decrement of nitric oxide availability. Arterioscler Thromb Vasc Biol 2003; 23: 1218-1223.
23) Miao R, Leng D, Liu M, Huang XX, Li JF, Gong JN, Liang Y, Zhai ZG, Yang YH, Wang Y, Wan J. Alteration of endothelial nitric oxide synthase expression in acute pulmonary embolism: a study from bench to bioinformatics. Eur Rev Med Pharmacol Sci 2017; 21: 827-836.