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Original Article

Effects of cytokines on the expression of angiotensin II type 1 receptors in vascular smooth muscle cells

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Abstract: Objective: The aim of this study was to investigate the effects of pro-inflammatory cytokines on the expression of angiotensin II type 1 receptors (AT1Rs) in vascular smooth muscle cells (VSMCs). Methods: Primary VSMCs obtained from the thoracic aorta of Sprague Dawley rats cultured in DMEM medium containing 10% fetal bovine serum were divided into the following groups: (1) control group; (2) group L: 10% (IL-1β 50 ng·mL^{-1}+TNF-α 100 ng·mL^{-1}+IFN-γ 500 ng·mL^{-1}, cytokines); (3) group N: 50% cytokines; (4) group H: 100% Cytokines; (5) group L-NAME (N-nitroso-L-arginine methyl ester): 100% cytokines+5 mmol·L^{-1} L-NAME; (6) group SNP: 500 μmol·L^{-1} SNP. After 12 h incubation, total RNA was extracted for mRNA expression detection by RT-PCR. AT1R expression was also detected by western blot. Results: cytokine complexes decreased the expression of VSMC AT1R mRNA and protein in a concentration-dependent manner ($P < 0.01$), and SNP significantly reduced the expression of VSMC AT1R mRNA and protein ($P < 0.01$). On the other hand, L-NAME significantly rebounded the expression of VSMC mRNA and protein ($P < 0.05$ or 0.01). Conclusions: The pro-inflammatory cytokines investigated in the current study inhibited the expression of VSMC mRNA and protein, and this effect was mediated by nitric oxide to some extent.

Keywords: Cytokines, angiotensin II type 1 receptor, vascular smooth muscle, septic shock

Introduction

Septic shock (SS) and multiple organ dysfunction syndrome (MODS) are currently the leading causes of death in intensive care wards, as well as the main practical clinical problems and challenges that need to be solved in contemporary critical care medicine. Data show that in the USA, 750,000 cases of severe infection occur each year, and with a mortality rate of approximately 20% to 63%, similar to the out-of-hospital mortality rate associated with acute myocardial infarction [1].

The basis for the hemodynamic changes that occur in SS lies in abnormal peripheral vascular systolic and diastolic functions, which might lead to irregular blood flow. After infection, an excessive release of a series of pro-inflammatory mediators, with an insufficient compensatory release of anti-inflammatory mediators, results in uncontrolled inflammation and changes in vascular permeability; in such cases, even removing the primary disease to prevent the progression and evolution of MODS would be difficult [2, 3].

Activation of the renin-angiotensin system (RAS) in SS leads to a significant increase in plasma renin activity and angiotensin II levels. Excessive activation of RAS might be involved in organ function damage, and renin activity and angiotensin II are correlated with disease severity. Thus, levels of these molecules have important roles in determining the prognosis and severity of a disease. During the infection process, however, the activation of renin-angiotensin was the Sword of Damocles, moderate activation was involved in maintaining the body circulation and water-electrolyte balance, while the excessive activation might lead to body's organ dysfunction [4, 5].

Cycle dysfunction is a dominant symptom in the pathological process of SS, and the critical pathological factor leading to MODS or even

death in patients with SS. RAS is abnormally activated in SS, however, the conflict between the generation and release of large amounts of angiotensin (vasoconstrictor) and the body's progressive intractable arterial low blood pressure is really puzzling [6, 7].

The current study cultured primary rat VSMCs, and observed AT1R expression in SS, aiming to investigate whether changes in its expression were associated with nitric oxide (NO) mediation.

Materials and methods

VSMC culture

Healthy 4-week-old Sprague Dawley rats (clean grade), weighing 120~130 g, were provided by the Experimental Animal Center of our University. Rats were sacrificed by cervical dislocation, the thoracic aorta was quickly removed under sterile conditions, and the medial smooth muscle layer was separated. The tissue was cut into approximately 1 mm^3-pieces, which were then adhered onto a culture bottle wall. The culture bottle was flipped 3~4 h later, and the solution within the bottle was changed to DMEM containing 20% fetal bovine serum (FBS) for culture. Scattered fusiform cells could be seen freeing from the surrounding tissue blocks 4~5 d later, whereby the culture medium was replaced. Two to 3 w later, the cells basically covered the bottom of the bottle, and cell growth could be observed under an inverted microscope. After the cells fused, they formed typical structures named "peaks and valleys". In addition, an electron microscope (EM) was used to identify the cultured cells. Trypsin (0.125%) was then used for digestion and passage, and 6~10-generation cells, which were in the logarithmic growth phase, were chosen for experiments. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Military Medical University.

Intervention and grouping of VSMCs

Cells in the logarithmic growth phase were transferred into 6-well plates with a 1:3 ratio. When cells were in the sub-confusion stage, the medium was changed to serum-free DMEM for 24-h culture, and cultured in DMEM containing 10%-FBS. Cells were then grouped according to the following interventions: (1) control group: control; (2) group L: 10% (IL-1β 50 ng·mL^{-1}+TNF-α 100 ng·mL^{-1}+IFN-γ 500 ng·mL^{-1}, cytokines); (3) group N: 50% cytokines; (4) group H: 100% cytokines; (5) group L-NAME (Shanghai Hao Ran Biotechnology Co., Ltd.): 100% cytokines+5 mmol·L^{-1} L-NAME; (6) group SNP: sodium nitroprusside (Guangdong Zhongsheng Pharmaceutical Co., Ltd.) 500 μmol·L^{-1}. Each group was repeated six times, and VSMCs in each group were incubated for 12 h before detection.

Expression of VSMC AT1R mRNA

The tripure-chloroform-isopropanol method was used to extract total RNA (Invitrogen, USA) from the intervened cells of each group. One μg of RNA sample was used for the reverse transcription-polymerase chain reaction (RT-PCR) to detect the VSMC AT1R mRNA expression in each group, and the PCR primer sequences were designed by the relative software and verified with the corresponding mRNA sequences in Gene Bank. AT1R (290 bp) primers: Upstream 5'-CTG GCA TTT TGT CTG GAT A-3', downstream 5'-CAG AAG AGT TAA GGG CCA T-3', β-actin (184 bp) primers: Upstream 5'-CAA TTC CAT CAT GAA GTG AC-3', downstream 5'-CCA CAC AGA GTA CTT GCG CTC-3'. PCR was performed referring to the kit instructions (TaKaRa Co.) for both target mRNA and β-actin: 50 ml of reaction system, pre-denaturation at 94°C for 3 min, then amplified under the following conditions: 94°C for 1 min, 55°C for 30 sec, 72°C for 1 min, with a total of 35 cycles, followed by 72°C for 7 min. The negative control used the reverse negative reaction mixture to replace the target mRNA, while the rest of the conditions were the same. After amplification, 5 ml of PCR products were run on a 2% agarose gel +0.5 × TBE electrophoresis. Image Master VDS software was used to scan and analyze the gel spectra. Total ab PCR electrophoresis image analysis software was used to scan and analyze the optical densities, which used β-actin as the internal reference for the relative amount analysis; the integral ratio of AT1R/β-actin represented the AT1R mRNA expression levels.

Protein expression of VSMC AT1R

The expression of VSMC AT1R protein was analyzed by western blot. After cell clearance was

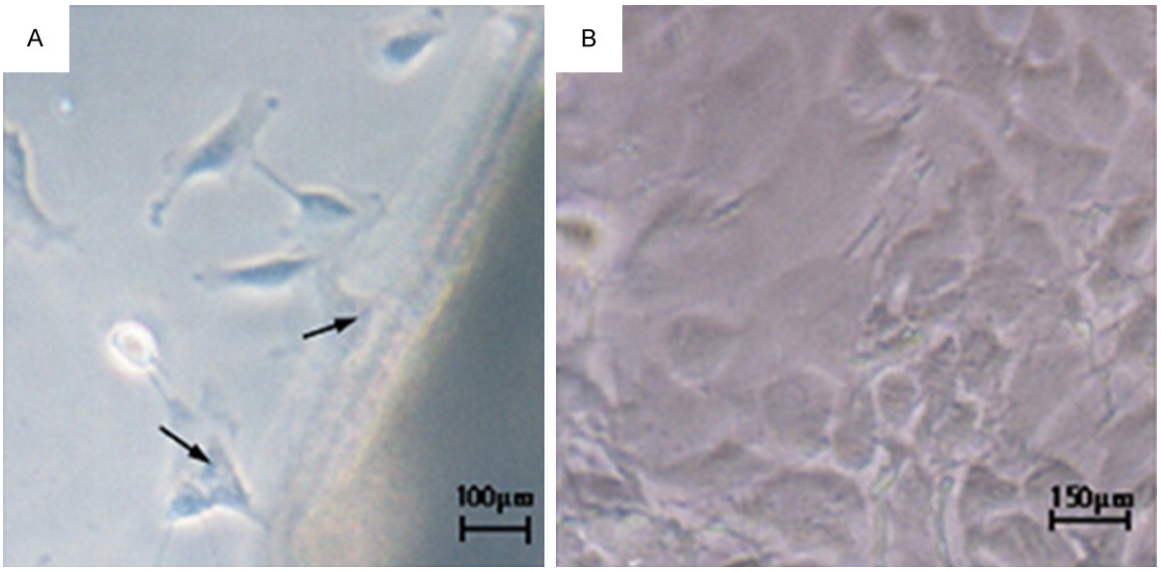

Figure 1. Pictures of phase contrast microscopy. A. Early stage of primary culture, the phase contrast microscopy revealed the scattered VSMCs freeing from the tissue block edges (× 400), and the arrows represent scattered VSMCs; B. Phase contrast microscopy revealed that after passage, VMSCs covered the whole bottom, partial region exhibited single layer of VMSCs, while partial region exhibited multi-overlapped layers, which was the typical "peak-like" feature (× 200).

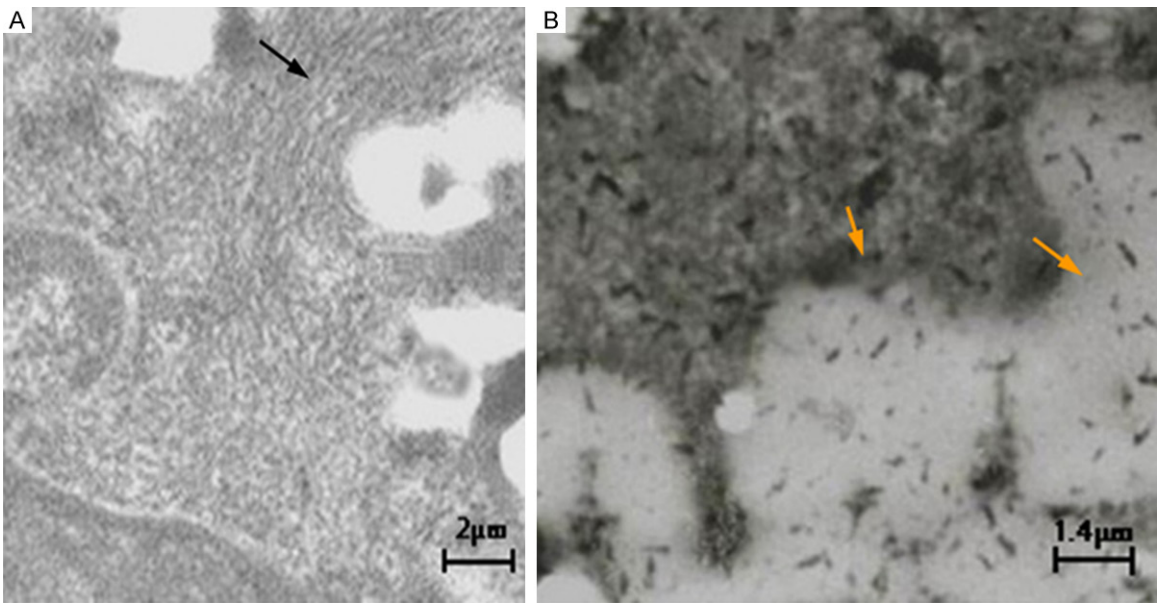

Figure 2. TME revealed that there exited streak-like dense myofilaments in VSMCs' cytoplasm (TEM × 20000) (A) and the characteristic dense plaques scattered on VMSCs membranes (TEM × 30000). (A) The arrows represent streak-like dense myofilaments; (B) The arrows represent characteristic dense plaques.

completed, the Coomassie blue staining method was used for protein quantification, and samples with the same concentrations were run on 10% polyacrylamide gel electrophoresis and electro-transferred onto nitrocellulose membranes. Membranes were incubated in 5% BSA at 4°C overnight and labeled with anti-AT1R monoclonal antibody (diluted 1:400, SC-579, Santa Cruz Inc.). Membranes were then washed with tris-buffered saline and Tween 20 (TBST), incubated with secondary antibody (diluted 1:1000), and washed with TBST. Finally,

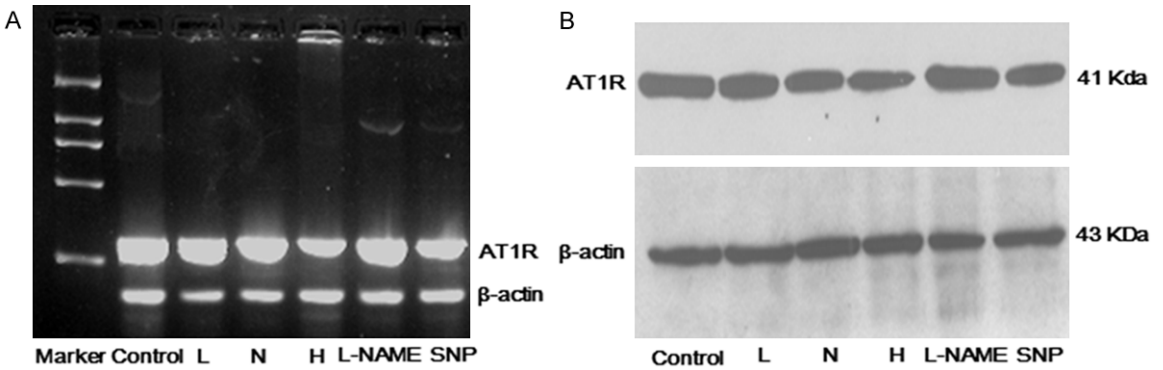

Figure 3. Expressions of VMSC AT1R mRNA (A) and protein (B) in each group.

Table 1. Comparison of VSMC AT1R mRNA and protein expressions among the groups ($\bar{x}$ ± s, n = 6)

Group	Control group	Group L	Group N	Group H	Group L-NAME	Group SNP
AT1R mRNA	302 ± 38	229 ± 19[b,c]	163 ± 21[b,d]	101 ± 20[b,d]	269 ± 34[a]	133 ± 21[b]
AT1R protein	239 ± 34	154 ± 22[b,c]	143 ± 28[b,d]	74 ± 19[b,d]	184 ± 17[b]	93 ± 15[b]

Note: Compared with control group, [a]$P < 0.05$, [b]$P < 0.01$; Compared with group L-NAME, [c]$P < 0.05$, [d]$P < 0.01$.

membranes were subjected to ECL film exposure, developed, and fixed. Images were obtained, and the density scan and area integral were determined. The integral ratio of AT1R/β-actin represented the expression levels of AT1R protein.

Statistical analysis

Data are expressed as mean ± standard deviation ($\bar{x}$ ± s), and the statistical software, SPSS13.0, was used for single factor analysis of variance (ANOVA). A one-way ANOVA was employed for intergroup comparisons, and a multi-comparison analysis (Tukey's test) was used for intragroup comparisons. A $P < 0.05$ was considered as statistically significant.

Results

Identification of VSMCs

Phase contrast microscopy revealed that early-cultured primary VSMCs grew from the edge of the tissue block (**Figure 1A**). After passage, VSMCs covered the bottom of the bottle, with one region exhibiting a single layer of VMSCs while the other region exhibited multiple overlapped layers. The latter layers were the typical "peak-like" features. VSMCs diversified into those with fusiform, diamond, and star shapes, etc. (**Figure 1B**).

TEM observation

Transmission EM (TEM) revealed streak-like dense myofilaments in the cytoplasm of the VSMCs (**Figure 2A**), with characteristic dense plaques scattered on their membranes (**Figure 2B**).

VSMC AT1R mRNA expression in each group

Compared with the control group, the mixture of pro-inflammatory factors inhibited the expression of VSMC AT1R mRNA in a concentration-dependent manner ($P < 0.01$). The expression of VSMC AT1R mRNA in group H was the lowest, while VSMCs co-incubated with the NO synthase (NOS) inhibitor, L-NAME, and the cytokine mixture significantly impaired the inhibitory effects of cytokines on the expression of VSMC AT1R mRNA ($P < 0.05$). Moreover, as the NO donor, SNP could exert similar effects as cytokines, which significantly inhibited the expression of AT1R mRNA ($P < 0.01$, **Figure 3A**; **Table 1**).

Expression of VSMC protein in each group

Compared with the control group, the mixture of pro-inflammatory cytokines inhibited the expression of VSMC AT1R protein in a dose-dependent manner ($P < 0.01$). SNP could also significantly inhibit the expression of VSMC AT1R protein ($P < 0.01$), while the expression of

VSMC AT1R protein in group L-NAME was significantly reversed when compared to the cytokine group at different concentrations ($P < 0.01$, **Figure 3B**; **Table 1**).

Discussion

As the main substrate of the RAS system, Ang II has four known receptor subtypes, namely AT1R, AT2R, AT3R, and AT4R. These receptors are members of the G-protein coupled receptor superfamily, with the primarily G protein-coupled subtype being AT1R, and play key roles in maintaining cardiovascular homeostasis [8]. AT1R is mainly distributed in the heart, VSMCs, adrenal gland, hypothalamus, platelets, and other tissues/organs. This subtype is the main mediator of vasoconstriction, increases the release of endothelin, catecholamines, and vasopressin, and promotes VSMC proliferation, oxidative stress, and inflammation [9, 10]. Studies have reported that AT1R expression in the hypothalamus or cardiac muscles is significantly reduced in rats with endotoxemia or SS [11, 12]; however, one study reported AT1R expression in the vascular system in a case of SS-induced circulatory failure. In the current report, we cultured primary rat thoracic aortic VSMCs in vitro and investigated expression changes in the AT1R gene and protein.

In SS, lipopolysaccharide stimulates the activation of nuclear factor-κB (NF-κB), and induces monocyte-macrophages, endothelial cells, and VSMCs to largely generate and release pro-inflammatory cytokines, which lead to uncontrolled inflammation and circulatory failure [13]. Thus, endotoxin-induced inflammatory responses are mainly mediated by pro-inflammatory cytokines, and the direct use of pro-inflammatory cytokines could create a model of SS. Accordingly, this experiment simulated the environment in which a large number of pro-inflammatory cytokines are released. Studies have shown that a single low dose of TNF-α or IL-1β can increase AT1R expression in cardiac fibroblasts [14], but when IL-1β, TNF-α, and IFN-γ co-act on mesangial cells, AT1R is downregulated [15]. Because the body releases more than one pro-inflammatory cytokine in SS, we selected IL-1β, TNF-α, and IFN-γ, as they are three relatively representative pro-inflammatory cytokines, for co-incubation with VSMCs. During the experiment, after the pro-inflammatory cytokine mixture was introduced to VSMCs, RT-PCR and western blot revealed that the expression levels of AT1R mRNA and protein were significantly reduced. Taken together, these findings indicate that largely released pro-inflammatory cytokines-caused downregulation of the VSMC AT1R gene and protein in SS, and that this might be one important mechanism of cycle dysfunction and low vascular reactivity in SS.

SS induces the activation of nitric oxide synthase (iNOS), leading to the excessive production of NO. The present study demonstrated that pro-inflammatory cytokines might directly promote iNOS activation in such parenchymal cells as in VSMCs. The activated iNOS would then promote the generation of NO, which would play the following roles through cGMP- and non-cGMP-dependent pathways: promoting the opening of K^+ channels and subsequent cell hyperpolarization, inhibiting the activation of BK channels, activating the Ca^{2+}-Mg^{2+} ATP enzyme, inhibiting L-type Ca^{2+} channels, promoting Na^+/Ca^{2+} exchange [16], inhibiting the formation of IP3 and the phosphorylation of the myosin light chain, and acting alongside the superoxide anion (O2•-) to form the peroxynitrite anion, thus leading to VSMC dysfunction [17, 18]. Therefore, it could be envisaged that NO mediates pro-inflammatory cytokines, thus downregulating the expression of AT1R.

In this study, the NOS inhibitor, L-NAME, was co-incubated with VSMCs and inflammatory cytokines. Under this condition, it was found that the expressions of VSMC AT1R mRNA and protein in different-concentration cytokine groups were reversed, to some extent. Schmidt [14] found that selectively silencing the NF-κB gene could improve the downregulation of AT1R in SS mice. Meanwhile, they showed that NF-κB can activate iNOS to generate excess NO, which is consistent with the conclusions of the current study. More specifically, the current study and the previous report suggest that inflammatory cytokines decrease AT1R expression, and that this is closely related to the large amounts of NO generated; however, exogenous NO SNP can simulate similar results.

The reasons why NO reduced the expression of the AT1R gene and protein remain unclear. It has been found that the regulation of AT1R mRNA expression does not depend on protein kinase C or Ca^{2+}-mediated signaling channels, but the 3' upstream non-coding region of AT1R mRNA plays a key role in regulating its expres-

sion [19]. NO can inhibit DNA-binding proteins to act on the AT1R proximal promoter by a cGMP-non-dependent pathway, thus downregulating the transcription and translation of AT1R [20]. The C-terminal end of AT1R plays major roles in mediating receptor retraction, desensitization, and receptor signal transduction. Pro-inflammatory cytokines could therefore activate a novel C-terminal protein which mainly acts on AT1R, known as AT1R-associated protein (ATRAP). After ATRAP activation in VSMCs, myocardial cells, and others bound with AT1R, activation of the c-fos promoter would be prevented. This would then reduce the gene transcription and protein synthesis of c-fos, inhibit p38 MAPK phosphorylation in the mitogen-activated protein kinase (MAPK) family, and decrease AT1 signal transduction. Furthermore, ATRAP could mediate, without binding to the G protein, AT1R entrapment, thus reducing the amount of AT1R on cell membranes [21, 22].

The results of this study showed that pro-inflammatory cytokines induced the downregulation of the AT1R gene and protein in VSMCs, and that this phenomenon could be simulated by exogenous NO. Meanwhile, NOS blockers could significantly reverse this low expression. Taken together, our results suggest that the release of a large number of pro-inflammatory cytokines in SS induces NO-associated low expression of AT1R. This might be one of the important mechanisms in cycle dysfunction and the low reactivity of blood vessels in SS, thus, the current study provides a theoretical basis for the treatment of SS.

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Disclosure of conflict of interest

None.

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References

[1] Green JM. Essentials of sepsis management. Surg Clin North Am 2015; 95: 355-365.
[2] Chong J, Dumont T, Francis-Frank L and Balaan M. Sepsis and septic shock: a review. Crit Care Nurs Q 2015; 38: 111-120.
[3] Sharawy N and Lehmann C. New directions for sepsis and septic shock research. J Surg Res 2015; 194: 520-527.
[4] Salgado DR, Rocco JR, Silva E and Vincent JL. Modulation of the renin-angiotensin-aldosterone system in sepsis: a new therapeutic approach? Expert Opin Ther Targets 2014; 14: 11-20.
[5] Corrêa TD, Jeger V, Pereira AJ, Takala J, Djafarzadeh S and Jakob SM. Angiotensin II in septic shock: effects on tissue perfusion, organ function, and mitochondrial respiration in a porcine model of fecal peritonitis. Crit Care Med 2014; 42: e550-e559.
[6] Opal SM and van der Poll T. Endothelial barrier dysfunction in septic shock. J Intern Med 2015; 277: 277-293.
[7] Zaky A, Deem S, Bendjelid K and Treggiari MM. Characterization of cardiac dysfunction in sepsis: an ongoing challenge. Shock 2014; 41: 12-24.
[8] Vogiatzi K, Apostolakis S, Vlata Z, Krabovitis E and Spandidos DA. Opposite effect of angiotensin receptor blockade on CXCL8 production and CXCR1/2 expression of angiotensin II-treated THP-1 monocytes. Exp Ther Med 2013; 5: 987-991.
[9] Rosei CA, Withers SB, Belcaid L, De Ciuceis C, Rizzoni D and Heagerty AM. Blockade of the renin-angiotensin system in small arteries and anticontractile function of perivascular adipose tissue. J Hypertens 2015; 33: 1039-1045.
[10] Sanchez-Lemus E, Murakami Y, Larrayoz-Roldan IM, Moughamian AJ, Pavel J, Nishioku T and Saavedra JM. Angiotensin II AT1 receptor blockade decreases lipopolysaccharide-induced inflammation in the ratadrenal gland. Endocrinology 2008; 149: 5177-5188.
[11] Chan JY, Wang LL, Ou CC and Chan SH. Downregulation of angiotensin subtype 1 receptor in rostral ventrolateral medulla during endotoxemia. Hypertension 2003; 42: 103-109.
[12] Sim MK and Min L. Desaspartate-angiotensin I and angiotensin AT1 receptors in rat cardiac ventricles. Regul Pept 2005; 129: 133-137.
[13] Cowling RT, Zhang X, Reese VC, Iwata M, Gurantz D, Dillmann WH and Greenberg BH. Effects of cytokine treatment on angiotensin II

type 1A receptor transcription and splicing in rat cardiac fibroblasts. Am J Physiol Heart Circ Physiol 2005; 289: H1176-H1183.

[14] Schmidt C, Höcherl K, Kurt B, Moritz S, Kurtz A and Bucher M. Blockade of multiple but not single cytokines abrogates downregulation of angiotensin II type-I receptors and anticipates septic shock. Cytokine 2010; 49: 30-38.

[15] Li X, Li Y, Shan L, Shen E, Chen R and Peng T. Over-expression of calpastatin inhibits calpain activation and attenuates myocardial dysfunction during endotoxemia. Cardiovasc Res 2009; 83: 72-79.

[16] Cauwels A. Nitric oxide in shock. Kidney Int 2007; 72: 557-565.

[17] Fernandes D and Assreuy J. Nitric oxide and vasular reactivity in sepsis. Shock 2008; 30: 10-13.

[18] Glenn DJ, Cardema MC, Ni W, Zhang Y, Yeghiazarians Y, Grapov D, Fiehn O and Gardner DG. Cardiac steatosis potentiates angiotensin II effects in the heart. Am J Physiol Heart Circ Physiol 2015; 308: H339-H350.

[19] Li N, Wang HX, Han QY, Li WJ, Zhang YL, Du J, Xia YL and Li HH. Activation of the cardiac proteasome promotes angiotension II-induced hypertrophy by down-regulation of ATRAP. J Mol Cell Cardiol 2015; 79: 303-314.

[20] Castrop H. A role for AT1 receptor-associated proteins in blood pressure regulation. Curr Opin Pharmacol 2015; 21: 43-47.

[21] Ishiguro S, Yoshimura K, Tsunedomi R, Oka M, Takao S, Inui M, Kawabata A, Wall T, Magafa V, Cordopatis P, Tzakos AG and Tamura M. Involvement of angiotensin II type 2 receptor (AT2R) signaling in human pancreatic ductal adenocarcinoma (PDAC): a novel AT2R agonist effectively attenuates growth of PDAC grafts in mice. Cancer Biol Ther 2015; 16: 307-316.

[22] Nakada TA, Russell JA, Boyd JH, McLaughlin L, Nakada E, Thair SA, Hirasawa H, Oda S and Walley KR. Association of angiotensin II type 1 receptor-associated protein gene polymorphism with increased mortality in septic shock. Crit Care Med 2011; 39: 1641-1648.