

Original Article

# Stimulation of the adenosine A<sub>3</sub> receptor reverses vascular hyporeactivity after hemorrhagic shock in rats

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**Aim:** To investigate whether adenosine A<sub>3</sub> receptors (A<sub>3</sub>AR) stimulation restore vascular reactivity after hemorrhagic shock through a ryanodine receptor (RyR)-mediated and large conductance calcium-activated potassium (BK<sub>Ca</sub>) channel-dependent pathway.

**Methods:** Rat hemorrhagic shock model (40 mmHg) and vascular smooth muscle cell (VSMC) hypoxic model were used. The expression of A<sub>3</sub>AR was determined by Western blot and RT-PCR. The effect of A<sub>3</sub>AR stimulation on RyR-mediated Ca<sup>2+</sup> release in VSMCs was analyzed by the Fura-3/AM loading Ca<sup>2+</sup> imaging. The modulation of vascular reactivity to norepinephrine (NE) by A<sub>3</sub>AR stimulation was monitored by an isolated organ tension instrument.

**Results:** Decrease of A<sub>3</sub>AR expression is consistent with the loss of vasoreactivity to NE in hemorrhagic shock rats. The stimulation of A<sub>3</sub>AR with a selective agonist, IB-MECA, could partly but significantly restore the vasoreactivity in the rats, and this restorative effect could be counteracted by MRS1523, a selective A<sub>3</sub>AR antagonist. In hypoxic VSMCs, RyR activation by caffeine significantly evoked the rise of [Ca<sup>2+</sup>] compared with the control cells, a phenomenon closely associated with the development of vascular hyporeactivity in hemorrhagic shock rats. The stimulation of A<sub>3</sub>AR with IB-MECA significantly blocked this over activation of RyR-mediated Ca<sup>2+</sup> release. RyR activation by caffeine and BK<sub>Ca</sub> channel activation by NS1619 attenuated the restoration of vasoreactivity to NE resulting from A<sub>3</sub>AR stimulation by IB-MECA after hemorrhagic shock; this attenuation effect could be antagonized by a selective BK<sub>Ca</sub> channel blocker.

**Conclusion:** These findings suggest that A<sub>3</sub>AR is involved in the modulation of vasoreactivity after hemorrhagic shock and that stimulation of A<sub>3</sub>AR can restore the decreased vasoreactivity to NE through a RyR-mediated, BK<sub>Ca</sub> channel-dependent signal pathway.

**Keywords:** hemorrhagic shock; vascular hyporeactivity; adenosine A<sub>3</sub> receptors (A<sub>3</sub>AR); Ca<sup>2+</sup> release; large conductance calcium-activated potassium (BK<sub>Ca</sub>); vascular smooth muscle cell (VSMC); ryanodine receptor (RyR)

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## Introduction

Vascular hyporesponsiveness is one of the most common complications of serious conditions such as trauma and hemorrhagic shock<sup>[1]</sup>. The decreased vascular reactivity to vasoactive substances, including NE and angiotensin II (Ang II), can result in systemic hypotension and poor perfusion to vital organs and finally lead to multiple organ dysfunction syndrome (MODS)<sup>[2]</sup>. Adenosine, produced by vascular endothelium, smooth muscle cells, and even neutrophils, is a potent endogenous protective mediator of the cardiovascular system through the activation of adenosine receptors (ARs)<sup>[3]</sup>. There are four types of ARs located in vascular smooth muscle (VSM): adenosine A<sub>1</sub> receptor (A<sub>1</sub>AR), A<sub>2a</sub>R, A<sub>2b</sub>R, and A<sub>3</sub>AR<sup>[4]</sup>.

A<sub>3</sub>AR is a member of the G-protein-coupled receptor superfamily; it couples to Gi1-3 and Gq/11. Many reports showed that the activation of A<sub>3</sub>AR confers partial cardioprotection, neuroprotection, renal protection, etc<sup>[5–7]</sup>, but whether A<sub>3</sub>AR stimulation can restore the decreased vascular reactivity after hemorrhagic shock and the possible mechanisms involved remain unknown.

NE induces the increased production of inositol 1,4,5-triphosphate (IP<sub>3</sub>), which binds to IP<sub>3</sub>R (inositol 1,4,5-triphosphate-sensitive receptor), a receptor abundant in the endoplasmic reticulum (ER) of VSMCs, triggers the Ca<sup>2+</sup> release from Ca<sup>2+</sup> store, and finally leads to the increase of intracellular calcium ion concentration ([Ca<sup>2+</sup>]) and vasoconstriction<sup>[8]</sup>. The Ca<sup>2+</sup> released from the Ca<sup>2+</sup> store also activates RyR-mediated Ca<sup>2+</sup> release, which could in turn activate the BK<sub>Ca</sub> channel in the nearby sarcolemma and negatively modulate vascular contraction<sup>[9]</sup>. It has been demonstrated that in hypoxic VSMCs,

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RyR-mediated  $\text{Ca}^{2+}$  release increases significantly<sup>[10]</sup>, which might contribute to the activation of the  $\text{BK}_{\text{Ca}}$  channel. Our previous studies suggested that over-activation of the  $\text{BK}_{\text{Ca}}$  channel is closely associated with vascular hyporeactivity after hemorrhagic shock in rats<sup>[11, 12]</sup>, and stimulation of  $\text{A}_3\text{AR}$  could inhibit RyR-mediated  $\text{Ca}^{2+}$  release<sup>[13]</sup>. Therefore, we hypothesized that  $\text{A}_3\text{AR}$  might be involved in the modulation of vasoreactivity through a RyR-mediated  $\text{Ca}^{2+}$  release and  $\text{BK}_{\text{Ca}}$  channel dependent signal pathway after hemorrhagic shock.

In this study, we examined: (1) whether  $\text{A}_3\text{AR}$  is involved in the modulation of vascular reactivity after hemorrhagic shock in rats and (2) whether  $\text{A}_3\text{AR}$  modulates the vascular reactivity to NE after hemorrhagic shock through a RyR-mediated  $\text{Ca}^{2+}$  release-dependent pathway. To the best of our knowledge, this is the first report to demonstrate that  $\text{A}_3\text{AR}$  is involved in the modulation of vasoreactivity after hemorrhagic shock and that stimulation of  $\text{A}_3\text{AR}$  could restore the decreased vasoreactivity to NE through a RyR-mediated,  $\text{BK}_{\text{Ca}}$  channel dependent signal pathway.

## Materials and methods

This study was approved by the Research Council and Animal Care and Use Committee of the Research Institute of Surgery, Daping Hospital, Third Military Medical University. All experiments conformed to the guidelines of the ethical use of animals, and all possible efforts were made to minimize animal suffering and to reduce the number of animals used.

## Drugs and reagents

The following reagents were purchased from Sigma Co (USA): an  $\text{A}_3\text{AR}$  agonist, IB-MECA (chloro-N6-(3-iodobenzyl) adenosine-5'-N-methyluronamide); an  $\text{A}_3\text{AR}$  antagonist, MRS1523 (3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate); a RyR agonist, caffeine (Caf); a RyR antagonist, ryanodine (Ry); an  $\text{IP}_3\text{R}$  agonist, adenophostin A (AdA); a  $\text{BK}_{\text{Ca}}$  channel blocker, tetraethylammonium (TEA); a  $\text{BK}_{\text{Ca}}$  channel opener, NS1619; pentobarbital sodium; and a sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase inhibitor, thapsigargin (Tha). NE was purchased from Shanghai Harvest Pharmaceutical Co (China). Trizol reagent was purchased from Gibco BRL (Grand Island, NY, USA). Fura-3/AM was purchased from Leiden (The Netherlands). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were provided by Hyclone Co (USA).

## Animal model

SD rats provided by the Animal Center of the Military Surgery Research Institute of the Third Military Medical University in China (SYXK2002-032), weighing  $220 \pm 20$  g, were anesthetized by pentobarbital sodium (40 mg/kg, ip). Their left femoral arteries were cannulated, connected to a pressure recorder for measuring the mean arterial pressure (MAP), and heparinized with sodium heparin (50 U/kg). The rats were then hemorrhaged, and the MAP was maintained at 40 mmHg for 0, 0.5, 1, 2, and 4 h via a femoral artery catheter.

## VSMC preparation

VSMCs were obtained by enzymatic digestion of the abdominal aorta from normal Wistar rats as previously described<sup>[14, 15]</sup>. Briefly, after the endothelium was scraped off, the abdominal aorta was digested in D-Hanks solution, containing collagenase I (2 mg/mL) and bovine serum albumin (BSA) (2 mg/mL), at 37 °C for 30 min. The VSMCs were then cultured in DMEM-F12 with 20% calf bovine serum for 5 to 7 d. Before each experiment, the third to fifth passages of VSMCs were serum-starved for 24 h. On the day of experiment, VSMCs were incubated in a hypoxic culture compartment with an  $\text{O}_2$  concentration less than 0.2% for 2 h as previously described and then used for the following experiment.

## Western blot of $\text{A}_3\text{AR}$

At different time points (0 min–4 h) after hemorrhage, the abdominal cavities of the rats were opened; the superior mesenteric arteries (SMAs) were obtained, and the protein level of  $\text{A}_3\text{AR}$  in SMA after hemorrhagic shock was determined by Western blot, as previously described<sup>[16]</sup>. Briefly, the tissues were put into pre-cooled RIPA (pH 7.6; Hepes 50 mmol/L, NaCl 150 mmol/L, EDTA 1 mmol/L, NP-40 1%,  $\beta$ -glycerophosphate 20 mmol/L,  $\text{Na}_3\text{VO}_4$  1 mmol/L, NaF 1 mmol/L, Benzamide 1 mmol/L, para-nitrophenylphosphate 5 mmol/L, DTT 1 mmol/L, and protein kinase inhibitor cocktail tablets), cut into pieces, and homogenized on ice. Then, the supernatants were collected, thawed on ice for 1 h, and centrifuged at 8000 g for 10 min at 4 °C. Afterward, the supernatants were collected from the lysates, and the protein concentrations were determined by the Bradford method. Aliquots of the lysates (120  $\mu\text{g}$  of protein) were boiled for 5 min and electrophoresed on a 10% SDS-polyacrylamide gel. Blots of the gel were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were blocked with 10% nonfat dry milk for 4 h and incubated with a primary  $\text{A}_3\text{AR}$  antibody (1:1200) for 4 h at room temperature. Then the membranes were further incubated with a horseradish peroxidase-conjugated secondary antibody, developed using an enhanced chemiluminescence Western blotting detection kit (Pierce, Rockland, IL, USA), and exposed to X-ray film. The intensity of the immunoreactive bands was quantified and the results were normalized to  $\beta$ -actin levels.

## Reverse transcriptase-polymerase chain reaction (RT-PCR) of $\text{A}_3\text{AR}$

At different time points (0 min–4 h) after hemorrhagic shock, the abdominal cavities of the rats were opened and the SMAs were obtained. Then the tissues were gently cut into pieces. One mL of Trizol reagent was added, and total RNA was extracted as per the manufacturer's protocol. RT-PCR was performed as previously described<sup>[17]</sup>. The PCR conditions for  $\text{A}_3\text{AR}$  and for the housekeeping gene,  $\beta$ -actin, were 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s. The primer pairs (synthesized by Shanghai Sangon Biological Engineering Technology & Services Co, Ltd China) were as follows (forward and reverse, respectively):

A<sub>3</sub>AR, (Fwd) 5'-GGTCCACTGGCCCCATACACA-3' and (Rev) 5'-CGTAGGTGATTTGCAAC CACA-3';  $\beta$ -actin, (Fwd) 5'-CACCCGCGAGTACAACCTTC-3' and (Rev) 5'-CCCAT-ACCCACCATCACACC-3'. The amplified products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

#### Experimental protocols

##### **Changes in vascular reactivity to NE in different vasculatures after hemorrhagic shock in rats**

The artery rings (2–3 mm in length) of the abdominal aorta, SMA stem and SMA branch were prepared from the hemorrhagic shock (40 mmHg for 2 h) rats. Each vascular ring was mounted in a 10 mL organ perfusion system filled with modified Krebs-Henseleit (K-H) solution (in mmol/L: NaCl 119, KCl 4.7, CaCl<sub>2</sub> 2.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.45, and glucose 11; pH 7.4) or Ca<sup>2+</sup>-free K-H solution (without Ca<sup>2+</sup> or Mg<sup>2+</sup>, but with 1 mmol/L EGTA in the K-H solution), continuously bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and maintained at 37 °C. Each mesenteric arterial or abdominal arterial ring was stretched to a passive force (preload) of about 0.6 g or 0.8 g and equilibrated for 2 h, respectively. The contractile response of each artery ring to NE was recorded by a Powerlab polygraph (AD instrument, Castle Hill, Australia) through a force transducer. NE was added cumulatively from 10<sup>-9</sup> to 10<sup>-5</sup> mol/L. The contractile force of each abdominal or SMA stem artery ring was calculated as the change in tension per g tissue (mN/g), and the contractile force of SMA branch was recorded as the change in tension (mN). The NE cumulative dose-response curve and the maximal contraction induced by 10<sup>-5</sup> mol/L NE (E<sub>max</sub>) were used to evaluate the vascular reactivity to NE. There were eight observations in each group.

##### **Involvement of A<sub>3</sub>AR in the modulation of vascular reactivity after hemorrhagic shock in rats**

The abdominal artery rings (2–3 mm in length) from the hemorrhagic shock (40 mmHg for 2 h) or the sham-operated control rats were prepared and randomly divided into the following 4 groups (*n*=8/group): sham-operated control, shock, shock+IB-MECA (4×10<sup>-6</sup> mol/L), and shock+IB-MECA+MRS1523 (10<sup>-7</sup> mol/L). Each vascular ring was mounted in a 10 mL organ perfusion system filled with K-H solution with or without Ca<sup>2+</sup>. The incubation time of IB-MECA was 20 min in the presence or absence of MRS1523. The contractile response of each artery ring to NE was recorded as described above.

##### **Modulation of A<sub>3</sub>AR on the RyR-mediated and the IP<sub>3</sub>R-mediated Ca<sup>2+</sup> release in hypoxic VSMCs**

To explore the effect of IB-MECA on RyR-mediated Ca<sup>2+</sup> release in the absence of extracellular Ca<sup>2+</sup>, the following experiments were conducted individually: 1) cells were randomly divided into 4 groups (*n*=6/group): control, hypoxia, control+NE (10<sup>-6</sup> mol/L), and hypoxia+NE (10<sup>-6</sup> mol/L), to examine the NE-induced changes in [Ca<sup>2+</sup>] in hypoxic cells; 2) cells were randomly divided into 3 groups (*n*=6 per group): control+caffeine (10<sup>-3</sup> mol/L), hypoxia+caffeine, and

hypoxia+caffeine+IB-MECA (4×10<sup>-6</sup> mol/L), to explore the effects of A<sub>3</sub>AR stimulation on the RyR-mediated Ca<sup>2+</sup> release in hypoxic VSMCs; 3) cells were randomly divided into 3 groups (*n*=6/group): control+adenophostin A (10<sup>-5</sup> mol/L), hypoxia+adenophostin A, and hypoxia+adenophostin A+IB-MECA (4×10<sup>-6</sup> mol/L), to explore the effects of A<sub>3</sub>AR stimulation on the IP<sub>3</sub>R-mediated Ca<sup>2+</sup> release in hypoxic VSMCs; and 4) in some hypoxia VSMCs, cells were pretreated with thapsigargin (10<sup>-8</sup> mol/L) before incubation with caffeine. The incubation time of each agent was 5 min. The single cell [Ca<sup>2+</sup>] was measured by a fluorescence indicator, Fura-3/AM, with confocal laser scanning microscopy as previously described<sup>[18]</sup>. Briefly, Fura-3/AM (10<sup>-5</sup> mol/L) was added and incubated for 30 min at 37 °C, and then the Fura-3/AM-loaded cells were placed on the stage of an inverted fluorescence microscope (Olympus, Japan). With the alternative illumination at 340 nm or 380 nm excitation, fluorescence images were obtained using a silicon-intensified-target video camera (C2400-8, Japan) and digitized by an image processor. The cellular [Ca<sup>2+</sup>] was represented by mean fluorescence intensity: the single cell mean fluorescence intensity of Ca<sup>2+</sup>=(the sum of the single fluorescence intensity of 1, 2, 3, ..., *n*)/ (the sum of the area of 1, 2, 3, ..., *n*).

##### **Involvement of RyR-mediated Ca<sup>2+</sup> release in the modulation of vascular hyporeactivity by A<sub>3</sub>AR stimulation after hemorrhagic shock in rats**

We further investigated whether stimulation of A<sub>3</sub>AR with IB-MECA regulates the contractile response to NE associated with RyR-evoked Ca<sup>2+</sup> release. One hundred and twelve abdominal artery rings from the rats subjected to either hemorrhagic shock (40 mmHg, 2 h) or sham-operated control treatment were randomized into the following 7 groups (*n*=8/group): sham-operated control, shock, shock+caffeine (10<sup>-3</sup> mol/L), shock+ryanodine (10<sup>-5</sup> mol/L), shock+IB-MECA (4×10<sup>-6</sup> mol/L), shock+IB-MECA+caffeine, and shock+IB-MECA+caffeine+ryanodine. The contractile response of each artery ring to NE was recorded both in the K-H solution with 2.2 mmol/L Ca<sup>2+</sup> and in the Ca<sup>2+</sup>-free K-H solution, as previously described. The incubation time and order of each group are described in Table 1.

##### **Involvement of the BK<sub>Ca</sub> pathway in the modulation of vascular hyporeactivity by A<sub>3</sub>AR stimulation after hemorrhagic shock in rats**

Last, we examined whether the stimulation of A<sub>3</sub>AR with IB-MECA regulates the contractile response to NE through a RyR-mediated Ca<sup>2+</sup> release, BK<sub>Ca</sub> channel dependent pathway. Fifty-six abdominal artery rings from rats subjected to either hemorrhagic shock (40 mmHg, 2 h) or sham-operated control treatment were randomized into the following 7 groups (*n*=8/group): sham-operated control, shock, shock+IB-MECA (4×10<sup>-6</sup> mol/L), shock+IB-MECA+caffeine (10<sup>-3</sup> mol/L), shock+IB-MECA+NS1619 (5×10<sup>-3</sup> mol/L), shock+IB-MECA+caffeine+TEA (0.1 mmol/L), and shock+IB-MECA+NS1619+TEA (0.1 mmol/L). The contractile response

of each artery ring to NE was recorded as previously described. The incubation time and order of each group are given in Table 1:

**Table 1.** Incubation time and order of each group.

Group	Incubation time and order
Shock control	
Shock+IB-MECA	IB-MECA ( $4 \times 10^{-6}$ mol/L) for 20 min
Shock+IB-MECA+MRS1523	IB-MECA for 20 min, followed by MRS1523 ( $10^{-7}$ mol/L) for 10 min
Shock+IB-MECA+caffeine	Pretreated with caffeine ( $10^{-3}$ mol/L) for 10 min, followed by IB-MECA for 20 min
Shock+IB-MECA+NS1619	Pretreated with NS1619 ( $5 \times 10^{-3}$ mol/L) for 10 min, followed by IB-MECA for 20 min
Shock+IB-MECA+caffeine+TEA	In the presence of TEA (0.1 mmol/L), pretreated with caffeine for 10 min and followed by IB-MECA for 20 min
Shock+IB-MECA+NS1619+TEA	In the presence of TEA (0.1 mmol/L), pretreated with NS1619 for 10 min and IB-MECA for 20 min

### Statistical analysis

All data are expressed as mean $\pm$ SD of "n" observations. The effect of A<sub>3</sub>AR stimulation (with IB-MECA) on vascular reactivity to NE *in vitro* was assessed by a one-factor analysis of variance, followed by *post-hoc* Tukey tests. A value of  $P < 0.05$  was considered statistically significant, and  $P < 0.01$  was considered highly significant.

## Results

### Decreased vascular reactivity to NE after hemorrhagic shock in rats

Three types of vasculatures (abdominal aorta, SMA stem, and SMA branch) were chosen for this study. As shown in Figure 1, in the abdominal aorta, the NE cumulative dose-response curve shifted to the right, and  $10^{-5}$  mol/L NE induced a decrease in maximal tension ( $E_{\max}$ ) from  $0.88 \pm 0.13$  mN/g to  $0.39 \pm 0.14$  mN/g ( $P < 0.01$ ) (Figure 1A). In SMA stem, the vascular reactivity to NE also decreased, characterized by a shift of the NE cumulative dose-response curve to the right and a decrease of the  $E_{\max}$  from  $0.91 \pm 0.18$  mN/g to  $0.50 \pm 0.19$  mN/g ( $P < 0.01$ ) (Figure 1B). In the SMA branch, the vascular reactiv-

ity to NE also significantly decreased: the NE cumulative dose-response curve shifted to the right, and the  $E_{\max}$  decreased from  $11.30 \pm 2.10$  mN/g to  $3.94 \pm 1.43$  mN/g ( $P < 0.01$ ) (Figure 1C).

### Decreased A<sub>3</sub>AR expression after hemorrhagic shock in rats

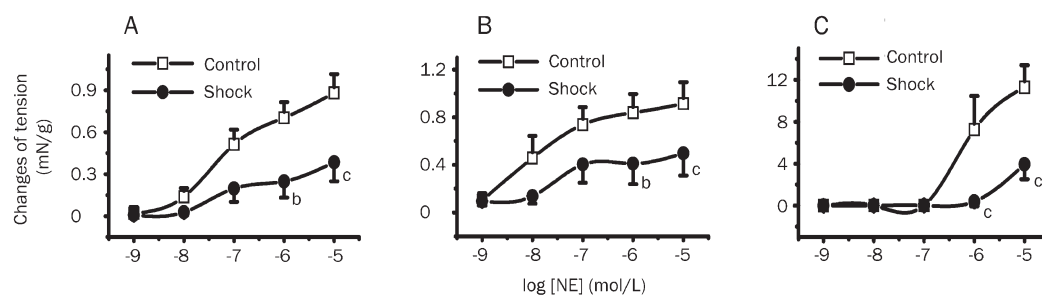
The results showed that after hemorrhagic shock (from 30 min to 4 h), there were no significant changes in the A<sub>3</sub>AR mRNA level in the hemorrhagic shock rats or in the controls (Figure 2A), while the A<sub>3</sub>AR protein level was significantly decreased 2–4 h after hemorrhagic shock (Figure 2B).

### Involvement of A<sub>3</sub>AR in the modulation of vasoreactivity to NE after hemorrhagic shock in rats

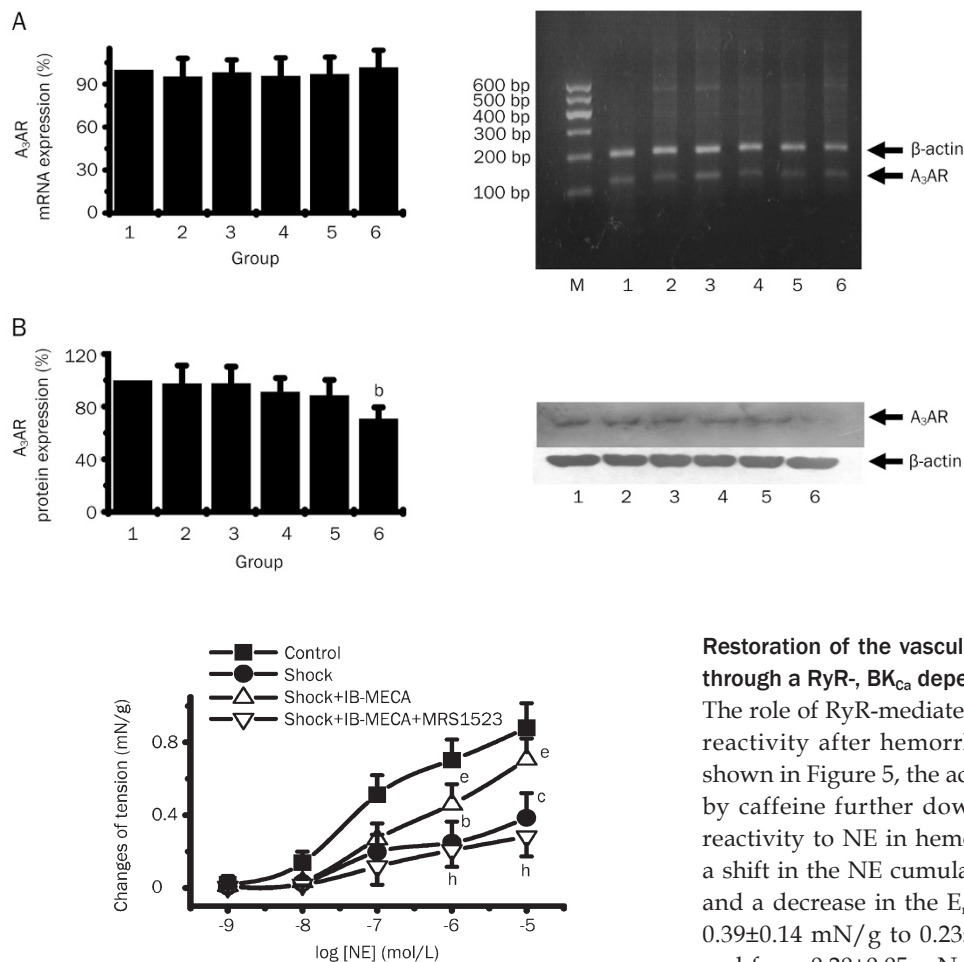
As shown in Figure 3, in the abdominal aorta, stimulation of A<sub>3</sub>AR with IB-MECA ( $4 \times 10^{-6}$  mol/L) improved the NE-induced contraction responses of the artery compared with the hemorrhagic shock group, resulting in a shift of the NE cumulative dose-response curve to the left and an increase in the  $E_{\max}$  of  $10^{-5}$  mol/L NE from  $0.39 \pm 0.14$  mN/g to  $0.71 \pm 0.12$  mN/g ( $P < 0.05$ ). In the presence of MRS1523 ( $10^{-7}$  mol/L), the effects of IB-MECA were significantly counteracted: the NE cumulative dose-response curve was shifted to the right compared with that of the IB-MECA only group, and the  $E_{\max}$  (induced by  $10^{-5}$  mol/L NE) decreased from  $0.71 \pm 0.12$  mN/g to  $0.28 \pm 0.11$  mN/g ( $P < 0.05$ ) (Figure 3).

### Effects of A<sub>3</sub>AR stimulation on the RyR-evoked Ca<sup>2+</sup> release in hypoxic VSMCs

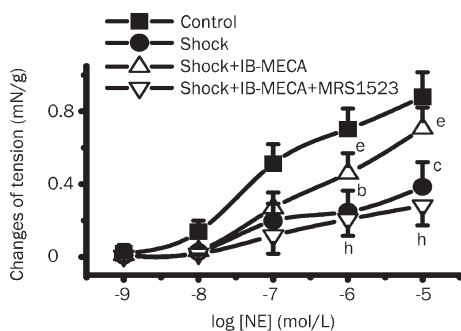
In abdominal aorta VSMCs, NE can induce the increase of cytosolic Ca<sup>2+</sup> concentration ( $[Ca^{2+}]$ ) resulting from the mobilization of Ca<sup>2+</sup> from ER in the absence of extracellular Ca<sup>2+</sup>. In hypoxic VSMCs, the NE-induced increase of  $[Ca^{2+}]$  decreased compared with the controls, but the change was not significant (Figure 4A). Furthermore, in the absence of extracellular Ca<sup>2+</sup>, the increase of  $[Ca^{2+}]$  induced by the activation of RyR with caffeine was significant in hypoxic VSMCs compared with the controls (from  $91.2 \pm 24.9$  to  $161.5 \pm 21.6$ ,  $P < 0.01$ ), while the increase in  $[Ca^{2+}]$  induced by the activation of IP<sub>3</sub>R with adenosine A decreased, although not significantly, in hypoxic VSMCs. The stimulation of A<sub>3</sub>AR with IB-MECA significantly inhibited the caffeine-induced upregulation of  $[Ca^{2+}]$  in hypoxic VSMCs (from  $161.5 \pm 21.6$  to  $112.8 \pm 19.0$ ,  $P < 0.05$ ) (Figure 4B), but there were no significant changes in adenosine A-induced Ca<sup>2+</sup> release resulting from the stimulation of A<sub>3</sub>AR



**Figure 1.** Changes in the vascular reactivity of the abdominal aorta (A), SMA stem (B), and SMA branch (C) to NE after hemorrhagic shock in rats (mean $\pm$ SD,  $n=8$ ). <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control group.



**Figure 2.** Analysis of A<sub>3</sub>AR mRNA (A) and protein (B) levels in SMA (mean±SD, n=5). 1: control; 2: shock for 0 min; 3: shock for 30 min; 4: shock for 1 h; 5: shock for 2 h; 6: shock for 4 h. <sup>b</sup>P<0.05 vs control group.

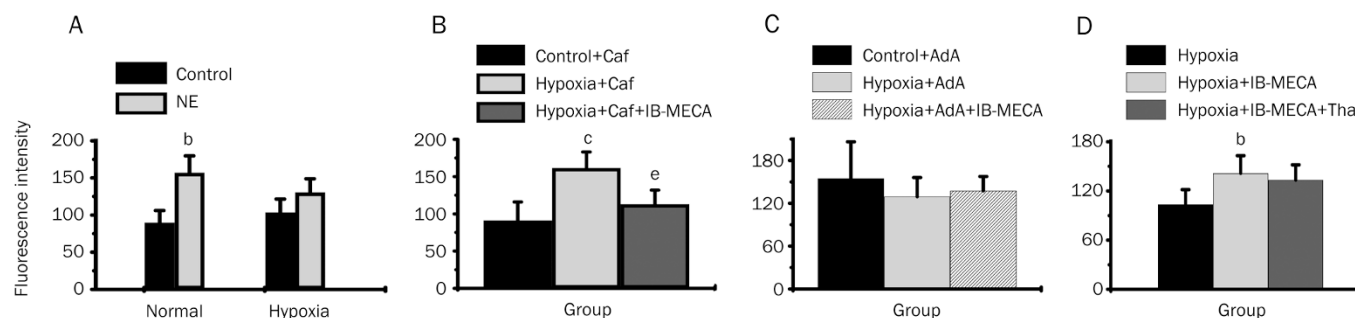


**Figure 3.** Effect of IB-MECA on the vasoreactivity to NE after hemorrhagic shock in rats (mean±SD, n=8). <sup>b</sup>P<0.05, <sup>c</sup>P<0.01 vs control group; <sup>e</sup>P<0.05 vs shock group; <sup>h</sup>P<0.05 vs shock+IB-MECA group.

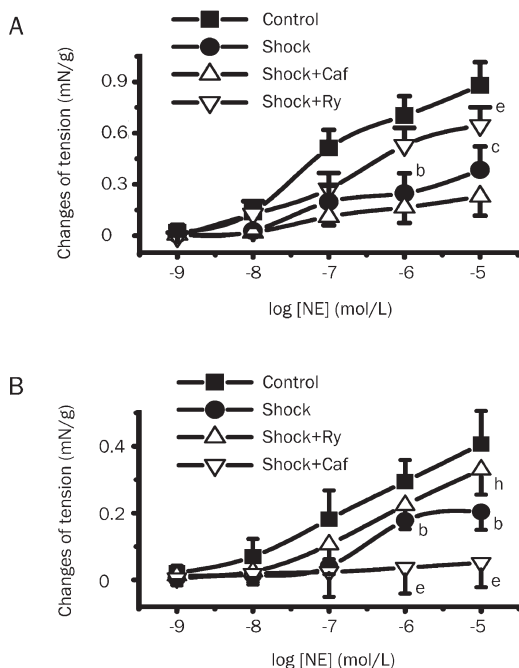
with IB-MECA (Figure 4C). In the presence of a Ca<sup>2+</sup>-ATPase inhibitor, thapsigargin (10<sup>-8</sup> mol/L), there was no significant effect of IB-MECA on [Ca<sup>2+</sup>] in hypoxic VSMCs (Figure 4D).

### Restoration of the vascular reactivity to NE by A<sub>3</sub>AR stimulation through a RyR-, BK<sub>Ca</sub> dependent signal pathway

The role of RyR-mediated Ca<sup>2+</sup> release in the decrease of vaso-reactivity after hemorrhagic shock was first explored. As shown in Figure 5, the activation of RyR-mediated Ca<sup>2+</sup> release by caffeine further down-regulated the decreased vascular reactivity to NE in hemorrhagic shock rats, characterized by a shift in the NE cumulative dose-response curve to the right and a decrease in the E<sub>max</sub> (induced by 10<sup>-5</sup> mol/L NE) from 0.39±0.14 mN/g to 0.23±0.11 mN/g in normal K-H solution and from 0.20±0.05 mN/g to 0.05±0.07 mN/g in the Ca<sup>2+</sup>-free K-H solution (P<0.05), respectively. In addition, the inhibition of RyR-mediated Ca<sup>2+</sup> release by ryanodine (10<sup>-5</sup> mol/L) at least partly restored vascular reactivity to NE after hemorrhagic shock: the NE cumulative dose-response curve shifted to the left, and the E<sub>max</sub> (induced by 10<sup>-5</sup> mol/L NE) increased from 0.39±0.14 mN/g to 0.65±0.11 mN/g in normal K-H solution (P<0.05) and from 0.20±0.05 mN/g to 0.33±0.07 mN/g in the Ca<sup>2+</sup>-free K-H solution (P<0.05), respectively (Figure 5).



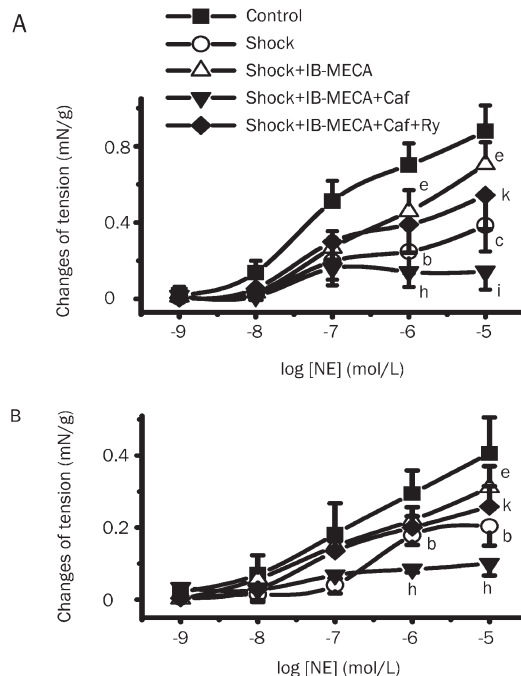
**Figure 4.** (A) Changes in [Ca<sup>2+</sup>] in hypoxic VSMCs in the absence of extracellular Ca<sup>2+</sup> (mean±SD, n=6). <sup>b</sup>P<0.05 vs control group. (B) Effect of the stimulation of A<sub>3</sub>AR with IB-MECA on the RyR-mediated Ca<sup>2+</sup> release in hypoxic VSMCs (mean±SD, n=6). The cells were incubated with IB-MECA or caffeine for 5 min after hypoxia insult and then loaded with Fura-3/AM. <sup>c</sup>P<0.01 vs control+Caf group; <sup>e</sup>P<0.05 vs hypoxia+Caf group. Caf: caffeine (10<sup>-3</sup> mol/L, a RyR agonist). (C) Effect of the stimulation of A<sub>3</sub>AR with IB-MECA on the IP<sub>3</sub>R-mediated Ca<sup>2+</sup> release in hypoxic VSMCs (mean±SD, n=6). AdA: adenophostin A (10<sup>-5</sup> mol/L, an IP<sub>3</sub>R agonist). (D) Effect of thapsigargin on [Ca<sup>2+</sup>] by the stimulation of A<sub>3</sub>AR with IB-MECA in hypoxic VSMCs (mean±SD, n=6). <sup>b</sup>P<0.05 vs hypoxia group. Tha: thapsigargin (10<sup>-8</sup> mol/L, an ER Ca<sup>2+</sup>-ATPase inhibitor).



**Figure 5.** Role of RyR-mediated Ca<sup>2+</sup> release in the development of vascular hyporeactivity after hemorrhagic shock in rats (mean±SD, *n*=8). A: in a K-H solution with 2.2 mmol/L [Ca<sup>2+</sup>]; B: in Ca<sup>2+</sup>-free K-H solution. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control group; <sup>e</sup>*P*<0.05 vs shock group; <sup>h</sup>*P*<0.05 vs shock+Caf group. Caf: caffeine; Ry: ryanodine.

Next, we investigated whether the inhibition of RyR-mediated Ca<sup>2+</sup> release by A<sub>3</sub>AR stimulation is involved in the improvement of vasoreactivity to NE in hemorrhagic shock rats. As shown in Figure 6, compared with the IB-MECA treated group, pre-treatment with a RyR agonist, caffeine (10<sup>-7</sup> mol/L), could partly reverse the improvement in vasoreactivity to NE by IB-MECA, as characterized by a significant shift of the NE cumulative dose-response curve to the right and a significant decrease in the E<sub>max</sub> from 0.71±0.12 mN/g to 0.14±0.12 mN/g (*P*<0.01) in normal K-H solution and from 0.31±0.06 mN/g to 0.10±0.03 mN/g in the Ca<sup>2+</sup>-free K-H solution, respectively. These effects could be antagonized by a RyR antagonist, ryanodine (10<sup>-5</sup> mol/L): the NE cumulative dose-response curve significantly shifted to the left and the E<sub>max</sub> increased from 0.14±0.10 mN/g to 0.54±0.18 mN/g (*P*<0.01) in normal K-H solution and from 0.10±0.03 mN/g to 0.26±0.06 mN/g (*P*<0.05) in the Ca<sup>2+</sup>-free K-H solution, respectively (Figure 6).

Last, we examined whether the stimulation of A<sub>3</sub>AR restores vasoreactivity through a RyR-mediated Ca<sup>2+</sup> release, BK<sub>Ca</sub> channel dependent signal pathway after hemorrhagic shock in rats. Our results showed that pretreatment with a BK<sub>Ca</sub> opener, NS1619, could partly counteract the restoration of vasoreactivity by IB-MECA, as characterized by a significant shift of NE cumulative dose-response curve to the right and a decrease in the E<sub>max</sub> from 0.71±0.12 mN/g to 0.25±0.09 mN/g (*P*<0.01). In addition, a selective BK<sub>Ca</sub> channel blocker, TEA (0.1 mmol/L), significantly antagonized the further decrease

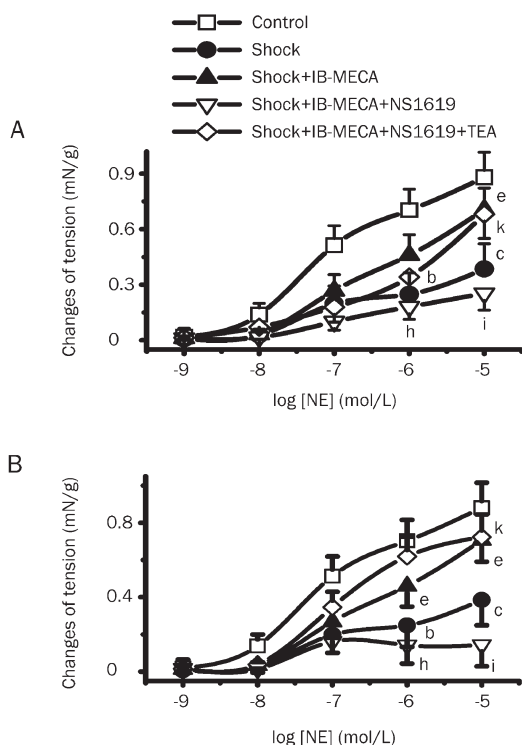


**Figure 6.** Role of RyR-mediated Ca<sup>2+</sup> release in the restoration of vascular reactivity to NE by A<sub>3</sub>AR stimulation after hemorrhagic shock in rats (mean±SD, *n*=8). A: in normal K-H solution; B: in Ca<sup>2+</sup>-free K-H solution. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control group; <sup>e</sup>*P*<0.05 vs shock group; <sup>h</sup>*P*<0.05, <sup>i</sup>*P*<0.01 vs shock+IB-MECA group; <sup>k</sup>*P*<0.05 vs shock+IB-MECA+Caf group. Caf: caffeine; Ry: ryanodine.

of vascular reactivity to NE induced by NS1619, characterized by a shift of the NE cumulative dose-response curve to the left and an increase in the E<sub>max</sub> from 0.25±0.09 mN/g to 0.68±0.13 mN/g (*P*<0.05, Figure 7A), without significant influence on the vascular basal tone (data not shown). TEA (0.1 mmol/L) also counteracted the further reduction of vasoreactivity to NE induced by caffeine: the NE cumulative dose-response curve shifted to the left, and the E<sub>max</sub> increased from 0.14±0.12 mN/g to 0.72±0.12 mN/g (*P*<0.05, Figure 7B).

## Discussion

The stimulation of A<sub>3</sub>AR has a protective effect against neurodegenerative diseases, myocardial injury<sup>[19, 20]</sup>, and the hypoxia-reoxygen-induced damage of vascular reactivity<sup>[21]</sup>. However, little is known about whether the stimulation of A<sub>3</sub>AR can improve decreased vascular reactivity after hemorrhagic shock. In this study, we first examined the effect of A<sub>3</sub>AR in decreased vascular reactivity to NE after hemorrhagic shock in rats. We found that the expression of A<sub>3</sub>AR was decreased 2–4 h after hemorrhagic shock (40 mmHg), consistent with the loss of vasoreactivity to NE. IB-MECA, a selective A<sub>3</sub>AR agonist, partly but significantly restored the decreased vasoreactivity in hemorrhagic shock rats; this restorative effect could be antagonized by MRS1523 (a selective A<sub>3</sub>AR antagonist). These results suggest that A<sub>3</sub>AR is involved in the modulation of vasoreactivity, and the stimulation of A<sub>3</sub>AR can at least partly restore vascular reactivity to



**Figure 7.** Involvement of RyR-mediated and BK<sub>Ca</sub> channel dependent pathway in the modulation of vascular reactivity to NE by A<sub>3</sub>AR stimulation after hemorrhagic shock in rats (mean±SD, n=8). <sup>b</sup>P<0.05, <sup>c</sup>P<0.01 vs control group; <sup>e</sup>P<0.05 vs shock group; <sup>h</sup>P<0.05, <sup>i</sup>P<0.01 vs shock+IB-MECA group; <sup>k</sup>P<0.05 vs shock+IB-MECA+NS1619 group. TEA: tetraethylammonium.

NE after hemorrhagic shock in rats.

There are two types of Ca<sup>2+</sup> release channels located on ER that are closely associated with the modulation of vascular tension: RyR (activated by Ca<sup>2+</sup> increase) and IP<sub>3</sub>R (activated by InsP<sub>3</sub>)<sup>[22, 23]</sup>. Many reports demonstrated that RyR-mediated Ca<sup>2+</sup> release (also called Ca<sup>2+</sup> spark) activates the BK<sub>Ca</sub> channel in plasmalemma, leads to VSM membrane hyperpolarization, and negatively regulates vascular tension<sup>[24]</sup>. Zheng *et al* reported that RyR-evoked Ca<sup>2+</sup> release is increased in hypoxic SMCs<sup>[10]</sup>, and our previous studies showed that over-activation of the BK<sub>Ca</sub> channel plays an important role in the occurrence of vascular hyporeactivity after hemorrhagic shock<sup>[12, 13]</sup>, suggesting that RyR-mediated Ca<sup>2+</sup> release might be related to the development of vascular hyporeactivity after hemorrhagic shock. Because the stimulation of A<sub>3</sub>AR could inhibit RyR-mediated Ca<sup>2+</sup> release from the ER, we then explored whether A<sub>3</sub>AR stimulation can reverse the decreased vascular hyporeactivity after hemorrhagic shock through a RyR-mediated Ca<sup>2+</sup> release and BK<sub>Ca</sub> channel dependent signal pathway.

To clarify the role of RyR-mediated Ca<sup>2+</sup> release in the restoration of vasoreactivity by A<sub>3</sub>AR stimulation, we first used hypoxia-insulted VSMCs to examine the modulation of ER Ca<sup>2+</sup> release by A<sub>3</sub>AR stimulation. Consistent with others' reports, our results showed that RyR-mediated Ca<sup>2+</sup> release is over-activated in hypoxic VSMCs, which might be owing to

the increased expression of RyR and the increased sensitivity of RyR to Ca<sup>2+</sup><sup>[25, 26]</sup>. Furthermore, the over-activation of RyR-mediated Ca<sup>2+</sup> release is closely associated with the occurrence of vascular hyporeactivity to NE in hemorrhagic shock rats. The stimulation of A<sub>3</sub>AR significantly antagonized the over-activation of RyR-mediated Ca<sup>2+</sup> release in hypoxic VSMCs, and the restoration of the vasoreactivity to NE by A<sub>3</sub>AR stimulation was partly but significantly counteracted by the activation of RyR-mediated Ca<sup>2+</sup> release by caffeine. These results suggest that RyR-mediated Ca<sup>2+</sup> release is indeed associated with the restoration of vasoreactivity by A<sub>3</sub>AR stimulation after hemorrhagic shock.

RyR-mediated Ca<sup>2+</sup> release contributes to the activation of several Ca<sup>2+</sup>-activated potassium (K<sub>Ca</sub>) channels in the nearby sarcolemma, induces membrane hyperpolarization, and leads to the reduction of voltage-dependent Ca<sup>2+</sup> channel activity and vascular smooth muscle relaxation<sup>[27, 28]</sup>. Meanwhile, our previous work showed that over-activation of the BK<sub>Ca</sub> channel plays an important role in the development of vascular hyporeactivity after hemorrhagic shock. Therefore, we conducted more pharmacological experiments to explore the role of the RyR-mediated, BK<sub>Ca</sub> channel dependent pathway in the modulation of vascular reactivity by A<sub>3</sub>AR stimulation. We used a selective BK<sub>Ca</sub> channel opener, NS1619, and a BK<sub>Ca</sub> channel blocker, TEA (0.1 mmol/L), to explore the role of the BK<sub>Ca</sub> channel in the restoration of vasoreactivity by A<sub>3</sub>AR stimulation. Our results showed that the selective A<sub>3</sub>AR agonist, IB-MECA, improved vascular reactivity to NE via a RyR-mediated Ca<sup>2+</sup> release and BK<sub>Ca</sub> channel dependent signal cascade, while the BK<sub>Ca</sub> selective opener, NS1619, and the RyR activator, caffeine, both counteracted the restoration of the vascular reactivity to NE by IB-MECA; this counteracting effect could be antagonized by 0.1 mmol/L TEA. These results suggest that the stimulation of A<sub>3</sub>AR improves the vascular reactivity to NE through a RyR-mediated Ca<sup>2+</sup> release dependent pathway, in which the BK<sub>Ca</sub> channel is closely involved.

In a nonpressurized vascular ring exposed to a vasoconstrictor, the frequency of RyR-mediated Ca<sup>2+</sup> release would be low, and vasodilation mechanisms via Ca<sup>2+</sup> spark activation would be blunted. The α1 adrenergic receptor agonist NE strongly inhibits the RyR-mediated Ca<sup>2+</sup> release during vasoconstriction under normal conditions, while in hypoxic VSMCs, RyR-mediated Ca<sup>2+</sup> release is over-activated<sup>[29]</sup>, which might contribute to the over-activation of BK<sub>Ca</sub> channel. Furthermore, our results suggest that the over-activation of RyR-mediated Ca<sup>2+</sup> release is at least partly involved in the occurrence of vascular hyporeactivity to NE after hemorrhagic shock. The restoration of vasoreactivity by A<sub>3</sub>AR stimulation after hemorrhagic shock is at least partly associated with the inhibition of the over-activation of RyR-mediated Ca<sup>2+</sup> release and BK<sub>Ca</sub> channel. Because RyR-mediated Ca<sup>2+</sup> release regulates BK<sub>Ca</sub> channel activity<sup>[30]</sup> and BK<sub>Ca</sub> channel is involved in the restoration of the vasoreactivity to NE by A<sub>3</sub>AR stimulation through a RyR-mediated Ca<sup>2+</sup> release dependent pathway, it is rational to conclude that the stimulation of A<sub>3</sub>AR restores vasoreactivity through a RyR-mediated, BK<sub>Ca</sub> channel dependent signal

pathway, although future efforts are warranted to explore the precise mechanisms whereby A<sub>3</sub>AR stimulation regulates BK<sub>Ca</sub> channel activity through RyR-mediated Ca<sup>2+</sup> release after hemorrhagic shock.

In summary, our results show that A<sub>3</sub>AR is closely involved in the modulation of vascular reactivity after hemorrhagic shock in rats, and stimulation of A<sub>3</sub>AR restores the vascular reactivity to NE after hemorrhagic shock through a RyR-mediated Ca<sup>2+</sup> release and BK<sub>Ca</sub> channel dependent pathway.

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### Author contribution

Rong ZHOU designed and performed research, analyzed data and wrote paper. Liang-ming LIU and De-yao HU constructed research. Feng CHEN and Qiang LI performed research.

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