Introduction

Vascular hyporesponsiveness is one of the most common complications of serious conditions such as trauma and hemorrhagic shock. 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). 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). There are four types of ARs located in vascular smooth muscle (VSM): adenosine A1 receptor (A1AR), A2aR, A2bR, and A3AR. A3AR 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 A3AR confers partial cardioprotection, neuroprotection, renal protection, etc., but whether A3AR 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 (IP3), which binds to IP3R (inositol 1,4,5-triphosphate-sensitive receptor), a receptor abundant in the endoplasmic reticulum (ER) of VSMCs, triggers the Ca2+ release from Ca2+ store, and finally leads to the increase of intracellular calcium ion concentration ([Ca2+]i) and vasoconstriction. The Ca2+ released from the Ca2+ store also activates RyR-mediated Ca2+ release, which could in turn activate the BKCa channel in the nearby sarcolemma and negatively modulate vascular contraction. It has been demonstrated that in hypoxic VSMCs,
RyR-mediated Ca2+ release increases significantly[10], which might contribute to the activation of the BKCa channel. Our previous studies suggested that over-activation of the BKCa channel is closely associated with vascular hyporeactivity after hemorrhagic shock in rats[11,12], and stimulation of A3AR could inhibit RyR-mediated Ca2+ release[13]. Therefore, we hypothesized that A3AR might be involved in the modulation of vasoreactivity through a RyR-mediated Ca2+ release and BKCa channel dependent signal pathway after hemorrhagic shock.

In this study, we examined: (1) whether A3AR is involved in the modulation of vascular reactivity after hemorrhagic shock in rats and (2) whether A3AR modulates the vascular reactivity to NE after hemorrhagic shock through a RyR-mediated Ca2+ release-dependent pathway. To the best of our knowledge, this is the first report to demonstrate that A3AR is involved in the modulation of vasoreactivity after hemorrhagic shock and that stimulation of A3AR could restore the decreased vasoreactivity to NE through a RyR-mediated Ca2+ release-dependent 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 A3AR agonist, IB-MECA (chloro-N6-(3-iodobenzyl)adenosine-5’-N-methyluronamide); an A3AR antagonist, MRS1523 (3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate); a RyR antagonist, caffeine (Ca2+); a RyR antagonist, ryanodine (Ry); an IP3R antagonist, adenophostin A (AdA); a BKCa channel blocker, tetraethylammonium (TEA); a BKCa channel opener, NS1619; pentobarbital sodium; and a sarcoplasmic/endoplasmic reticulum Ca2+-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 (SVYX2002-032), weighing 220±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[14,15]. 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 O2 concentration less than 0.2% for 2 h as previously described and then used for the following experiment.

**Western blot of A3AR**

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 A3AR in SMA after hemorrhagic shock was determined by Western blot, as previously described[16]. Briefly, the tissues were put into pre-cooled RIPA (pH 7.6; Heps 50 mmol/L, NaCl 150 mmol/L, EDTA 1 mmol/L, NP-40 1%, β-glycerophosphate 20 mmol/L, Na2VO3 1 mmol/L, NaF 1 mmol/L, Benzamidine 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 µg 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 A3AR 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 β-actin levels.

**Reverse transcriptase-polymerase chain reaction (RT-PCR) of A3AR**

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[17]. The PCR conditions for A3AR and for the housekeeping gene, β-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):
A3AR, (Fwd) 5'-GGTCCACTGCCCATACACA-3' and (Rev) 5'-CTAGGTGATTGCAAC CACA-3'; β-actin, (Fwd) 5'-CACCCGAGTACAACCTTTCC-3' and (Rev) 5'-CCCCATAACCCATCATCACACC-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, CaCl2 2.2, NaHCO3 25, KH2PO4 1.2, MgSO4 1.2, glucose 11; pH 7.4) or Ca2+-free K-H solution (without Ca2+ or Mg2+, but with 1 mmol/L EGTA in the K-H solution), continuously bubbled with 95% O2 and 5% CO2, 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 10-9 to 10-5 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 contractive force of SMA branch was recorded as the change in tension (mN). The NE cumulative dose-response curve and the maximal contraction induced by 10-5 mol/L NE (Emax) were used to evaluate the vascular reactivity to NE. There were eight observations in each group.

Involvement of A3AR in the modulation of vascular reactivity after hemorrhagic shock in rats

The abdominal artery rings (2-3 mm in length) from the hemorraghic shock (40 mmHg for 2 h) or sham-operated control rats were prepared and randomly divided into the following 4 groups (n=8/group): sham-operated control, shock, shock+IB-MECA (4×10-6 mol/L), and shock+IB-MECA+MRS1523 (10-7 mol/L). Each vascular ring was mounted in a 10 mL organ perfusion system filled with K-H solution with or without Ca2+. The incubation time of each agent was 5 min. The single cell [Ca2+] was measured by a fluorescence indicator, Fura-3/AM, with confocal laser scanning microscopy as previously described[18]. Briefly, Fura-3/AM (10-5 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 [Ca2+] was represented by mean fluorescence intensity: the single cell mean fluorescence intensity of Ca2+= (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 Ca2+ release in the modulation of vascular hyporeactivity by A3AR stimulation after hemorrhagic shock in rats

We further investigated whether stimulation of A3AR with IB-MECA regulates the contractile response to NE associated with RyR-evoked Ca2+ release. One hundred and twelve abdominal arterial 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 (10-5 mol/L), shock+ryanodine (10-5 mol/L), shock+IB-MECA (4×10-5 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 Ca2+ and in the Ca2+-free K-H solution, as previously described. The incubation time and order of each group are described in Table 1.

Involvement of the BKCa pathway in the modulation of vascular hyporeactivity by A3AR stimulation after hemorrhagic shock in rats

Last, we examined whether the stimulation of A3AR with IB-MECA regulates the contractile response to NE through a RyR-mediated Ca2+ release, BKCa 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 (4×10-5 mol/L), shock+IB-MECA+caffeine (10-5 mol/L), shock+IB-MECA+NS1619 (5×10-3 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                | IB-MECA (4×10^{-6} mol/L) for 20 min                     |
| Shock+IB-MECA                | IB-MECA for 20 min, followed by MRS1523 (10^{-3} mol/L) for 10 min |
| Shock+IB-MECA+MRS1523        | Pretreated with MRS1523 (10^{-3} mol/L) for 10 min       |
| Shock+IB-MECA+caffeine       | Pretreated with caffeine (10^{-3} mol/L) for 10 min       |
| Shock+IB-MECA+NS1619         | Pretreated with NS1619 (5×10^{-3} mol/L) for 10 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±SD of “n” observations. The effect of A3AR 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±0.13 mN/g to 0.39±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±0.18 mN/g to 0.50±0.19 mN/g (P<0.01) (Figure 1B). In the SMA branch, the vascular reactivity to NE also significantly decreased: the NE cumulative dose-response curve shifted to the right, and the E_{max} decreased from 11.30±2.10 mN/g to 3.94±1.43 mN/g (P<0.01) (Figure 1C).

Decreased A3AR 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 A3AR mRNA level in the hemorrhagic shock rats or in the controls (Figure 2A), while the A3AR protein level was significantly decreased 2–4 h after hemorrhagic shock (Figure 2B).

Involvement of A3AR in the modulation of vasoreactivity to NE after hemorrhagic shock in rats
As shown in Figure 3, in the abdominal aorta, stimulation of A3AR with IB-MECA (4×10^{-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±0.14 mN/g to 0.71±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±0.12 mN/g to 0.28±0.11 mN/g (P<0.05) (Figure 3).

Effects of A3AR stimulation on the RyR-evoked Ca^{2+} release in hypoxic VSMCs
In abdominal aorta VSMCs, NE can induce the increase of cytosolic Ca^{2+} concentration ([Ca^{2+}]) resulting from the mobilization of Ca^{2+} from ER in the absence of extracellular Ca^{2+}. 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^{2+}, 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±24.9 to 161.5±21.6, P<0.01), while the increase in [Ca^{2+}] induced by the activation of IP_{3}R with adenophostin A decreased, although not significantly, in hypoxic VSMCs. The stimulation of A3AR with IB-MECA significantly inhibited the caffeine-induced upregulation of [Ca^{2+}] in hypoxic VSMCs (from 161.5±21.6 to 112.8±19.0, P<0.05) (Figure 4B), but there were no significant changes in adenophostin A-induced Ca^{2+} release resulting from the stimulation of A3AR.
with IB-MECA (Figure 4C). In the presence of a Ca2+-ATPase inhibitor, thapsigargin (10^-8 mol/L), there was no significant effect of IB-MECA on [Ca2+] in hypoxic VSMCs (Figure 4D).

**Restoration of the vascular reactivity to NE by A3AR stimulation through a RyR-, BKCa dependent signal pathway**

The role of RyR-mediated Ca2+ release in the decrease of vaso-reactivity after hemorrhagic shock was first explored. As shown in Figure 5, the activation of RyR-mediated Ca2+ 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 Emax (induced by 10^-5 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 Ca2+-free K-H solution (P<0.05), respectively. In addition, the inhibition of RyR-mediated Ca2+ release by ryanodine (10^-5 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 Emax (induced by 10^-5 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 Ca2+-free K-H solution (P<0.05), respectively (Figure 5).

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

**Figure 4.** (A) Changes in [Ca2+] in hypoxic VSMCs in the absence of extracellular Ca2+ (mean±SD, n=6). b P<0.05 vs control group. (B) Effect of the stimulation of A3AR with IB-MECA on the RyR-mediated Ca2+ 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. c P<0.01 vs control+Caf group; d P<0.05 vs hypoxia+Caf group. Caf: caffeine (10^-3 mol/L, a RyR agonist). (C) Effect of the stimulation of A3AR with IB-MECA on the IP3R-mediated Ca2+ release in hypoxic VSMCs (mean±SD, n=6). AdA: adenosphostin A (10^-5 mol/L, an IP3R agonist). (D) Effect of thapsigargin on [Ca2+] by the stimulation of A3AR with IB-MECA in hypoxic VSMCs (mean±SD, n=6). e P<0.05 vs hypoxia group. Tha: thapsigargin (10^-8 mol/L, an ER Ca2+-ATPase inhibitor).

**Figure 2.** Analysis of A3AR 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. b P<0.05 vs control group.
Next, we investigated whether the inhibition of RyR-mediated Ca^{2+} release by A3AR 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^{-7} 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_{\text{max}} from 0.71\pm0.12 mN/g to 0.14\pm0.12 mN/g (P<0.01) in normal K-H solution and from 0.31\pm0.06 mN/g to 0.10\pm0.03 mN/g in the Ca^{2+}-free K-H solution, respectively. These effects could be antagonized by a RyR antagonist, ryanodine (10^{-5} mol/L): the NE cumulative dose-response curve significantly shifted to the left and the E_{\text{max}} increased from 0.14\pm0.09 mN/g to 0.54\pm0.18 mN/g (P<0.01) in normal K-H solution and from 0.26\pm0.06 mN/g to 0.68\pm0.13 mN/g (P<0.05) in the Ca^{2+}-free K-H solution, respectively (Figure 6).

Last, we examined whether the stimulation of A3AR restores vasoreactivity through a RyR-mediated Ca^{2+} release, BK_{Ca} channel dependent signal pathway after hemorrhagic shock. Our results showed that pretreatment with a BK_{Ca} 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_{\text{max}} from 0.71\pm0.12 mN/g to 0.25\pm0.09 mN/g (P<0.01). In addition, a selective BK_{Ca} channel blocker, TEA (0.1 mmol/L), significantly antagonized the further decrease 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_{\text{max}} from 0.25\pm0.09 mN/g to 0.68\pm0.13 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_{\text{max}} increased from 0.25\pm0.09 mN/g to 0.72\pm0.12 mN/g (P<0.05, Figure 7B).

**Discussion**

The stimulation of A3AR has a protective effect against neurodegenerative diseases, myocardial injury[19, 20], and the hypoxia-reoxygen-induced damage of vascular reactivity[21]. However, little is known about whether the stimulation of A3AR can improve decreased vascular reactivity after hemorrhagic shock. In this study, we first examined the effect of A3AR in decreased vascular reactivity to NE after hemorrhagic shock in rats. We found that the expression of A3AR was decreased 2–4 h after hemorrhagic shock (40 mmHg), consistent with the loss of vasoreactivity to NE. IB-MECA, a selective A3AR agonist, partly but significantly restored the decreased vasoreactivity in hemorrhagic shock rats; this restorative effect could be antagonized by MRS1523 (a selective A3AR antagonist). These results suggest that A3AR is involved in the modulation of vasoreactivity, and the stimulation of A3AR can at least partly restore vascular reactivity to
The increased expression of RyR and the increased sensitivity of RyR to Ca$^{2+}$[25, 26]. Furthermore, the over-activation of RyR-mediated Ca$^{2+}$ release is closely associated with the occurrence of vascular hyporeactivity to NE in hemorrhagic shock rats. The stimulation of A$_3$AR significantly antagonized the over-activation of RyR-mediated Ca$^{2+}$ release in hypoxic VSMCs, and the restoration of the vasoreactivity to NE by A$_3$AR stimulation was partly but significantly counteracted by the activation of RyR-mediated Ca$^{2+}$ release by caffeine. These results suggest that RyR-mediated Ca$^{2+}$ release is indeed associated with the restoration of vasoreactivity by A$_3$AR stimulation after hemorrhagic shock.

RyR-mediated Ca$^{2+}$ release contributes to the activation of several Ca$^{2+}$-activated potassium (K$_{Ca}$) channels in the nearby sarcolemma, induces membrane hyperpolarization, and leads to the reduction of voltage-dependent Ca$^{2+}$ channel activity and vascular smooth muscle relaxation[27, 28]. Meanwhile, our previous work showed that over-activation of the BK$_{Ca}$ 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$_{Ca}$ channel dependent pathway in the modulation of vascular reactivity by A$_3$AR stimulation. We used a selective BK$_{Ca}$ channel opener, NS1619, and a BK$_{Ca}$ channel blocker, TEA (0.1 mmol/L), to explore the role of the BK$_{Ca}$ channel in the restoration of vasoreactivity by A$_3$AR stimulation. Our results showed that the selective A$_3$AR agonist, IB-MECA, improved vascular reactivity to NE via a RyR-mediated Ca$^{2+}$ release and BK$_{Ca}$ channel dependent signal cascade, while the BK$_{Ca}$ 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$_3$AR improves the vascular reactivity to NE through a RyR-mediated Ca$^{2+}$ release dependent pathway, in which the BK$_{Ca}$ channel is closely involved.

In a nonpressurized vascular ring exposed to a vasoconstrictor, the frequency of RyR-mediated Ca$^{2+}$ release would be low, and vasodilation mechanisms via Ca$^{2+}$ spark activation would be blunted. The a1 adrenergic receptor agonist NE strongly inhibits the RyR-mediated Ca$^{2+}$ release during vasoconstriction under normal conditions, while in hypoxic VSMCs, RyR-mediated Ca$^{2+}$ release is over-activated[29], which might contribute to the over-activation of BK$_{Ca}$ channel. Furthermore, our results suggest that the over-activation of RyR-mediated Ca$^{2+}$ release is at least partly involved in the occurrence of vascular hyporeactivity to NE after hemorrhagic shock. The restoration of vasoreactivity by A$_3$AR stimulation after hemorrhagic shock is at least partly associated with the inhibition of the over-activation of RyR-mediated Ca$^{2+}$ release and BK$_{Ca}$ channel. Because RyR-mediated Ca$^{2+}$ release regulates BK$_{Ca}$ channel activity[30] and BK$_{Ca}$ channel is involved in the restoration of the vasoreactivity to NE by A$_3$AR stimulation through a RyR-mediated Ca$^{2+}$ release dependent pathway, it is rational to conclude that the stimulation of A$_3$AR restores vasoreactivity through a RyR-mediated, BK$_{Ca}$ channel dependent signal pathway.
pathway, although future efforts are warranted to explore the precise mechanisms whereby A3AR stimulation regulates BKCa channel activity through RyR-mediated Ca\textsuperscript{2+} release after hemorrhagic shock.

In summary, our results show that A3AR is closely involved in the modulation of vascular reactivity after hemorrhagic shock in rats, and stimulation of A3AR restores the vascular reactivity to NE after hemorrhagic shock through a RyR-mediated Ca\textsuperscript{2+} release and BKCa 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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