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ERK and miRNA-1 target Cx43 expression and phosphorylation to modulate the vascular protective effect of angiotensin II

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ABSTRACT

Aims and methods: We previously reported that angiotensin II (AngII) restores the vascular reactivity diminished by hemorrhagic shock. In this study, we investigated whether the beneficial effects of AngII are related to regulation of gap junctions (GJs) and connexin43 (Cx43), and the implication of MAPK signaling and microRNA (miR-1) in this process.

Key findings: Our results show that after hemorrhagic shock or hypoxia, the blockade of GJs or knockdown of Cx43 inhibits the AngII-induced increase in vascular reactivity of superior mesenteric arteries and the contractile response of vascular smooth muscle cells (VSMCs). AngII treatment increases Cx43 expression and phosphorylation at Ser262, and restores gap-junctional communication (GJIC) between VSMCs after hypoxia. The AngII-induced up-regulation of Cx43 expression and phosphorylation is blocked in cells transduced with ERK-siRNA, but is not blocked in cells transduced with p38-siRNA. miR-1 levels are elevated after hypoxia; AngII treatment reverses the up-regulation of miR-1, while ERK-siRNA abolishes that effect of AngII. In hypoxic cells, transfection of a miR-1 mimic into VSMCs decreases Cx43 expression and VSMC reactivity, whereas a miR-1 inhibitor increases both. Also in hypoxic cells, miR-1 eliminates the restoration effects of AngII on Cx43 expression and VSMC reactivity.

Significance: AngII provides protection of vascular function through the restoration of the expression and phosphorylation of Cx43 and its mediated GJIC in VSMCs. It is ERK that mediates the AngII-induced phosphorylation of Cx43 at Ser262. Additionally, miR-1 is involved in this process, and AngII may exert its protective effect partially by inhibiting miR-1 elevation via ERK signaling.

1. Introduction

Vascular hyporeactivity contributes to refractory hypotension and hemodynamic disorder in severe hemorrhagic shock, a major cause of death in injured patients [1–3]. Vascular hyporeactivity is characterized by a reduced response of blood vessels to vasoconstrictors, and is possibly related to receptor desensitization, membrane hyperpolarization, and calcium desensitization of vascular smooth muscle cells (VSMCs) [2–6]. We have demonstrated that low doses of angiotensin II (AngII) can improve shock-induced vascular hyporeactivity by enhancing the vasoconstrictor effects of norepinephrine (NE, a clinically used vasoconstrictor), but not by directly inducing vasoconstriction [7]. We also found that the beneficial effect of AngII was partially mediated by improving calcium sensitivity in VSMCs, possibly in conjunction with other mechanisms.

Gap junctions (GJs) enable direct cell-to-cell communication between vascular endothelial cells and/or smooth muscle cells, a process that is fundamental in the control of vascular function. GJs play an important role in the regulation of vascular tone and contractility [8–10]. GJs are composed of connexins (Cxs), a family of transmembrane proteins, and multiple Cxs have been identified in the vascular system, such as Cx37, Cx40, Cx43, Cx45 and Cx46 [11,12]. We recently showed that GJs and Cx43 are involved in the vascular reactivity to bradykinin and vasopressin under conditions of hemorrhagic shock [13,14]. Using the renin-dependent model of hypertension, Alonso et al. [15] found that AngII increases the expression of Cx43 in the aortic SMCs of mice. However, little is known about whether GJs and Cx43 contribute to the beneficial effects of AngII on vascular reactivity after shock, or what mechanisms may be involved.

Evidence suggests that mitogen-activated protein kinase (MAPK)

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pathways are important mediators of AngII-induced VSMC growth and migration [16,17]. MAPKs also participate in the regulation of vascular contractility and tone induced by AngII. We previously demonstrated that AngII increases the activity of MAPKs and thereby enhances the contractile response of smooth muscle to NE after hemorrhagic shock [7]. In addition, Cx43 phosphorylation has been shown to affect Cx protein expression and gap junctional communication (GJIC) [18,19]. MAPKs can phosphorylate Cx43 at specific serine residues (e.g. Ser262) [20]. Whether MAPK-dependent Cx43 phosphorylation pathways are involved in the vascular effects of AngII is unknown.

Recent studies have highlighted the role of microRNAs (miRNAs/miRs) in cardiovascular biology and diseases [21,22]. MiRs are a family of endogenous, highly-conserved, small non-coding RNAs (~22 nucleotides) that regulate gene expression at the post-transcriptional level. Among the miRNAs, miR-1 is highly enriched in cardiac and skeletal muscle tissues, and plays critical roles in the regulation of muscle growth and differentiation. miR-1 inhibits the expression of Cx43 protein in myocardial cells in coronary artery disease, and three-prime untranslated region (3′-UTRs) of Cx43 gene contains binding sites for miR-1, suggesting that Cx43 is a direct negative target for miR-1 [23]. However, it is unknown whether miR-1 is involved in the regulation of Cx43 when VSMCs are treated with AngII, or if miR-1 contributes to the pathological processes underlying shock-induced vascular hyporeactivity.

Based on the literature and our previous findings, we hypothesized that AngII may improve shock-induced vascular hyporeactivity through MAPK-dependent phosphorylation of Cx43 and the associated miR-1 pathway. To test this hypothesis, we used superior mesenteric arteries (SMAs) from rats subjected to hemorrhagic shock, as well as hypoxia-treated vascular smooth muscle cells. We then investigated the roles of GJs and Cx43 in AngII-regulated vascular reactivity after hemorrhagic shock and the involvement of MAPKs signaling and miR-1 in this process.

2. Materials and methods

2.1. Ethics

The investigation conformed to *Guide for the Care and Use of Laboratory Animals* (Eighth Edition, 2011, Washington, D.C., National Academies Press, USA). The study protocol was approved by the Research Council and Animal Care and Use Committee of the Research Institute of Surgery, Daping Hospital (Third Military Medical University, Chongqing, P. R. China),

2.2. Animal models and blood vessel preparation

Male and female adult Sprague-Dawley (SD) rats, weighing 200–220 g, were anesthetized with sodium pentobarbital (initial dosage 30 mg/kg, i.p.) and Jingsongling (xylidinothiazole, initial dosage 0.1 mg/kg, i.m.). After rats reached ideal anesthesia, the right femoral artery was penetrated with a heparinized catheter for blood pressure monitoring and bleeding. To subject rats to hemorrhagic shock, blood was withdrawn through the arterial catheter until the mean arterial pressure (MAP) decreased to 40 mmHg. Sham-operated animals underwent the same operation but without the withdrawal of blood. Animals were maintained in either state for 4 h and then euthanized with a pentobarbital-based solution (Sleepaway, 2 mL, i.v., Fort Dodge, IA) [24]. The superior mesenteric arteries (SMAs) were rapidly excised and placed in ice-cold Krebs-Henseleit solution for subsequent experiments.

2.3. Vascular reactivity studies

SMAs were sectioned into rings 2–3 mm long, and the endothelium was denuded by gently rubbing the intimal surface as previously described [24]. SMA rings were suspended in isolated organ chambers containing Krebs-Henseleit solution at 37 °C, bubbled with 95% O_2/5% CO_2. After equilibration for 2 h, NE (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} mol/L) was added to the organ chambers, and SMA ring tension was measured using a Power Lab System via a force transducer (AD Instruments, Castle Hill, NSW, Australia). Some preparations were treated with AngII (10^{-7} mol/L, Sigma, St. Louis, MO, USA) and/or GJ channel blockers carbenoxolone (CBX, 10^{-4} mol/L, Sigma) and octanol (OCT, 10^{-3} mol/L, Sigma) for 60 min before inducing contractions. Maximal contraction (Emax) and NE concentration-response curves were used to compare the vascular reactivity.

2.4. Primary culture of VSMCs and hypoxic treatment

Rat VSMCs were obtained from adult SD rats by the explant method as described previously [14]. The superior mesenteric arteries were excised, cleaned of connective tissue, and cut into small pieces. The endothelium was carefully removed, and the pieces were placed in a petri flask containing Dulbecco-modified Eagle medium-F12 supplemented with 20% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% antibiotics. The preparations were incubated 5–7 days at 37 °C in 5% CO_2/95% air. Tissue pieces were then removed, and cells were passaged. Cells from passages 3 to 5 were used in the study. For hypoxic challenge, VSMCs were transferred into a hypoxia culture compartment (MIC-101, Billups-Rothenberg Inc., Del Mar, CA, USA), bubbled with 95% N_2 and 5% CO_2 at 10 L/min for 15 min, and then equilibrated for 10 min. This procedure was repeated 5 times until the O_2 concentration in the chamber was < 0.2%. VSMCs were maintained under hypoxic conditions for 4 h, and then used for subsequent experiments.

2.5. VSMC reactivity studies

VSMCs were seeded on Transwell inserts (0.4 μm pore size, Corning, NY, USA) and grown to confluence. After being subjected to hypoxia and/or treated with AngII (10^{-7} mol/L), CBX (10^{-4} mol/L), or OCT (10^{-3} mol/L), fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA, 5 mg/mL, Sigma) and NE (10^{-5} mol/L) were added to the upper compartment of the Transwell. After 30 min, 100 μL of medium from the lower compartment was collected and fluorescence measured. The contractile response of VSMCs to NE was expressed as the infiltration rate of FITC-BSA from the upper to lower compartment of the Transwell.

2.6. Lentiviral overexpression of Cx43

A lentivirus expression construct containing the rat Cx43 gene was obtained from Genechem Co., Ltd. (Shanghai, China). An empty vector was used as a negative control. Rat VSMCs were infected with Cx43-expressing lentivirus or the empty vector for 12 h, following the manufacturer's instructions. VSMCs were then washed and cultured in culture medium without antibiotics for 48 h. Overexpression of Cx43 was verified by determining levels of Cx43 protein using western blot analysis.

2.7. Transduction of small interfering RNA

A small interfering RNA (siRNA) against rat Cx43 was designed and packaged into an adenovirus expression system by Genechem Co., Ltd. VSMCs were seeded the day before transduction and grown to 50% confluence. VSMCs were infected with adenovirus (either expressing the Cx43-siRNA or the empty vector) following the manufacturer's instructions. After 48 h, cells were subjected to further experiments. The knockdown efficiency was determined by western blotting.

siRNAs targeting two major members of the MAPK family, ERK (extracellular-signal regulated kinase) and p38 (p38 mitogen-activated protein kinase), were purchased from Santa Cruz (CA, USA). A siRNA

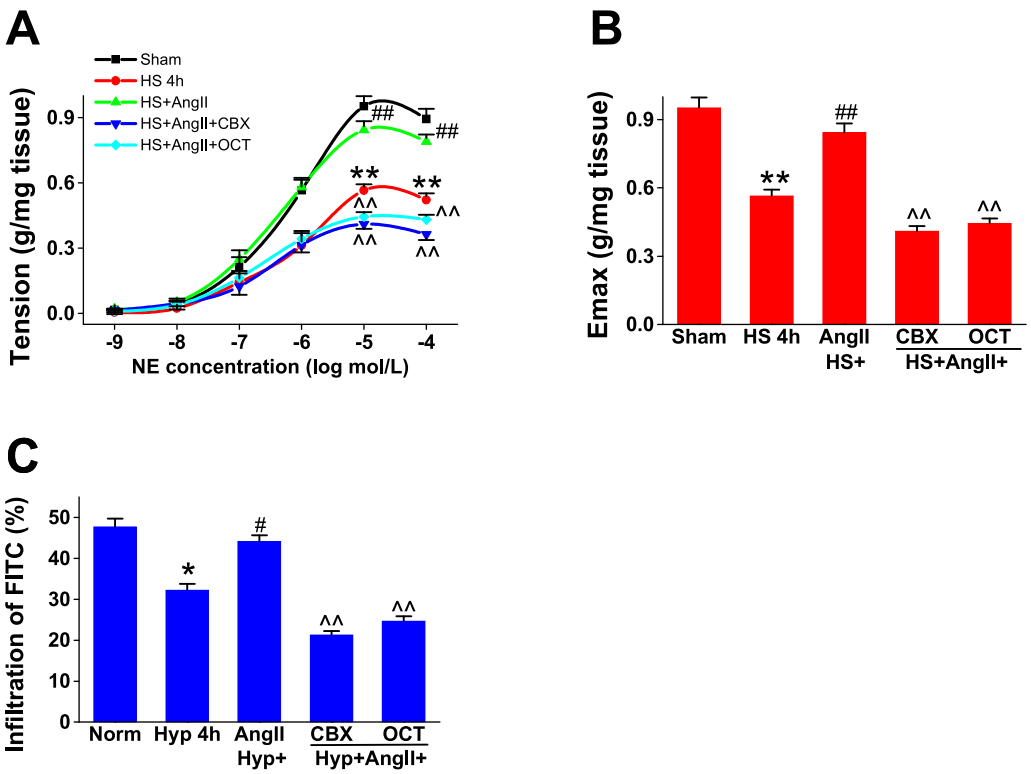

Fig. 1. Gap junction blockers CBX and OCT inhibit the AngII-induced increase of vascular reactivity of SMAs and the contractile response of VSMCs after hemorrhagic shock/hypoxia.
A and B: effect of CBX and OCT on AngII-regulated vascular reactivity. Left, the concentration-response curves of SMAs to NE; right, maximal contraction (Emax) of SMAs to NE. **C:** effect of CBX and OCT on AngII-regulated VSMC reactivity (expressed as the infiltration rate of FITC-BSA). Values are means ± SEM; n = 8/group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with sham-operated or normal groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with shock or hypoxia groups; $^{\wedge}P < 0.01$ compared with shock + AngII or hypoxia + AngII groups. Sham, sham-operated; HS, hemorrhagic shock; Norm, normal; Hyp, hypoxia; CBX, carbenoxolone; OCT, octanol.

with a non-targeting sequence (scrambled siRNA) was used as a negative control. VSMCs were transfected with siRNA or the control using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

2.8. Western blot analysis

Western blots were performed as described previously [24]. Briefly, rat VSMCs were lysed in buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L sodium vanadate, 5 mmol/L sodium pyrophosphate, 1 mmol/L EDTA, 0.25% sodium deoxycholate, 1% NP-40 and protease inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. After incubation with specific primary antibodies and horseradish peroxide-conjugated secondary antibody (1:8000, Pierce, Rockford, IL, USA), blots were visualized by enhanced chemiluminescence (ECL kit, Pierce). Protein levels were quantified using the Quantity One application (Bio-Rad, Hercules, CA, USA). The following antibodies were used: total Cx43 (1:1000, Sigma), phospho-Cx43 at Ser262 (1:200, Santa Cruz, CA), ERK (1:1000, Pierce), p38 (1:1500, Sigma), and β-actin (1:5000, Sigma).

2.9. Gap junction dye transfer assay

A dye transfer assay (parachute assay) was used to measure gap-junction intercellular communication (GJIC) in VSMCs. The assay measures the transfer of fluorescent dye (calcein) from a donor cell (cell preloaded with calcein) to adjacent cells (recipient cells) [25]. On the day before the experiment, recipient cells were detached with trypsin, resuspended in culture medium, and equal numbers of cells were placed in the confocal culture dish and incubated until the next day. On the day of the experiment, donor cells were trypsinized, loaded with 2 μmol/L calcein-AM (Invitrogen) for 20 min at 37 °C, then washed thrice with fresh culture medium to remove residual extracellular dye. Recipient cells were subjected to hypoxia or treated with AngII (10^{-7} mol/L) as described above. Thereafter, a small number of the calcein-loaded donor cells were layered on the recipient cells and incubated for 1.5 h at 37 °C. Fluorescence images were captured using a Leica confocal microscope and the number of calcein-positive cells surrounding parachuted donor cells was counted. At least ten random microscopic fields per condition were photographed and counted, and experiments were repeated three times.

2.10. Quantification of miR-1 levels

Total miRNA from rat VSMCs was extracted using TRIzol (Sigma) according to the manufacturer's protocol. Mature miR-1 levels were measured using the All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia). Quantitative real-time PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Relative miR-1 expression levels were normalized against U6 expression as an endogenous control, and were calculated using the $2^{-\Delta\Delta Ct}$ method. RT-PCR was performed in triplicate in each experiment.

2.11. Statistical analysis

Data are summarized as means ± standard error (SEM). Statistical calculations were performed using SPSS 18.0 (SPSS Inc., Chicago, IL). Differences between experimental groups were analyzed by one or two-factor analyses of variance, followed by *post-hoc* Tukey tests. $P < 0.05$ was the threshold for statistical significance.

3. Results

3.1. Effect of GJ blockers on the AngII-induced increase of vascular reactivity following hemorrhagic shock

We first examined whether GJs between VSMCs participate in the regulation of vascular reactivity and VSMC reactivity by AngII during shock or hypoxia. Vascular reactivity of SMAs to NE was significantly reduced after hemorrhagic shock. Treatment with AngII increased the vascular reactivity of SMAs from rats subjected to shock. Both CBX and

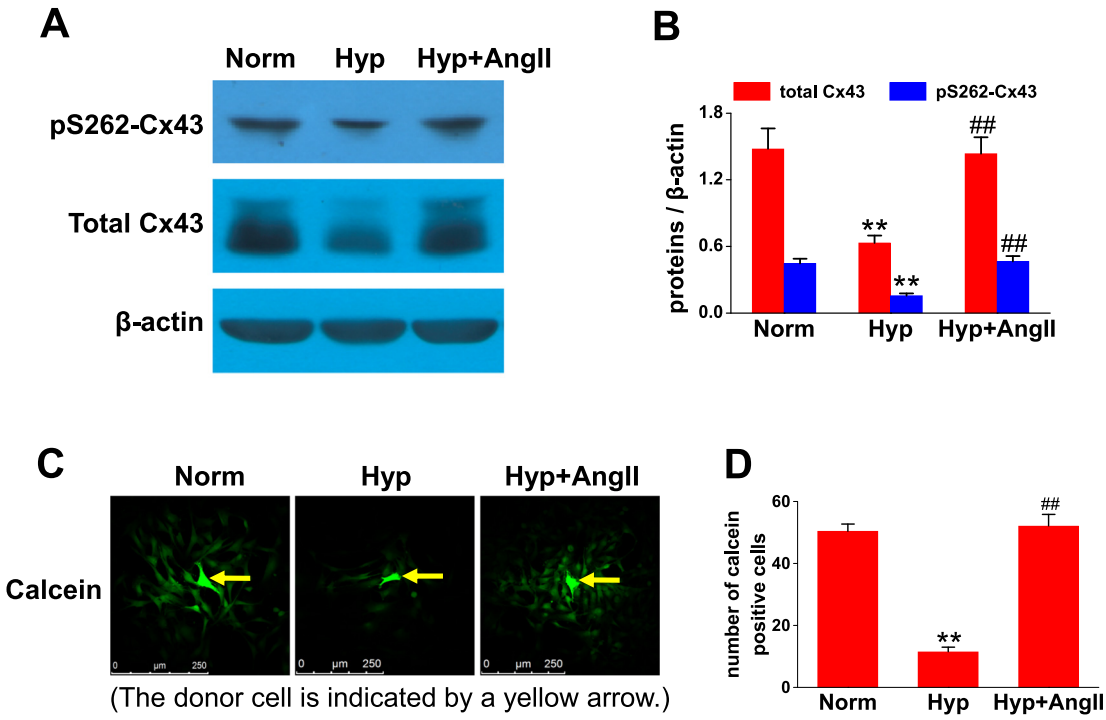

Fig. 2. AngII reverses the reduction of Cx43 expression and phosphorylation and GJIC in hypoxic-VSMCs.
A: representative western blot of Cx43 protein expression and phosphorylation at Ser262. **B:** ratios of the optical density for total Cx43 or phospho-S262-Cx43 to β-actin. Immunoblot analyses were repeated thrice. **C:** representative confocal images show GJIC in VSMCs by a parachute assay, which measures dye transfer from a calcein-loaded donor cell (indicated by the arrows in the figure) to adjacent cells. **D:** the levels of GJIC were quantified by counting dye-filled neighbors. At least ten random microscopic fields from three independent experiments were photographed and counted. Values are means ± SEM. $^{**}P < 0.01$ compared with normal group; $^{\#\#}P < 0.01$ compared with hypoxia group. Norm, normal; Hyp, hypoxia.

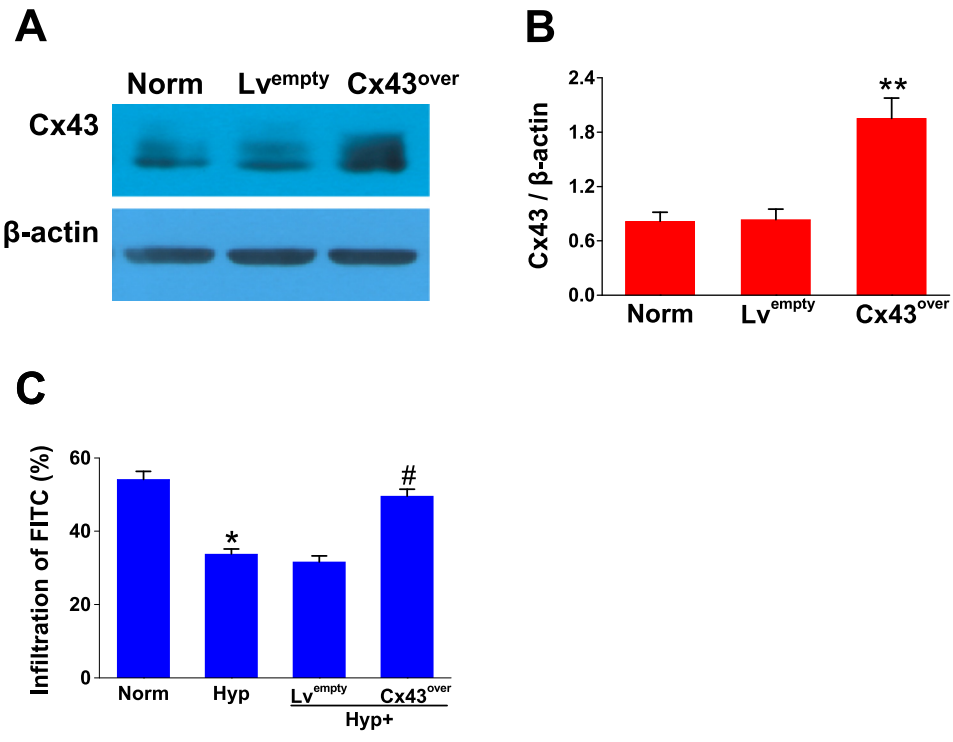

Fig. 3. Overexpression of Cx43 enhances the contractile response of VSMCs under hypoxic conditions.
A: representative western blot of the Cx43 protein levels in cells transduced by Cx43-expressing lentivirus or empty vector (negative control). **B:** ratio of the optical density of Cx43/β-actin. Immunoblot analyses were repeated thrice. **C:** effect of Cx43 overexpression on the contractile response of VSMCs after hypoxia (n = 8/group). Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with normal group; $^{\#}P < 0.05$ compared with hypoxia group. Norm, normal; Lv^{empty}, lentiviral empty vector; $Cx43^{over}$, Cx43-expressing lentivirus; Hyp, hypoxia.

OCT, two different GJ blockers, antagonized the AngII-induced restoration of vascular hyporeactivity following hemorrhagic shock ($P < 0.01$) (Fig. 1, A and B). Similar results were obtained under conditions of hypoxia. Blockade of gap junctions with CBX and OCT also significantly inhibited AngII-induced increase of the contractile response of VSMCs to NE under hypoxic conditions ($P < 0.01$). (Fig. 1C).

3.2. Effect of AngII on Cx43 expression and phosphorylation, and gap-junction intercellular communication in VSMCs after hypoxia

We next determined whether AngII regulates the expression and phosphorylation of Cx43, a predominant GJ protein, and its mediated GJIC. In our in vitro studies, hypoxia-treated VSMCs were used for mimicking the hypoxic-ischemic conditions of hemorrhagic shock. Immunoblot analysis revealed that in VSMCs, 4-h hypoxia caused a significant decrease in the protein expression of Cx43 and the phosphorylation of the serine in amino acid residue 262 (pS262-Cx43) of Cx43 ($P < 0.01$). AngII treatment significantly increased Cx43 expression and phosphorylation at Ser262 ($P < 0.01$) (Fig. 2A and B). GJIC was determined by parachute assay, which measures the dye transfer (calcein, a GJ permeable fluorescent dye) from donor cells to adjacent recipient cells. The confocal images showed that hypoxia reduced the spread of calcein from a central donor cell to surrounding cells, suggesting a hypoxia-induced decrease in GJIC. Treatment with AngII restored calcein diffusion in hypoxic-VSMCs, which was close to the normal level (Fig. 2C and D).

3.3. Effect of Cx43 overexpression on the contractile response of VSMCs after hypoxia

To determine whether Cx43 expression has a role in the regulation of vascular reactivity under hypoxic-ischemic conditions of shock, we overexpressed Cx43 in VSMCs using a lentivirus expression system. Immunoblotting revealed that Cx43 protein levels were significantly increased in VSMCs infected with the Cx43-expressing lentivirus over cells infected with the empty vector (negative control) (Fig. 3A and B). Remarkably, under hypoxic conditions, VSMCs infected with Cx43-expressing lentivirus had a higher infiltration rate of FITC-BSA than cells infected with the empty vector; suggesting that overexpression of Cx43 enhances the contractile response of hypoxic-VSMCs (Fig. 3C).

3.4. Effect of Cx43 silencing on AngII regulating VSMC reactivity after hypoxia

To further determine whether Cx43 is involved in the regulation of AngII on the contractile response of VSMCs under hypoxic conditions, Cx43 was knocked down using an adenovirus expressing Cx43-siRNA. In VSMCs infected with the Cx43-siRNA adenovirus, there was significant reduction in Cx43 protein (by ~80%), compared to cells infected by adenovirus carrying the empty vector or sham infected cells (Fig. 4A and B). As shown in Fig. 4C, silencing Cx43 expression significantly inhibited the AngII-induced increase of the contractile response of VSMCs under hypoxic conditions. Together these results demonstrate the important role of Cx43 in action of AngII on vascular and VSMC reactivity.

3.5. Role of the MAPK signaling pathway in AngII-induced Cx43 expression and phosphorylation

We then investigated the potential signaling pathways in Ang II-regulating Cx43 expression and phosphorylation in VSMCs. AngII induces activation of MAPK signaling including ERK and p38 in VSMCs, as stated in our previous report [7]. Here we show that VSMCs transduced with siRNA for ERK or p38, expressed significantly less ERK (~74%) and p38 (~76%) protein than cells transduced with scrambled siRNA or sham transduced cells (Fig. 5A–D). In hypoxic-VSMCs, ERK-siRNA significantly blocked an AngII-induced increase in Cx43 expression and phosphorylation, whereas p38-siRNA did not affect Cx43 levels following AngII (Fig. 5E and F). These results suggest that the ERK pathway, but not the p38 pathway, mediates AngII-induced up-regulation of Cx43 inVSMCs.

3.6. The role of miR-1 in Ang II-regulation of VSMC reactivity and Cx43 expression

We investigated whether hypoxic-ischemic conditions cause a change in miR-1 expression and whether AngII, through the ERK signaling pathway, affects miR-1 expression. Quantitative RT-PCR revealed that miR-1 expression was elevated (~3-fold) in rat VSMCs after hypoxia, while miR-1 levels in AngII treated hypoxic-VSMCs were at background levels, (i.e. levels in non-hypoxic cells). This effect of AngII on miR-1levels in hypoxic cells was abolished by ERK-siRNA (Fig. 6A).

The role of miR-1 in the AngII-regulation of VSMC reactivity and Cx43 expression was further investigated by transfecting a miR-1 mimic or inhibitor into VSMCs. We found that the miR-1 mimic further decreased the contractile response of VSMCs after hypoxia ($P < 0.05$), and the miR-1 inhibitor increased the contractile response of VSMCs, to about background levels, after hypoxia ($P < 0.05$). Moreover, the miR-1 mimic offset the beneficial effects of AngII on VSMC reactivity ($P < 0.01$) (Fig. 6B). Western blot analysis revealed that Cx43 protein levels were reduced after 4-h hypoxia, and treatment with the miR-1 mimic caused a further reduction in Cx43 levels ($P < 0.05$). In contrast, treatment with the miR-1 inhibitor resulted in levels of Cx43 to almost those of non-hypoxic cells ($P < 0.01$) (Fig. 6C and D). In addition, treatment with the miR-1 mimic abolished the restoration of Cx43 levels by AngII ($P < 0.01$) (Fig. 6E and F). These results suggest that AngII suppresses the up-regulation of miR-1 via the ERK pathway and subsequently restores Cx43 expression in hypoxic-VSMCs.

4. Discussion

This study demonstrates the important role of GJs and a constituent protein, Cx43, in the activity of AngII following hemorrhagic shock. First, blockade of GJs or knockdown of Cx43 levels inhibits the AngII-induced increase of vascular reactivity and VSMC contractility after shock or hypoxia. Second, AngII treatment increases Cx43 expression and phosphorylation at Ser262, and restores GJIC between VSMCs after hypoxia. Third, ERK mediates AngII-induced up-regulation of Cx43 expression and phosphorylation. Fourth, miR-1 is also involved in AngII-induced Cx43 protein expression. These findings help us to understand the mechanisms underlying the protective effects of AngII, and provide potential therapeutic targets (such as miRNAs) for the treatment of critical illness.

Angiotensin II (AngII) is a powerful vasoconstrictor and is a critical factor in the pathophysiology of cardiovascular disease [26,27]. Our previous studies showed that under shock conditions, treatment with low-dose AngII did not directly induce vasoconstriction, but produced a restoration of vascular reactivity by enhancing the vasoconstrictor effects of catecholamine [7]; the mechanism of action remains unknown. There is increasing evidence that GJs and their connexins are involved in the control and coordination of vascular function [8]. Studies from our laboratory and others have demonstrated that GJs and Cxs play a role in the regulation of vascular tone, blood pressure, and shock-induced vascular hyporeactivity [9,14]. Hence, we began by asking whether GJs are involved in the protective effect of AngII on vascular reactivity following hemorrhagic shock. We used two classic GJ blockers [28]: carbenoxolone, a semisynthetic derivative of glycyrrhetinic acid, and octanol, a long carbon chain n-alkanols. Our results show that both blockers inhibited the AngII-induced increase of the contractile response of SMAs from rats subjected to shock. This result suggesting that gap junctions do have a function in AngII-induced restoration of vascular reactivity.

Cx43 is the most prominent GJ protein expressed in the smooth muscle cells [11]. We therefore used RNA interference to investigate the link between to Cx43 and AngII. Transduction of a siRNA targeting Cx43 into VSMCs caused a 75% reduction in Cx43 protein and abolished the AngII-induced increase of the contractile response of VSMCs under hypoxia. Moreover, we found that hypoxia decreased Cx43

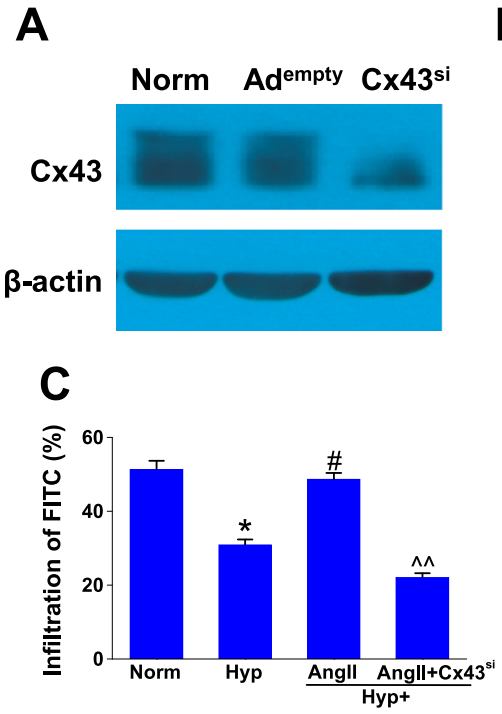

Fig. 4. Knockdown of Cx43 inhibits the AngII-induced increased contractile response of VSMCs after hypoxia.
A: representative western blot of Cx43 protein levels in the presence of Cx43-siRNA adenovirus or adeno-empty vector (negative control). **B:** ratio of the optical density of total Cx43/β-actin. Immunoblot analyses were repeated thrice. **C**: effect of Cx43-siRNA on AngII regulation of VSMC reactivity after hypoxia (n = 8/group). Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with normal group; $^{\#}P < 0.05$ compared with hypoxia group; $^{\sim\sim}P < 0.01$ compared with hypoxia + AngII group. Norm, normal; Ad^{empty}, adeno-empty vector; Cx43^{si}, adeno-Cx43-siRNA; Hyp, hypoxia.

expression and GJ-mediated communication between VSMCs, these conditions were reversed by treatment with AngII. Overexpression of Cx43 in hypoxic-VSMCs also enhanced the cell contractility. These results suggest that Cx43 and the GJIC it mediates, play a critical role in the vascular protective effects of AngII.

Phosphorylation of Cxs has been shown to control the Cx lifecycle (biosynthesis, trafficking, degradation), GJ structure (assembly, disassembly,) and function (channel gating, GJIC) [11,12,18]. As the most widely expressed GJ protein, the phosphorylation of Cx43 has received much attention. The carboxy terminus of Cx43 contains a number of phosphorylation sites, and is phosphorylated the various sites by protein kinase A, protein kinase C, MAPK, casein kinase 1 and pp60Src [19]. Serine 262 (Ser262) of Cx43 is a phosphorylation site known to be associated with GJ structure and function, and is a MAPK family substrate [20,29]. In our previous study, we demonstrated that phosphorylation at Ser262 contributed to vasopressin-induced

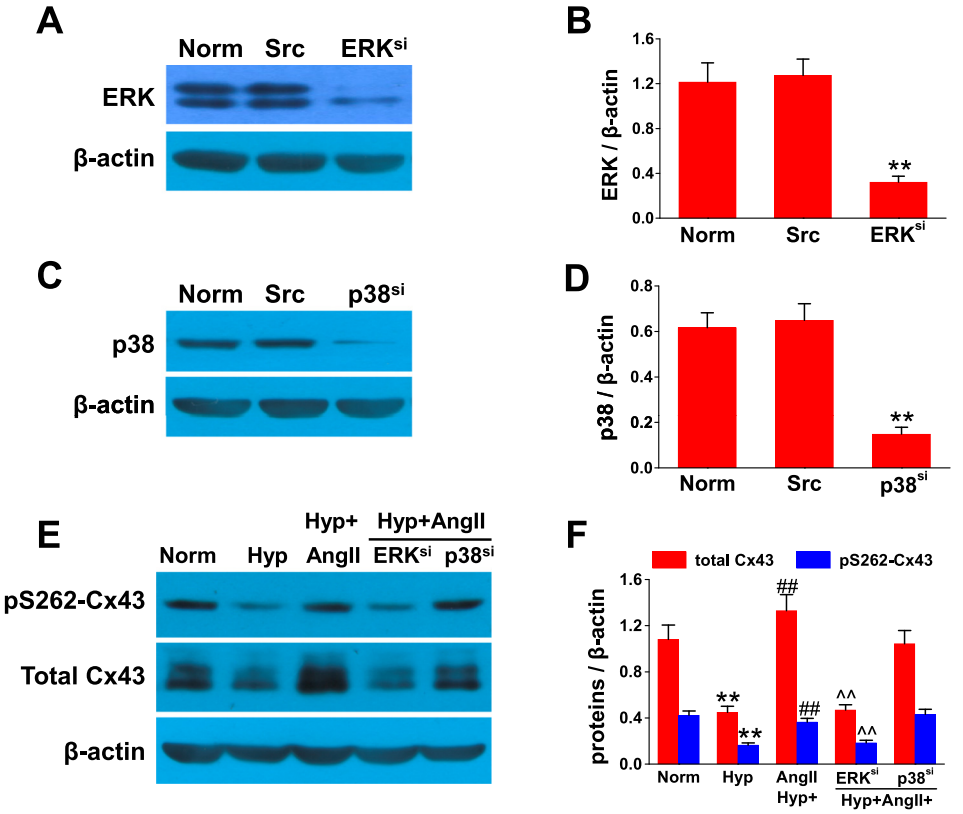

Fig. 5. AngII regulation of Cx43 expression and phosphorylation is via the MAPK signaling pathway.
A and C: western blot analyses demonstrate the knockdown efficiency of ERK-siRNA and p38-siRNA. A scrambled siRNA was used as a negative control. **B and D**: ratio of optical densities of ERK or p38/β-actin. **E:** effect of siRNAs targeting ERK or p38 on AngII regulation of Cx43 expression and phosphorylation at S262 in VSMCs. **F:** ratio of optical densities of total Cx43 or phospho-S262-Cx43/β-actin. Immunoblot analyses were repeated thrice. Values are means ± SEM. $^{**}P < 0.01$ compared with normal group; $^{\#\#}P < 0.01$ compared with hypoxia group; $^{\sim\sim}P < 0.01$ compared with hypoxia + AngII group. Norm, normal; Scr, scrambled siRNA; ERK^{si}, ERK-siRNA; p38^{si}, p38-siRNA; Hyp, hypoxia.

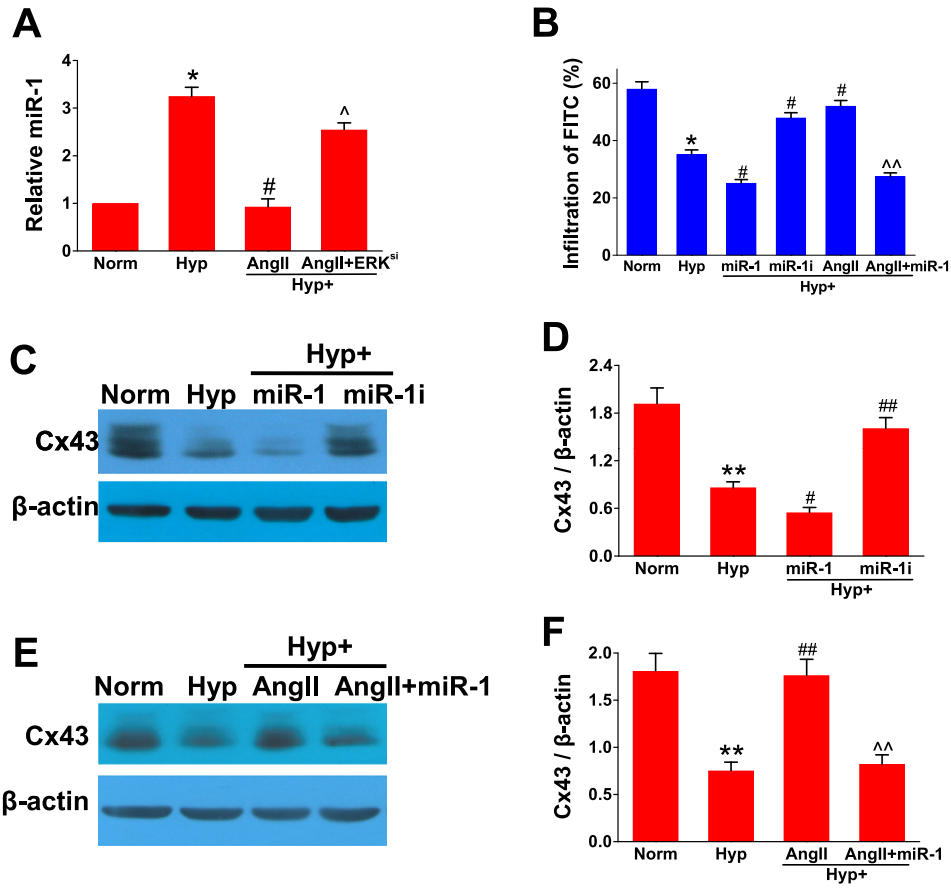

Fig. 6. Role of miR-1 in AngII-regulation of VSMC reactivity and Cx43 expression.
A: changes in miR-1 levels in VSMCs after hypoxia and the roles of AngII and ERK-siRNA. miR-1 levels were quantified by and normalized by the expression of U6. Experiments were performed in triplicate. **B:** effects of miR-1 on AngII-regulation of VSMC reactivity after hypoxia (n = 8/group). **C and E**: effect of miR-1 on the protein levels of Cx43 in hypoxic-VSMCs, and role of miR-1 in regulation of Cx43 expression by AngII. Representative immunoblots showing the protein levels of Cx43. **D and F**: ratio of the optical density of Cx43/β-actin. Immunoblot analyses were repeated thrice. Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with hypoxia group; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ compared with hypoxia + AngII group. Norm, normal; Hyp, hypoxia; ERK^{si}, ERK-siRNA; miR-1, miR-1 mimic; miR-1i, miR-1 inhibitor.

vasoconstriction after hemorrhagic shock [14]. We have also shown that AngII can activate the MAPK (especially ERK and p38) pathway to enhance the vascular reactivity after shock [7]. MAPK is one of the major downstream targets of AngII [16,17,30]. This led us to hypothesize that AngII produces protective effects against hemorrhagic shock, possibly through MAPK-dependent phosphorylation of Cx43 at MAPK sites such as Ser262. We found that both phosphorylation of Cx43 at Ser262 and the Cx43 protein expression were significantly decreased after hypoxia and this decrease was reversed by AngII, which is similar to the changes in GJIC. Furthermore, transduction of VSMCs with ERK-siRNA blocked the AngII-induced increase in Cx43 expression and phosphorylation at Ser262, although transduction with p38-siRNA had no significant effect. These findings suggest that ERK-mediated phosphorylation of Ser262 in Cx43 may be a novel molecular mechanism by which AngII restores vascular reactivity following hemorrhagic shock.

Another finding of this study is that miR-1 is involved in the protective effects of AngII. Our data show that miR-1 levels were elevated after hypoxia, that AngII treatment reduced miR-1 expression, and this effect was abolished by ERK-siRNA. Several studies have indicated that Cx43 is a direct target of miR-1 [22,31]. We used a miR-1 mimic and inhibitor to further investigate the link between miR-1, Cx43 and AngII. Transfection of the miR-1 mimic into VSMCs decreased Cx43 expression, whereas the miR-1 inhibitor increased the level of Cx43. Moreover, transfection of miR-1 eliminated the restoration effects of AngII on the expression of Cx43 under hypoxic conditions. These findings indicate that part of the protective effect of AngII is due to its inhibition of miR-1 elevation, via ERK pathway.

The discovery of miRNAs is regarded as the most important breakthrough in gene expression regulation [32]. Increasing evidence points to a critical role for miR-1 in cardiovascular biology and physiology. Yang et al. [23] reported that levels of miR-1 are elevated in the hearts of patients with coronary artery disease and of rats with experimental myocardial infarction. miR-1 may be an arrhythmogenic factor. Mondejar-Parreño et al. [33] showed that miR-1 expression was increased in the pulmonary arteries of hypoxia-induced pulmonary arterial hypertension rats. Taken together with our present study, these findings strongly suggest miR-1 as a potentially important biomarker and therapeutic target for cardiovascular diseases.

This study leaves open several important questions. For example, miRNAs often regulate multiple genes. Does miR-1 regulate other smooth muscle contractile proteins, or play a role in the protective effects conferred by AngII? Cx43 is regulated by other miRNAs such as miR-206 [34] and miR-1298 [35] in VSMCs. Are these miRs involved in the action of AngII? Answering such questions will help reveal the complex network of miRNAs, target genes and their mechanisms of action in vascular function.

5. Conclusion

In summary, our study demonstrates that angiotensin II protects vascular function against ischemic-hypoxic injury by restoring the expression and phosphorylation of Cx43, and thus its ability to mediate gap junctional communication in VSMCs. This protective effect is associated with ERK-dependent phosphorylation of Cx43 at Ser262. AngII also exerts its protective effect by inhibiting the elevation of miR-1 via ERK signaling, thereby restored Cx43 expression in VSMCs. These findings advance our understanding of the underlying mechanisms in vascular hyporeactivity, and suggest miRNAs as novel therapeutic targets. More effort to investigate the importance of miR-1 and elucidate its mechanisms of action is warranted.

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Disclosures

The authors declare that there are no conflicts of interest.

Author contributions

Y. L. performed experiments, analyzed data, and drafted the manuscript. X. P. and T.L. performed experiments and analyzed data. L. L. contributed in experimental design and data analysis. G. Y. conceived and designed the research, analyzed data, prepared figures, and finalized the manuscript. All authors approved final version of manuscript.

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