Original Paper

The Enhancement of Cx45 Expression and Function in Renal Interlobar Artery of Spontaneously Hypertensive Rats at Different Age

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Key Words

Connexin • Gap junction • Renal interlobar artery • Vascular smooth muscle cell • Spontaneously hypertensive rats • Vasoconstriction

ABSTRACT

Background/Aims: This study was designed to investigate the expression and function of gap junction protein connexin 45 (Cx45) in renal interlobar artery (RIA) of spontaneously hypertensive rats (SHR), and the association between hypertension and enhanced vasoconstrictive response in SHR. **Methods:** Western blot analysis and pressure myography were used to examine the differences in expression and function of Cx45 in vascular smooth muscle cells (VSMCs) of RIA between SHR and normotensive Wistar-Kyoto (WKY) rats. **Results:** Our results demonstrated that 1) whole-cell patch clamp measurements showed that the membrane capacitance and conductance of in-situ RIA VSMCs of SHR were significantly greater than those of WKY rats ($p < 0.05$, $n = 6$), suggesting that the coupling of gap junction between VSMCs of RIA was enhanced in SHR; 2) the KCl or phenylephrine (PE)-stimulated RIA constriction was more pronounced in SHR than that in WKY rats ($p < 0.05$, $n = 10$). After applying a gap junction inhibitor 18 β -glycyrrhetinic acid (18 β -GA), the inhibitory effect of 18 β -GA on KCl or PE-induced vasoconstriction was greater in SHR ($p < 0.05$, $n = 10$); and 3) the expression of Cx45 in RIA of SHR was greater than that in WKY rats ($p < 0.05$, $n = 3$) at 4,

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12 and 48 wks of age. **Conclusions:** The hypertension-induced elevation of Cx45 may affect communication between VSMCs and coupling between VSMCs and endothelium, which results in an increased vasoconstrictive response in renal artery and might contribute to the development of hypertension.

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Introduction

Hypertension can cause damage in heart, brain, kidney and vascular dysfunction, and is an important risk factor for the rapid progression of renal failure. Cross transplantation of kidneys between spontaneously hypertensive rats (SHR) and normotensive rats leads to development of hypertension in normotensive rats and normalization of the blood pressure (BP) in SHR [1-3]. Renal circulation plays a crucial role in the regulation of arterial blood pressure. Changes in structure and function of renal vasculature are associated with the development of hypertension [4]. Afferent arterioles originate from interlobular arteries and deliver blood to the glomeruli [4]. Cells in renal arterioles are coupled with each other and express several connexins (Cxs) in a cell-specific pattern [5]. Four isoforms of the Cxs, Cx37, Cx40, Cx43, and Cx45 are expressed in renal vasculature of mice, rats, rabbits and humans, with predominant expression of Cx40 in endothelial cells (ECs) and Cx45 in vascular smooth muscle cells (VSMCs) [4, 6, 7]. These Cxs play important roles in regulation of secretion of renin (Cx40/Cx43/Cx45), signaling in juxtaglomerular apparatus (JGA)(Cx40/Cx45), regulation of renal blood flow (Cx40), and coupling of VSMCs and ECs (Cx40/Cx43) [6]. The interactions between the different isoforms of Cxs form homomeric and heteromeric connexons [5]. Heterotypic channels are formed between Cx45 and Cx40/Cx43 in several cell types and each combination has unique feature in the mesangium and likely the granular cells [6, 8]. Compared to most membrane proteins, the turnover rate of Cxs is fast (half-life of 1.5–5 hrs) [9], which implies that modulation of the Cx degradation rate might be an important mechanism for controlling the level of gap junction to regulate intercellular communication under physiological and pathophysiological conditions [10]. The regulation of gap junction assembly and turnover can be modulated by several proteins that interact with Cxs, such as structural proteins (e.g. zona occludens-1 [ZO-1] and microtubules), protein kinase and phosphatases [11]. Among them, ZO-1 participates in the regulation of gap junction assembly by facilitating the coupling of connexon with the actin cytoskeleton [12]. Thus, the Cxs-ZO-1 interaction may be an important regulator of gap junction formation and maintenance on the membrane.

The changes in expression and function of Cxs in renal vasculature are associated with the development of vascular diseases, including hypertension, hyperreninemia, atherosclerosis, and restenosis [4]. Cxs in kidney likely exert an essential function in renal autoregulatory mechanisms (tubuloglomerular feedback) and in vasomotor responses [5]. In renal circulation, the role of Cx40 in modulating renin secretion and blood pressure is well documented. Deletion of Cx40 results in hyperplasia of renin-producing cells in kidney, hyperreninemia, and hypertension [13, 14]. Further, Cx45 can functionally replace Cx40 for regulation of renin secretion and blood pressure when Cx45 was knocked into the genome of Cx40 knock out mice [15], indicating the two isoforms may be functionally interchangeable [15].

Gap junction can also affect the vasomotor activity by regulating vasomotor tone [16, 17]. Vascular conductive responses coordinate microcirculatory control in vascular beds, and these responses are the consequence of direct intercellular communication between cells on the vascular wall [18-20]. As a consequence of intercellular communication, local vascular constrictive or dilatory stimulation can be propagated along the length of the

vascular wall, leading to constrictive or dilatory responses at sites several millimeters from the stimulation site [21-23]. Intercellular communication travels through gap junctions on the cell membrane..

An increased communication between cells has been demonstrated in the renal microvasculature of SHR. An increase in activity of the tubuloglomerular feedback mechanism [24-26] has been shown in young SHR, resulting in renal vasoconstriction and sodium retention [27]. The increased tubuloglomerular feedback response in SHR is associated with a stronger nephron-nephron coupling in SHR [28].

It is known that the structure and function of blood vessels change during aging. However, the expression of gap junction proteins on renal blood vessels at different ages, especially during the development of hypertension, has not been studied. The goal of the present study is to test whether an increase in gap junction communication is associated with the increase in Cx45 expression on renal blood vessels of SHR during the development of hypertension. We used whole-cell patch clamping technique, pressure myography and Western blot analysis to study the differences in function and expression of gap junctions between VSMCs of renal interlobar artery (RIA) of SHR and control normotensive Wistar-Kyoto (WKY) rats at different ages.

Materials and Methods

Animals

4, 12 and 48 wks old male SHR and WKY rats were used in this study. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical College of Shihezi University and were consistent with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Public Health Service Policy on Humane Care and Use of Animals, DHEW Publication No. 96-01, PHS Policy revised in 2002). BP was measured by the tail cuff method. To collect RIA, rats were anesthetized with an injection (i.m., 1 ml/kg) of a mixture of ketamine/xylazine/acepromazine (500/20/10 mg in 8.5 ml H₂O) and subsequently euthanized. RIA was dissected out from the upper renal artery, and surrounding connective and adipose tissue were removed for whole-cell patch clamp recording, pressure myographic measurement, and Western blot analysis.

Whole-cell patch clamp recording

RIA (~ 0.4 mm in length, 200 μ m in diameter) was placed on a glass-bottom petri dish which was filled with oxygen saturated external solution containing (in mM): NaCl 138, KCl 5, CaCl₂ 21.6, MgCl₂ 21.2, Na-HEPES 5.0, HEPES 6.0 and glucose 7.5. RIA segments were treated with external solution containing collagenase A (1 mg/ml) for 15 min at 37°C. After washed out collagenase with external solution twice the segments were further cleaned to remove adventitial tissue. RIA segments were anchored to the bottom by placing platinum strips on each end. The petri dish was then placed onto the stage of an inverted microscope equipped with micromanipulators. The segments were continuously perfused with external solution (0.2 ml/min) at room temperature.

To isolate VSMCs from RIAs, RIAs were incubated in a low-Ca²⁺ solution for 20 min containing (in mM): NaCl 142, KCl 5, CaCl₂ 0.05, MgCl₂ 1.0, Na-HEPES 4.0, HEPES 5.0 and glucose 7.5, and cut into 1 mm segments and digested with low-Ca²⁺ solution containing papain (1.5 mg/ml), collagenase A (2 mg/ml), bovine serum albumin (BSA, 3.75 mg/ml) and DL-dithiothreitol (0.3 mg/ml) for 20-25 min at 37°C. After centrifuging (67x g for 5 min) and replacing the supernatant with enzyme-free low-Ca²⁺ solution three times, the preparation was triturated with a Pasteur pipette. The cell-rich suspension was transferred to a petri dish with a poly-L-lysine-coated coverslip at the bottom. After the dispersed cells attached to the coverslip, the dish was placed onto the stage of an inverted microscope and perfused with extracellular solution for whole-cell recording.

Conventional whole-cell patch clamp recording was performed using an Axon 700B amplifier (Axon Instruments, Union City, CA, USA) as described previously [29]. The pipette had a resistance of approximately 5 M Ω after being filled with internal solution containing (mM): K-gluconate 130, NaCl 10, CaCl₂ 2, MgCl₂ 1.2, HEPES 10, ethylene glycol-bis [β -aminoethylether] N,N,N',N'-tetraacetic acid 5 and glucose 7.5. The seal

resistance usually reached 1–20 GΩ before rupture of the membrane. The membrane current or voltage signal were filtered at 10 kHz and recorded on a PC equipped with a Digidata 1440A AD-interface and pClamp 10.2 software (Axon Instruments, Union City, CA, USA) at a sampling interval of 10, 20 or 100 ms. A Minidigi digitizer and Axoscope 10.2 software (Axon Instruments) were used to perform gap-free recording at a sampling interval of 50 ms.

The transient current over the membrane input capacitance (C_{input}) was uncompensated to calculate the access resistance (R_a) and the membrane parameters on-line or off-line. The off-line calculation was performed with an exponential fit to the capacitive current transients by employing commonly used equations. The C_{input} values for in-situ cells were calculated according to $C=Q/V$, in which the charge (Q) was obtained by a four-term exponential fit to the current transient elicited by a voltage step. The voltage clamping error introduced by the current (I) passing the R_a was corrected according to the equation $V_m = V_c / I R_a$ (in which V_m is the actual clamping membrane voltage and V_c is the apparent command voltage).

Pressure myographic measurement

RIA segments were placed in an 4°C oxygen saturation physiological solution containing (in mM): NaCl 118.9, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, and glucose 5.5. RIA segment was tied to a glass tube using 12-0 nylon monofilament sutures, and placed in a microvascular chamber (Pressure Myograph System, DMT, Denmark) [30]. The chamber was perfused with physiological solution (pH 7.4, bubbled with 95% O₂ and 5% CO₂) and heated to 37°C. RIA segment was pressurized to a constant transmural pressure of 60 mmHg. The diameter was continuously determined using a video dimension analyzer and recorded using the DMT Vessel Acquisition Suite. RIA segment was treated with progressively increasing doses of PE (0.01–30 μM) and following by increasing doses of KCl (10–100 mM). The results were evaluated according to the changes in vascular diameter recorded on the DMT [30].

Western blot analysis

RIA was homogenized in RIPA buffer (at a ratio of 100 mg of tissue to 200 μl of RIPA buffer) with addition of freshly prepared protease inhibitor cocktail. Tissue homogenate was incubated at 4°C for 30 min and centrifuged at 12,000x g at 4°C for 15 min. The supernatant was collected, and the protein concentration was determined. Protein aliquots (40 μg) were subjected to 4–15% Tris-glycine denaturing gradient gel electrophoresis and then transferred to a NC membrane. The membrane was hybridized with specific primary antibodies against Cx45 at 4°C overnight. The membrane was then incubated with a fluorescein-conjugated secondary antibody at room temperature for 1 hr. Immunoreactive bands were detected using the ECL chemiluminescence reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Membrane was stripped and re-labeled with β-actin antibody as an internal control. The intensity of protein bands was analyzed using a Quantity One software (Bio-Rad, Hercules, CA, USA) [31].

Reagents

Primary antibodies and the horseradish peroxidase-conjugated secondary antibody were obtained from Santa Cruz Biotechnology (Dallas, Texas USA). The BCA protein assay kit was obtained from Pierce (Thermo Fisher Scientific Inc. Rockford, IL USA). RIPA buffer, PE and 18β-GA were purchased from Sigma (Sigma-Aldrich China, Inc. Shanghai, PRC). 18β-GA was dissolved in dimethyl sulfoxide (DMSO) as a stock solution before being further diluted with external solution to achieve the final concentration. The final DMSO concentration in solution was ≤0.1%, which had no detectable effect on vasomotor activity.

Statistical analysis

SHR and WKY rats were age matched to minimize individual differences. Results are expressed as means ± SEM. Statistical analysis was performed using the SPSS (Statistical Package for the Social Sciences) 17.0 statistical software package. The primary statistical tests was performed by 2-tailed paired or unpaired Student's t test, or 1 and 2-way ANOVA, if not stated otherwise. When the overall F test result of analysis of variance was significant, a multiple comparison Tukey test was applied. Student's t-test was used for comparisons between two means. The differences were considered significant when the p values were <0.05.

Results

Basal parameters

The body weight $\pm$ SEM of SHR (227.2 $\pm$ 4.5 g) and WKY rats (242.1 $\pm$ 7.3 g) were not different through 4~48 weeks of age. The systolic pressure of SHR was significantly higher than that of WKY rats at 12 and 48 weeks of age (Table 1). The outer diameter of RIA of SHR was significantly less than that of WKY rats at 4, 12 and 48 weeks of age (Table 2).

Cx45 expression increased in the RIA of SHR

It has been reported that Cx45 is expressed in VSMCs of small arterioles and large conducting vessels (A. gracilis, femoral, and mesenteric arteries), but not in ECs [32]. In the current study, we demonstrated that, Cx45 was highly expressed in VSMCs of RIA and cardiac muscle of SHR and WKY rats. Further, a significant up-regulation of Cx45 expression was observed in both 12-wk-old SHR and WKY rats compared to that of 4-wk-old and 48-wk-old SHR and WKY rats (data not shown). A marked increase of Cx45 protein was also detected in the RIA and cardiac muscle of SHR compared to that of age-matched WKY rats (Figure 1).

Membrane properties of VSMCs in RIA of SHR and WKY rats

Whole-cell recordings on in-situ or dispersed RIA VSMCs of SHR and WKY rats were shown in Figure 2. Step and ramp voltage commands from a holding potential of -40 mV were applied to measure the membrane properties. The current transients showed a time course that fit poorly to a single-term exponential function in the in-situ VSMCs of SHR and WKY rats (Figure 2A). However, these current transients fit well to a four-term exponential function (not shown), indicating a multiple source in charging the circuit or an electrical coupling of multiple cells in the vessel. Treatment with gap junction inhibitor 18 β -glycyrrhetinic acid

Table 1. Blood pressure of SHR and WKY rats at 4, 12 and 48 weeks of age

Age (wks)	N	Systolic Blood Pressure (mmHg)	
		WKY	SHR
4	8	111.3 $\pm$ 4.0	115.7 $\pm$ 5.4
12	8	117.6 $\pm$ 3.2	181.3 $\pm$ 7.6*#
48	8	121.4 $\pm$ 5.2	198.3 $\pm$ 2.1*#

Results are means $\pm$ SEM. * $p < 0.01$, compared to the same age WKY; # $p < 0.01$, compared to 4-wk old SHR

Table 2. Size of renal interlobar artery (RIA) of SHR and WKY rats at 4, 12 and 48 weeks of age

Age (wks)	N	Outer diameter of RIA (μ m)	
		WKY	SHR
4	8	356.7 $\pm$ 6.5	308.6 $\pm$ 5.6*
12	8	362.5 $\pm$ 5.7	311.4 $\pm$ 7.2*
48	8	363.2 $\pm$ 6.8	312.3 $\pm$ 5.4*

Results are means $\pm$ SEM. * $p < 0.01$, compared to the same age WKY

Fig. 1. Western blot analysis showed Cx45 protein levels in RIA of 12-wk old SHR and WKY rats. Results are means $\pm$ SEM, n=6/group. * $p < 0.05$, compared to WKY rats. Cardiac muscle was used as a positive control.

Fig. 2. The electrical coupling of in-situ VSMCs in renal interlobar artery (RIA) segment of 12-wk old SHR and WKY rats. A) Whole-cell current traces induced by voltage steps from the holding potential at -40 mV in 20 mV increments on in-situ RIA VSMCs of WKY rats and SHR. Expanded time scale of the initial part of currents (red tracing) showed that a single term exponential function fitted poorly ($r < 0.90$) with the transient current (black thin line). B) Gap junction inhibitor 18β-GA (100 μM) caused significant reduction in the steady-state current (Iss). Expanded time scale of the initial part of currents (red tracing) showed that a single term exponential function fitted well ($r > 0.98$) after the treatment with 18β-GA. “ τ ” is the time constant of the bottom trace.

(18β-GA) caused significant reduction in the steady-state current (Figure 2B). Expanded time scale of the initial part of currents showed that a single term exponential function fitted well after the treatment with 18β-GA.

The results of R_{input} , G_{input} , C_{input} and τ values of in-situ and dispersed RIA VSMCs are summarized in Table 3. The R_{input} value of in-situ VSMCs in SHR rats was lower than that of WKY rats, but the G_{input} , C_{input} and τ values of in-situ VSMCs in SHR rats were greater than those of VSMCs in WKY rats, respectively. The R_{input} values of the dispersed VSMCs of SHR and WKY rats were 28 and 8 times greater than those of the in-situ VSMCs of SHR and WKY rats, respectively. The G_{input} , C_{input} and τ values of in-situ VSMCs were 20, 32, 41 and 9, 17, 28 times greater than those of the dispersed VSMCs of SHR and WKY rats, respectively. The R_{input} , G_{input} , C_{input} and τ values of the dispersed VSMCs of SHR rats were the same of the dispersed VSMCs of WKY rats. These results suggested a tighter coupling in the recording circuit in RIA of SHR compared to that of WKY rats, further supporting the tighter electrical coupling among VSMCs in RIA of SHR.

Vasoconstrictive response to vasoconstrictors of RIAs of SHR and WKY rats

To test the vasoconstrictive responses to KCl or PE, RIAs of SHR and WKY rats were stimulated with KCl (from 10 to 100 mM) or PE (from 0.1 to 30 μM). The myographic measurement showed that the vasoconstrictive responses of RIA to either KCl or PE were greater in SHR than that of WKY rats (Figure 3). The EC_{50} value of PE stimulation was significantly smaller in SHR ($EC_{50} = 0.37$ μM) than that in WKY rats ($EC_{50} = 0.49$ μM) ($p < 0.05$, $n = 10$ /group), but the EC_{50} value of KCl stimulation was not significantly different between SHR ($EC_{50} = 31.3$ μM) and WKY rats ($EC_{50} = 38.1$ μM) ($p > 0.05$, $n = 10$ /group).

Pre-treated RIA with gap junction inhibitor 18β-GA for 20 min inhibited the KCl- or PE-

Table 3. Whole cell membrane properties of renal interlobar artery (RIA) vascular smooth muscle cells (VSMCs) of SHR and WKY rats at 12 weeks of age

Membrane property		<i>in situ</i> VSMCs (n=8)	dispersed VSMCs (n=10)
WKY	R_{input} (M Ω) ^a	352±79**	2865±229
	G_{input} (nS) ^a	3.37±0.98*	0.37±0.04
	C_{input} (pF) ^b	168±26*	9.7±0.8
	τ (ms) ^b	5.5±0.6**	0.35±0.03
SHR	R_{input} (M Ω) ^a	97±17**#	2713±207
	G_{input} (nS) ^a	7.9±1.1**#	0.39±0.04
	C_{input} (pF) ^b	369±61**#	11.32±0.62
	τ (ms) ^b	15.4±0.5**#	0.37±0.07

Results are means±SEM. * $p < 0.05$ and ** $p < 0.01$, compare to dispersed VSMCs. # $p < 0.05$ and ## $p < 0.05$, compared to WKY.
^a Measured from -60 and -40 mV. ^b Measured from -40 to -100 mV

Fig. 3. Effects of gap junction inhibitor 18 β -glycyrrhetinic acid (18 β -GA) on vasoconstriction induced by A) KCl or B) phenylephrine (PE) of renal interlobar artery (RIA) of 12-wk old SHR and WKY rats. The inhibitory effects of 18 β -GA inhibitory on KCl and PE-induced vasoconstriction were greater in SHR. The level of contraction was normalized by value of WKY rats as 1. Results are means±SEM, n=10 RIA segments/group. * $p < 0.05$, compared to respective WKY groups; # $p < 0.05$, effects of 18 β -GA between SHR and WKY rats.

induced vasoconstriction and right shifted the concentration-dependent responsive curves. The inhibitory effects of 18 β -GA on KCl- or PE-induced vasoconstriction were greater in SHR than that in WKY rats (Figure 3). After 18 β -GA treatment, the EC_{50} value of PE stimulation was significantly greater in SHR (EC_{50} =1.02 μ M) than that in WKY rats (EC_{50} =0.63 μ M) ($p < 0.05$, n=10/group), but the EC_{50} value of KCl stimulation was not significantly different between SHR (EC_{50} =41.7 μ M) and WKY rats (EC_{50} =38.2 μ M) ($p > 0.05$, n=10/group).

Age-dependent vasoconstrictive responses to KCl or PE in SHR and WKY rats

We also measured the vasoconstrictive responses to KCl or PE in RIAs of SHR and WKY rats at different age. Dose-response to KCl (from 10 to 100 mM) or PE (from 0.1 to 30 μ M) stimulation elicited concentration-dependent contraction in the RIA of SHR and WKY rats

Fig. 4. The KCl- or PE-induced vasoconstriction of renal interlobar artery (RIA) of SHR (A and C) and WKY (B and D) rats at different age. The level of contraction was normalized by the value of respective 4-wk group as 1. Results are means $\pm$ SEM, $n=10$ RIA segments/group. * $p < 0.05$, comparison between 4-wk and 12-wk groups; # $p < 0.05$, comparison between 4-wk and 48-wk groups; \$ $p < 0.05$, comparison between 12-wk and 48-wk groups.

at different ages (Figure 4). The vasoconstrictive responses to KCl or PE were significant different at 4, 12 and 48 weeks of age in both SHR and WKY rats. At 12-wk of age, the KCl- or PE-stimulated contraction of RIAs was greater than that at 4- and 48-wk of age in both WKY and SHR ($p < 0.05$, $n=10$ /group). The KCl- and PE-stimulated contraction was the same between 4- and 48-wk of age in both WKY and SHR rats. The peak constrictive responses of RIAs to KCl or PE were significantly greater in SHR compared to those in age-matched WKY rats ($p < 0.05$, $n=10$ /group).

Expression of Cx45 in RIAs of SHR and WKY rats at different age

Western blot analysis showed that the expression of Cx45 in RIA was significantly different at different age. Cx45 expression was significantly increased from 4 to 12 weeks and then decreased from 12 to 48 wks of age (Figure 5). RIAs of SHR had higher Cx45 protein levels than that in WKY rats at all ages studied.

Discussion

Hypertension is a major risk factor for the development of cardiovascular disease and chronic renal disease [33]. Majority of hypertensive cases are classified as essential or primary hypertension. Essential hypertension is considered to be originated from the interaction between genetic and environmental factors, and is characterized by an increase in blood pressure together with peripheral vascular resistance [33, 34]. Both human and hypertensive animal models are associated with abnormal changes in remodeling of vascular wall and vascular activity [34]. The changes in vascular structure result from hypertrophy or a rearrangement of the smooth muscle layers, which are associated with reducing in the production of vasodilatory factors [34]. Recent studies demonstrate that gap junctions among VSMCs can affect vascular tone by regulate vasomotor activity, and play a

key role in the development of hypertension [16, 34]. Several studies have indicated that the abnormal Cxs expression are associated with the development of hypertension [6]. Deletion of the Cx40 or the Cx45 genes or altered Cxs expression in mice results in hypertension and hyperreninemia [15]. Besides the effects on vasoconstriction, high expression of Cx45 in ECs and VSMCs is required for normal vascular development during embryogenesis [35]. Global knockout of Cx45 in mice causes vascular malformations and is embryonic lethal [36]. Animals with Cx45-deficiency fail to form a smooth muscle layer surrounding the artery [16]. These findings demonstrated the Cx45-dependent gap junctional coupling among vascular cells are essential for maturation of blood vessels. Since gap junctions play a critical role in the propagation of vasoconstriction [16, 34], in this study, we investigate the effect of changes in gap junction protein expression and properties of gap junction in VSMCs of RIAs on the development of hypertension in SHRs.

The principal findings of our study are summarized as: 1) the membrane properties of VSMCs of RIA were significantly difference between SHRs and WKY rats, the membrane C_{input} and G_{input} were increased and R_{input} was decreased in SHRs; 2) compare to WKY rats, the vasoconstrictive responses of RIA by KCl and PE were significantly greater in SHRs, 18 β -GA treatment inhibited the vasoconstrictive response to KCl and PE, and the inhibitory effect of 18 β -GA was greater in SHRs than in WKY rats; 3) the age-dependent vasoconstrictive responses to KCl and PE was greater in SHRs than in WKY rats. In both SHR and WKY rats, 12-wk old animals had the strongest responses to KCl or PE stimulation; and 4) a significant up-regulation of Cx45 protein expression was observed in 12-wk-old SHR and WKY rats compared to that in 4- and 48-wk-old rats. The expression of Cx45 protein levels in RIA of SHR was significantly higher than that of WKY rats. These findings suggest that enhanced

Fig. 5. Western-blot analysis of Cx45 protein expression in renal interlobar arteries (RIAs) of SHR and WKY rats at 4-, 12- and 48-weeks of age. Results are means $\pm$ SEM, n=3/group. * p < 0.05 and ** p < 0.01, compared to the same age of WKY; # p < 0.05, compared to respective 4-wk groups.

expression of Cx45 and gap junction communication among VSMCs in RIA of SHR may contribute the development of hypertension in SHR.

Gap junctions regulates vasomotor tone and vasomotor activity [21, 37]. When there is local stimulation of small arteries or arterioles, the gap junctions can spread the contractive signals along the vessels by several millimeters [22, 23]. Vascular wall is mainly made up of VSMCs and endothelial cells, they work together to coordinate contraction of blood vessels. The gap junction could ensure the signal transmission rate, to make the blood vessels quickly and unified contraction or relaxation. When the body is in pathological states or during aging, the structure, function and expression of gap junction will change, which will cause the synchronization vasomotor activity obstacles of the vascular wall cells [38].

In the current study, whole-cell recordings of VSMCs still embedded in RIA were used to measure gap junction communication. This novel technique allowed us to determine the function of gap junctions among VSMCs under a more physiologically condition (compared to dispersed VSMCs) [39, 40]. Table 3 showed that the G_{input} and C_{input} of in-situ VSMCs were greater than those of dispersed VSMCs, suggesting a tight electrical coupling among VSMCs in the recording circuit in vivo. Further, the G_{input} and C_{input} were greater in SHR versus WKY rats, suggesting a tighter electrical coupling among VSMCs in RIA of SHR versus WKY rats, supporting the results in Figure 2. The coupling between various cell types, the number of junctional channels and the channel opening probability are modulated by multiple mechanisms, including the synthesis of Cxs, the intracellular trafficking of Cx proteins, the assembly of Cxs into plasma membrane, and the turnover rate of Cx proteins [10]. Recent study has shown that the coupling between nephrons is increased in SHR, suggesting that changes in nephron coupling may be associated with the development of hypertension [41]. The enhanced coupling between VSMCs and ECs has also been observed in SHR [23]. The greater VSMC-EC coupling has been assumed to be a compensatory mechanism to maintain blood pressure regulation through the endothelium-derived hyperpolarizing factor (EDHF) signaling in this hypertensive model [16]. Thus, increased electrical coupling in RIA artery of SHR may be accompanied by tighter VSMC-EC gap junctions that formed by Cx40 and Cx43.

The time course of the current transients during the voltage stepping fit well with a single-term exponential function in the dispersed VSMCs, however, fit poorly with a single-term exponential function in the in-situ VSMCs, indicating multiple membrane sources in the circuit or an electrical coupling of multiple cells in-situ. Increased electrical coupling among VSMCs of SHR resulted in longitudinal electrical conduction accompanied by membrane potential changes, and produces more uniform contractile responses. Change in membrane potential results in the opening of voltage-sensitive Ca^{2+} channels, and this in turn enhanced vasomotor tone over vessel segments within several millimeters in length [16]. ATP induces Ca^{2+} mobilization in neighboring cells via hemichannels, which may stimulate purinergic receptors on cells and give rise to a propagated Ca^{2+} wave [6]. Therefore, increased expression of Cx45 may lead to enhanced Ca^{2+} wave and secretion of ATP in RIAs of SHR, thus results in increasing in vascular tone and hypertension. Hypertension can lead to hypertrophy of VSMCs [42], and the C_{input} of a cell is positively correlated with the cell size. Although the C_{input} of in-situ VSMCs of SHR was much higher than that of WKY rats, the C_{input} of a single VSMC of SHR was the same as that of a single VSMC of WKY rat, suggesting that the hypertrophy of VSMCs had little effect on gap junction communication.

RIAs of both SHR and WKY rats showed a dose-dependent vasoconstrictive response to KCl or PE stimulation. The contractive response stimulated by KCl and PE was greater in SHR than in WKY rats. Previous studies have shown that there are differences in vascular function between SHR and WKY rats [31]. KCl-stimulated vascular contraction was enhanced in preglomerular vessels of SHR compared to normotensive rats [43]. Early studies in our laboratory have demonstrated contractive response of mesenteric artery was significantly increased in SHR compared to that in WKY rats [31]. Combined with the results of the whole-cell patch-clamp measurement, our current findings suggest that the increased gap junction communication was involved in the greater vasoconstrictive response and the development

of hypertension in SHR. 18β -GA also can interfere with Ca^{2+} wave propagation [6], which is consistent with the inhibitory effect of 18β -GA on the contractive response stimulated by KCl and PE was greater in SHRs than in WKY rats. It has been shown that pretreatment with 18β -GA has hepatoprotective effects through attenuation of oxidative stress, inflammation and hyper-proliferation, which imply that 18β -GA may have therapeutic effect on hypertension by anti-inflammation and by inhibition of VSMC hyper-proliferation [44].

Many experiments have demonstrated that gap junction proteins were expressed in VSMCs in various vascular beds [45-47]. In this study, we showed that Cx45 protein was expressed in RIAs of SHR and WKY rats. The levels of Cx45 were significantly higher in SHR than in WKY rats. Xing Li and J. Marc Simard found that Cx45 expression is increased in cerebral arterial VSMCs of SHR [17, 48, 49]. In DOCA-salt, Goldblatt 2 kidney-1 clip (2K1C) and NO synthase inhibition models of hypertension, the blood pressure, increased angiotensin II and reduced NO are associated with the change in vascular Cx protein levels [50]. The function and turnover of gap junction is regulated by its interaction with many proteins, including protein kinases and phosphatases [51]. Interaction among Cxs may triggered alteration of the expression of themselves [51]. In this study, the increased Cx45 expression in RIA of SHR may result from reduced phosphorylation of Cx45 and altered interaction between Cx45 and Cx-associated proteins such as ZO-1 [12], which could prolonged the turnover rate of Cx45 [11], thus resulted in changes in activity of other Cxs in renal vasculature, and enhanced vasoconstriction and electrical coupling in RIA of SHR.

Our study indicated that propagated vasoconstriction in response to local application of KCl was inhibited in mesenteric arterioles of Cx37 knockout mice [6]. Study of Schmidt et al. demonstrated that the amplitude of K^{+} initiated endothelium-independent vasoconstriction declines in Cx45 deficient mice [32]. In the current study, we found that there are age-dependent changes in vasoconstrictive responses to KCl or PE in SHR and WKY rats. Higher blood pressure was associated with increase in expression of Cx45 in RIAs of SHR at 12-wk of age compared to that of WKY rats at the same age, which might contribute to greater vasoconstriction when RIAs were stimulated by KCl or PE. These results suggested that Cxs are dispensable for the conduction of vasomotor responses and regulation of blood pressure. To date, few data are available on the vasoconstrictive effect of Cx45 in humans. A recent study shows that there was a remarkable resemblance of the distribution of Cx37, Cx40, Cx43 and Cx45 between the human and rodent kidneys [7, 13], suggesting that the expression of vascular Cxs in kidney may be rather conserved among the mammalian species [7]. In future studies, we will investigate whether this phenomenon is correlated with the expression and function of gap junction.

Conclusion

Our current study demonstrated that the hypertension-induced elevation of Cx45 may affect communication between VSMCs and coupling between VSMCs and ECs, which resulted in enhancement of vasoconstrictive response in renal artery and contributed to the development of hypertension.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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References

- 1 Rettig R, Stauss H, Folberth C, Ganten D, Waldherr B, Unger T: Hypertension transmitted by kidneys from stroke-prone spontaneously hypertensive rats. *Am J Physiol* 1989;257:F197-203.
- 2 Patschan O, Kuttler B, Heemann U, Uber A, Rettig R: Kidneys from normotensive donors lower blood pressure in young transplanted spontaneously hypertensive rats. *Am J Physiol* 1997;273:R175-180.
- 3 Brisset AC, Isakson BE, Kwak BR: Connexins in vascular physiology and pathology. *Antioxid Redox Signal* 2009;11:267-282.
- 4 Kurtz A: Renal connexins and blood pressure. *Biochim Biophys Acta* 2012;1818:1903-1908.
- 5 Wagner C: Function of connexins in the renal circulation. *Kidney Int* 2008;73:547-555.
- 6 Hanner F, Sorensen CM, Holstein-Rathlou NH, Peti-Peterdi J: Connexins and the kidney. *Am J Physiol Regul Integr Comp Physiol* 2010;298:R1143-1155.
- 7 Kurtz L, Madsen K, Kurt B, Jensen BL, Walter S, Banas B, Wagner C, Kurtz A: High-level connexin expression in the human juxtaglomerular apparatus. *Nephron Physiol* 2010;116:p1-8.
- 8 Rackauskas M, Verselis VK, Bukauskas FF: Permeability of homotypic and heterotypic gap junction channels formed of cardiac connexins mCx30.2, Cx40, Cx43, and Cx45. *Am J Physiol Heart Circ Physiol* 2007;293:H1729-1736.
- 9 Brücher BL, Jamall IS: Cell-cell communication in the tumor microenvironment, carcinogenesis, and anticancer treatment. *Cell Physiol Biochem* 2014;34:213-243.
- 10 Herve JC, Derangeon M, Bahbouhi B, Mesnil M, Sarrouilhe D: The connexin turnover, an important modulating factor of the level of cell-to-cell junctional communication: comparison with other integral membrane proteins. *J Membr Biol* 2007;217:21-33.
- 11 Giepmans BN: Gap junctions and connexin-interacting proteins. *Cardiovasc Res* 2004;62:233-245.
- 12 Laing JG, Koval M, Steinberg TH: Association with ZO-1 correlates with plasma membrane partitioning in truncated connexin45 mutants. *J Membr Biol* 2005;207:45-53.
- 13 Takenaka T, Inoue T, Kanno Y, Okada H, Meaney KR, Hill CE, Suzuki H: Expression and role of connexins in the rat renal vasculature. *Kidney Int* 2008;73:415-422.
- 14 Kurtz L, Gerl M, Kriz W, Wagner C, Kurtz A: Replacement of connexin 40 by connexin 45 causes ectopic localization of renin-producing cells in the kidney but maintains in vivo control of renin gene expression. *Am J Physiol Renal Physiol* 2009;297:F403-409.
- 15 Schweda F, Kurtz L, de Wit C, Janssen-Bienhold U, Kurtz A, Wagner C: Substitution of connexin40 with connexin45 prevents hyperreninemia and attenuates hypertension. *Kidney Int* 2009;75:482-489.
- 16 Figueroa XF, Duling BR: Gap junctions in the control of vascular function. *Antioxid Redox Signal* 2009;11:251-266.
- 17 Figueroa XF, Isakson BE, Duling BR: Vascular gap junctions in hypertension. *Hypertension* 2006;48:804-811.
- 18 Muraki K, Watanabe M, Imaizumi Y: Nifedipine and nisoldipine modulate membrane potential of vascular endothelium via a myo-endothelial pathway. *Life Sci* 2000;67:3163-3170.
- 19 Figueroa XF, Paul DL, Simon AM, Goodenough DA, Day KH, Damon DN, Duling BR: Central role of connexin40 in the propagation of electrically activated vasodilation in mouse cremasteric arterioles in vivo. *Circ Res* 2003;92:793-800.
- 20 Looft-Wilson RC, Payne GW, Segal SS: Connexin expression and conducted vasodilation along arteriolar endothelium in mouse skeletal muscle. *J Appl Physiol* (1985) 2004;97:1152-1158.

- 21 Segal SS, Duling BR: Propagation of vasodilation in resistance vessels of the hamster: development and review of a working hypothesis. *Circ Res* 1987;61:II20-25.
- 22 Gustafsson F, Holstein-Rathlou NH: Angiotensin II modulates conducted vasoconstriction to norepinephrine and local electrical stimulation in rat mesenteric arterioles. *Cardiovasc Res* 1999;44:176-184.
- 23 Wagner AJ, Holstein-Rathlou NH, Marsh DJ: Internephron coupling by conducted vasomotor responses in normotensive and spontaneously hypertensive rats. *Am J Physiol* 1997;272:F372-379.
- 24 Dilley JR, Arendshorst WJ: Enhanced tubuloglomerular feedback activity in rats developing spontaneous hypertension. *Am J Physiol* 1984;247:F672-679.
- 25 Holstein-Rathlou NH, Leyssac PP: TGF-mediated oscillations in the proximal intratubular pressure: differences between spontaneously hypertensive rats and Wistar-Kyoto rats. *Acta Physiol Scand* 1986;126:333-339.
- 26 Persson AE, Boberg U: Renal abnormalities in experimental models of hypertension: the SHR versus the Milan HR. *J Cardiovasc Pharmacol* 1988;12:S27-35.
- 27 Dilley JR, Stier CT, Jr., Arendshorst WJ: Abnormalities in glomerular function in rats developing spontaneous hypertension. *Am J Physiol* 1984;246:F12-20.
- 28 Chen YM, Yip KP, Marsh DJ, Holstein-Rathlou NH: Magnitude of TGF-initiated nephron-nephron interactions is increased in SHR. *Am J Physiol* 1995;269:F198-204.
- 29 Li XZ, Ma KT, Guan BC, Li L, Zhao L, Zhang ZS, Si JQ, Jiang ZG: Fenamates block gap junction coupling and potentiate BKCa channels in guinea pig arteriolar cells. *Eur J Pharmacol* 2013;703:74-82.
- 30 Li L, Wang R, Ma KT, Li XZ, Zhang CL, Liu WD, Zhao L, Si JQ: Differential effect of calcium-activated potassium and chloride channels on rat basilar artery vasomotion. *J Huazhong Univ Sci Technolog Med Sci* 2014;34:482-490.
- 31 Ma KT, Li XZ, Li L, Jiang XW, Chen XY, Liu WD, Zhao L, Zhang ZS, Si JQ: Role of gap junctions in the contractile response to agonists in the mesenteric artery of spontaneously hypertensive rats. *Hypertens Res* 2014;37:110-115.
- 32 Schmidt VJ, Jobs A, von Maltzahn J, Worsdorfer P, Willecke K, de Wit C: Connexin45 is expressed in vascular smooth muscle but its function remains elusive. *PLoS One* 2014;7:e42287.
- 33 Singh MV, Chapleau MW, Harwani SC, Abboud FM: The immune system and hypertension. *Immunol Res* 2014;59:243-253.
- 34 Rummery NM, Hill CE: Vascular gap junctions and implications for hypertension. *Clin Exp Pharmacol Physiol* 2004;31:659-667.
- 35 Fang JS, Dai C, Kurjiaka DT, Burt JM, Hirschi KK: Connexin45 regulates endothelial-induced mesenchymal cell differentiation toward a mural cell phenotype. *Arterioscler Thromb Vasc Biol* 2013;33:362-368.
- 36 Kruger O, Plum A, Kim JS, Winterhager E, Maxeiner S, Hallas G, Kirchhoff S, Traub O, Lamers WH, Willecke K: Defective vascular development in connexin 45-deficient mice. *Development* 2000;127:4179-4193.
- 37 Looft-Wilson RC, Haug SJ, Neuffer PD, Segal SS: Independence of connexin expression and vasomotor conduction from sympathetic innervation in hamster feed arteries. *Microcirculation* 2004;11:397-408.
- 38 Azzam EI, de Toledo SM, Little JB: Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells. *Proc Natl Acad Sci USA* 2001;98:473-478.
- 39 Guan BC, Si JQ, Jiang ZG: Blockade of gap junction coupling by glycyrrhetic acids in guinea pig cochlear artery: a whole-cell voltage- and current-clamp study. *Br J Pharmacol* 2007;151:1049-1060.
- 40 Ma KT, Guan BC, Yang YQ, Nuttall AL, Jiang ZG: 2-Aminoethoxydiphenyl borate blocks electrical coupling and inhibits voltage-gated K⁺ channels in guinea pig arteriole cells. *Am J Physiol Heart Circ Physiol* 2011;300:H335-346.
- 41 Arensbak B, Mikkelsen HB, Gustafsson F, Christensen T, Holstein-Rathlou NH: Expression of connexin 37, 40, and 43 mRNA and protein in renal preglomerular arterioles. *Histochem Cell Biol* 2001;115:479-487.
- 42 Lacolley P, Regnault V, Nicoletti A, Li Z, Michel JB: The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. *Cardiovasc Res* 2012;95:194-204.
- 43 Yamamoto Y, Fukuta H, Nakahira Y, Suzuki H: Blockade by 18beta-glycyrrhetic acid of intercellular electrical coupling in guinea-pig arterioles. *J Physiol* 1998;511:501-508.

- 44 Hasan S, Khan R, Ali N, Khan A, Rehman M, Tahir M, Lateef A, Nafees S, Mehdi S, Rashid S, Shahid A, Sultana S: 18-beta Glycyrrhetinic acid alleviates 2-acetylaminofluorene-induced hepatotoxicity in Wistar rats: Role in hyperproliferation, inflammation and oxidative stress. *Hum Exp Toxicol* 2014, in press.
- 45 de Wit C, Griffith TM: Connexins and gap junctions in the EDHF phenomenon and conducted vasomotor responses. *Pflugers Arch* 2010;459:897-914.
- 46 Yamamoto Y, Klemm MF, Edwards FR, Suzuki H: Intercellular electrical communication among smooth muscle and endothelial cells in guinea-pig mesenteric arterioles. *J Physiol* 2001;535:181-195.
- 47 Yamazaki J, Kitamura K: Intercellular electrical coupling in vascular cells present in rat intact cerebral arterioles. *J Vasc Res* 2003;40:11-27.
- 48 McGuire PG, Twietmeyer TA: Aortic endothelial junctions in developing hypertension. *Hypertension* 1985;7:483-490.
- 49 Li X, Simard JM: Increase in Cx45 gap junction channels in cerebral smooth muscle cells from SHR. *Hypertension* 2002;40:940-946.
- 50 Haefliger JA, Demotz S, Braissant O, Suter E, Waeber B, Nicod P, Meda P: Connexins 40 and 43 are differentially regulated within the kidneys of rats with renovascular hypertension. *Kidney Int* 2001;60:190-201.
- 51 Figueroa XF, Isakson BE, Duling BR: Connexins: gaps in our knowledge of vascular function. *Physiology (Bethesda)* 2004;19:277-284.