Enhanced expression of Cx43 and gap junction communication in vascular smooth muscle cells of spontaneously hypertensive rats

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Abstract. Niflumic acid (NFA) is a novel gap junction (GJ) inhibitor. The aim of the present study was to investigate the effect of NFA on GJ communication and the expression of connexin (Cx) in vascular smooth muscle cells (VSMCs) of mesenteric arterioles of spontaneously hypertensive rats (SHR). Whole-cell patch clamp recording demonstrated that NFA at $1x10^{-4}$ M significantly inhibited the inward current and its effect was reversible. The time for charging and discharging of cell membrane capacitance (C_{input}) reduced from 9.73 to 0.48 ms (P<0.05; n=6). Pressure myograph measurement showed that NFA at $3x10^{-4}$ M fully neutralized the contraction caused by phenylephrine. The relaxation responses of normotensive control Wistar Kyoto (WKY) rats were significantly higher, compared with those of the SHRs (P<0.05; n=6). Western blot and reverse transcription-quantitative polymerase chain reaction analyses showed that the mRNA and protein expression levels of Cx43 of the third-level branch of mesenteric arterioles of the SHRs and WKY rats were higher, compared with those of the first-level branch. The mRNA and protein expression levels of Cx43 of the primary and third-level branches of the mesenteric arterioles in the SHRs were higher, compared with those in the WKY rats (P<0.05; n=6). The mRNA levels of Cx43 in the mesenteric arterioles were significantly downregulated by NFA in a concentration-dependent manner (P<0.01; n=6). The protein levels of Cx43 in primary cultured VSMCs isolated from the mesenteric arterioles were also significantly downregulated by NFA in a concentration-dependent manner (P<0.01; n=6). These results showed that the vasorelaxatory effects of GJ inhibitors were reduced in the SHRs, which was associated with a higher protein expression level of Cx43 in the mesenteric arterioles of the SHRs. NFA also relaxed the mesenteric arterioles by reducing the expression of Cx43, which decreased blood pressure. Therefore, regulation of the expression of GJs may be a therapeutic target for the treatment of hypertension.

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Introduction

Hypertension is the most common risk factor for cardiovascular diseases (1). One of the causes of increasing blood pressure is the continuous contraction or spasm of systemic arterioles. The gap junctions (GJs) formed by connexin (Cx) protein subunits serve as a direct pathway to enable the exchange of information between cells and provides important channels for intercellular communication (2). Alterations of GJs are closely associated with changes in vascular tone (3,4). Four types of Cx proteins, Cx37, Cx40, Cx43 and Cx45, are expressed on blood vessels (5). The distribution of Cx proteins depends on the size and location of the blood vessels, and the species of animal. Cx43 is the most abundant Cx protein in vascular smooth muscle cells (VSMCs) (6,7). Generally, GJs are constantly expressed in specific vascular beds. Changes in the number and function of GJs can alter vascular tone and blood pressure (8-10).

GJ communication is essential for regulating cardiovascular function. Several studies have shown that changes in the expression of Cxs lead to abnormal function of GJ communication, which can induce hypertension, atherosclerosis and a number of other vascular diseases (11-13). Cx43 has been regarded as a pressure receptor, which is sensitive to hemodynamic changes (14). The expression of Cx43 is upregulated on

the aorta of spontaneously hypertensive rats (SHR) and renal hypertensive rats (15,16). Increased expression of Cx43 may be an adaptation to the changes occurring in the remodeling of vascular structure, which further demonstrates that alteration in the expression of Cx43 is closely associated with the development of hypertension (17,18).

The application of GJ inhibitors can be crucial for the treatment of vascular-associated diseases (19). The expression of Cx43 is upregulated in different hypertensive states. Treatment with GJ inhibitors can further determine whether the upregulated expression of Cx43 is important in hypertension. 18β-glycyrrhetintic acid (18β-GA) is a GJ inhibitor, which can non-selectively inhibit GJs between VSMCs and endothelial cells of small arterioles (20). Heptanol is a GJ inhibitor, which is commonly used in electrophysiological experiments to inhibit GJ and increase axial resistance between myocytes, and has been shown to decrease the mRNA and protein expression levels of Cx43 (21-23). Niflumic acid (NFA) is a novel reversible GJ inhibitor. A study by Harks *et al* (24) indicated that NFA can regulate GJ communications. However, the mechanisms underlying how NFA regulates GJ communication remain to be fully elucidated. The present study investigated the effects of NFA on GJ communication at the transcription and translation level in mesentery arterioles and VSMCs. The effects of NFA on systolic and diastolic function, and the expression of Cx43 in mesenteric arterioles were investigated in SHRs and normotensive control Wistar Kyoto (WKY) rats. The current study also provide evidence that GJ may serve as potential therapeutic target for regulation of vascular tone and inhibition of blood pressure elevation by altering GJ activities and expression.

Materials and methods

Animals. Male SHR and WKY rats (~250 g; 20-week-old; n=18 per group) obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA) were used in the present study. Laboratory conditions were maintained at constant humidity (60±5%), temperature (24±1˚C) and light cycle (6.00 a.m-6.00 p.m.) and fed a standard rat pellet diet *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee at Shihezi University Medical College (Shihezi, China) and were consistent with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MA, USA). The blood pressure of the rats was measured using a BP-6 non-invasive blood pressure monitor system.

Whole-cell patch clamp. The rats were anesthetized and then sacrificed by exsanguination. The anesthesia was performed by intramuscular injection of a mixture (1 ml/kg) of ketamine (500 mg), xylazine (20 mg) and acepromazine (10 mg) in 8.5 ml H_2O. The mesenteric arterioles were rapidly removed and pinned in a dissecting dish filled with physiological saline solution (PSS) containing NaCl (138.0 mM), KCl (5.0 mM), $CaCl_2$ (1.6 mM), $MgCl_2$ (1.2 mM), Na-HEPES (5.0 mM), HEPES (6.0 mM) and glucose (7.5 mM). The arteries were digested in PSS containing collagenase A (1.5 mg/ml, Roche, Mannheim, Germany) for 15 min at 37˚C, and then returned to the PSS. Vascular connective tissue was further removed under a microscope and the vascular segment was transferred to an inverted phase contrast microscope (Olympus Corporation, Tokyo, Japan) for whole-cell patch clamp recording using an Axon 700B amplifier (Molecular Devices, LLC, Sunnyvale, CA, USA) at room temperature. The recording pipettes were pulled with a P-97 pipette puller (Sutter Instrument Co., Novato, CA, USA) and had a resistance of 3-5 MΩ. The pipette was filled with an internal solution containing K-gluconate (130.0 mM), NaCl (10.0 mM), $CaCl_2$ (2.0 mM), $MgCl_2$ (1.2 mM), HEPES (10.0 mM), EGTA (5.0 mM) and glucose (7.5 mM), and contacted with cells with negative pressure. The seal resistance typically reached 1-20 GΩ before rupture of the membrane. Membrane rupture was achieved by a high-frequency buzz current and/or suction pressure from the pipette. The transient current over the membrane input capacitance (C_{input}) was routinely uncompensated to monitor and calculate the access resistance and the membrane parameters on-line or off-line. The drug perfusion device was applied to the whole-cell patch clamp recording, as described previously (25).

Pressure myograph system. Mesenteric arterioles with a diameter <300 μm from the SHRs and WKY rats were used in the present study. The vascular segment was pinned in a dissecting dish filled with solution containing NaCl (118.9 mM), KCl (4.69 mM), $MgSO_4 \cdot 7H_2O$ (1.17 mM), KH_2PO_4 (1.18 mM), $CaCl_2$ (2.5 mM), $NaHCO_3$ (25.0 mM), EDTA (0.26 mM) and glucose (5.5 mM). The initial transmural pressure was set at 20 mmHg at the import end and 5 mmHg at the export end using a pressure myograph system, and allowed to equilibrate for 3 min to wash away residual blood. The segment was pressurized with a 10 mmHg stepwise increase in transmural pressure to 60 mmHg, with 5 min of equilibration at each pressure. The vessel was stabilized for 1 h once the intraluminal pressure reached 60 mmHg and the bath solution was replaced every 20 min. Phenylephrine (PE; $1x10^{-5}$ M) was then added to the arteriole to stimulate contraction. On reaching maximal contraction, acetylcholine ($1x10^{-3}$ M) was added to relax the vessel. If the dilatation rate was >70%, indicating the integrity of the endothelium, the vessel was used for experiments, as described previously (26,27). To perform the experiment, $1x10^{-5}$ M PE was added to pre-contract the vessel, following which NFA (10^{-5}, $3x10^{-5}$, $5x10^{-5}$, 10^{-4}, $3x10^{-4}$ M) was successively added. The vasomotor responses to PE and NFA were recorded. In order to investigate the effect of the large conductance calcium-activated potassium channels (BK_{Ca}) on vasodilation, following pre-contraction of the blood vessel with PE, the vessel was incubated with tetraethylammonium (TEA; $1x10^{-3}$ M). NFA ($3x10^{-5}$ M) was then added to observe the vasodilator response.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was isolated from mesenteric arterioles using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) from WKY and SHR rats. To remove genomic DNA, extracted RNA was digested with DNase I (Promega Corporation, Madison, WI, USA), followed by first-strand cDNA synthesis using 500 g total RNA, oligo(dT)16 primers and M-MLV reverse transcriptase according to the manufacturer's protocol (Promega Corporation). To analyze

gene expression, the Cx43 cDNA was quantified and normalized using GAPDH as a reference gene. All semi-quantitative PCR amplification were performed in a total volume of 25 μl under the following PCR conditions in a thermal cycler (TP650; Takara Biotechnology Co., Ltd., Dalian, China): 94˚C for 5 min, followed by 94˚C for 40 sec, 58˚C for 40 sec, and finally 72˚C for 10 min for 26 (GAPDH) or 30 (CX42) cycles, respectively. The rat Cx43 genome sequence was obtained from GenBank (GenBank accession no. NM_012567.2). The following gene-specific primers were used: Cx43, forward 5'-aagttagacgaagtccacgtagag-3', reverse 5'-aaccacaga gagcgaaacttgtag-3'; GAPDH, forward 5'-caaggtcatccatgacaa ctttg-3', reverse 5'-gtccaccaccctgttgctgtag-3'. PCR products were separated by agarose gel (1.0%) electrophoresis and observed by ethidium bromide staining. Semi-quantitative analysis was performed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following scanning of agarose gel electrophoretic images.

VSMC culture. The mesenteric arterioles from the SHRs were rapidly dissected and transferred into a petri dish with Krebs solution containing NaCl (138.0 mM), KCl (5.0 mM), $CaCl_2$ (1.6 mM), $MgCl_2$ (1.2 mM), Na HEPES (5.0 mM), HEPES (6.0 mM) and glucose (7.5 mM) at pH 7.4. The perivascular adipose tissue and residual blood were cleaned out using a fine forceps under a microscope and the shredded vessel was digested using Krebs solution with 0.1% collagenase. Following centrifugation at 168 x *g* for 5 min at 4˚C, the precipitated cells ($5x10^5$ cells/ml) were plated in dishes with culture medium containing 20% fetal bovine serum. As a marker of VSMCs, α-smooth muscle actin (α-SMA) was determined using immunohistochemistry.

Western blot analysis. The mesenteric arterioles were homogenized in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Inc.) (ratio of 10 mg tissue to 100 μl RIPA buffer) with freshly added protease inhibitor, phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The homogenates were incubated at 4˚C for 30 min and centrifuged at 12,000 x *g* for 15 min at 4˚C. The supernatant was collected, and the protein concentration in the supernatant was determined using a bicinchoninic acid assay. Protein aliquots (40 μg/lane) were separated by Tris-glycine denaturing gradient gel electrophoresis on a 10% SDS-PAGE gel. The proteins were then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBS-Tween buffer (pH 8.0, 10 mmol/l Tris-HCl, 150 mmol/l NaCl and 0.2% Tween 20) for 1 h at room temperature, and then probed the following primary antibodies: Anti-Cx43 polyclonal antibody (1:1,000; cat. no. 3512; Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-β-actin monoclonal antibody (1:10,000; cat. no. A5316; Sigma-Aldrich; Merck Millipore) overnight at 4˚C. Following incubation of primary antibodies, the blots were washed three times with TBS-Tween for 5 min and incubated with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. A0545) or goat anti-mouse (cat. no. A9044) secondary antibodies (1:20,000; Sigma-Aldrich; Merck Millipore) for 2 h at room temperature. The blots were washed five times with TBS-Tween, 5 min each time, and visualized on X-ray film using the ECL chemiluminescence reagents (Thermo Fisher Scientific, Inc.). The optical density of each target protein band was assessed with Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.) and normalized to the corresponding β-actin bands in the same sample.

Statistical analysis. The results are expressed as the mean ± standard error of the mean. Statistical analysis was performed using the SPSS 17.0 statistical package (SPSS, Inc., Chicago, IL, USA). The statistical tests used were one-way analysis of variance (ANOVA), Student's *t*-test and q-test. When the overall F test of the ANOVA was significant, Tukey's multiple-comparison test was applied. Student's *t*-test was used to compare between two means. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of NFA on membrane properties of VSMCs from WKY rats. The voltage step induced whole-cell current tracings prior to the application of NFA (Fig. 1A). The electrophysiological characteristics of the VSMCs were similar to a single VSMC following the addition of NFA (Fig. 1B). The charging and discharging of VSMCs in a single exponential equation were applied to a current curve prior to (Fig. 1C) and during (Fig. 1D) application of NFA, which indicated that simulation of a single exponential equation was improved following the application of NFA and cell C_{input} charge-discharge time decreased significantly from 9.73 to 0.48 ms. NFA at $1x10^{-4}$ M decreased VSMC membrane C_{input} from 87±16 to 11.2±0.64 pF ($P<0.05$; n=6; Fig. 1E) and decreased membrane conductance (G_{input}) from 3.31±1.15 to 0.39±0.11 nS ($P<0.05$; n=6; Fig. 1F).

NFA induces the relaxation of mesenteric arterioles of SHRs and WKY rats. Following pre-contraction of the blood vessels with $1x10^{-5}$ M PE, various concentrations (30, 50 and 100 μM) of NFA were added to compare the relaxation of mesenteric arterioles of WKY rats (Fig. 2A) with SHRs (Fig. 2B). The relaxation response of the mesenteric arterioles of the WKY rats was significantly higher, compared with that in the SHRs. The half maximal effective concentration (EC_{50}) of NFA was also higher in the WKY rats (EC_{50}=192 μM), compared with that in the SHRs (EC_{50}=366 μM; $P<0.05$; n=6), as shown in Fig. 2C. To assess the contribution of BK_{Ca} to the vasodilator reactivity of the mesenteric arteriole during application of NFA, $1x10^{-3}$ M TEA was applied to inhibit BK_{Ca} channels following pre-contraction of the blood vessel with PE. No significant change in the diameter of the blood vessels was observed following the application of TEA (142.3±22.7 μm diameter). However, the vasodilator reactivity of the mesenteric arterioles was significant (210.5±18.6 μm diameter; $P<0.05$; n=6) following the addition of $3x10^{-5}$ M NFA (data not shown).

mRNA and protein expression levels of Cx43 in the primary and third-level branches of mesenteric arterioles of SHRs and WKY rats. The mRNA (Fig. 3A) and protein (Fig. 3B) levels of Cx43 in the third-level branches of the mesenteric arterioles were significantly higher, compared with those in the primary

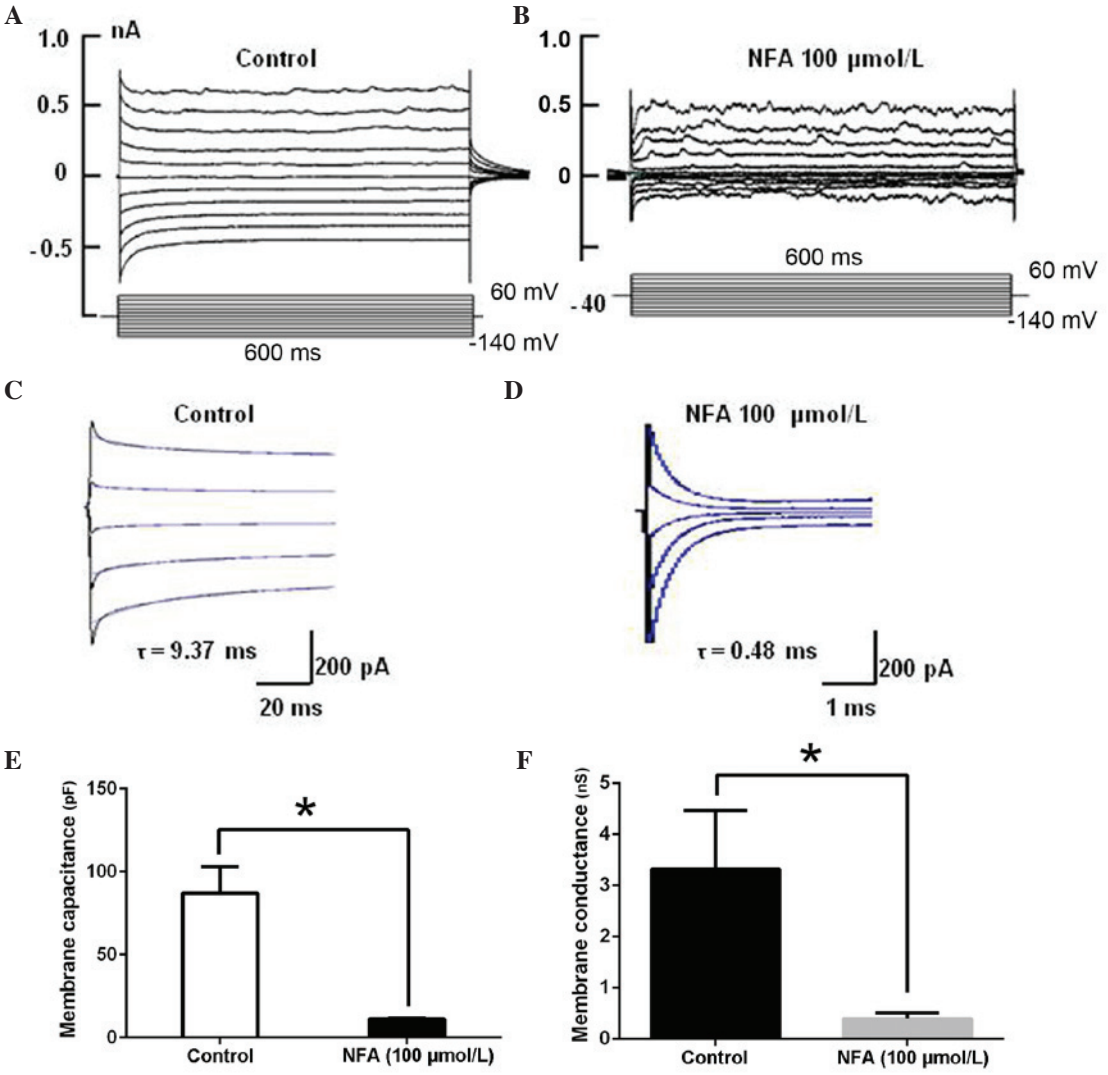

Figure 1. Inhibitory effect of NFA on the electrophysiological properties of VSMC. Voltage step-induced whole-cell current tracings (A) prior to and (B) during application of NFA, respectively. (C) Simulation of cell charge-discharge with single exponential equation prior to application of NFA. (D) Expanded time scale of the initial part of the currents showed that a single term exponential function fitted well following the application of NFA. The horizontal axes represent duration of time and vertical axes represent electric current. The blue lines represent a single exponential curve following fitting. 'τ' represents the time constant of the bottom trace. (E) Statistical results for the effect of NFA on VSMC membrane capacitance (C_{input}). (F) Statistical results for the effect of NFA on VSMC membrane conductance (G_{input}). *P<0.05, comparison indicated by brackets. VSMC, vascular smooth muscle cells; NFA, niflumic acid.

branch of the mesenteric arterioles from the SHRs and WKY rats (P<0.05; n=6; Fig. 3C and D). When comparing the same branch of mesenteric arterioles from the SHRs with those of the WKY rats, the mRNA (Fig. 3A) and protein (Fig. 3B) levels of Cx43 in the SHRs were significantly higher, compared with those in the WKY rats (P<0.05; n=6; Fig. 3C and D).

mRNA and protein expression levels of Cx43 in SHR VSMCs treated with NFA. Following treatment with 30, 50 and 100 μM NFA, the mRNA levels of Cx43 (Fig. 4A) were significantly decreased in the VSMCs of the SHRs in a concentration-dependent manner (P<0.05; n=6; Fig. 4B).

Similar to mRNA, the protein levels of Cx43 (Fig. 5A) were significantly reduced in the VSMCs of the SHRs in a concentration-dependent manner (P<0.05; n=6; Fig. 5B). The expression of Cx43 was almost completely inhibited in the VSMCs when the NFA concentration reached 100 μM.

Discussion

The arterioles form a complex microcirculatory network, which is an important factor in controlling vascular resistance (28,29). The enhancement of vascular tone is key in the pathogenesis of hypertension. The contraction and relaxation of arterioles depends on communication between VSMCs to regulate vascular activity. GJs form connection channels between two adjacent cells, which allow molecules, including cAMP and Ca^{2+}, of <1 KD in size to pass through (30,31). Through GJs, the adjacent cells are engaged in the exchanging of information and material, in addition to electrical and metabolic coupling (32). It is known that GJs have important regulatory effects in VSMC proliferation, vascular tone and blood pressure (33-36). However, the association between the expression of Cxs on resistant arterioles and the development of hypertension remains to be fully elucidated. The results

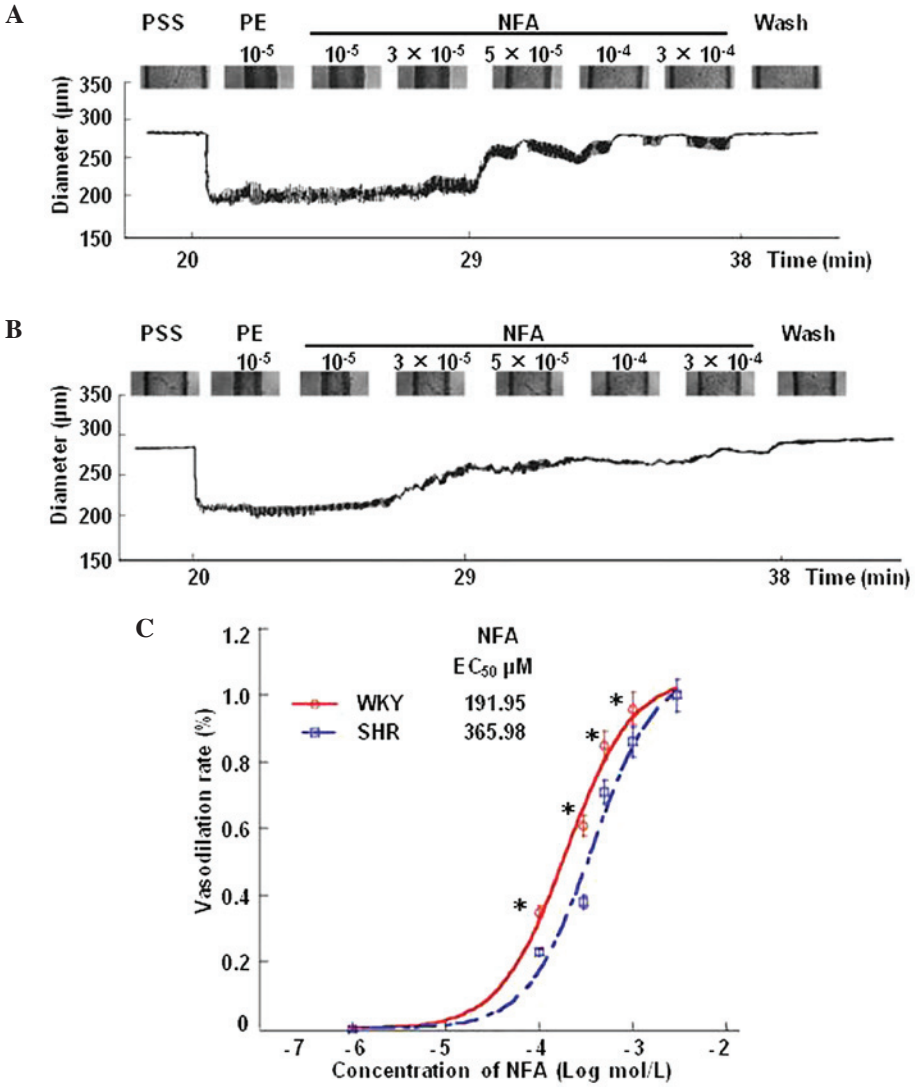

Figure 2. Inhibitory effects of NFA on PE-induced vasoconstriction of mesentery arterioles of SHRs and normotensive control WKY rats. (A) Response to NFA of mesentery arterioles of WKY rats; (B) response to NFA of mesentery arteriole of SHRs. Images are mesentery arterioles at x100 magnification (NFA concentrations are in mol/l and PE in mmol/l). (C) Inhibitory effects of NFA on PE-induced vasoconstriction were higher in the in WKY rats, compared with those in the SHRs. The level of contraction was normalized by WKY rats with a value of 1. Results are presented as the mean ± standard error of the mean (n=6/group). *P<0.05, compared with the WKY rats. NFA, niflumic acid; PSS, physiological saline solution; PE, phenylephrine; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat; EC_{50}, half maximal effective concentration.

from the present study showed that the vasorelaxatory effect of GJ inhibitors was lower in SHRs, which was associated with a higher expression level of the GJ protein, Cx43, in the mesenteric arterioles of the SHRs. This suggested that regulation of the expression of GJs is a potential target for the treatment of hypertension.

The regulation of GJs predominantly involves the expression of GJ protein and adjustment of GJ protein channels (37). The adjustment of GJ protein channels predominantly depends on the level of phosphorylation, which is primarily regulated by the levels of intracellular calcium. The phosphorylation of Cx43 can lead to changes in the 3D structure of Cx43 protein, which can alter the opening probability, opening time, conductivity and permeability of GJ channels (38). In DOCA/NaCl and unilateral renal artery ligation hypertensive rats, the mRNA and protein expression levels of Cx43 are increased in arteriolar VSMCs (39,40). These results suggest that the abnormal expression and altered phosphorylation of Cx43 may affect the regulation of GJ function on resistant vessels and lead to hypertension.

As the distribution of Cx43 on vascular beds is different among different species, the present study selected mesenteric arterioles of SHRs and WKY rats to investigate the expression and function of Cx43 on VSMCs of resistant vessels. The results showed that the mRNA and protein expression levels of Cx43 in the third-level branches of mesenteric arterioles from SHRs and WKY rats were higher, compared with those in the first-level branches of mesenteric arterioles. This suggested that Cx43 was more important in regulating the function of small arterioles. In addition, the expression of Cx43 was markedly higher in the primary and third-level branches of the mesenteric arterioles of SHRs, compared with those of WKY rats. This suggested that the increased expression of Cx43 on small arterioles may be one of the factors causing hypertension. Therefore, reducing the expression of Cx43 may be a novel program for treating hypertension.

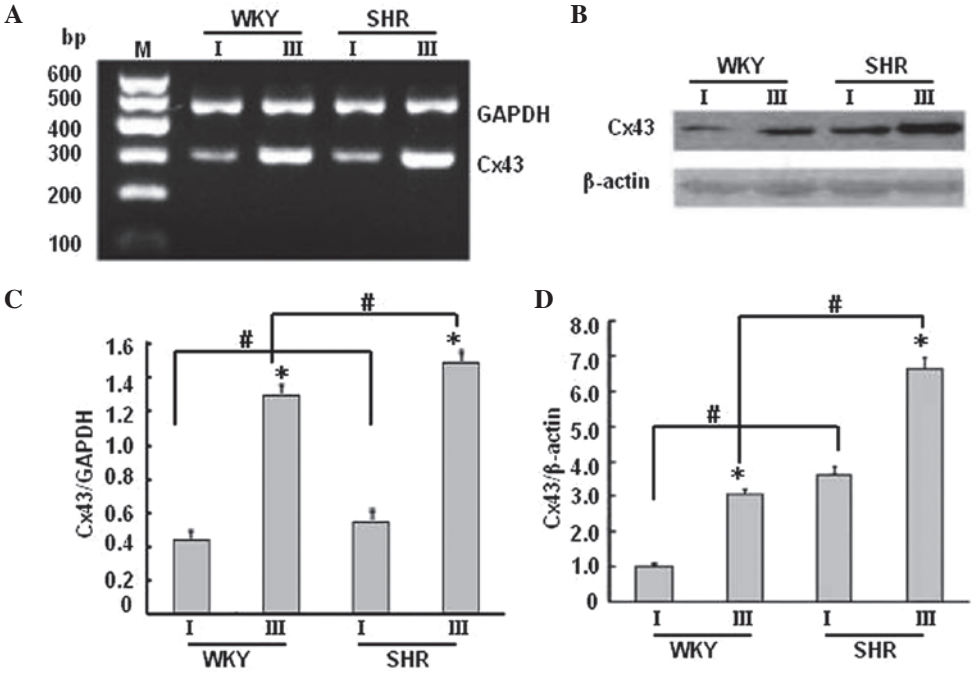

Figure 3. mRNA and protein expression levels of Cx43 in primary (I) and third-level (III) branches of mesenteric arterioles of WKY rats and SHRs. mRNA and protein levels were determined using RT-qPCR and western blot analyses. (A) Representative results of RT-qPCR analysis. (B) Representative western blot. The bars graphs show semiquantitative results of the (C) mRNA and (D) protein levels. Results show the average of six experiments per group and are presented as the means ± standard error of the mean. *P<0.05, compared with the respective WKY groups; #P<0.05, compared with the respective I or III level branches of mesentery arterioles. NFA, niflumic acid; Cx43, connexin 43; SHRs, spontaneously hypertensive rats; WKY, Wistar Kyoto rats; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; M, marker.

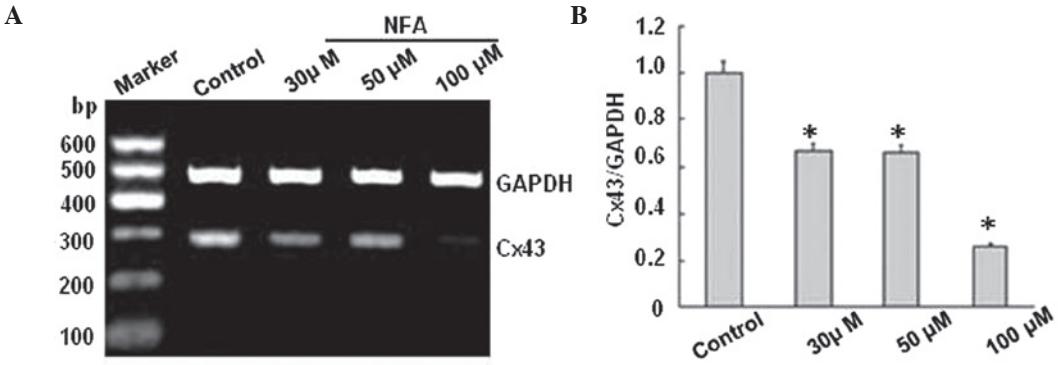

Figure 4. Effects of NFA on the mRNA expression of Cx43 in mesenteric arterioles. (A) Representative results of RT-qPCR analysis. (B) Semiquantitation of RT-qPCR data. Results are presented as the mean ± standard error of the mean (n=6/group). *P<0.05, compared with the control. NFA, niflumic acid; Cx43, connexin 43; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

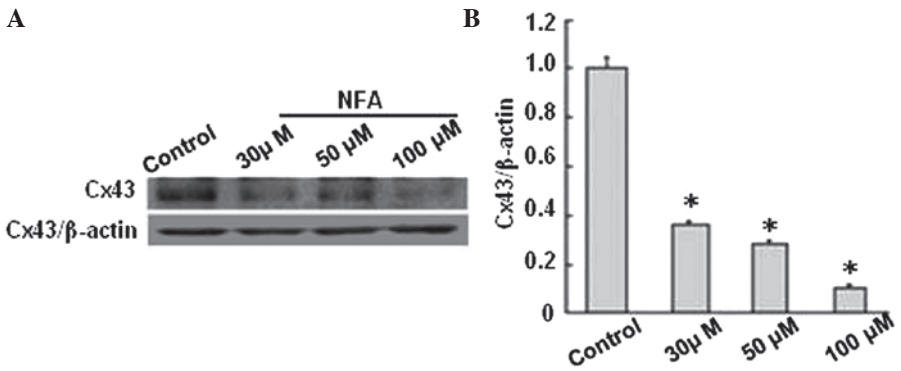

Figure 5. Western blot analysis of the dose-dependent effects of NFA on the protein expression of Cx43 in cultured vascular smooth muscle cells. (A) Representative western blot. (B) Bar graph showing the average values of six experiments per group. Results are presented as the mean ± standard error of the mean (n=6/group). *P<0.05, compared with the control. NFA, niflumic acid; Cx43, connexin 43.

Previous studies have shown that heptanol, a GJ inhibitor, can downregulate the mRNA and protein levels of Cx43 (23), and vasoconstriction of the renal interlobar artery is reduced by pretreatment with 18β-GA (41). Harks *et al* (24) demonstrated that 100 μM mefenamic acid inhibits GJ communication between renal fibroblasts of normal rats (24). These studies indicate that the inhibition of GJ channels may downregulate the expression of GJ mRNA and protein, and decrease GJ communication between cells. NFA, a fenamate, which inhibits cyclooxygenase-2, is commonly used as a non-steroidal anti-inflammatory drug in patients. In previous studies, it was found that NFA at $3x10^{-4}$ M completely inhibited the leakage current and strengthened current components of BK_{Ca} (42). The whole-cell patch clamp experiment performed in the present study demonstrated that C_{input} and G_{input} were reduced by NFA, further indicating that NFA was able to inhibit the GJ communication among VSMCs. Pressure myograph measurement also showed that NFA treatment enhanced the vasorelaxatory responses of mesenteric arterioles of the WKY rats, compared with those of the SHRs. NFA at $3x10^{-4}$ M fully neutralized the contraction caused by PE. In order to investigate the effect of BK_{Ca} channels on vasodilation, TEA was applied to inhibit BK_{Ca} during NFA treatment. NFA dilated mesenteric arterioles under this condition. Therefore, the relaxation of VSMCs caused by NFA was independent from BK_{Ca} activation. These results suggested that GJ channels were involved in the process of vasomotor activity. It was also found that the mRNA and protein levels of Cx43 in the primary cultured mesenteric VSMCs of SHRs were significantly downregulated, in a concentration-dependent manner, by NFA. This suggested that GJ inhibitors decreased GJ expression, reduced the permeability or deactivated the GJ channels, which inhibited the contraction of VSMCs and caused vasodilation (43-46).

The results of the present study demonstrated that increased vascular tone and peripheral resistance due to high expression levels of Cx43 in mesenteric arterioles may be involved in the development of hypertension. NFA, a GJ inhibitor, relaxed arterioles by decreasing the expression of Cx43. Therefore, GJs in resistant vessels may provide a target for the development of effective drugs for the treatment of hypertension.

The present study showed that changes in the expression of GJs and communication among VSMCs are important in the development of hypertension. The GJ inhibitor, NFA, was able to relax blood vessels, decrease arteriole spasm and reduce the expression of Cx43 in mesenteric arterioles. These findings provide a theoretical basis for GJs to be used as a therapeutic target for the treatment of hypertension and cardiovascular diseases.

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References

1. Staessen JA, Wang J, Bianchi G and Birkenhäger WH: Essential hypertension. Lancet 361: 1629-1641, 2003.
2. Hanner F, von Maltzahn J, Maxeiner S, Toma I, Sipos A, Krüger O, Willecke K and Peti-Peterdi J: Connexin45 is expressed in the juxtaglomerular apparatus and is involved in the regulation of renin secretion and blood pressure. Am J Physiol Regul Integr Comp Physiol 295: R371-R380, 2008.
3. Krattinger N, Capponi A, Mazzolai L, Aubert JF, Caille D, Nicod P, Waeber G, Meda P and Haefliger JA: Connexin40 renin regulates production and blood pressure. Kidney Int 72: 814-822, 2007.
4. Schweda F, Kurtz L, de Wit C, Janssen-Bienhold U, Kurtz A and Wagner C: Substitution of connexin40 with connexin45 prevents hyperreninemia and attenuates hypertension. Kidney Int 75: 482-489, 2009.
5. Figueroa XF, Isakson BE and Duling BR: Vascular gap junctions in hypertension. Hypertension 48: 804-811, 2006.
6. De Wit C: Connexins pave the way for vascular communication. News Physiol Sci 19: 148-153, 2004.
7. de Wit C, Hoepfl B and Wölfle SE: Endothelial mediators and communication through vascular gap junctions. Biol Chem 387: 3-9, 2006.
8. Kurtz A: Connexins, renin cell displacement and hypertension. Curr Opin Pharmacol 21: 1-6, 2015.
9. Figueroa XF and Duling BR: Gap junctions in the control of vascular function. Antioxid Redox Signal 11: 251-266, 2009.
10. Brisset AC, Isakson BE and Kwak BR: Connexin in vascular physiology and pathology. Antioxid Redox Signal 11: 267-282, 2009.
11. Rummery NM and Hill CE: Vascular gap junctions and implications for hypertension. Clin Exp Pharmacol Physiol 31: 659-667, 2004.
12. Severs NJ, Rothery S, Dupont E, Coppen SR, Yeh HI, Ko YS, Matsushita T, Kaba R and Halliday D: Immunocytochemical analysis of connexin expression in the healthy and diseased cardiovascular system. Microsc Res Tech 52: 301-322, 2001.
13. Chadjichristos CE, Matter CM, Roth I, Sutter E, Pelli G, Lüscher TF, Chanson M and Kwak BR: Reduced connexin43 expression limits neointima formation after balloon distension injury in hypercholesterolemic mice. Circulation 113: 2835-2843, 2006.
14. Watts SW and Webb RC: Vascular gap junctional communication is increased in mineralocoriocoid-salt hypertension. Hypertension 28: 888-893, 1996.
15. Haefliger JA, Castillo E, Waeber G, Bergonzelli GE, Aubert JF, Sutter E, Nicod P, Waeber B and Meda P: Hypertension increases connexin-43 in a tissue-specific manner. Circulation 95: 1007-1014, 1997.
16. Haefliger JA, Demotz S, Braissant O, Suter E, Waeber B, Nicod P and Meda P: Connexins 40 and 43 are differentially regulated within the kidneys of rats with renovascular hypertension. Kidney Int 60: 190-201, 2001.
17. Dlugosova K, Okruhlicova L, Mitasikova M, Sotnikova R, Bernatova I, Weismann P, Slezak J and Tribulova N: Modulation of connexin-43 by omega-3 fatty acids in the aorta of old spontaneously hypertension rats. J Physiol Pharmacol 60: 63-69, 2009.
18. Seki A, Nishii K and Hagiwara N: Gap junctional regulation of pressure, fluid force, and electrical fields in the epigenetics of cardiac morphogenesis and remodeling. Life Sci 129: 27-34, 2015.
19. Lagaud G, Karicheti V, Knot HJ, Christ GJ and Laher I: Inhibitors of gap junctions attenuate myogenic tone in cerebral arteries. Am J Physiol Heart Circ Physiol 283: H2177-H2186, 2002.
20. Matchkov VV, Rahman A, Peng H, Nilsson H and Aalkjaer C: Junctional and nonjunctional effects of heptanol and glycyrrhetinic acid derivates in rat mesenteric small arteries. Br J Pharmacol 142: 961-972, 2004.
21. Mao HJ, Chen BP, Ren GY, Jin JS, Fan FY, Gao Q, Bruce I and Xia Q: The Effects of Heptanol on electrical coupling during ischemia in the perfused isolated rat heart. Conf Proc IEEE Eng Med Bio Soc 1: 122-125, 2005.
22. Saltman AE, Aksehirli TO, Valiunas V, Gaudette GR, Matsuyama N, Brink P and Krukenkamp IB: Gap junction uncoupling protects the heart against ischemia. J Thorac Cardiovasc Surg 124: 371-376, 2002.
23. Sun B, Qi X and Jiang J: Heptanol decreases the incidence of ischemia-induced ventricular arrhythmias through altering electrophysiological properties and connexin43 in rat hearts. Biomed Rep 2: 349-353, 2014.

24. Harks EG, de Roos AD, Peters PH, de Haan LH, Brouwer A, Ypey DL, van Zoelen EJ and Theuvenet AP: Fenamates: A novel class of reversible gap junction blockers. J Pharmacol Exp Ther 298: 1033-1041, 2001.
25. Li L, Ma KT, Zhao L and Si JQ: Niflumic acid hyperpolarizes the smooth muscle cells by opening BK(Ca) channels through ryanodine-sensitive Ca(2+) release in spiral modular artery. Sheng Li Xue Bao 60: 743-750, 2008.
26. Ma KT, Li XZ, Li L, Jiang XW, Chen XY, Liu WD, Zhao L, Zhang ZS and Si JQ: Role of gap junctions in the contractile response to agonists in the mesenteric artery of spontaneously hypertensive rats. Hypertens Res 37: 110-115, 2014.
27. Li L, Wang R, Ma KT, Li XZ, Zhang CL, Liu WD, Zhao L and Si JQ: Differential effect of calcium-activated potassium and chloride channels on rat basilar artery vasomotion. J Huazhong Univ Sci Technolog Med Sci 34: 482-490, 2014.
28. le Noble FA, Stassen FR, Hacking WJ and Struijker Boudier HA: Angiogenesis and hypertension. J Hypertens 16: 1563-1572, 1998.
29. Segal SS: Integration of blood flow control to skeletal muscle: Key role of feed arteries. Acta Physiol Scand 168: 511-518, 2000.
30. Evans WH and Martin PE: Gap junctions: Structure and function (review) Mol Membr Biol 19: 121-136, 2002.
31. Saez JC, Berthoud VM, Branes MC, Martinez AD and Beyer EC: Plasma membrane channels formed by connexins: Their regulation and functions. Physiol Rev 83: 1359-1400, 2003.
32. Figueroa XF, Isakson BE and Duling BR: Connexins: Gaps in our knowledge of vascular function. Physiology (Bethesda) 19: 277-284, 2004.
33. Song D, Liu X, Liu R, Yang L, Zuo J and Liu W: Connexin 43 hemichannel regulates H9c2 cell proliferation by modulating intracellular ATP and [Ca^{2+}]. Acta Biochim Biophys Sin (Shanghai) 42: 472-482, 2010.
34. Azzam EI, de Toledo SM and Little JB: Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage singals from alpha-particle irradiated to nonirradiated cells. Proc Natl Acad Sci USA 98: 473-478, 2001.
35. de Wit C, Roos F, Bolz SS, Kirchhoff S, Krüger O, Willecke K and Pohl U: Impaired conduction of vasodilation along arterioles in connexin40-deficient mice. Circ Res 86: 649-655, 2000.
36. de Wit C, Roos F, Bolz SS and Pohl U: Lack of vascular connexin 40 is associated with hypertension and irregular arteriolar vasomotion. Physiol Genomics 13: 169-177, 2003.
37. Lampe PD and Lau AF: Regulation of gap junctions by phosphorylation of connexins. Arch Biochem Biophys 384: 205-215, 2000.
38. Moreno AP and Lau AF: Gap junction channel gating modulated through protein phosphorylation. Prog Biophys Mol Biol 94: 107-119, 2007.
39. Haefliger JA and Meda P: Chronic hypertension alters the expression of Cx43 in cardiovascular muscle cells. Braz J Med Biol Res 33: 431-438, 2000.
40. Haefliger JA, Castillo E, Waeber G, Aubert JF, Nicod P, Waeber B and Meda P: Hypertension differentially affects the expression of the gap junction protein connexin43 in cardiac myocytes and aortic smooth muscle cells. Adv Exp Med Biol 432: 71-82, 1997.
41. Li L, Zhang W, Shi WY, Ma KT, Zhao L, Wang Y, Zhang L, Li XZ, Zhu H, Zhang ZS, *et al*: The enhancement of Cx45 expression and function in renal interlobar artery of spontaneously hypertensive rats at different age. Kidney Blood Press Res 40: 52-65, 2015.
42. Li XZ, Ma KT, Guan BC, Li L, Zhao L, Zhang ZS, Si JQ and Jiang ZG: Fenamates block gap junction coupling and potentiate BKCa channels in guinea pig arteriolar cells. Eur J Pharmacol 703: 74-82, 2013.
43. Boittin FX, Alonso F, Le Gal L, Allagnat F, Bény JL and Haefliger JA: Connexins and M3 muscarinic receptors contribute to heterogeneous Ca(2+) signaling in mouse aortic endothelium. Cell Physiol Biochem 31: 166-178, 2013.
44. Rocha ML, Kihara AH, Davel AP, Britto LR, Rossoni LV and Bendhack LM: Blood pressure variability increases connexin expression in the vascular smooth muscle of rats. Cardiovasc Res 80: 123-130, 2008.
45. Takeda Y, Ward SM, Sanders KM and Koh SD: Effects of the gap junction blocker glycyrrhetinic acid on gastrointestinal smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 288: G832-G841, 2005.
46. Tare M, Coleman HA and Parkington HC: Glycyrrhetinic derivatives inhibit hyperpolarization in endothelial cells of guinea pig and rat arteries. Am J Physiol Heart Circ Physiol 282: H335-H341, 2002.