ORIGINAL ARTICLE

Rho-kinase Contributes to Pressure-induced Constriction of Renal Microvessels

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Renal afferent arterioles (AFF) regulate glomerular capillary pressure through two main mechanisms: the myogenic response (MYO) and tubuloglomerular feedback (TGF). Because Rho-kinase and nitric oxide synthase (NOS) are established factors that modulate vascular tone, we examined the role of these factors in pressure-induced AFF tone in Wistar-Kyoto rats and in spontaneously hypertensive rats (SHR) using an intravital CCD camera. Elevated renal perfusion pressure elicited marked AFF constriction that was partially inhibited by gadolinium, furosemide and fasudil, which inhibit MYO, TGF and Rho-kinase, respectively; however, this AFF constriction was completely blocked by combined treatment with fasudil+gadolinium or fasudil+furosemide. *S*-methyl-L-thiocitrulline (SMTC) partially reversed the fasudil-induced inhibition of TGF-mediated, but not that of MYO-mediated, AFF constriction. In SHR, the pressure-induced AFF response was enhanced, and MYO- and TGF-induced constriction were exaggerated. In the presence of gadolinium, SMTC partially mitigated the fasudil-induced inhibition of TGF-mediated AFF constriction. Immunoblot analyses demonstrated that both Rho-kinase activity and neuronal NOS were augmented in SHR kidneys. In conclusion, Rho-kinase contributes to MYO- and TGF-mediated AFF responses, and these responses are enhanced in SHR. Furthermore, neuronal NOS-induced nitric oxide modulates the TGF mechanism. This mechanism constitutes a target for Rho-kinase in TGF-mediated AFF constriction. (doi: 10.2302/kjm.2013-0001-OA ; Keio J Med 63 (1) : 1–12, March 2014)

***Keywords*: Rho-kinase, myogenic response, tubuloglomerular feedback, nNOS, intravital CCD camera**

Introduction

Rho/Rho-kinase plays an important role in the regulation of vascular tone by enhancing calcium sensitivity.[1] A growing body of evidence has indicated that activated Rho/Rho-kinase in vascular beds is responsible for pathophysiological conditions, including hypertension[2] and coronary vasospasm.[3] Furthermore, the Rho/Rho-kinase pathway has been demonstrated to contribute to the development of renal injury in various models of renal disease.[4,5] Additionally, in the renal microcirculation, the myogenic response and the tubuloglomerular feedback mechanism (TGF) constitute the two main mechanisms responsible for renal autoregulation and are mediated by active adjustments of vascular smooth muscle tone, primarily in the afferent arterioles (AFF).[6] Recently, Rho/Rho-kinase was found to be responsible for the generation of renal arteriolar tone, and Y-27632, a Rho/Rho-kinase inhibitor, was found to decrease basal renal arteriolar tone and impair the myogenic response of AFF in

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hydronephrotic kidneys (in which AFF tone is regulated mainly by the myogenic response of renal arterioles) both in vivo[7] and in vitro[8]. Nevertheless, the role of Rho/Rho-kinase in the TGF mechanism remains to be determined.

Nitric oxide (NO) is produced by three isoforms of NO synthase (NOS): endothelial NOS (eNOS), inducible NOS and neuronal NOS (nNOS). Although it has been postulated that in the kidney eNOS predominantly synthesizes NO, recent studies have shown that nNOS is abundantly expressed in macula densa cells, Bowman's capsules, cells of the thick ascending limb and endothelial cells of renal microvessels.[9] Furthermore, nNOS-derived NO plays an important role in counteracting the modulation of TGF-mediated AFF constriction.[10] Recently, an interaction between NO and the Rho/Rho-kinase pathway was demonstrated. Shin et al.[11] reported that Rho-kinase negatively regulates eNOS activity in the acutely ischemic brain. Furthermore, the Rho/Rho-kinase signaling pathway is amplified in kidneys from eNOS-deficient mice.[12] In this regard, it has been demonstrated that the Rho-kinase pathway participates in adenosine-induced contraction of renal arterioles.[13] Because adenosine has been established as a factor mediating TGF, there exists a complex relationship among Rho-kinase, NOS and adenosine in the renal microcirculatory milieu. Although these interactions have attracted much interest, no studies have examined the mutual actions among these components.

In the present study, we examined the role of Rho/Rho-kinase in mediating pressure-induced changes in renal arteriolar tone in rat kidneys using an intravital needle-type CCD camera technique to directly visualize the renal microcirculation. Furthermore, the interaction between Rho/Rho-kinase and nNOS in mediating pressure-induced AFF constriction was also investigated. Finally, we evaluated these responses in kidneys from Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) to clarify whether hypertension modifies these responses.

Materials and Methods

Intravital, pencil-lens-probe, charge-coupled-device videomicroscopy

The intravital videomicroscope used in the present study was a modification of a needle-lens probe previously detailed elsewhere.[14] Images on the CCD were converted into black-and-white video signals every 33 ms and recorded on videocassette tape. The spatial resolution of this system was determined by the use of a United States Air Force 1951 test target to be 0.9 μm for ×600 magnification on a 15-inch video monitor.

Animal preparation

All experimental procedures were conducted in accordance with the guidelines of the Animal Care Committee of Keio University. Nine- to eleven-week-old male WKY and SHR (Charles-River Japan, Kanagawa, Japan) were fed a standard laboratory chow. The animals were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneal injection) and the left kidney was exposed via a flank incision and isolated from the surrounding tissues. The tip of the CCD camera probe was introduced into the renal cortex, and AFF diameters were assessed. The left carotid and femoral arteries were cannulated (PE50, Becton Dickinson, Franklin Lakes, NJ) to measure arterial blood pressure with the use of a pressure transducer (TP4009, Nihon Koden, Tokyo, Japan). The pressure measured at the carotid artery was assumed to be the renal perfusion pressure (RPP).[15]

Measurement of microvascular diameter

After the surgical procedure and instrumentation, the pencil-lens probe was introduced into the superficial cortex.[14] The probe was moved inside slowly to obtain clear images of glomeruli and the adjoining afferent arterioles using a three-dimensional micromanipulator. After determination of the microvascular area for observation, the probe was pulled back in several increments of 10 μm to prevent direct compression of the glomerular network. AFF were distinguished by the direction of the blood flow. Video images were continuously recorded on videocassette tape.

The sequential images of renal microvessels were analyzed by a computer (840AV; Apple Computer, Cupertino, CA, USA) using a freeze-frame modality. The density in gray-scale mode was digitized to an arbitrary unit along the scanning line across the vessel. The difference in the gray scale between the peak value and the background noise level was divided into quarters. The position with the density value a quarter higher than the noise level was identified as the inner wall of the vessel, as described previously.[16] The diameters were determined by averaging at least five measurements during the plateau of the response.

Immunoblotting of Rho-kinase and nNOS

Renal cortical tissues were lysed and sonicated in solubilization buffer. Immunoblotting was performed as described previously,[17,18] using specific antibodies against phospho-MYPT1 (Upstate Biochemistry, Lake Placid, NY, USA) and nNOS (Transduction Laboratories, Lexington, KY, USA). Immunoreactive bands were detected using an ECL detection kit (Amersham Biosciences, Uppsala, Sweden).

Experimental protocols

Protocol 1: Effect of elevated RPP on renal microvascular tone

One hour was allowed after stabilization of renal hemodynamics. After observation of the basal AFF diameters in WKY and SHR, RPP was elevated by clamping the celiac artery and the superior mesenteric artery. Then, RPP was further elevated by clamping the descending aorta at the peripheral level of renal arteries. The AFF diameters were measured at each RPP.

Protocol 2: Effect of gadolinium and furosemide on pressure-induced changes in AFF tone

The roles of TGF and the myogenic response in mediating pressure-induced AFF vasoconstriction in vivo was evaluated using gadolinium (Gd) and furosemide in WKY and SHR. Gd is a relatively selective blocker of mechanosensitive cation channels and thereby abolishes myogenic constriction.[19,20] In contrast, loop diuretics such as furosemide cause significant diuresis and natriuresis by inhibiting the Na-K-2Cl cotransporter in the thick ascending limb of Henle's loop, including the macula densa, and induce vasodilation through inhibition of the TGF mechanism.[21] Initially, Gd was infused intravenously (5 or 10 mg/kg; Sigma Chemicals, St. Louis, MO, USA). After 5 min, the effect of the stepwise RPP elevation on AFF diameter was assessed. Similarly, AFF responses to elevated RPP were examined 5 min after initiation of intravenous infusion of furosemide (10 or 16 μg/kg/min; Sigma Chemicals).

Protocol 3: Role of Rho-kinase in pressure-induced AFF tone

The participation of Rho-kinase in pressure-induced AFF constriction was evaluated in WKY and SHR. Initially, fasudil (Asahi Kasei, Tokyo, Japan) was infused intravenously at 0.3 or 0.6 mg/kg for 5 min. Thereafter, the pressure-induced changes in AFF diameter were assessed in WKY and SHR.

We further evaluated the role of nNOS-associated nitric oxide in Rho-kinase-mediated AFF constriction. *S*-methyl-L-thiocitrulline (SMTC; Sigma Chemicals), an nNOS-selective inhibitor,[22] was infused at a rate of 20 μg/kg/min. Then, the inhibitory action of fasudil (0.6 mg/kg) on pressure-induced AFF constriction was evaluated in WKY and SHR.

To further clarify whether SMTC-associated NO inhibition affected myogenic or TGF inhibition by fasudil, we examined the effect of fasudil on pressure-induced AFF tone under pretreatment with SMTC (20 μg/kg/min) and Gd (10 mg/kg) or furosemide (16 μg/kg/min). Thus, fasudil was injected intravenously in the presence of Gd or furosemide, and the effect of fasudil on the pressure-induced changes in renal arteriolar tone was evaluated.

Statistical analysis

Data are expressed as the mean ± SEM. Data were analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison post hoc test. $P < 0.05$ was defined as statistically significant.

Results

Protocol 1: Effect of elevated RPP on renal microvascular tone

Figure 1A shows representative images of AFF responses to elevated RPP in WKY. On elevation of RPP, AFF manifested marked constriction, whereas no constrictor response was observed in the efferent arteriole. The responses of AFF to pressure are summarized in **Figure 1B**. Elevation of RPP from 78 ± 6 to 106 ± 6 mmHg tended to reduce AFF diameter (from 12.6 ± 0.5 μm to 11.9 ± 0.5 μm, n =10, $P > 0.20$), and elevation to 140 ± 8 mmHg elicited 17.5 ± 2.0% constriction (from 12.6 ± 0.5 μm to 10.6 ± 0.4 μm, n =10, $P < 0.05$).

Protocol 2: Effect of Gd and furosemide on pressure-induced changes in AFF tone

Neither Gd (5 and 10 mg/kg) nor furosemide (10 and 16 μg/kg/min) had any effect on the basal AFF diameter in WKY (data not shown). The addition of Gd (5 and 10 mg/kg) blunted the pressure-induced changes in AFF diameter in a dose-dependent manner in WKY, (n =10, **Fig. 2**). Similarly, furosemide (10 and 16 μg/kg/min) significantly inhibited the pressure-induced constriction of AFF (n =10). Furthermore, combined treatment with Gd (10 mg/kg) and furosemide (16 μg/kg/min) completely abolished this response (n =10).

Protocol 3: Role of Rho-kinase in pressure-induced AFF tone

As shown in **Figure 3A**, the administration of fasudil dilated AFF in a dose-dependent manner at baseline RPP in WKY (baseline, 11.5 ± 0.5 μm; 0.3 mg/kg, 12.2 ± 0.5 μm; 0.6 mg/kg, 13.3 ± 0.5 μm; $P < 0.05$; n =10).

In the presence of fasudil (0.6 mg/kg), pressure-induced AFF constriction was blunted, with only 1.1 ± 1.1% ($P <$ 0.05 vs. control) and 4.3 ± 0.8% decrements ($P < 0.01$ vs. control) observed at 110 ± 8 and 143 ± 6 mmHg, respectively (**Fig. 3B**, lower).

The ability of fasudil to inhibit the TGF-mediated and myogenic AFF tone was evaluated in WKY. In the presence of Gd (10 mg/kg), fasudil (0.6 mg/kg) abolished the AFF constriction induced by elevated RPP (1.1 ± 0.8% vs. 9.5 ± 1.2% decrease in diameter) (**Fig. 4**). Similarly, in the

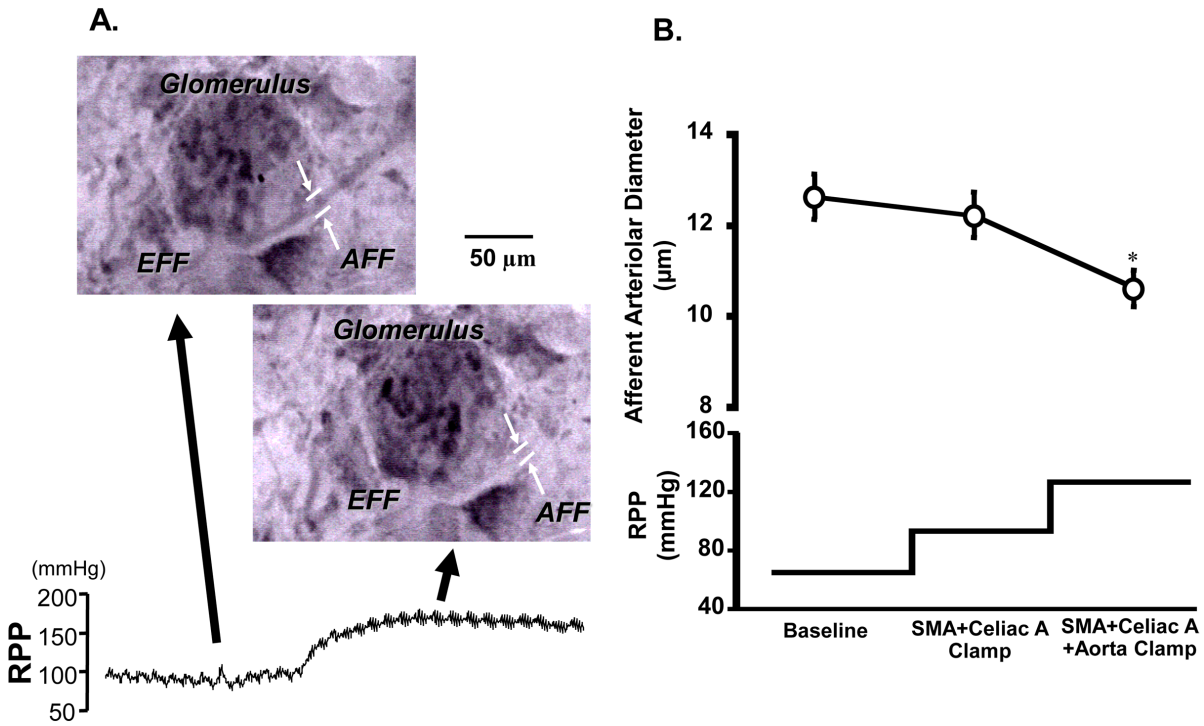

Fig. 1 Visualization of renal microcirculation *in vivo* and responses to elevated renal perfusion pressure in Wistar-Kyoto rats (WKY). An intravital CCD camera allowed direct visualization of afferent arteriolar responses to elevated renal perfusion pressure (RPP) (A). Stepwise elevation in RPP by clamping the superior mesenteric artery (SMA)+celiac artery and SMA+celiac artery+aorta elicited RPP-dependent constriction of afferent arterioles (AFF) (B). EFF; efferent arterioles. **P*<0.05 vs. baseline.

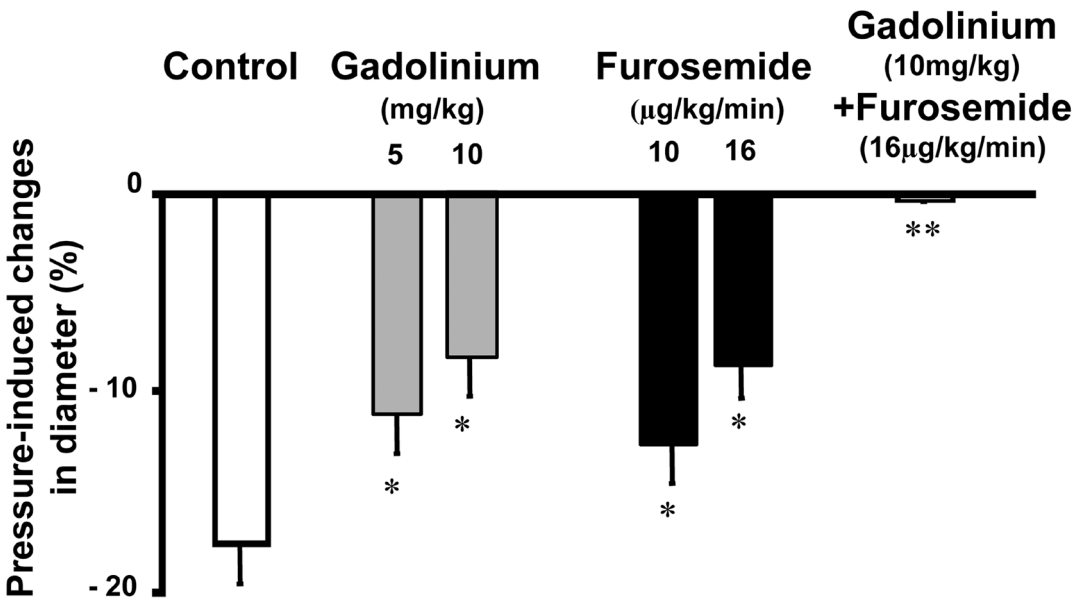

Fig. 2 Myogenic and tubuloglomerular feedback (TGF)-mediated constriction of the afferent arteriole in WKY.
Gadolinium and furosemide blunted pressure-induced afferent arteriolar constriction in a dose-dependent manner. Pretreatment with both gadolinium and furosemide completely abolished the response. **P* <0.05 vs. control, ***P* <0.01 vs. control.

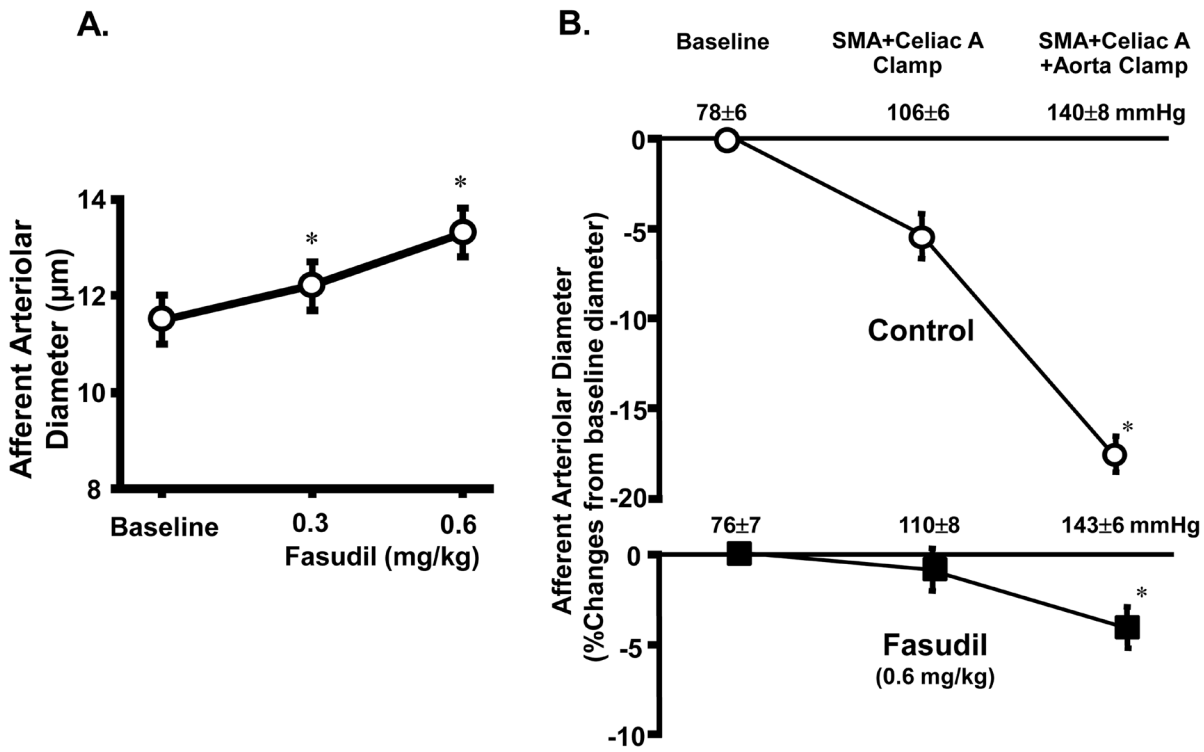

Fig. 3 Effect of fasudil on basal and pressure-induced tone of afferent arterioles in WKY.
Under basal conditions, fasudil dilated afferent arterioles in a dose-dependent manner (A). Fasudil markedly blunted the pressure-induced vasoconstriction of afferent arterioles (B). RPP was elevated stepwise by clamping the SMA+celiac artery and SMA+celiac artery+aorta. *P <0.05 vs. baseline.

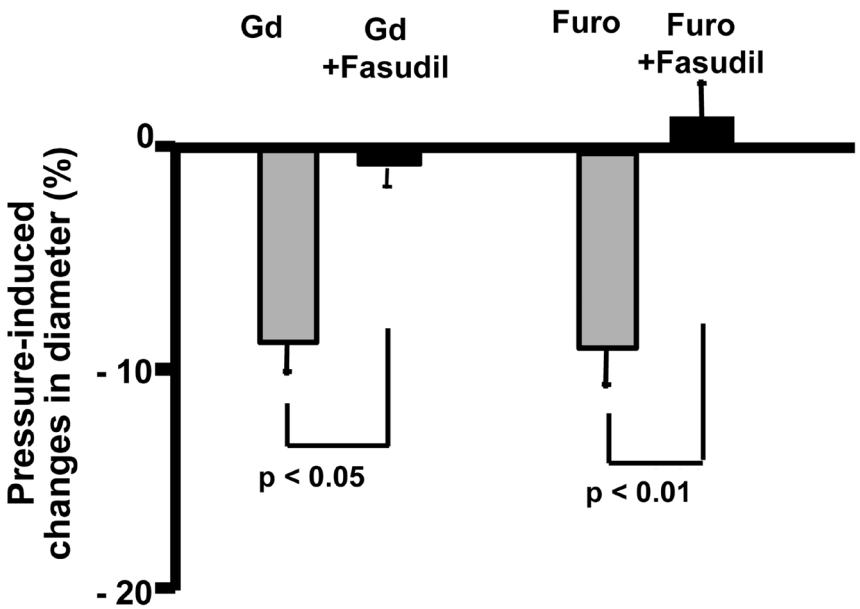

Fig. 4 Effect of fasudil on pressure-induced constriction of the afferent arteriole in the presence of gadolinium or furosemide in WKY.
Fasudil (0.6 mg/kg) completely abolished the pressure-induced constriction of afferent arterioles treated with gadolinium (Gd, 10 mg/kg) or furosemide (Furo, 16 μg/kg/min).

presence of furosemide (16 μg/kg/min), fasudil (0.6 mg/kg) abolished the RPP-induced AFF constriction (1.2 ± 2.0% increase vs. 7.8 ± 0.8% decrease in diameter).

Next, the role of nNOS in fasudil inhibition of pressure-induced AFF constriction was examined in WKY. SMTC (20 μg/kg/min), an nNOS-selective inhibitor, had no effect on basal AFF tone but enhanced pressure-induced constriction (23.2 ± 1.6% vs. 17.5 ± 2.0% decrease in diameter, $P < 0.05$, $n = 10$, **Fig. 5A**). When the effect of SMTC on fasudil-induced inhibition was assessed, SMTC markedly restored the AFF response to elevated RPP (12.0 ± 1.6% vs. 4.3 ± 0.8% decrease in diameter, $P < 0.05$, $n = 10$).

We further examined whether the effects of SMTC on fasudil-induced inhibition were mediated by a myogenic or TGF mechanism. In the presence of Gd (10 mg/kg), fasudil (0.6 mg/kg) completely abolished the AFF constriction induced by elevated RPP (1.1 ± 0.8% decrease in diameter, **Fig. 5B**). The addition of SMTC (20 μg/kg/min) reversed the fasudil-induced inhibition of RPP-induced AFF constriction (9.1 ± 0.6%, $P < 0.05$) to the level observed in the presence of Gd (i.e., 9.5 ± 1.2%, P

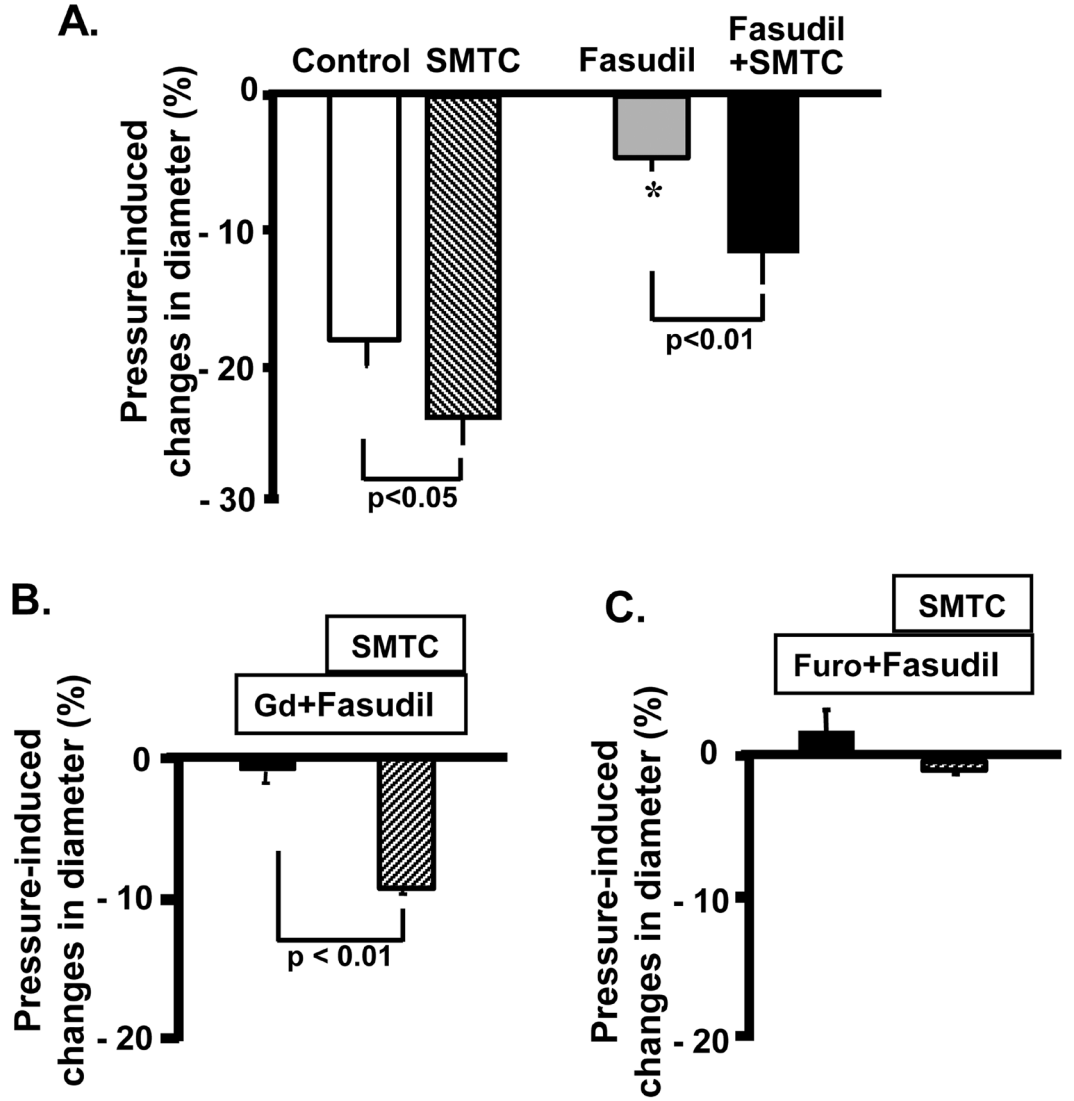

Fig. 5 Role of neuronal nitric oxide synthase (nNOS) in myogenic and tubuloglomerular feedback-induced afferent arteriolar tone in WKY.

S-methyl-L-thiocitrulline (SMTC), an nNOS-selective inhibitor, augmented pressure-induced afferent arteriolar constriction. Fasudil (0.6 mg/kg)-induced inhibitory effects on the pressure-constricted afferent arterioles were substantially mitigated by SMTC (A). SMTC potently reversed the fasudil-induced inhibition in the presence of gadolinium (Gd, 10 mg/kg; B), but not in the presence of furosemide (Furo, 16 μg/kg/min; C). *P <0.05 vs. control

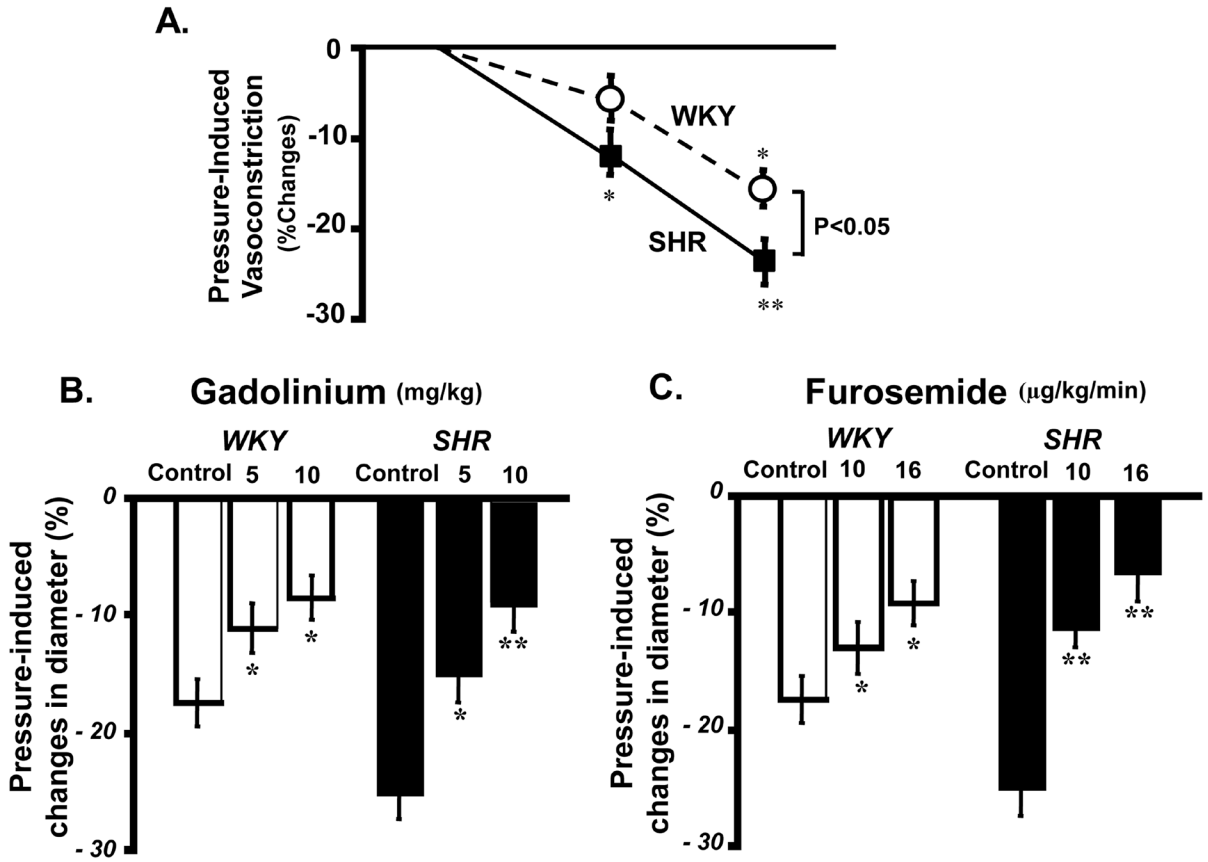

Fig. 6 Pressure-induced constrictor responses of the afferent arteriole in SHR and WKY.
The same magnitude of elevation in renal perfusion pressure caused a larger vasoconstrictor response in SHR than in WKY (A). When the inhibitory action of gadolinium or furosemide on pressure-induced constriction was evaluated, the ability of gadolinium to inhibit afferent arteriolar constriction tended to be greater in SHR than in WKY (B). Similarly, the inhibition of pressure-induced constriction by furosemide was greater in SHR (C). *P <0.05 vs. control, **P <0.01 vs. control.

> 0.5). Simultaneous treatment with furosemide (16 μg/kg/min) and fasudil (0.6 mg/kg) abolished RPP-induced AFF constriction (1.2 ± 2.0% increase in diameter, **Fig. 5C**). In this setting, however, SMTC failed to reverse the fasudil-induced inhibition of AFF vasoconstriction.

Protocol 4: RPP-induced constriction in SHR

The basal AFF diameter in SHR (10.0 ± 0.4 μm, n =10) was slightly less than that in WKY (12.6 ± 0.5 μm, P < 0.05). AFF of SHR exhibited 12.2 ± 2.0% and 24.9 ± 2.3% decreases in diameter (n =10, P < 0.01) in response to elevated RPP (**Fig. 6A**). It is of note that the AFF constriction in SHR was greater than that in WKY in response to the same magnitude of elevated RPP.

The ability of Gd and furosemide to inhibit elevated RPP-induced AFF constriction was compared in WKY and SHR. Both Gd and furosemide inhibited RPP-induced AFF vasoconstriction in a dose-dependent manner in WKY and SHR (**Figs. 6B** and **C**). Of note, the inhibitory action of Gd tended to be greater in SHR than in WKY, and the ability of furosemide to inhibit RPP-induced AFF vasoconstriction was actually enhanced in SHR.

The administration of fasudil at baseline RPP dilated AFF from 9.3 ± 0.5 μm to 11.0 ± 0.5 μm (0.3 mg/kg; P < 0.05, n =10) and 12.5 ± 0.6 μm (0.6 mg/kg; P < 0.01, n =10) in SHR, which corresponded to 18.3 ± 3.3% and 23.7 ± 4.0% vasodilation, respectively (**Fig. 7A**); the vasodilator responses to fasudil were greater in SHR than in WKY (P < 0.05).

Fasudil markedly inhibited the pressure-induced constriction of AFF in SHR (control, 24.9 ± 2.3% decrease; fasudil, 7.3 ± 1.5% decrease; **Fig. 7B**, black bars). It is of note that fasudil eliminated the difference in RPP-induced AFF vasoconstriction between WKY and SHR.

Pretreatment with SMTC enhanced the pressure-induced AFF constriction in both SHR and WKY (**Fig. 7C**; see also **Fig. 7B**, control). Furthermore, SMTC restored the fasudil-induced impairment in the AFF constrictor response to pressure more greatly in SHR than

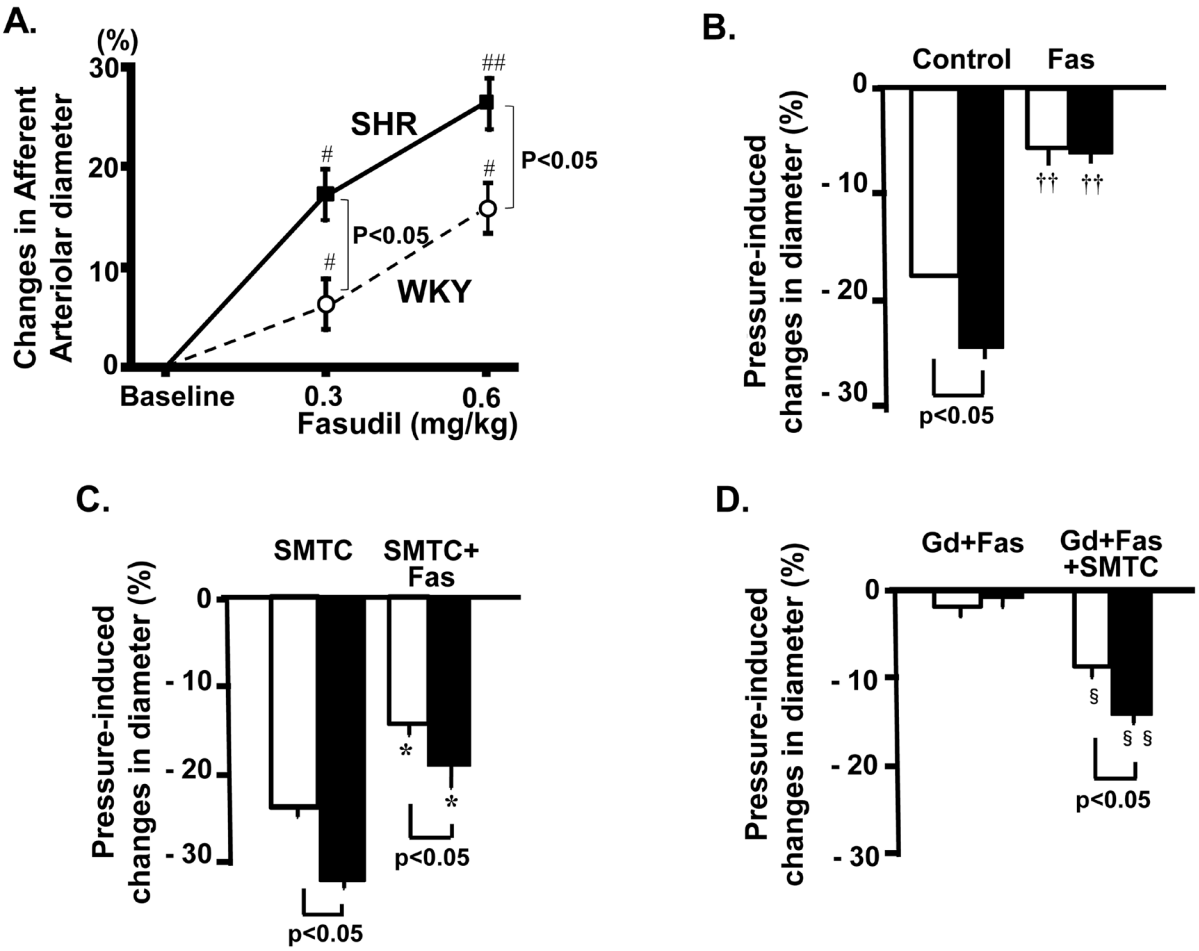

Fig. 7 Effects of fasudil on basal and pressure-induced afferent arteriolar constriction in SHR.
The vasodilator effect of fasudil on basal afferent arteriolar tone was larger in SHR than in WKY (A). Although the pressure-induced constrictor response was greater in SHR, fasudil (Fas, 0.6 mg/kg) prevented this response to the same extent in SHR and WKY (B). In contrast, pretreatment with SMTC mitigated the inhibitory action of fasudil (Fas) during pressure-induced AFF constriction, and this mitigation effect was more pronounced in SHR (C). SMTC restored the pressure-induced AFF constriction that was almost completely inhibited by gadolinium (Gd, 10 mg/kg)+Fas, and this restorative action was greater in SHR (D). White bars, WKY; black bars, SHR. # P <0.05 vs. baseline, ## P <0.01 vs. baseline. †† P <0.01 vs. control. * P <0.05 vs. SMTC. § P <0.05 vs. Gd+Fas, §§ P <0.01 vs. Gd+Fas.

in WKY (**Fig. 7C**; see also **Fig. 7B**, Fas). An enhanced AFF responses in SHR was also apparent when the effect of SMTC was examined in the presence of Gd; the fasudil+Gd-induced inhibition of pressure-induced AFF constriction was identical in WKY and SHR, whereas in the presence of SMTC, a greater constrictor response was observed in SHR (14.3 ± 0.9%, n =10) than in WKY (8.6 ± 1.1%, n =10) (**Fig. 7D**). These observations suggest that fasudil-induced inhibition of pressure-induced AFF constriction is mediated in part by nNOS-induced NO and the resulting TGF inhibition, and this mechanism is exaggerated in SHR. In the presence of furosemide, however, SMTC failed to reverse the inhibition by fasudil of the AFF constrictor response to pressure (data not shown).

Figure 8A illustrates the comparison of Rho-kinase activity (as assessed by phosphorylation of MYPT) in kidneys from WKY and SHR. It was clearly demonstrated that Rho-kinase activity was greater in SHR than in WKY (n =3). Similarly, intrarenal nNOS was upregulated in SHR relative to WKY (n =3, **Fig. 8B**).

Discussion

In the present study, we confirmed that both myogenic and TGF-mediated AFF constriction can be visualized in an *in vivo*, *in situ* and relatively intact setting (**Figs. 1, 2**). Furthermore, fasudil was found to elicit dose-dependent relaxation of the basal and pressure-constricted tone of AFF (**Fig. 3**). These findings indicate that the Rho-kinase pathway is involved not only in the generation of basal

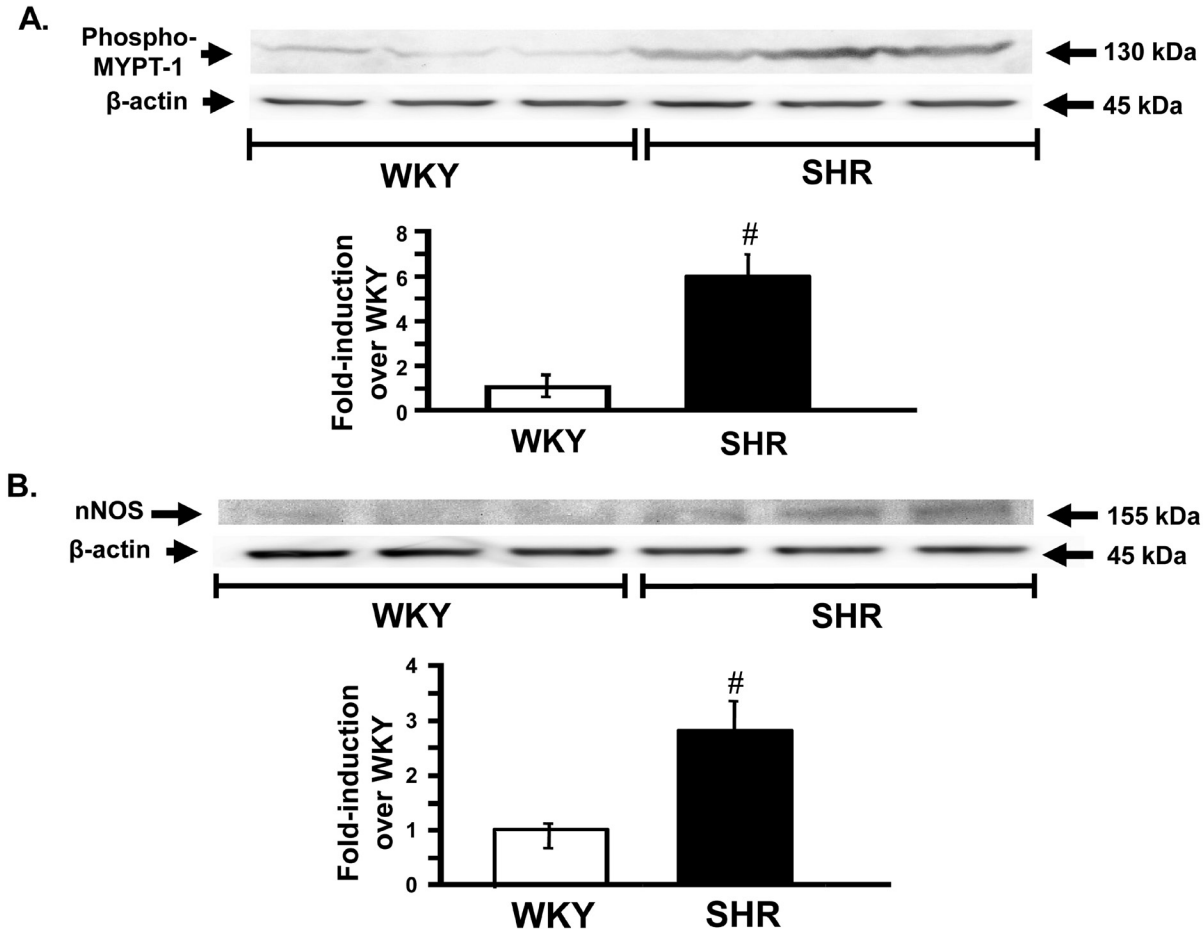

Fig. 8 Comparison of Rho-kinase activity and nNOS expression in WKY and SHR.
Renal Rho-kinase activity, as assessed by phosphorylation of MYPT-1, was enhanced in SHR (A). Similarly, nNOS expression was greater in SHR than in WKY (B). # P <0.01 vs. WKY.

tone but also in the development of pressure-induced tone in the renal microcirculation. Moreover, further inhibition of pressure-induced constriction by fasudil during the inhibition of myogenic and TGF-induced tone by Gd and furosemide, respectively, suggests that Rho-kinase makes a substantial contribution to both of these vasoconstrictor mechanisms (**Fig. 4**). In addition, we recently demonstrated that Y-27632 (a selective Rho-kinase inhibitor) prevents pressure-induced AFF constriction in the isolated perfused hydronephrotic rat kidney[8] in which the TGF-mediated AFF constrictor mechanism is absent.[23] This finding suggests a crucial role for Rho-kinase in the myogenic mechanism of pressure-induced AFF constriction. The present study further indicates the involvement of Rho-kinase in TGF-mediated AFF tone in addition to its role in myogenic tone. Thus, our current study clearly supports the concept that Rho-kinase constitutes a determinant of both myogenic and TGF-induced AFF tone.

It is well established that the TGF mechanism involves multiple components that affect AFF tone in a positive or negative manner. Among these components, NO is assumed to be a modulator of TGF that counters TGF-mediated AFF constriction.[10,22] Indeed, we and other laboratories previously reported that elevated RPP elicited increases in renal NO levels.[24,25] Immunohistochemical studies have revealed the presence of nNOS in the kidney, with expression primarily localized to the cells of the macula densa,[26] and selective nNOS inhibition causes a 32–42% reduction in renal cortical and medullary NO levels in normotensive rats.[27] These observations suggest that nNOS-dependent NO production constitutes a significant portion of the total NO generation within the kidney. Collectively, our study is consistent with the premise that TGF is influenced by nNOS-induced NO.

The present study shows that the inhibition of nNOS by SMTC enhances pressure-induced AFF constriction (**Fig. 5A**). Furthermore, the ability of SMTC to restore pressure-induced AFF constriction is preserved during the treatment with fasudil (12.0 ± 1.6% vs. 4.3 ± 0.8%, P < 0.01). It follows, therefore, that the inhibition by fa-

sudil of pressure-induced AFF constriction is modulated in part by the vasodilator action of nNOS-generated NO. We carried out further experiments to delineate the role of Rho-kinase in TGF-mediated AFF constriction. Thus, in the presence of Gd, in which TGF is assumed to be a main constrictor mechanism, fasudil completely abolished pressure-induced AFF constriction. In this setting, SMTC pronouncedly reversed this response (**Fig. 5B**). These observations strongly suggest that the inhibition of TGF by fasudil is mediated largely by the activation of nNOS. It is known that Rho-kinase exerts multifaceted effects that are necessary to maintain various cellular functions. In addition to the Ca-sensitizing action of Rho-kinase in vascular smooth muscle cells, Rho-kinase activation causes the suppression of eNOS activity.[11,28] Furthermore, our finding that SMTC reverses the fasudil-induced suppression of TGF lends support to the hypothesis that Rho-kinase modulates nNOS activity and the subsequent production of NO.

In contrast, in the presence of furosemide, SMTC fails to affect the fasudil-induced inhibition of pressure-induced AFF constriction (**Fig. 5C**). This observation suggests that nNOS-induced NO appears to exert only modest action on myogenic AFF constriction. In this regard, by using nNOS-knockout mice, Dautzenberg et al.[29] showed that nNOS-generated NO had no significant effect on the myogenic response. Several studies have examined the effect of NO on the myogenic response of the renal microcirculation. An *in vivo* study revealed that NO inhibition fails to alter the autoregulatory efficiency of renal blood flow, although basal blood flow was reduced.[30] These results suggest that NO does not impair the myogenic AFF response. Similarly, nitro-L-arginine methyl ester (a L-NAME) has no effect on the myogenic AFF responses in the *in vivo* hydronephrotic kidney[31] or fails to enhance the efficiency of myogenic AFF constriction.[32] Finally, nitroprusside, cyclic GMP, and atrial natriuretic peptide, which shares its intracellular signal transduction pathway with NO, failed to inhibit myogenic AFF constriction.[33] In contrast, recent studies have demonstrated that TGF-mediated NOS activation modifies myogenic autoregulation[34] and the AFF response to pressure.[35] Because the dilator action of NO is mediated in part by protein kinase G and the inhibition of Rho A,[36] this mechanism might explain how NO could modulate the myogenic AFF response. A schematic illustrating the possible roles of Rho-kinase in the myogenic response and in TGF and the association between Rho-kinase and nNOS is shown in **Figure 9**.

Several studies have shown that the renal microvascular response to pressure is altered in hypertension. Thus, myogenic constriction was reported to be enhanced in AFF from SHR.[37] Furthermore, Welch et al.[38] showed that TGF is augmented in AFF from SHR. In the present study, we demonstrated that the AFF of SHR kidneys manifest exaggerated responses to elevated pressure (**Fig. 6A**). Furthermore, both TGF and myogenic components are enhanced in SHR compared to those in WKY (**Figs. 6B** and **C**). These *in vivo* findings are therefore consistent with the previous *in vitro* observations described above and support the concept that an enhanced AFF constrictor response to pressure protects against high systemic blood pressure in hypertensive animals.

The mechanisms underlying the augmented AFF constrictor response to pressure in hypertension remain undetermined. One possible mechanism is enhanced Rho-kinase activity in hypertensive animals. Uehata et al.[2] demonstrated that Y-27632, a Rho-kinase inhibitor, elicited an exaggerated depressor action when administered in hypertensive animals. In the present study, we examined the role of Rho-kinase in renal microvascular tone in SHR and observed an enhanced vasodilator effect of fasudil on the AFF (**Fig. 7A**). Furthermore, fasudil abolished the exaggerated pressure-induced AFF constrictor response observed in SHR (**Fig. 7B**). These observations suggesting enhanced Rho-kinase activity in SHR kidneys were confirmed by the immunoblotting of MYPT-1 (**Fig. 8A**). It follows, therefore, that Rho-kinase activity constitutes an important determinant of the enhanced pressure-induced AFF constriction in SHR.

The role of nNOS in mediating the renal microvascular action of Rho-kinase in hypertensive animals remains unclear. In contrast to the effect of fasudil on pressure-induced AFF constriction (**Fig. 7B**), pretreatment with SMTC mitigated the inhibitory action of fasudil on pressure-induced AFF constriction, and this effect was more pronounced in SHR (**Fig. 7C**). Thus, SMTC restored the difference in pressure response between WKY and SHR. Furthermore, in the presence of Gd, i.e., when TGF is predominantly mediating the pressure-induced AFF constriction, the reversal by SMTC of fasudil-induced inhibition of the pressure response is exaggerated in SHR (**Fig. 7D**). These observations raise the possibility that augmented Rho-kinase in SHR suppresses nNOS activity and NO production, which subsequently enhances TGF signaling. Finally, renal nNOS expression is upregulated in SHR (**Fig. 8B**), which may serve to counter suppressed NO activity or bioavailability.[39]

In conclusion, we demonstrated that the Rho/Rho-kinase pathway is involved in the pressure-induced tone of the renal microcirculation. This novel mechanism exerts its action through several vasomotor components within the renal microcirculation, including the TGF and myogenic tone, and is further affected at least in part by nNOS-associated TGF modulation. Finally, the exaggerated pressure-induced tone in hypertensive animals is attributable to the enhanced activity of Rho-kinase. It awaits further investigations to determine whether exaggerated Rho-kinase activity protects against glomerular hypertension or contributes to the progression of renal injury in hypertension.

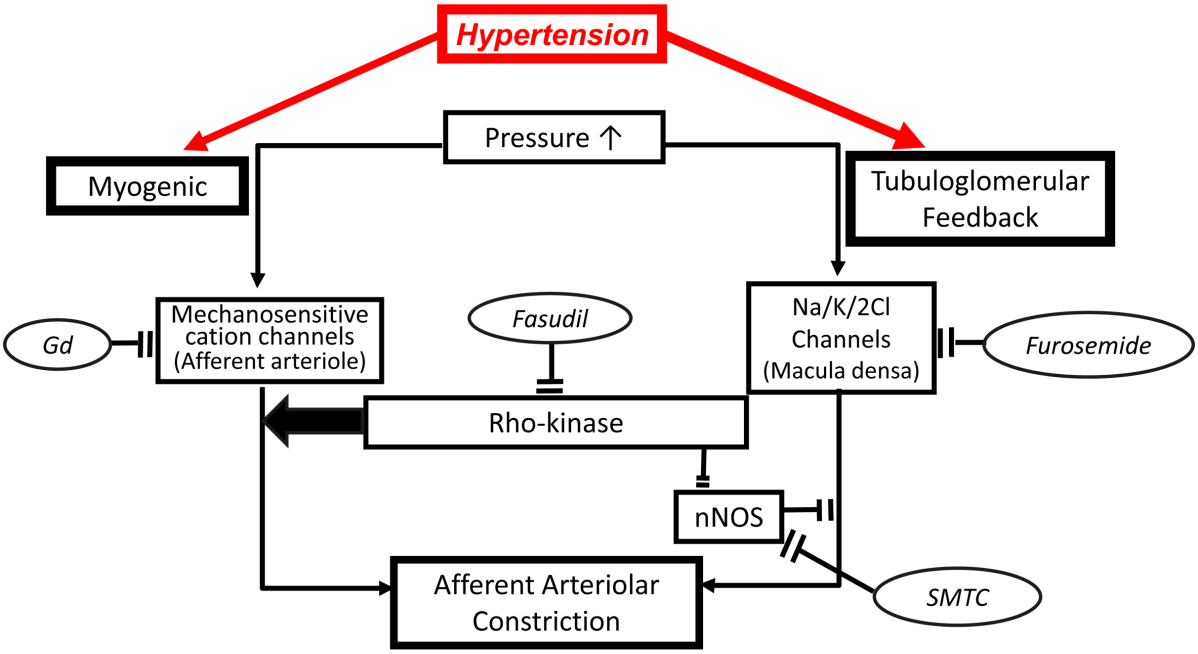

Fig. 9 Schematic illustrating the role of Rho-kinase in myogenic and tubuloglomerular feedback-mediated afferent arteriolar vasoconstriction.
Elevated renal perfusion pressure enhances both myogenic and TGF mechanisms and causes afferent arteriolar vasoconstriction. Rho-kinase mediates or modulates the myogenic mechanism. TGF is under the negative control of nNOS-mediated nitric oxide (NO). Rho-kinase is assumed to suppress nNOS activity and NO production, leading to augmentation of TGF-mediated afferent arteriolar vasoconstriction. Gadolinium (Gd) is a selective inhibitor for mechanosensitive cation channels that mediate myogenic vasoconstriction. Furosemide inhibits the Na/K/2Cl cotransporter in the macula densa that plays an important role in TGF. *S*-methyl-L-thiocitrulline (SMTC) is a blocker of nNOS activity and suppresses NO production.

References

1. Narumiya S: The small GTPase Rho: cellular functions and signal transduction. J Biochem 1996; **120**: 215–228. [Medline] [CrossRef]
2. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S: Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 1997; **389**: 990–994. [Medline] [CrossRef]
3. Shimokawa H: Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. J Cardiovasc Pharmacol 2002; **39**: 319–327. [Medline] [CrossRef]
4. Kanda T, Wakino S, Hayashi K, Homma K, Ozawa Y, Saruta T: Effect of fasudil on Rho-kinase and nephropathy in subtotally nephrectomized spontaneously hypertensive rats. Kidney Int 2003; **64**: 2009–2019. [Medline] [CrossRef]
5. Nishikimi T, Akimoto K, Wang X, Mori Y, Tadokoro K, Ishikawa Y, Shimokawa H, Ono H, Matsuoka H: Fasudil, a Rho-kinase inhibitor, attenuates glomerulosclerosis in Dahl salt-sensitive rats. J Hypertens 2004; **22**: 1787–1796. [Medline] [CrossRef]
6. Hayashi K, Epstein M, Saruta T: Altered myogenic responsiveness of the renal microvasculature in experimental hypertension. J Hypertens 1996; **14**: 1387–1401. [Medline] [CrossRef]
7. Cavarape A, Endlich N, Assaloni R, Bartoli E, Steinhausen M, Parekh N, Endlich K: Rho-kinase inhibition blunts renal vasoconstriction induced by distinct signaling pathways in vivo. J Am Soc Nephrol 2003; **14**: 37–45. [Medline] [CrossRef]
8. Nakamura A, Hayashi K, Ozawa Y, Fujiwara K, Okubo K, Kanda T, Wakino S, Saruta T: Vessel- and vasoconstrictor-dependent role Rho/Rho-kinase in renal microvascular tone. J Vasc Res 2003; **40**: 244–251. [Medline] [CrossRef]
9. Mount PF, Power DA: Nitric oxide in the kidney: functions and regulation of synthesis. Acta Physiol (Oxf) 2006; **187**: 433–446. [Medline] [CrossRef]
10. Ren YL, Garvin JL, Carretero OA: Role of macula densa nitric oxide and cGMP in the regulation of tubuloglomerular feedback. Kidney Int 2000; **58**: 2053–2060. [Medline] [CrossRef]
11. Shin HK, Salomone S, Potts EM, Lee SW, Millican E, Noma K, Huang PL, Boas DA, Liao JK, Moskowitz MA, Ayata C: Rho-kinase inhibition acutely augments blood flow in focal cerebral ischemia via endothelial mechanisms. J Cereb Blood Flow Metab. 2006 Oct 11.
12. Williams J, Bogwu J, Oyekan A: The role of the RhoA/Rho-kinase signaling pathway in renal vascular reactivity in endothelial nitric oxide synthase null mice. J Hypertens 2006; **24**: 1429–1436. [Medline] [CrossRef]
13. Lai EY, Martinka P, Fahling M, Mrowka R, Steege A, Gericke A, Sendeski M, Persson PB, Persson AE, Patzak A: Adenosine restores angiotensin II-induced contractions by receptor-independent enhancement of calcium sensitivity in renal arterioles. Circ Res 2006; **99**: 1117–1124 Epub 2006 Oct 12. [Medline] [CrossRef]
14. Matsuda H, Hayashi K, Arakawa K, Naito M, Kubota E, Honda M, Matsumoto A, Suzuki H, Yamamoto T, Kajiya F, Saruta T: Zonal heterogeneity in action of angiotensin-converting enzyme

inhibitor on renal microcirculation: role of intrarenal bradykinin. J Am Soc Nephrol 1999; **10**: 2272–2282. [Medline]
15. Cowley AW Jr, Mattson DL, Lu S, Roman RJ: The renal medulla and hypertension. Hypertension 1995; **25**: 663–673. [Medline] [CrossRef]
16. Yada T, Hiramatsu O, Kimura A, Goto M, Ogasawara Y, Tsujioka K, Yamamori S, Ohno K, Hosaka H, Kajiya F: In vivo observation of subendocardial microvessels of the beating porcine heart using a needle-probe videomicroscope with a CCD camera. Circ Res 1993; **72**: 939–946. [Medline] [CrossRef]
17. Wakino S, Hayashi K, Kanda T, Tatematsu S, Homma K, Yoshioka K, Takamatsu I, Saruta T: Peroxisome proliferator-activated receptor gamma ligands inhibit Rho/Rho kinase pathway by inducing protein tyrosine phosphatase SHP-2. Circ Res 2004; **95**: e45–e55. [Medline] [CrossRef]
18. Wakino S, Hayashi K, Tatematsu S, Hasegawa K, Takamatsu I, Kanda T, Homma K, Yoshioka K, Sugano N, Saruta T: Pioglitazone lowers systemic asymmetric dimethylarginine by inducing dimethylarginine dimethylaminohydrolase in rats. Hypertens Res 2005; **28**: 255–262. [Medline] [CrossRef]
19. Yang XC, Sachs F: Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions. Science 1989; **243**: 1068–1071. [Medline] [CrossRef]
20. Wellner M.C., Isenberg G: Stretch-activated non-selective cation channels in urinary bladder myocytes: importance for pacemaker potentials and myogenic response. Non-Selective Cation Channels: Pharmacology, Physiology and Biophysics, pp 93–99. Birkhauser Verlag Basal, Switzerland.
21. Wright FS, Schnermann J: Interference with feedback control of glomerular filtration rate by furosemide, triflocin, and cyanide. J Clin Invest 1974; **53**: 1695–1708. [Medline] [CrossRef]
22. Ichihara A, Navar LG: Neuronal NOS contributes to biphasic autoregulatory response during enhanced TGF activity. Am J Physiol 1999; **277**: F113–F120. [Medline]
23. Steinhausen M, Blum M, Fleming JT, Holz FG, Parekh N, Wiegman DL: Visualization of renal autoregulation in the split hydronephrotic kidney of rats. Kidney Int 1989; **35**: 1151–1160. [Medline] [CrossRef]
24. Fujiwara K, Hayashi K, Matsuda H, Kubota E, Honda M, Ozawa Y, Saruta T: Altered pressure-natriuresis in obese Zucker rats. Hypertension 1999; **33**: 1470–1475. [Medline] [CrossRef]
25. Majid DS, Omoro SA, Chin SY, Navar LG: Intrarenal nitric oxide activity and pressure natriuresis in anesthetized dogs. Hypertension 1998; **32**: 266–272. [Medline] [CrossRef]
26. Tojo A, Welch WJ, Bremer V, Kimoto M, Kimura K, Omata M, Ogawa T, Vallance P, Wilcox CS: Colocalization of demethylating enzymes and NOS and functional effects of methylarginines in rat kidney. Kidney Int 1997; **52**: 1593–1601. [Medline] [CrossRef]
27. Mattson DL, Higgins DJ: Influence of dietary sodium intake on renal medullary nitric oxide synthase. Hypertension 1996; **27**: 688–692. [Medline] [CrossRef]
28. Shiga N, Hirano K, Hirano M, Nishimura J, Nawata H, Kanaide H: Long-term inhibition of RhoA attenuates vascular contractility by enhancing endothelial NO production in an intact rabbit mesenteric artery. Circ Res 2005; **96**: 1014–1021. [Medline] [CrossRef]
29. Dautzenberg M, Keilhoff G, Just A: Modulation of the myogenic response in renal blood flow autoregulation by NO depends on endothelial nitric oxide synthase (eNOS), but not neuronal or inducible NOS. J Physiol 2011; **589**: 4731–4744. [Medline]
30. Racasan S, Joles JA, Boer P, Koomans HA, Braam B: NO dependency of RBF and autoregulation in the spontaneously hypertensive rat. Am J Physiol Renal Physiol 2003; **285**: F105–F112. [Medline]
31. Hoffend J, Cavarape A, Endlich K, Steinhausen M: influence of endothelium-derived relaxing factor on renal microvessels and pressure-dependent vasodilation. Am J Physiol 1993; **265**: F285–F292. [Medline]
32. Hayashi K, Suzuki H, Saruta T: Nitric oxide modulates but does not impair myogenic vasoconstriction of the afferent arteriole in spontaneously hypertensive rats. Studies in the isolated perfused hydronephrotic kidney. Hypertension 1995; **25**: 1212–1219. [Medline] [CrossRef]
33. Hayashi K, Epstein M, Loutzenhiser R: Determinants of renal actions of atrial natriuretic peptide. Lack of effect of atrial natriuretic peptide on pressure-induced vasoconstriction. Circ Res 1990; **67**: 1–10. [Medline] [CrossRef]
34. Just A, Arendshorst WJ: Nitric oxide blunts myogenic autoregulation in rat renal but not skeletal muscle circulation via tubuloglomerular feedback. J Physiol 2005; **569**: 959–974. [Medline] [CrossRef]
35. Shi Y, Wang X, Chon KH, Cupples WA: Tubuloglomerular feedback-dependent modulation of renal myogenic autoregulation by nitric oxide. Am J Physiol Regul Integr Comp Physiol 2006; **290**: R982–R991. [Medline]
36. Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM, Bertoglio J, Chardin P, Pacaud P, Loirand G: Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle. J Biol Chem 2000; **275**: 21722–21729. [Medline] [CrossRef]
37. Ito S, Juncos LA, Carretero OA: Pressure-induced constriction of the afferent arteriole of spontaneously hypertensive rats. Hypertension 1992; **19**(Suppl): II164–II167. [Medline] [CrossRef]
38. Welch WJ, Tojo A, Wilcox CS: Roles of NO and oxygen radicals in tubuloglomerular feedback in SHR. Am J Physiol Renal Physiol 2000; **278**: F769–F776. [Medline]
39. Tojo A, Onozato ML, Fujita T: Role of macula densa neuronal nitric oxide synthase in renal diseases. Med Mol Morphol 2006; **39**: 2–7. [Medline] [CrossRef]