Rho Kinase Activation and Gene Expression Related to Vascular Remodeling in Normotensive Rats With High Angiotensin I–Converting Enzyme Levels

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Abstract—The RhoA/Rho kinase (ROCK) pathway is a new mechanism of remodeling and vasoconstriction. Few data are available regarding ROCK activation when angiotensin I–converting enzyme is high and blood pressure is normal. We hypothesized that ROCK is activated in the vascular wall in normotensive rats with genetically high angiotensin I–converting enzyme levels, and it causes increased vascular expression of genes promoting vascular remodeling and also oxidative stress. Aortic ROCK activation, mRNA and protein levels (of monocyte chemoattractant protein-1, transforming growth factor [TGF]-β_1, and plasminogen activator inhibitor-1 [PAI-1]), NADPH oxidase activity, and $O_2^{\cdot-}$ production were measured in normotensive rats with genetically high (Brown Norway [BN]) and low (Lewis) angiotensin-I–converting enzyme levels and in BN rats treated with the ROCK antagonist fasudil (100 mg/kg per day) for 7 days. ROCK activation was 12-fold higher in BN versus Lewis rats ($P<0.05$) and was reduced with fasudil by 100% ($P<0.05$). Aortic TGF-β1, PAI-1, and monocyte chemoattractant protein-1 mRNA levels were higher in BN versus Lewis rats by 300%, 180%, and 1000%, respectively ($P<0.05$). Aortic TGF-β1, PAI-1, and monocyte chemoattractant protein-1 protein levels were higher in BN versus Lewis rats ($P<0,05$). Fasudil reduced TGF-β1 and PAI-1 mRNA and TGF-β1, PAI-1, and monocyte chemoattractant protein-1 protein aortic levels to those observed in Lewis rats. Aortic reduced nicotinamide-adenine dinucleotide phosphate oxidase activity and $\cdot O_2^-$ production were increased by 88% and 300%, respectively, in BN rats ($P<0.05$) and normalized by fasudil. In conclusion, ROCK is significantly activated in the aortic wall in normotensive rats with genetically high angiotensin-I–converting enzyme and angiotensin II, and it causes activation of genes that promote vascular remodeling and also increases vascular oxidative stress.

Key Words: Rho kinase ■ angiotensin-converting enzyme ■ angiotensin ■ remodeling ■ fasudil ■ NADPH ■ hypertension

Different molecules produced by the vascular wall are responsible for remodeling and vasoconstriction in hypertension, heart failure, and atherosclerosis. One novel mechanism promoting both vascular remodeling and vasoconstriction is the intracellular signaling pathway RhoA and its Rho kinase, or ROCK (ρ-associated, coiled-coil–containing protein kinase [ROCK]).[1–3] ROCK plays an important role in mediating various cellular functions, such as contraction of vascular smooth muscle cells,[3,4] actin cytoskeleton organization,[5–7] cell adhesion and motility,[8] cytokinesis,[9] and expression of genes involved in vascular remodeling.[2,10]

Contraction and relaxation of the vessels are importantly regulated by phosphorylation reactions of the myosin light chain phosphatase through the effect of the myosin phosphatase target protein-1 (MYPT1[11,12]). ROCK modulates MYPT1 phosphorylation.[12,13] When ROCK is activated, MYPT1 is phosphorylated in Thr696, and its phosphatase activity is inhibited. MYPT1 inhibition subsequently raises phosphorylated myosin light chain levels and vessel contraction increases.[14]

ROCK also mediates upregulation of several proinflammatory,[12,15–18] thrombogenic,[19,20] and fibrogenic molecules.[20–22] In addition, it mediates hypoxia-induced downregulation of endothelial NO synthase.[23] Thus, when ROCK is activated, inflammation, thrombosis, and tissue fibrosis are accelerated, whereas endothelial NO production is inhibited.[2]

The deleterious role of angiotensin II (Ang II) in remodeling and hypertension has been established during the last 2 decades. Ang II promotes vasoconstriction, aldosterone secretion, procoagulation, and atherosclerosis, as well as cardiovascular fibrosis, hypertrophy, inflammation, and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activation. Most of these effects are mediated by the Ang II type 1 receptor.[24] Because of these pathophysiological

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effects, Ang II type 1 receptor blockers and angiotensin I–converting enzyme (ACE) inhibitors make up a cornerstone therapy in hypertension and heart failure. In hypertension, ROCK is substantially involved in the vascular effects of Ang II.[25,26] In hypertensive rats, long-term ROCK inhibition suppresses Ang II–induced cardiovascular hypertrophy[25] and Ang II–induced monocyte chemoattractant protein-1 (MCP-1) expression in vascular smooth muscle cells.[16] However, in normotensive animals, there are no data available on the effect of high Ang II levels on ROCK activation.

One experimental model to assess the role of constantly elevated Ang II levels and normal blood pressure on the RhoA/ROCK pathway is the rat with genetically high ACE levels (the Brown Norway [BN] rat), an experimental model similar to humans with the D allele and high ACE expression.[27–29] In the BN rats, normal blood pressure, high circulating and tissue levels of ACE, high plasma Ang II levels, and low Ang-1-7 levels are observed.[30–34] Compared with rats with genetically low ACE levels (Lewis rats), the BN rats have higher levels of vascular oxidative stress[31] and develop higher levels of chronic hypertension in response to experimental renal artery stenosis.[32] There are no studies assessing the role of genetically increased ACE expression and Ang II levels on the Rho/ROCK pathway activation.

We have hypothesized here that, in normotensive rats with genetically increased ACE (and Ang II) levels, the Rho/ROCK pathway is significantly activated in the vascular wall and also that the activation of this pathway induces vascular expression of genes and proteins contributing to vascular remodeling, specifically of inflammation (MCP-1), fibrosis (transforming growth factor [TGF]-β_1), and coagulation (plasminogen activator inhibitor [PAI]-1) and higher levels of oxidative stress. With this aim, we determined aortic levels of ROCK activation, mRNA and protein (of MCP-1, TGF-β_1, and PAI-1), and oxidative stress in normotensive rats with genetically high (BN) or low (Lewis) levels of ACE and Ang II and also in BN rats treated with the ROCK inhibitor fasudil.

Methods

Experimental Design

This investigation was compliant with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication No. 85-23, revised 1985), and it was approved by our institutional review committee. Normotensive male rats, homozygous F_2 Lewis and BN, with contrasting levels of plasma ACE, were used.[30–34] Systolic blood pressure (SBP) was measured by the tail-cuff method. To select homozygous BN and Lewis rats, the PCR was performed in all of the animals[30–36] (data not shown).

To further investigate the role of ROCK in this experimental model, BN rats were treated for 7 days by gavage with the ROCK inhibitor fasudil (100 mg/kg per day; LC Laboratories). Animals were euthanized and the whole aorta was excised, weighed, washed in saline buffer, and stored at −80°C until processing.

Plasma ACE Activity

Was determined by spectrofluorometry using Z-phenyl-L-histidyl-L-leucine (Bachem Bioscience Inc) as substrate and expressed in units per milliliters (1 unit=1 nmol of L-histidyl-L-leucine formed per minute).[31,33,34]

Preparation of Aorta Extracts

The aorta was homogenized in cold lysis buffer (Tris HCl at 50 mmol/L, Nonidet P-40 at 1%, NaCl at 150 mmol/L, Na-deoxycholate at 0.25%, EDTA at 1 mmol/L, sodium dodecyl sulfate at 0.1%, aprotinin at 1 μg/mL, leupeptin at 1 μg/mL, and PMSF at 1 mmol/L). Samples were centrifuged at 4°C, and protein content of supernatants was determined by Bradford assay using BSA as the standard.[37] Soluble fractions were heated at 95°C with 0.33 vol of 4× SDS-PAGE sample buffer for Western blot analysis.

Analysis for MYPT1

The levels of aortic phosphorylated MYPT1 (phospho-MYPT1), downstream target of Rho kinase, an index of ROCK activation,[11,38–40] were assessed by Western blot. Aorta extracts were matched for protein, separated by SDS-PAGE on 6% polyacrylamide gels, and electrotransferred to nitrocellulose using a Trans-blot unit (Bio-Rad) for 1.5 hours at 300 mA. Membranes were blocked with 1% nonfat milk in PBS containing 0.005% Tween-20 [PBST] at room temperature. Anti-phospho-thr696-MYPT1 (Upstate Biotechnology, Millipore) or anti-MYPT1 (BD Transduction Laboratories) primary antibodies were diluted in blocking solution (1:2000 and 1:1000, respectively). Nitrocellulose membranes were incubated with primary antibody overnight at 4°C. After washing in PBST, blots were incubated at room temperature with horseradish peroxidase–linked secondary antibody. Blots were washed again in PBST, and specific binding was detected using enhanced chemiluminescence with exposure to Kodak film. Each blot was quantified by scanning densitometry with the Un-Scan-It software.

mRNA Levels of TGF-β_1, PAI-1, and MCP1

DNAase-treated total RNA isolated from aorta with Trizol[31] was quantified by ultraviolet spectroscopy. The RT-PCR assay was performed using published primer sequences for TGF-β_1, PAI-1, and MCP-1.[41] Amplification conditions for TGF-β_1 were as follows: denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, and elongation at 72°C for 10 minutes. For PAI-1 the conditions were as follows: denaturation at 94°C for 1 minute, annealing at 58°C for 0.5 minutes, and elongation at 72°C for 10 minutes. The cycle numbers for TGF-β_1, PAI-1, and MCP1 were 33, 33, and 40, respectively. Finally, an elongation at 72°C for 10 minutes was performed. For MCP-1 the conditions were as follows: denaturation at 92°C for 1 minute, annealing at 53°C for 1 minute, and elongation at 72°C for 10 minutes. The cycle numbers for TGF-β_1, PAI-1, and MCP1 were 33, 33, and 38, respectively. After PCR, the amplification products were fractionated on a 1.5% (weight/weight) agarose gel and visualized by staining with ethidium bromide. Band intensities were quantified by computerized densitometry and normalized with respect to 18S RNA.

Protein Levels of TGF-β and PAI-1 in the Aorta

The levels of TGF-β_1 and PAI-1 were assessed by Western blot. Aorta extracts were matched for protein, separated by SDS-PAGE on 12% polyacrylamide gels, and electrotransfered to nitrocellulose. Membranes were blocked with 5% nonfat milk in PBS containing 0.005% Tween-20 at 4°C overnight. Anti-TGF-β_1 or anti-PAI-1 (Santa Cruz Biotechnology) primary antibodies were diluted in 0.5% nonfat milk in PBS containing 0.005% Tween-20 (PBST; 1:1000 and 1:500, respectively). Nitrocellulose membranes were incubated with primary antibody for 6 hours at room temperature. After washing in PBST, blots were incubated with horseradish peroxidase–linked secondary antibody (1:10 000) for 2 hours. Blots were washed again in PBST, and specific binding was detected using ECL with exposure to Kodak film. Each blot was quantified by scanning densitometry with the Un-Scan-It software.

NADPH Oxidase Activity and $O_2^{\cdot-}$ Production

Aorta homogenates were centrifuged at 4°C, and the pellet was resuspended in lysis buffer and manually homogenized on ice. NADPH oxidase activity was measured by a luminescence assay containing lucigenin as the electron acceptor and NADPH as the

Table. Body Weight, Blood Pressure, Left Ventricular Mass, and Plasma ACE Activity

Parameter	Lewis (n=8)	BN (n=8)	BN-F (n=8)
BW, g	161±2	150±1	163±2
SBP, mm Hg	111±1	109±1	107±2
RLVM, mg*100 g BW	243±2	231±5	263±2
Plasma ACE activity, units/mL	145±13*	221±9	184±9†

Data are mean±SEM. BN+F indicates BN rats treated with fasudil (100 mg/kg per day for 7 days); BW, body weight; SBP, systolic blood pressure; RLVM, relative left ventricular mass.

*$P<0.05$ vs BN (after significant ANOVA).

†$P<0.05$ vs BN and Lewis (after significant ANOVA).

substrate.[31] To verify the specificity of the signal, each assay was performed with and without apocynin (2.5 mmol/L), and the counts were subtracted to determine the final counts. Luminescence measurements were obtained every 15 seconds for 5 minutes. Aortic $\cdot O_2^-$ production was estimated with lucigenin-enhanced chemiluminescence. Lucigenin count was expressed as counts per second per gram of protein.

Statistical Analysis

Results (mean±SEM) were compared by 1-factor ANOVA followed by Student-Newman-Keuls test. A $P\leq0.05$ was considered statistically significant.

Results

SBP, Left Ventricular Mass, and Plasma ACE Activity

SBP levels in both BN and Lewis rats were within the usual normal blood pressure levels observed in normotensive rats (Table).[30–33] The administration of the ROCK inhibitor fasudil (100 mg/kg per day) to normotensive BN rats did not modify SBP. Left ventricular mass was similar among the groups. Plasma ACE was significantly increased in BN compared with Lewis rats (not modified by fasudil).

Phospho-MYPT1 Aortic Levels

The phospho-MYPT1/MYPT1 ratio, index of ROCK activation[11,37–39] was 12-fold higher in the aortic wall of the normotensive BN rats compared with the Lewis rats (12.9±2.4 versus 1.0±0.2 units of optical density, respectively; $P<0.05$; Figure 1). Fasudil reduced the aortic phospho-MYPT1/MYPT1 ratio to the same levels observed in the Lewis rats.

TGF-β_1, PAI-1, and MCP-1 mRNA Aortic Levels

Aortic mRNA levels of TGF-β1, PAI-1, and MCP-1 mRNA were significantly higher in the BN compared with the Lewis rats by 300%, 180%, and 1000%, respectively ($P<0.05$). Treatment with fasudil significantly reduced the aortic mRNA levels of TGF-β_1 and PAI-1 to levels similar to those observed in the Lewis rats ($P<0.05$). The higher expression of the MCP1 gene observed in BN rats showed a trend to be reduced by fasudil (Figures 2 to 4).

Protein Levels of TGF-β_1, PAI-1, and MCP-1 in the Aorta

Protein levels of TGF-β1, PAI-1, and MCP-1 mRNA were significantly higher in the BN compared with the Lewis rats

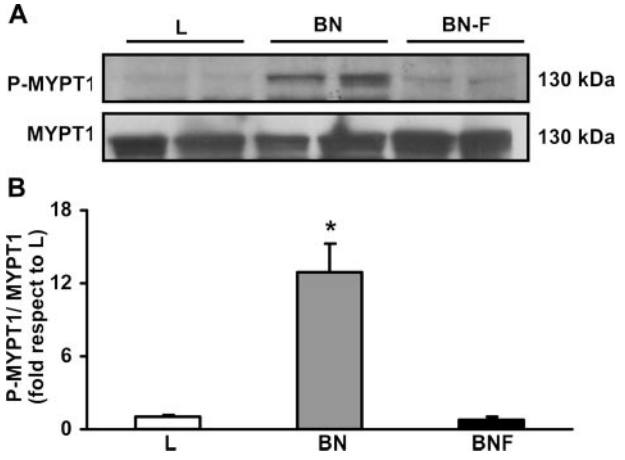

Figure 1. Levels of phospho-MYPT1 in the aorta of rats with genetically distinct plasma ACE levels and effect of fasudil (100 mg/kg per day for 7 days). The levels of phospho-MYPT1 and MYPT1 were determined by Western blot as described in the Methods in the aortic wall of the Lewis or BN rats or in BN rats treated for 7 days with fasudil (100 mg/kg per day; BN-F). A, Representative photographs of Western blots for phospho-MYPT1 and MYPT1 in the 3 experimental groups. B, phospho-MYPT1/MYPT1 ratio. The values are mean±SEM; n=8 to 10 per group. P-MYPT1 indicates phospho-MYPT1. *$P<0.05$ vs Lewis (after significant ANOVA).

by 230%, 92%, and 210%, respectively ($P<0.05$; Figure 5). Fasudil significantly reduced the protein levels of TGF-β_1, PAI-1, and MCP-1 to levels similar to those observed in Lewis rats ($P<0.05$).

NADPH Oxidase Activity and $O_2^{\cdot-}$ Production

NADPH oxidase activity was increased significantly by 88% in the BN rats compared with the Lewis rats, and it was

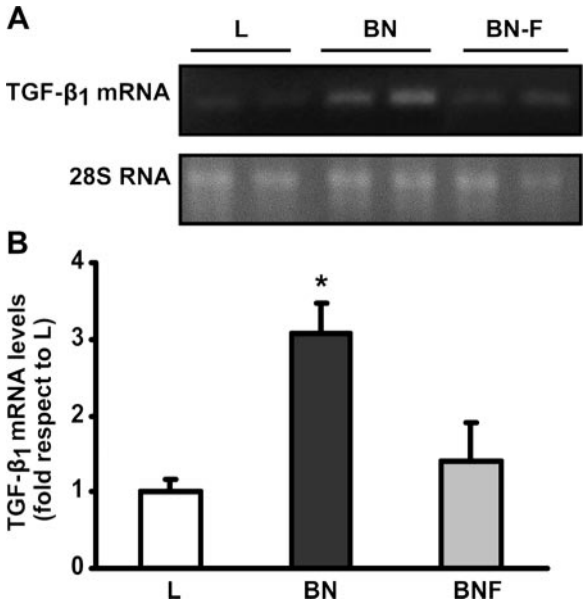

Figure 2. TGF-β_1 mRNA levels in the aortic wall of Lewis or BN rats or in BN rats treated for 7 days with fasudil (100 mg/kg per day; BN-F). A, DNAase-treated total RNA was isolated with Trizol, and integrity of 26S RNA was assessed on agarose gel. B, Band intensities were quantified by computerized densitometry and normalized with respect to 26S RNA. Data are mean±SEM; n=8 to 10 per group. *$P<0.05$ vs Lewis (after significant ANOVA).

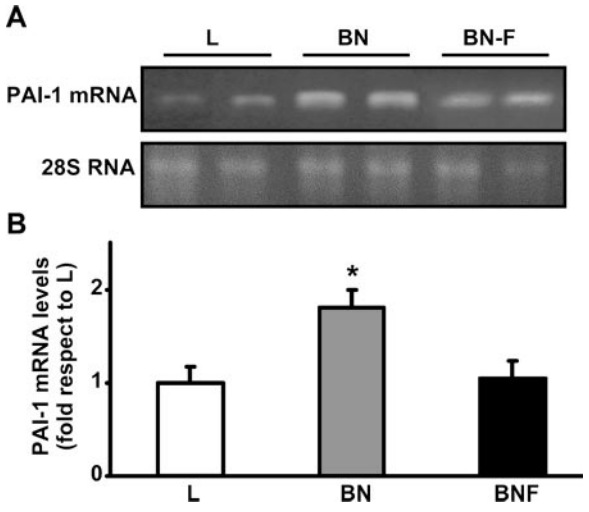

Figure 3. PAI-1 mRNA levels in the aortic wall of Lewis or BN rats or in BN rats treated for 7 days with fasudil (100 mg/kg per day; BN-F). A, DNAase-treated total RNA was isolated with Trizol, and integrity of 26S RNA was assessed on agarose gel. B, Band intensities were quantified by computerized densitometry and normalized with respect to 26S RNA. Data are mean±SEM; n=8 to 10 per group. *P<0,05 vs Lewis (after significant ANOVA).

normalized by fasudil (1±0.05, 1.88±0.41, and 1.2±0.28 relative light units, respectively; $P<0.05$). Aortic $O_2^{\cdot-}$ production was increased significantly by 300% in the BN rats compared with the Lewis rats, and it was normalized by fasudil (1±0.06, 3±0.98, and 0.9±0.07 relative light units, respectively; $P<0.05$).

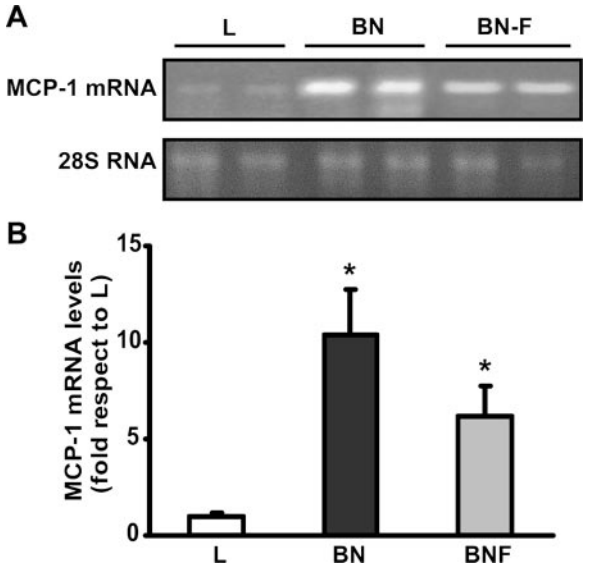

Figure 4. MCP-1 mRNA levels in the aortic wall of Lewis or BN rats or in BN rats treated for 7 days with fasudil (100 mg/kg per day; BN-F). A, DNAase-treated total RNA was isolated with Trizol, and integrity of 26S RNA was assessed on agarose gel. B, Band intensities were quantified by computerized densitometry and normalized with respect to 26S RNA. Data are mean±SEM; n=8 to 10 per group. *P<0.05 vs Lewis (after significant ANOVA).

Discussion

Our main results were that the RhoA/ROCK pathway is significantly activated (by 12-fold) in the aortic wall in normotensive rats with genetically high ACE and Ang II. In this experimental model, ROCK activation was associated with increased expression of genes and proteins that promote vascular remodeling and high oxidative stress. In BN rats (with genetically high ACE and Ang II levels and low Ang-1-7 levels),[31–33] the aortic mRNA and protein levels of TGF-β_1, PAI-1, and MCP-1 were significantly higher compared with the "control" Lewis rats (with genetically low levels of ACE and Ang II and high levels of Ang-1-7) and were "normalized" to levels observed in Lewis rats by the ROCK inhibitor fasudil.

The causative role of the RhoA/ROCK pathway in the activation of the TGF-β1, PAI-1, and MCP-I genes, as well as in higher vascular NADPH oxidase activity and $\cdot O_2^-$ in this model, was established by administering the specific ROCK inhibitor fasudil.[1–3] Fasudil also exerts nonspecific inhibitory effects on other serine/threonine kinases, such as protein kinase A and protein kinase C.[42] In BN rats, fasudil reduced the higher levels of phospho-MYPT-1 to the similar "normal" levels observed in the Lewis rats. In addition, in the BN rats, fasudil significantly reduced the levels of aortic mRNA and protein levels of TGF-β1, PAI-1, and MCP-1 to the levels observed in the Lewis rats (the higher levels of aortic MCP-1 mRNA observed in the BN rats showed a trend to be reduced with fasudil). In addition, fasudil normalized the high levels of vascular oxidative stress observed in the BN rats.

The RhoA/ROCK pathway is related to the cytoskeleton organization, and it regulates the expression of several genes. In experimental hypertension, the selective ROCK inhibitor Y-27632 inhibits PAI-1 gene expression,[43] and it also inhibits TGF-β_1 and MCP-1 gene expression in the vascular smooth muscle cell.[16,44] These data are consistent with our observations in the aortic wall where higher mRNA gene expression of PAI-1, MCP-1, and TGF-β1 in the normotensive BN rats was detected.

The current observations suggest that in this normotensive experimental model with high ACE and Ang II levels, direct ROCK inhibition normalizes the higher profibrotic, procoagulant, and proinflammatory vascular gene expression. These data also suggest that the high aortic wall levels of both phospho-MYPT1 and gene expression of TGF-β1, PAI-1, and MCP-1, because of high ACE and Ang II, are not only a consequence of constant Ang II type 1 receptor stimulation. In this model (and also in humans with the ACE deletion/deletion genotype), low circulating levels of Ang-1-7 and neutral endopeptidase 24.11 are observed,[28,33,34] but a precise relationship with the RhoA/ROCK pathway remains unknown.

By blocking the ROCK pathway, the molecular vascular remodeling induced by Ang II, assessed here by the gene and protein expression of PAI-1, TGF-β1, and MCP-1, is prevented and strongly supports its role in pathological vascular remodeling because of Ang II. Our results agree with observations on the effect of ROCK on Ang II-induced cardiovascular damage in hypertension. In hypertensive rats induced by Ang II infusion, fasudil suppressed the coronary vascular

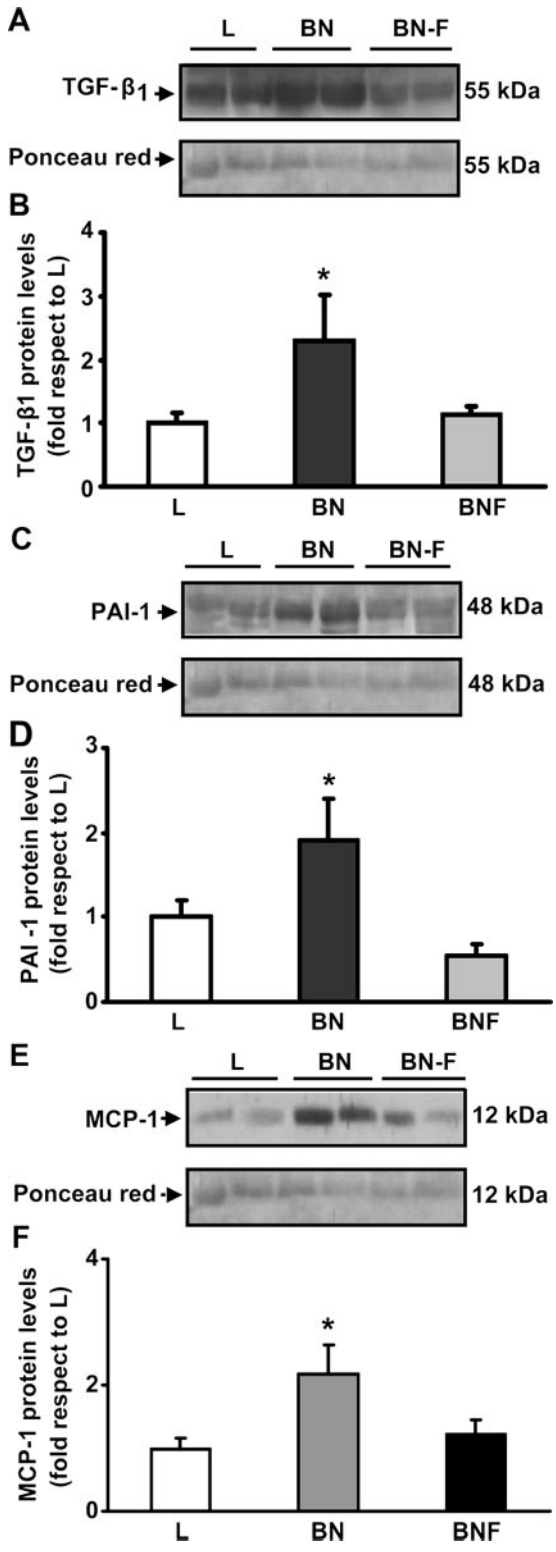

Figure 5. Protein expression (Western blot) of TGF-β_1 (A and B), PAI-1 (C and D), and MCP-1 (E and F) in the aortic wall of Lewis (L) or BN rats or in BN rats treated for 7 days with fasudil (100 mg/kg per day; BN-F). Aorta lysates were inmunoblotted with anti TGF-β_1 (A), anti PAI-1 (B), or with anti-MCP-1 antibody (C), respectively, and the proteins were stained with Ponceau red. TGF-β_1, PAI-1, and MCP-1 band intensities were quantified by computerized densitometry and normalized with respect to the protein stained with Ponceau red (B, D, and F, respectively). Data are mean±SEM; n=6 to 8 per group. *$P<0.05$ vs Lewis (after significant ANOVA).

lesion formation, normalized endothelial NADPH oxidase activity, endothelial production of superoxide anions, and endothelial function.[25] In addition, fasudil improved Ang II–induced vascular injury through p27-dependent and p27-independent mechanisms[45] in hypertensive mice and suppressed Ang II–induced cardiovascular hypertrophy, NADPH oxidase upregulation, and superoxide production in rats.[25] In apolipoprotein E–deficient mice, fasudil attenuated the Ang II–induced aortic abdominal aneurysm inhibiting both apoptosis and proteolysis pathways.[46]

The clinical use of ROCK inhibitors could have significant vasculoprotective effects in diseases with elevated Ang II levels, such as hypertension, heart failure, and also in atherosclerosis beyond lowering blood pressure. In patients with coronary artery disease, fasudil increased endothelium-dependent vasodilation, suggesting that ROCK activation promotes endothelial dysfunction in atherosclerosis.[47] In patients with stable angina, the intracoronary vasodilatory effect of fasudil surpassed that of nitroglycerin in concentric coronary stenosis, suggesting that the RhoA/ROCK pathway regulates the coronary vasomotion of atherosclerotic lesions.[48]

Recent data point to a substantial role of the RhoA/ROCK pathway activation in hypertension and show that different upstream signals can converge toward ROCK in hypertensive vascular diseases.[49] In our normotensive BN rats, increased aortic ROCK activity was not correlated with hypertension; however, we have observed previously that BN rats have persistent higher levels of hypertension than Lewis rats in the Goldblatt model of chronic hypertension.[32]

Some limitations of our study are as follows: (1) Lewis rats were not treated with fasudil because of the clear differences in the aortic levels of phospho-MYPT1 and gene expression between the BN and Lewis rats; (2) no dose-response experiments were performed; (3) no specific cell contribution within the aortic wall for increased MYPT-1 activity was evaluated; and (4) no end points, such as measures of inflammation or thrombosis, were determined. In this sense, the current experiments provide expression results and biochemical data, but functional evidence that the RhoA/ROCK system plays a role in the BN rats at this age is lacking. These limitations do not invalidate the current observations and conclusions but should be addressed in more extensive studies.

Perspectives

Because this is a genetic model of increased ACE and Ang II levels, similar to humans with the D allele,[27–29] it is quite possible that, in people with this condition, vascular ROCK is enhanced. In these people, RhoA/ROCK activation could explain why they develop a higher incidence of vascular diseases, such as hypertension in men[50,51] or restenosis after coronary angioplasty,[52,53] and to block this pathway.

In conclusion, the intracellular ROCK pathway is activated in the aortic wall in normotensive rats with genetically higher levels of ACE and Ang II. The activation of this signaling pathway induces the expression of genes and proteins contributing to pathological vascular remodeling and increased oxidative stress and can be blocked by the specific ROCK inhibitor fasudil.

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Disclosures

None.

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