# Crucial role of Rho-nuclear factor-κB axis in angiotensin II-induced renal injury

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## Abstract

This study was performed to determine the effectiveness of the Rho kinase inhibitor and NF-κB inhibitor in renal injury of ANG II-infused hypertensive rats. Male Sprague-Dawley rats, maintained on a normal diet, received either a sham operation ($n = 7$) or continuous ANG II infusion (120 ng/min) subcutaneously via minipumps. The ANG II-infused rats were further subdivided into three subgroups ($n = 7$ each) to receive one of the following treatments during the entire period: vehicle, Rho kinase inhibitor (fasudil; 3 mg·kg$^{-1}$·day$^{-1}$ ip), or NF-κB inhibitor (parthenolide; 1 mg·kg$^{-1}$·day$^{-1}$ ip). After 12 days of ANG II infusion, systolic blood pressure (BP; $208 \pm 7$ vs. $136 \pm 3$ mmHg), Rho kinase activity, NF-κB activity, renal ANG II contents ($160 \pm 25$ vs. $84 \pm 14$ pg/g), monocytic chemotactic protein (MCP) 1 mRNA, interstitial macrophage infiltration, transforming growth factor-β1 (TGF-β1) mRNA, interstitial collagen-positive area, urinary protein excretion ($43 \pm 6$ vs. $11 \pm 2$ mg/day), and urinary albumin excretion were significantly enhanced compared with the Sham group. While fasudil or parthenolide did not alter systolic BP ($222 \pm$ and $190 \pm 21$, respectively), both treatments completely blocked ANG II-induced enhancement of NF-κB activity, renal ANG II contents ($103 \pm 11$ and $116 \pm 21$ pg/g, respectively), MCP1 mRNA, interstitial macrophage infiltration, TGF-β1 mRNA, interstitial collagen-positive area, urinary protein excretion ($28 \pm 6$ and $23 \pm 3$ mg/day, respectively), and urinary albumin excretion. Importantly, parthenolide did not alter ANG II-induced Rho kinase activation although fasudil abolished ANG II-induced Rho kinase activation. These data indicate that the Rho-NF-κB axis plays crucial roles in the development of ANG II-induced renal injury independently from BP regulation.

## Keywords

hypertension

Recent findings related to the intrarenal renin-angiotensin system (RAS), which is one of the most important regulatory mechanisms for blood pressure (BP) homeostasis, have provided us with an improved understanding of the pathophysiology of hypertension (22,50-52,71). However, these findings have also led to unique concepts and questions that need to be investigated in more depth. More detailed investigations can now be performed to

characterize the mechanisms responsible for these alterations which lead to the development of hypertension and, either directly or indirectly, to progressive renal damage.

Increased peripheral vascular resistance plays an important role in the pathogenesis of hypertension. Recent studies report that ANG II and a small GTPase, Rho, are involved in these mechanisms (47,67,79). Rho kinase is activated by Rho, and it induces vascular contractions via two mechanisms. First, Rho kinase phosphorylates myosin light chain, which causes vascular smooth muscle cell contraction. Second, Rho kinase inhibits myosin phosphatase activity, and this inhibits dephosphorylation of myosin light chain, which sustains vascular smooth muscle cell contraction. A Rho kinase inhibitor is now clinically developed and used for the treatment of brain ischemia as a vasodilator and is being evaluated for the treatment of angina pectoris. In signaling pathways of ANG II, Rho kinase has a potent role to regulate effects of ANG II. Yamakawa et al. (79) demonstrated that ANG II-induced hypertrophy is regulated by the Rho kinase pathway in rat aortic smooth muscle cells. In skeletal dynamics, Shome et al. (67) demonstrated that small GTPases modulate the ANG II-induced activation of phospholipase D in cultured vascular smooth muscle cells. In renal microvasculature, Nakamura et al. (47) demonstrated that the Rho kinase pathway mediates the basal tone and its inhibitor diminishes ANG II-induced vasoconstriction in a hydronephrotic kidney model. It is suggested that Rho kinase can be one of the important modulators in ANG II-dependent hypertension.

Although many kinds of transcriptional factors are involved in the ANG II signaling pathway, NF-κB has pivotal roles in the ANG II-induced inflammatous change (43,58). Muller et al. (43) demonstrated that inhibition of NF-κB ameliorates ANG II-induced cardiac and renal damages. Ruiz-Ortega et al. (58) demonstrated that NF-κB is activated by ANG II in rat thoracic aorta vascular smooth muscle cells. Interestingly, Perona et al. (55) demonstrated that Rho induces the transcriptional activity of NF-κB by stimulating the phosphorylation of the IκBα in Ser-32 and Ser-36 residues. Thus the Rho-NF-κB pathway may have the important role in ANG II-induced inflammatous change in ANG II-dependent hypertensive nephropathy.

We have recently reported that NF-κB-dependent upregulation of monocytic chemotactic protein (MCP) 1 and transforming growth factor-β1 (TGF-β1) plays an important role in the development of renal injury in ANG II-dependent hypertension (54). However, it has not been established whether a Rho kinase inhibitor and/or NF-κB inhibitor exerts renoprotective effects in ANG II-dependent hypertension. Therefore, this study was performed to determine the effectiveness of the Rho kinase inhibitor fasudil and the NF-κB inhibitor parthenolide in renal injury of ANG II-infused hypertensive rats.

## MATERIALS AND METHODS

### Preparation of animals

The experimental protocol was approved by the Animal Care and Use Committees of Tulane University. Male Sprague-Dawley rats (~250 g, Charles River), maintained on a normal diet, received either a sham operation ($n = 7$) or continuous ANG II infusion (120 ng/min) subcutaneously via minipumps (Alzet). The ANG II-infused rats were further subdivided into three subgroups ($n = 7$ each) to receive one of the following treatments during the entire period: vehicle, Rho kinase inhibitor (fasudil; 3 mg·kg$^{-1}$·day$^{-1}$ ip, Asahi Kasei), or NF-κB inhibitor (parthenolide; 1 mg·kg$^{-1}$·day$^{-1}$ ip, Biomol). All rats were monitored up to 12 days of ANG II infusion with free access to a regular diet and water. Systolic BP was measured in conscious rats using tail-cuff plethysmography (Visitech) every 3 days as previously described (28-30,33,36). Twenty-four-hour urine samples were collected the day before the

tissue harvesting, and the protein concentration and albumin concentration in urine samples were measured as previously described (28-30,33,36).

## Sample collection

Kidney samples were harvested by decapitation after 12 days of ANG II infusion. Immediately after removal, one kidney was homogenized in cold methanol and renal ANG II was measured as previously described (28-30,33,36).

The contralateral kidneys were separated into four pieces. The first piece was immersed in RNAlater (Ambion) for total RNA extraction. The second piece was immersed in zinc-saturated formalin (Anatech) for tissue fixation. The third piece and the last piece were immersed in liquid nitrogen in Cryotubes (Nalgene) for protein extraction and nuclear protein extraction, respectively.

## Quantitative real-time RT-PCR

Total RNA extraction from rat kidneys and quantitative real-time RT-PCR for RelA, MCP1, and TGF-β1 mRNA were performed as previously described (34,44,54,56,57). Data of quantitative real-time RT-PCR were normalized by GAPDH mRNA expression. The sequence information of the primers and the probes for real-time RT-PCR are summarized in Table 1.

## Interstitial macrophage/monocyte infiltration

Using zinc-saturated formalin-fixed paraffin-embedded renal sections, the numbers of macrophages/monocytes were examined by immunohistochemistry with a commercially available antibody against CD68 (Serotec) as previously described (26,34,54). Immunohistochemistry was performed by a robotic system (Dako) as previously described (34,36,54,56) and counterstained with hematoxylin-eosin. Twenty consecutive microscopic fields were examined for each rat, and CD68-positive cells (brown) were counted in the interstitium in each microscopic field. The averaged numbers of macrophages/monocytes in the interstitium were then obtained for each rat.

## Interstitial collagen-positive area

Using zinc-saturated, formalinfixed, paraffin-embedded renal sections, the extent of interstitial collagen-positive areas was quantitatively evaluated by an automatic image analysis occupied by interstitial tissue staining positively for collagen in picro-Sirius red-stained sections (Mass Histology) as previously described (15,34,54). The fraction of renal cortex occupied by interstitial tissue was performed using Image-Pro plus software (Media Cybernetics). For each microscopic field, the collagen-positive area (pink) was automatically calculated by the software, and this affected area was, in turn, divided by the total area of the microscopic field. Twenty consecutive microscopic fields were examined for each rat, and the averaged percentages of the collagen-positive lesions were obtained for each rat.

The above-mentioned histological analyses were performed by an outsourcing company (Mass Histology) or a robotic system (Dako) with automatic image-analysis software (Media Cybernetics) in a blind manner to avoid any biases.

## Rho kinase activity assay

Protein extraction, sample purification, and Rho kinase activity assay were performed with rat kidneys using a commercially available kit (CycLex) according to the manufacturer's instructions.

### Electromobility shift assay

Nuclear protein extraction and electromobility shift assay for NF-κB were done with rat kidneys using a commercially available kit (Panomics) according to the manufacturer's instructions as previously described (31,54).

### Western blot analysis

Protein extraction and Western blot analysis were performed with rat kidneys as previously described (32,34-36,54,70) using an infrared imaging system (LI-COR Biosciences). Polyclonal primary antibodies against phosphorylated IκBα, phosphorylated IκBKα, and phosphorylated IκBKβ were purchased from Cell Signaling Technology. Polyclonal primary antibody against phosphorylated NF-κB-inducing kinase was purchased from Santa Cruz Biotechnology. Polyclonal primary antibody against β-actin was purchased from Abcam. Appropriate secondary antibodies were purchased from LI-COR Biosciences.

### Statistical analysis

Statistical analysis was performed using a one-way factorial ANOVA with post hoc Scheffé's $F$-test. All data are presented as means ± SE. $P < 0.05$ was considered significant.

## RESULTS

### Body weight

Body weight was similar among the four groups before the treatments. As previously described, chronic ANG II infusion in rats significantly suppressed the increase in body weight may be due to increased peripheral metabolism that is independent of elevations in BP (18). However, fasudil or parthenolide treatment did not show any additional effect on body weight in ANG II-infused rats (Table 2).

### Systolic BP

Systolic BP (Fig. 1A) was similar among the four groups before the treatments. However, systolic BP progressively and significantly increased ($208 \pm 7$ for ANG II vs. $136 \pm 3$ mmHg for Sham at *day 12*). Fasudil or parthenolide did not alter systolic BP ($222 \pm 8$ and $190 \pm 21$ at *day 12*, respectively).

### Rho kinase activity

As shown in Fig. 1B, chronic ANG II infusion significantly increased Rho kinase activity ($2.23 \pm 0.20$ for ANG II vs. $1.00 \pm 0.12$ arbitrary units for Sham). Importantly, while fasudil abolished ANG II-induced Rho kinase activation, parthenolide did not alter ANG II-induced Rho kinase activation ($0.98 \pm 0.22$ for ANG II+fasudil and $2.07 \pm 0.25$ arbitrary units for ANG II+parthenolide, respectively).

### RelA mRNA

For the evaluation of NF-κB expression, mRNA levels of RelA (p65), a part of the NF-κB complex, were measured by real-time RT-PCR. As shown in Fig. 1C, chronic ANG II infusion significantly increased RelA mRNA levels ($1.60 \pm 0.18$ for ANG II vs. $1.00 \pm 0.11$ arbitrary units for Sham). Both treatments completely blocked ANG II-induced enhancement of RelA mRNA levels ($0.95 \pm 0.11$ for ANG II+fasudil and $0.68 \pm 0.06$ arbitrary units for ANG II+parthenolide, respectively).

### Electromobility shift assay

NF-κB activity was evaluated by electromobility shift assay. As shown in Fig. 1D, chronic ANG II infusion significantly increased NF-κB activity ($2.25 \pm 0.18$ for ANG II vs. $1.00 \pm 0.19$ arbitrary units for Sham). Both treatments completely blocked ANG II-induced enhancement of NF-κB activity ($0.98 \pm 0.13$ for ANG II+fasudil and $0.95 \pm 0.10$ arbitrary units for ANG II+parthenolide, respectively).

### Kidney ANG II levels

As demonstrated in Fig. 1E, chronic ANG II infusion significantly increased kidney ANG II levels ($160 \pm 25$ for ANG II vs. $84 \pm 14$ pg/g for Sham). Both treatments completely blocked ANG II-induced enhancement of kidney ANG II levels ($103 \pm 11$ for ANG II+fasudil and $116 \pm 21$ pg/g for ANG II+parthenolide, respectively).

### MCP1 mRNA

As demonstrated in Fig. 2A, chronic ANG II infusion significantly increased intrarenal MCP1 mRNA levels ($1.55 \pm 0.15$ for ANG II vs. $1.00 \pm 0.13$ arbitrary units for Sham). Both treatments completely blocked ANG II-induced enhancement of intrarenal MCP1 mRNA levels ($1.16 \pm 0.13$ for ANG II+fasudil and $0.90 \pm 0.05$ arbitrary units for ANG II +parthenolide, respectively).

### Interstitial macrophage/monocyte infiltration

The interstitial macrophage/monocyte infiltration was evaluated by CD68-positive cell number, which is a surface marker for macrophages and monocytes, using zinc-saturated, formalin-fixed, paraffin-embedded kidney samples from the Sham (Fig. 2B), ANG II (Fig. 2C), ANG II+fasudil (Fig. 2D), and ANG II+parthenolide (Fig. 2E) groups. CD68-positive cells are stained brown. Figure 2F demonstrated that CD68-positive cell numbers were significantly increased by chronic ANG II infusion ($28 \pm 2$ cells/mm$^2$) compared with Sham ($16 \pm 1$ cells/mm$^2$). Both treatments completely blocked ANG II-induced enhancement of interstitial macrophage/monocyte infiltration ($23 \pm 2$ for ANG II+fasudil and $18 \pm 1$ for ANG II+parthenolide, respectively).

### TGF-β1 mRNA

As demonstrated in Fig. 3A, chronic ANG II infusion significantly increased intrarenal TGF-β1 mRNA levels ($1.52 1 \pm 0.16$ for ANG II vs. $1.00 \pm 0.08$ arbitrary units for Sham). Both treatments completely blocked ANG II-induced enhancement of intrarenal TGF-β1 mRNA levels ($0.95 \pm 0.12$ for ANG II+fasudil and $0.74 \pm 0.07$ arbitrary units for ANG II +parthenolide, respectively).

### Interstitial collagen-positive area

The interstitial collagen-positive area was stained by Picro-sirius red using zinc-saturated, formalin-fixed, paraffin-embedded kidney samples from Sham (Fig. 3B), ANG II (Fig. 3C), ANG II+fasudil (Fig. 3D), and ANG II±parthenolide (Fig. 3E) groups. The collagen-positive area is stained pink. With the use of an established computer-aided semiautomatic quantification system, Fig. 3F demonstrated that the interstitial collagen-positive area was significantly increased by chronic ANG II infusion ($1.00 \pm 0.04\%$) compared with Sham ($0.82 \pm 0.05$). Both treatments completely blocked ANG II-induced enhancement of the interstitial collagen-positive area ($0.84 \pm 0.02$ for ANG II+fasudil and $0.81 \pm 0.04$ for ANG II+parthenolide, respectively).

### Urinary protein excretion

As demonstrated in Fig. 4A, chronic ANG II infusion significantly increased urinary protein excretion ($43 \pm 6$ for ANG II vs. $11 \pm 2$ mg/day for Sham). Both treatments completely blocked ANG II-induced enhancement of urinary protein excretion ($28 \pm 6$ for ANG II +fasudil and $23 \pm 3$ mg/day for ANG II+parthenolide, respectively).

### Urinary albumin excretion

As demonstrated in Fig. 4B, chronic ANG II infusion significantly increased urinary albumin excretion ($6.9 \pm 1.7$ for ANG II vs. $2.7 \pm 0.5$ mg/day for Sham). Both treatments completely blocked ANG II-induced enhancement of urinary albumin excretion ($2.3 \pm 0.4$ for ANG II+fasudil and $3.5 \pm 1.7$ mg/day for ANG II+parthenolide, respectively).

### Western blot analysis

As demonstrated in Fig. 5A, chronic ANG II infusion significantly increased phosphorylation of IκBα ($1.49 \pm 0.09$ for ANG II vs. $1.00 \pm 0.07$ arbitrary units for Sham). Both treatments completely blocked ANG II-induced phosphorylation of IκBα ($1.03 \pm 0.02$ for ANG II+ fasudil and $1.05 \pm 0.07$ arbitrary units for ANG II+ parthenolide, respectively). As demonstrated in Fig. 5B (85 kDa), chronic ANG II infusion did not change phosphorylation of IκBKα ($0.99 \pm 0.07$ for ANG II vs. $1.00 \pm 0.09$ arbitrary units for Sham). Either treatment did not alter phosphorylation of IκBKα ($1.00 \pm 0.05$ for ANG II+fasudil and $1.08 \pm 0.07$ arbitrary units for ANG II+parthenolide, respectively). As demonstrated in Fig. 5B (87-kDa), chronic ANG II infusion did not change phosphorylation of IκBKβ ($0.94 \pm 0.05$ for ANG II vs. $1.00 \pm 0.07$ arbitrary units for Sham). Either treatment did not alter phosphorylation of IκBKβ ($0.93 \pm 0.09$ for ANG II+fasudil and $1.00 \pm 0.07$ arbitrary units for ANG II+parthenolide, respectively). As demonstrated in Fig. 5C, chronic ANG II infusion did not change phosphorylation of NF-κB-inducing kinase ($0.90 \pm 0.05$ for ANG II vs. $1.00 \pm 0.07$ arbitrary units for Sham). Either treatment did not alter phosphorylation of NF-κB-inducing kinase ($0.89 \pm 0.07$ for ANG II+fasudil and $0.94 \pm 0.07$ for ANG II +parthenolide, respectively). To verify equal loading, membranes were reprobed with β-actin antibody. As demonstrated in Fig. 5D, similar densities were observed among the four groups.

## DISCUSSION

A small GTPase, Rho, and its downstream effector molecule, Rho kinase, play an important role in various cellular functions (49). Rho/Rho kinase inhibits myosin phosphatase by phosphorylating its myosin-binding subunit, favoring accumulation of phosphorylated myosin light chain and enhanced contraction of vascular smooth muscle cell (46). It has been established that Rho/Rho kinase participates in cell adhesion/ migration and proliferation (1,64) and is involved in various models of cardiovascular disorders, independently of systemic BP (66). Thus the inhibition of Rho kinase is reported to suppress the neointimal formation of balloon-injured rat carotid arteries (63) and attenuate the formation of the coronary injury induced by chronic treatment with MCP1 and/or low-density lipoprotein (40). Recently, a couple of studies have examined the role of Rho kinase in renal cells in culture (12,20). Endlich et al. (12) found that a novel Rho kinase inhibitor, Y-27632, inhibited the reorganization of cytoskeleton induced by mechanical stress in cultured renal podocytes. Furthermore, the inhibition of Rho kinase activity is reported to prevent the TGF-β1-induced increase in connective tissue growth factor accumulation in cultured human renal fibroblast cells (20). Although these in vitro observations strongly suggest a substantial role of Rho kinase in mediating the progression of renal injury, direct in vivo evidence for the contribution of Rho kinase to the development of renal disease is insufficient. We have recently reported that NF-κB-dependent upregulation of MCP1 and

TGF-β1 plays an important role in the development of renal injury in ANG II-dependent hypertension (54). Interestingly, Perona et al. (55) demonstrated that Rho induces the transcriptional activity of NF-κB by stimulating the phosphor-ylation of the IκBα in Ser-32 and Ser-36 residues. However, it has not been established whether a Rho kinase inhibitor and/or NF-κB inhibitor exerts renoprotective effects on ANG II-dependent hypertension. Therefore, the present study was performed to determine the effectiveness of Rho kinase inhibitor fasudil and NF-κB inhibitor parthenolide in renal injury in ANG II-infused hypertensive rats. Our study provides a firm foundation that fasudil as well as parthenolide markedly attenuated the progression of renal injury in ANG II-infused hyper-tensive rats.

Emerging evidence has demonstrated that macrophage/ monocyte infiltration is one of the key mechanisms in the progression of renal fibrosis (34,78). Consistent with a previous study (54), chronic treatment with ANG II resulted in enhancement of CD68-positive macrophage/monocyte infiltration with an increase in expression of MCP1 mRNA as a potent chemotactic factor of macrophage/monocytes (34,78). In the present study, we demonstrated that the inhibition of Rho kinase with fasudil strongly suppressed the expression of MCP1 mRNA and macrophage/monocyte infiltration. These findings are in agreement with those of previous studies indicating the protective effects of Rho kinase inhibitors on macrophage/monocyte infiltration and interstitial fibrosis in different renal disease models (26,69). Furthermore, in vitro studies using chemotactic factors have demonstrated that neutrophil chemotaxis is significantly inhibited by fasudil (61). Collectively, these data suggest that the renoprotective effects of Rho kinase inhibitor on ANG II-induced renal injury are mediated, at least in part, by inhibition of macrophage/ monocyte infiltration.

Renal fibrosis is characterized mainly by an excessive synthesis and accumulation of extracellular matrix components, including collagen (48). Kagami et al. (25) demonstrated that ANG II stimulates extracellular matrix protein synthesis through induction of TGF-β1 expression in rat glomerular cells and this induction is via an ANG II type 1 receptor-dependent mechanism. The cytokine TGF-β1 has been shown to play roles in the process of fibrogenesis and collagen synthesis via multiple pathways such as $G_1$ phase arrest, cell-size enlargement, protein synthesis induction, an inhibitory effect on proteinase activity, and extracellular matrix enhancement (6,80). Consistent with a previous study (54), the present study demonstrated that in chronic ANG II-infused rats, renal fibrosis is associated with increases in collagen-positive area accompanied by increases in TGF-β1 mRNA expression. These data suggest a possible contribution of the TGF-β1 pathway to ANG II-induced renal fibrosis. Of interest, recent studies also have indicated that Rho kinase plays a role in mediating renal fibrosis through a TGF-β1-dependent mechanism (20,45,53,60). Nishikimi et al. (53) showed that increased TGF-β1 and collagen expression in injured kidneys of Dahl salt-sensitive hypertensive rats was associated with augmented gene expression of several Rho kinase families. Furthermore, treatment with a subdepressor dose of fasudil attenuated augmentation of TGF-β1 and collagen expression and improved renal injury in these animals (53). Similarly, another specific Rho kinase inhibitor, Y27632, suppressed interstitial fibrosis and augmentation of TGF-β1 and collagen expression in mouse kidneys with unilateral ureteral obstruction (45). Recent in vitro studies also demonstrated that Rho kinase is an essential factor for mechanical stretch-induced TGF-β1 synthesis in hepatic stel-late cells (60). Furthermore, the inhibition of Rho kinase activity prevents TGF-β1-induced increases in collagen accumulation in cultured human renal fibroblasts (20). In our study, Rho kinase inhibition with fasudil significantly attenuated ANG II-induced renal fibrosis and augmentation of TGF-β1 mRNA expression as well as collagen in the kidney. These data suggest that Rho kinase plays an important role in mediating ANG II-induced activation of the TGF-β1-dependent pathway, leading to collagen accumulation and the progression of renal fibrosis.

In the present study, treatment with a Rho kinase inhibitor did not alter BP but markedly attenuated the progression of renal injury in ANG II-infused rats, suggesting a potential contribution of ANG II-induced Rho kinase activation to renal injury independently of BP changes. A BP-independent renoprotective effect of Rho kinase inhibitors is also recently demonstrated in a different model of renal injury (69). Sun el al. (69) reported that treatment with fasudil did not alter BP but significantly ameliorated proteinuria and renal injury in rats that received aldosterone infusion. It is well known that ANG II is one of the most potent vasoconstrictor in the body, and it is well established that ANG II-mediated vascular tone constitutes an important determinant of glomerular hemodynamics (50). However, ANG II has multiple effects and the ANG II-induced vasoconstriction and high BP are only a small part of the roles of ANG II. For example, ANG II causes aldosterone secretion (52), cell infiltration and migration (11), thrombosis (74), and superoxide production (16,17,77). ANG II also modulate transporters (4,5) and channels (9,76) in proximal tubules as well as distal tubules. All of these factors are involved in ANG II-induced renal injury independently of the hypertension-induced renal injury. The variety of roles of ANG II may account for the BP-independent renoprotective effect of Rho kinase inhibitors in this study.

We selected 3 mg·kg$^{-1}$·day$^{-1}$ as the dose of fasudil treatment in the present study, because we previously reported that this dose of fasudil treatment had no effect on systolic BP and a higher dose (10 mg·kg$^{-1}$·day$^{-1}$) decreased systolic BP in rats (26). It is reported that relatively higher doses of fasudil (14–48 mg·kg$^{-1}$·day$^{-1}$) produce a blocking action against calcium entry (2,23) as well as an inhibitory effect on protein kinase C (3,23). We did not examine the intracellular calcium levels or protein kinase C levels in this study. However, according to the BP profiles, it seems unlikely that a relatively low dose of Fasudil exerts such nonspecific actions in this study.

Parthenolide is a sesquiterpene lactone and an active constituent derived from the Mexican Indian medicinal herb feverfew (*Tanacetum parthenium*). We selected 1 mg·kg$^{-1}$·day$^{-1}$ as the dose of parthenolide treatment in the present study, because it was previously reported that this dose of parthenolide treatment exerted anti-inflammatory effects without altering the normal behavior of mice and rats (24,38). A recent report demonstrates that parthenolide directly alkylates the p65 subunit of NF-κB, thereby inhibiting DNA binding (14). Moreover, it is shown that parthenolide directly binds to and inhibits IκBKβ, the kinase subunit known to play a critical role in cytokine-mediated signaling (37). The inhibitory effects of parthenolide seem to be specific for NF-κB because they did not influence the activity of other transcription factors (19).

The effectiveness of a Rho kinase inhibitor in renal and cardiovascular injury in experimental animal models is also reported in previous studies (26,27,42,45,53,59,62,72). Mukai et al. (42) provided evidence that upregulation of Rho kinase plays a key role in the pathogenesis of hypertensive vascular disease in spontaneously hypertensive rats. Kataoka et al. (27) presented an important role of Rho kinase in the pathogenesis of cardiovascular inflammation and remodeling induced by long-term blockade of nitric oxide synthesis in rats through the MCP1 and TGF-β1 pathways. Nagatoya et al. (45) demonstrated that a Rho kinase inhibitor prevents tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction through suppressing the migration of macrophages. Satoh et al. (62) also exhibited that Rho kinase inhibition produces a reduction of macrophage infiltration and attenuates interstitial fibrosis in rat kidneys with unilateral ureteral obstruction. Kanda et al. (26) showed evidence that fasudil improved glomerular and tubulointerstitial injury in subtotally nephrectomized, spontaneously hypertensive rats through an upregulation of p27kip1, a cyclin-dependent kinase inhibitor, and the subsequent inhibition of cell proliferation. Nishikimi et al. (53) revealed that fasudil attenuates glomerulosclerosis in Dahl salt-sensitive rats fed a high-salt diet with the reduction of mRNA expression levels of

TGF-β1, collagen I, and collagen III in the renal cortex. Teraishi et al. (72) reported that the Rho kinase pathway plays a key role in the pathogenesis of ischemia-reperfusion-induced acute renal failure in rats through the suppression of the enhanced myeloperoxidase activity. Ruperez et al. (59) illustrated that the Rho kinase pathway is involved in renal damage in rats caused by ANG II through the regulation of proinflammatory and profibrotic mediators. Our results extend these findings and provide novel information by showing that the Rho-NF-κB axis plays an important role through NF-κB-dependent upregulation of the MCP1 and TGF-β1 pathways in the development of renal injury in ANG II-dependent hypertensive rats independently of changes in BP.

Several authors have also shown the effectiveness of NF-κB inhibitors in models of renal damage (13,38,39,43,73,75). Muller et al. (43) provided the evidence that NF-κB inhibition ameliorates inflammatory renal injury through prevention of the NF-κB-dependent transactivation of the intercellular adhesion molecule and inducible nitric oxide synthase in double-transgenic rats harboring both human renin and angiotensinogen genes. Lopez-Franco et al. (38) demonstrated that an NF-κB inhibitor prevented proteinuria in an anti-Thy 1.1 nephritis rat model through the diminished renal expression of MCP1 and inducible nitric oxide synthase. Volpini et al. (75) presented an important role of NF-κB in the pathogenesis of tubulointerstitial nephritis induced by gentamicin in rats through the attenuation of macrophage infiltration. Tugcu et al. (73) also exhibited that NF-κB inhibitor ameliorates the nephrotoxicity induced by gentamicin in rats via the suppression of inducible nitric oxide synthase levels. Mitaka et al. (39) showed evidence that a blockade of NF-κB activation prevents hypodynamic shock and gastric hypoperfusion induced by endotoxin in anesthetized dogs, maybe through the suppression of inducible nitric oxide synthase levels. Fujihara et al. (13) revealed that the activation of the NF-κB system plays an important role in the pathogenesis of renal injury in the $\frac{5}{6}$ renal ablation model of rats through the prevention of interstitial macrophage infiltration. Our results are supported by these findings by showing that an NF-κB inhibitor attenuates the MCP1 mRNA levels as well as interstitial macrophage infiltration and thus ameliorates renal injury in ANG II-dependent hypertensive rats.

It is reported that NF-κB activation is related to ANG II type 1 receptor-mediated pathways and is believed to be dependent on activation of the Rho proteins that regulate intracellular signaling (68). It is also demonstrated that treatment with a Rho kinase inhibitor diminished ANG II-induced NF-κB DNA binding activity in the kidneys of rats (59). Our data are consistent with these findings. Moreover, our data clearly indicated that parthenolide did not alter ANG II-induced Rho kinase activation although fasudil abolished ANG II-induced Rho kinase activation. Our findings are supported by previous in vitro studies (7,10,21,55,65,81). Perona et al. (55) demonstrated that Rho-induced NF-κB activation stimulates translocation of RelA (p65) to the nucleus in NIH-3T3 murine fibroblast cells. They also reported that Rho-dependent activation of NF-κB is mediated by phosphorylation of IκBα and is reversed by overexpression of IκBα (55). Hodge et al. (21) provided evidence that Rho activation is required for increased NF-κB activity in PC-3 human prostate cancer cells. Zhao et al. (81) exhibited that neurotensin, a neuropeptide, stimulates IL-8 expression in NCM460 human colonic epithelial cells through Rho-mediated NF-κB pathways. Chen et al. (10) showed that a Rho exchange factor mediates NF-κB activation in human peripheral blood monocytes. Boyer et al. (7) presented that Rho GTPase instructs NF-κB activation by conveying IκBα to the ruffling membranes in HEp-2 epithelial carcinoma cells. Shimizu et al. (65) reveled that NF-κB activation is involved through the Rho/Rho kinase pathway in lipopolysaccharide-induced IL-8 production in cultured human cervical stromal cells. These data clearly indicate that there is a mechanistic linkage between Rho and NF-κB. Taken together, the present data suggest that the Rho-NF-κB axis plays a crucial role in the development of ANG II-induced renal injury independently of BP regulation.

To elucidate at which level ANG II-induced Rho activation stimulates the NF-κB pathway, related kinase activities were evaluated by Western blot analysis. As demonstrated in Fig. 5, ANG II-induced Rho activation stimulates the NF-κB pathway at phosphorylation of IκBα levels and this mechanism is independent from IκBKα, IκBKβ, and NF-κB-inducing kinase. These data are supported by the previous findings in vitro. Perona et al. (55) and Montaner et al. (41) demonstrated that Rho-induced NF-κB activation stimulates phosphorylation of IκBα. Moreover, Cammarano et al. (8) presented that whereas Rac, another Rho family GTPase, stimulates the activity of the IκBKβ, Rho activates NF-κB without activating either IκBKα or IκBKβ. These data clearly indicated that ANG II-induced Rho activation stimulates the NF-κB pathway at phosphorylation of IκBα levels and this mechanism is independent from IκBKα, IκBKβ, and NF-κB-inducing kinase.

In summary, this study was performed to determine the effectiveness of a Rho kinase inhibitor and NF-κB inhibitor in renal injury in ANG II-infused hypertensive rats. After 12 days of ANG II infusion, systolic BP (Fig. 1A), Rho kinase activity (Fig. 1B), NF-κB activity (Fig. 1,C and D), renal ANG II contents (Fig. 1E), MCP1 mRNA (Fig. 2A), interstitial macrophage/monocyte infiltration (Fig. 2, B-F), TGF-β1 mRNA (Fig. 3A), interstitial collagen-positive area (Fig. 3, B-F), urinary protein excretion (Fig. 4A), urinary albumin excretion (Fig. 4B), and phosphorylation of IκBα (Fig. 5A) were significantly enhanced compared with the Sham group. While fasudil or parthenolide did not alter systolic BP, both treatments completely blocked ANG II-induced enhancement of NF-κB activity, renal ANG II contents, MCP1 mRNA, interstitial macrophage/monocyte infiltration, TGF-β1 mRNA, interstitial collagen-positive area, urinary protein excretion, urinary albumin excretion, and phosphorylation of IκBα. Importantly, parthenolide did not alter ANG II-induced Rho kinase activation although fasudil abolished ANG II-induced Rho kinase activation. Moreover, ANG II infusion did not alter phosphorylation of IκBKα, IκBKβ, or NF-κB-inducing kinase. These data indicate that the Rho-NF-κB axis plays a crucial role in the development of ANG II-induced renal injury independently of BP regulation. Although the mechanistic linkage between Rho/Rho kinase and NF-κB is reported in previous studies in vitro and in vivo, this study is providing for the first time in vivo evidence that NF-κB is located downstream of the Rho/Rho kinase pathway by virtue of the fact that ANG II-induced Rho activation stimulates the NF-κB pathway at phosphorylation of IκBα levels and that this mechanism is independent of IκBKα, IκBKβ, and NF-κB-inducing kinase. A Rho kinase inhibitor and NF-κB inhibitor exert renoprotective effects, at least partially, through BP-independent mechanisms in ANG II-infused rats.

## Acknowledgments

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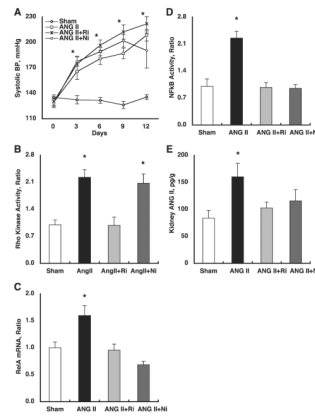
**Fig. 1.**

*A*: temporal profile of systolic blood pressure (BP). Systolic BP was similar among the 4 groups before the treatments. However, systolic BP progressively and significantly increased (208 ± 7 for ANG II vs. 136 ± 3 mmHg for Sham at *day 12*). Fasudil (Ri) or parthenolide (Ni) did not alter systolic BP (222 ± 8 and 190 ± 21 mmHg at *day 12*, respectively). *$P < 0.05$ compared with the corresponding Sham group at that time period and $P < 0.05$ compared with the corresponding group at *day 0. B*: chronic ANG II infusion significantly increased Rho kinase activity (2.23 ± 0.20 for ANG II vs. 1.00 ± 0.12 arbitrary units for Sham). Importantly, while fasudil abolished ANG II-induced Rho kinase activation, parthenolide did not alter ANG II-induced Rho kinase activation (0.98 ± 0.22 for ANG II+fasudil and 2.07 ± 0.25 for ANG II+parthenolide, respectively). *$P < 0.05$ compared with the Sham group. *C*: for evaluation of NF-κB expression, mRNA levels of RelA (p65), a part of the NF-κB complex, were measured by real-time-PCR. Chronic ANG II infusion significantly increased RelA mRNA levels (1.60 ± 0.18 for ANG II vs. 1.00 ± 0.11 arbitrary units for Sham). Both treatments completely blocked ANG II-induced enhancement of RelA mRNA levels (0.95 ± 0.11 for ANG II+fasudil and 0.68 ± 0.06 for ANG II+parthenolide, respectively). *$P < 0.05$ compared with the Sham group. *D*: chronic ANG II infusion significantly increased NF-κB activity (2.25 ± 0.18 for ANG II vs. 1.00 ± 0.19 arbitrary units for Sham). Both treatments completely blocked ANG II-induced enhancement of NF-κB activity (0.98 ± 0.13 for ANG II+fasudil and 0.95 ± 0.10 for ANG II +parthenolide, respectively). *$P < 0.05$ compared with the Sham group. *E*: chronic ANG II infusion significantly increased kidney ANG II levels (160 ± 25 for ANG II vs. 84 ± 14 pg/g for Sham). Both treatments completely blocked ANG II-induced enhancement of kidney ANG II levels (103 ± 11 for ANG II+fasudil and 116 ± 21 for ANG II+parthenolide, respectively). *$P < 0.05$ compared with the Sham group.
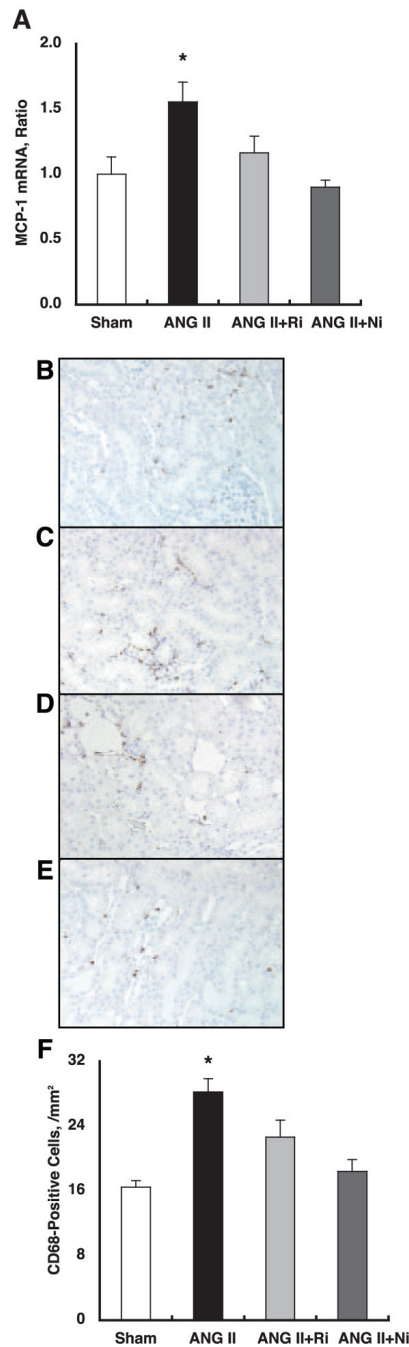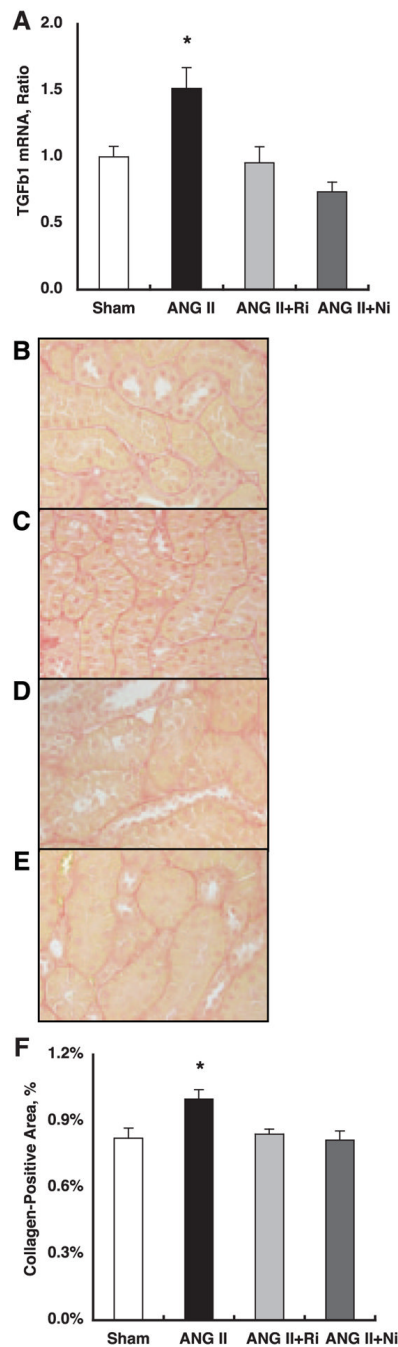
**Fig. 2.**
*A*: chronic ANG II infusion significantly increased intrarenal MCP1 mRNA levels ($1.55 \pm 0.15$ for ANG II vs. $1.00 \pm 0.13$ arbitrary units for Sham). Both treatments completely blocked ANG II-induced enhancement of intrarenal MCP1 mRNA levels ($1.16 \pm 0.13$ for ANG II+fasudil and $0.90 \pm 0.05$ for ANG II+parthenolide, respectively). *$P < 0.05$ compared with the Sham group. *B–E*: interstitial macrophage/monocyte infiltration was evaluated by CD68-positive cell number, which is a surface marker for macrophages and monocytes, using zinc-saturated, formalin-fixed, paraffin-embedded kidney samples from Sham (*B*), ANG II (*C*), ANG II+fasudil (*D*), and ANG II+parthenolide (*E*) groups. CD68-positive cells are stained brown. *F*: CD68-positive cell numbers were significantly increased

by chronic ANG II infusion ($28 \pm 2$ cells/mm$^2$) compared with Sham ($16 \pm 1$). Both treatments completely blocked ANG II-induced enhancement of interstitial macrophage/monocyte infiltration ($23 \pm 2$ for ANG II+fasudil and $18 \pm 1$ for ANG II+parthenolide, respectively). *$P < 0.05$ compared with the Sham group.

**Fig. 3.**
*A*: chronic ANG II infusion significantly increased intrarenal transforming growth factor
(TGF)-β1 mRNA levels (1.52 ± 0.16 for ANG II vs. 1.00 ± 0.08 arbitrary units for Sham).
Both treatments completely blocked ANG II-induced enhancement of intrarenal TGF-β1
mRNA levels (0.95 ± 0.12 for ANG II+fasudil and 0.74 ± 0.07 for ANG II+parthenolide,
respectively). *$P < 0.05$ compared with the Sham group. *B–E*: interstitial collagen-positive
area was stained by Picro-sirius red using zinc-saturated, formalinfixed, paraffin-embedded
kidney samples from Sham (*B*), ANG II (*C*), ANG II+fasudil (*D*), and ANG II+parthenolide
(*E*) groups. Collagen-positive area is stained pink. *F*: an established computer-aided
semiautomatic quantification system demonstrates that the interstitial collagen-positive area

was significantly increased by chronic ANG II infusion ($1.00 \pm 0.04\%$) compared with Sham ($0.82 \pm 0.05$). Both treatments completely blocked ANG II-induced enhancement of interstitial collagen-positive area ($0.84 \pm 0.02$ for ANG II+fasudil and $0.81 \pm 0.04$ for ANG II+parthenolide, respectively). *$P < 0.05$ compared with the Sham group.
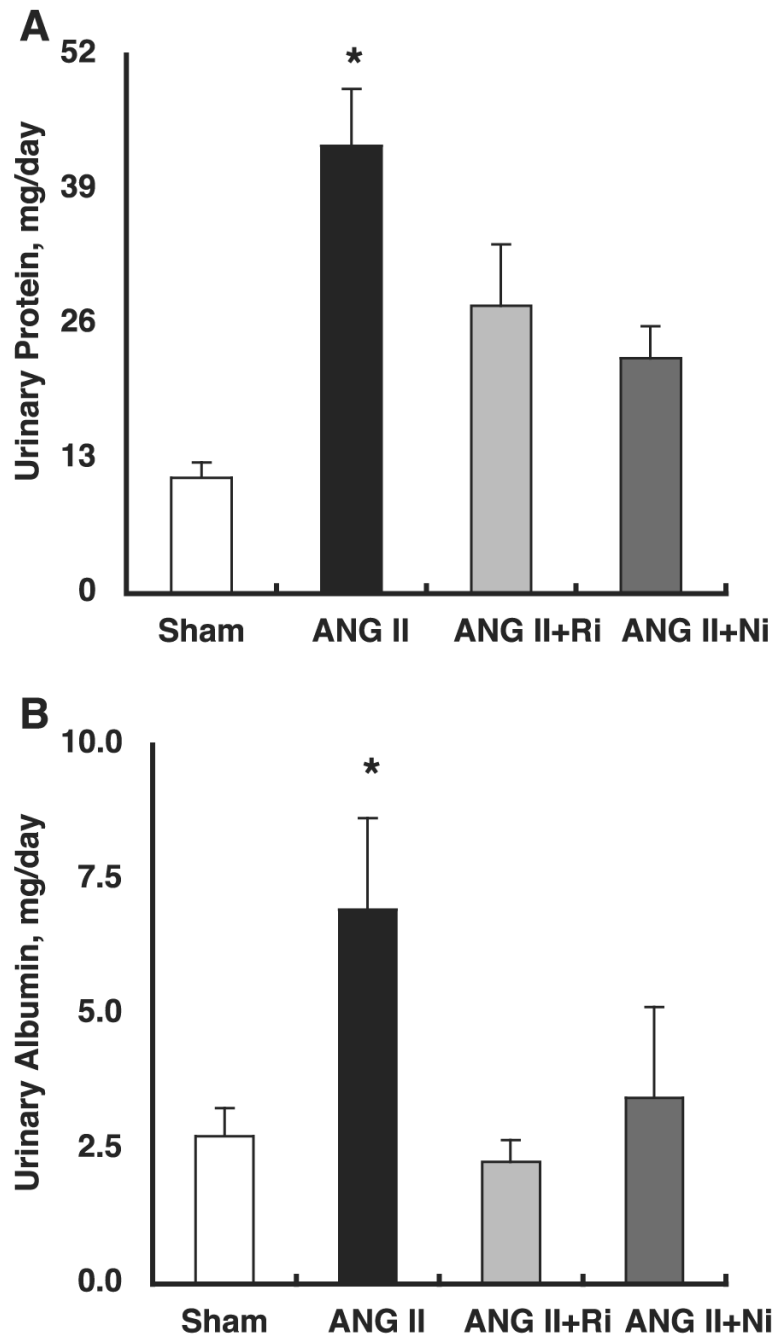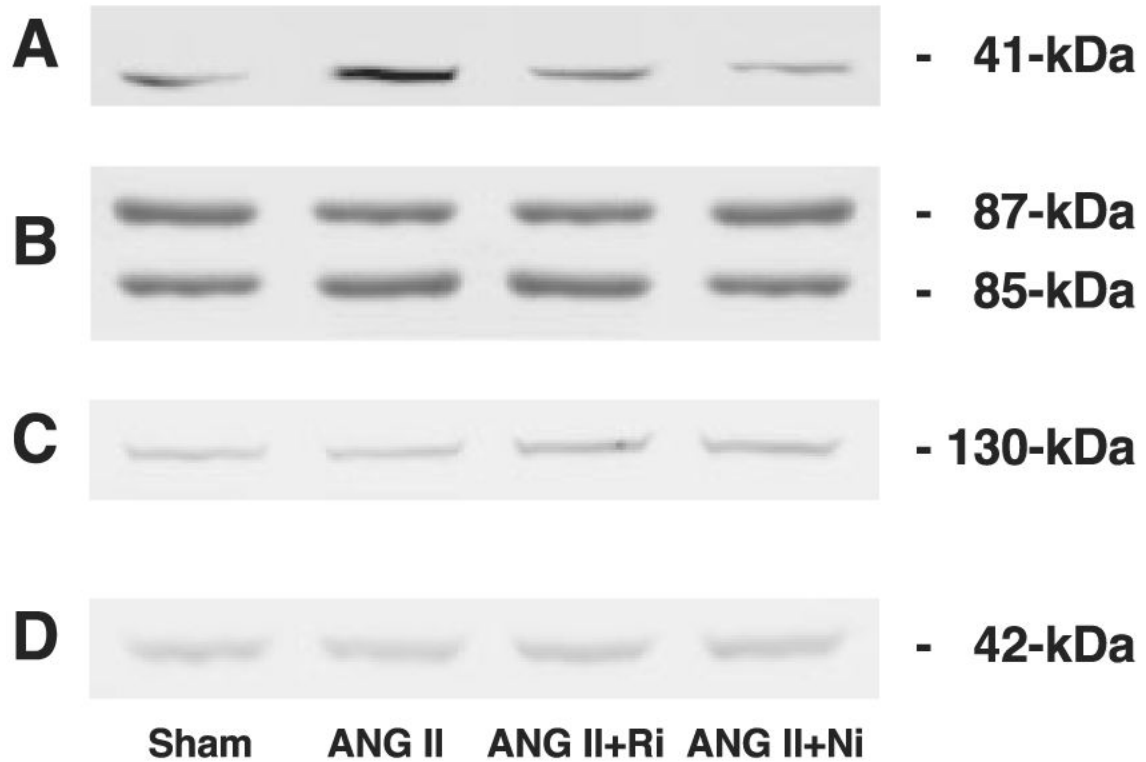
**Fig. 4.**
*A*: chronic ANG II infusion significantly increased urinary protein excretion ($43 \pm 6$ for ANG II vs. $11 \pm 2$ mg/day for Sham). Both treatments completely blocked ANG II-induced enhancement of urinary protein excretion ($28 \pm 6$ for ANG II+fasudil and $23 \pm 3$ for ANG II +parthenolide, respectively). *B*: chronic ANG II infusion significantly increased urinary albumin excretion ($6.9 \pm 1.7$ for ANG II vs. $2.7 \pm 0.5$ mg/day for Sham). Both treatments completely blocked ANG II-induced enhancement of urinary albumin excretion ($2.3 \pm 0.4$ for ANG II+fasudil and $3.5 \pm 1.7$ for ANG II+parthenolide, respectively). *$P < 0.05$ compared with the Sham group.

**Fig. 5.**
Representative Western blot analysis. To elucidate at which level ANG II-induced Rho activation stimulates the NF-κB pathway, related kinase activities were evaluated by Western blot analysis. Chronic ANG II infusion significantly increased phosphorylation of IκBα ($A$; $1.49 \pm 0.09$ for ANG II vs. $1.00 \pm 0.07$ arbitrary units for Sham). Both treatments completely blocked ANG II-induced phosphorylation of IκBα ($1.03 \pm 0.02$ for ANG II +fasudil and $1.05 \pm 0.07$ for ANG II+parthenolide, respectively). Chronic ANG II infusion did not change phosphorylation of IκBKα ($B$; 85 kDa, $0.99 \pm 0.07$ for ANG II vs. $1.00 \pm 0.09$ arbitrary units for Sham). Either treatment did not alter phosphorylation of IκBKα ($1.00 \pm 0.05$ for ANG II+fasudil and $1.08 \pm 0.07$ for ANG II+parthenolide, respectively). Chronic ANG II infusion did not change phosphorylation of IκBKβ ($B$; 87 kDa, $0.94 \pm 0.05$ for ANG II vs. $1.00 \pm 0.07$ arbitrary units for Sham). Either treatment did not alter phosphor-ylation of IκBKβ ($0.93 \pm 0.09$ for ANG II+fasudil and $1.00 \pm 0.07$ for ANG II+parthenolide, respectively). Chronic ANG II infusion did not change phosphorylation of NF-κB-inducing kinase ($C$; $0.90 \pm 0.05$ for ANG II vs. $1.00 \pm 0.07$ arbitrary units for Sham). Either treatment did not alter phosphor-ylation of NF-κB-inducing kinase ($0.89 \pm 0.07$ for ANG II+fasudil and $0.94 \pm 0.07$ for ANG II+parthenolide, respectively). To verify equal loading, membranes were reprobed with β-actin antibody. Similar densities were observed among the 4 groups ($D$). These data clearly indicate that ANG II-induced Rho activation stimulates NF-κB pathway at phosphorylation of IκBα levels and that this mechanism is independent of IκBKα, IκBKβ, and NF-κB-inducing kinase.

**Table 1**

Sequence information for primers and probes for quantitative real-time PCR

| Gene | Sense Primer | Antisense Primer | Probe |
| --- | --- | --- | --- |
| GAPDH | CAGAACATCATCCCTGCATC | CTGCTTCACCACCTTCTTGA | CCTGGAGAAACCTGCCAAGTATGATGA |
| RelA | CATCAAGATCAATGGCTACA | CACAAGTTCATGTGGATGAG | AACAGTTCGAATCTCCCTGGTCAC |
| MCP1 | AGCACCTTTGAATGTGAACT | AGAAGTGCTTGAGGTGGTT | CCCATAAATCTGAAGCTAATGCATCC |
| TGF-β1 | TACCATGCCAACTTCTGTC | AAGGACCTTGCTGTACTGTGT | CCCTACATTTGGAGCCTGGAC |

MCP, monocyte chemotactic protein; TGF, transforming growth factor.

**Table 2**

Body weight of different groups at day 12

|  | Sham | ANG II | ANG II+Ri | ANG II+Ni |
|---|---|---|---|---|
| Body weight, g | 302±5 | 289±3[*] | 290±3[*] | 291±5[*] |

Ri, Rho kinase inhibitor; Ni, NF-κB inhibition.

[*]$P < 0.05$ compared with Sham group.