

# Rho kinase inhibition activates the homologous angiotensin-converting enzyme-angiotensin-(1–9) axis in experimental hypertension

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**Background** Angiotensin II (Ang II) levels depend on renin, angiotensin-converting enzyme (ACE), and on the homologous angiotensin-converting enzyme (ACE2). Increased ACE and Ang II levels are associated with higher Rho kinase activity. However, the relationship between Rho kinase activation and ACE2 in hypertension is unknown.

**Objective** The role of the Rho kinase signaling pathway in both enzymatic activity and aortic gene expression of ACE2 in deoxycorticosterone acetate (DOCA) hypertensive rats was assessed in the present study.

**Methods and results** Compared with sham animals, Rho kinase activity was higher by 400% ( $P < 0.05$ ) in the aortic wall of the DOCA hypertensive rats. In addition to blood pressure reduction, the specific Rho kinase inhibitor fasudil reduced aortic Rho kinase activity to levels observed in the sham control group and increased ACE2 enzymatic activity (by 83% in plasma and by 52% in the aortic wall,  $P < 0.05$ ), ACE2, and endothelial nitric oxide synthase (eNOS) aortic mRNA levels (by 340 and 40%, respectively,  $P < 0.05$ ) with respect to the untreated hypertensive DOCA rats. Fasudil also increased significantly plasma levels of Ang-(1–9) in normotensive and in the hypertensive rats. Aortic mRNA and protein levels of transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), plasminogen activator inhibitor 1 (PAI-1), and monocyte chemoattractant protein 1 (MCP-1) were significantly ( $P < 0.05$ ) higher in the untreated DOCA rats and were normalized by fasudil administration.

**Conclusion** In experimental hypertension, Rho-associated, coiled-coil containing protein kinase (ROCK) inhibition reduces blood pressure and increases ACE2 levels and

activity. At the same time, ROCK inhibition reduces angiotensin II and increases Ang-(1–9) plasma levels. Fasudil also increases vascular eNOS mRNA levels and reduces aortic overexpression of the remodeling promotion proteins TGF- $\beta_1$ , PAI-1, and MCP-1. This effect might additionally contribute to the antihypertensive and antiremodeling effects of ROCK inhibition in hypertension.

**Keywords:** angiotensin-converting enzyme, homologous angiotensin-converting enzyme, angiotensin-(1–9), arterial wall, fasudil, hypertension, renin-angiotensin system, Rho kinase

**Abbreviations:** ACE, angiotensin-converting enzyme; ACE2, homologous angiotensin-converting enzyme; Ang, angiotensin; DOCA, deoxycorticosterone acetate; eNOS, endothelial nitric oxide synthase; LV, left ventricle; LVMI, left ventricle mass index; MCP1, monocyte chemoattractant protein 1; MLC, myosin light chain; MYPT1, myosin phosphatase target protein-1; PAI-1, plasminogen activator inhibitor 1; RAS, renin-angiotensin system; ROCK, Rho-associated coiled-coil containing protein kinase; TGF- $\beta_1$ , transforming growth factor  $\beta_1$

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## Introduction

Activation of the renin-angiotensin system (RAS), with higher levels of angiotensin-converting enzyme (ACE) and angiotensin (Ang) II, is a major determinant of hypertension, cardiovascular remodeling, and dysfunction as well as of impaired vasodilatation in the resistance arteries. Both ACE and Ang II are relevant therapeutic targets in the treatment of hypertension, heart failure, and kidney disease.

More recently, it has been observed that Ang II levels depend not only on renin and ACE, but also on the

homologous angiotensin-converting enzyme or homologous angiotensin-converting enzyme (ACE2) [1,2]. This carboxypeptidase cleaves Ang I to form Ang-(1–9) and also Ang II to form Ang-(1–7) [3]. By producing both Ang-(1–7) and Ang-(1–9), ACE2 antagonizes the effects of ACE [2,4]. We have proposed that Ang-(1–9) rather than Ang-(1–7) acts as a counterregulator of Ang II [5]. ACE2 does not hydrolyze bradykinins and its enzymatic activity is not inhibited by ACE inhibitors [1]. There are interesting observations on the functional significance of ACE2 in cardiovascular pathophysiology. In knockout ACE2 mice, increased levels of Ang II, cardiac contractility disturbances as well as increased hypoxia-induced gene expression are observed [4,6]. In contrast, in mice

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overexpressing the ACE2 gene, lower SBP is observed [4]. ACE2 seems to be then a possible therapeutic target in the treatment of hypertension and other cardiovascular diseases.

On the other side, one novel mechanism promoting both vasoconstriction and vascular remodeling is the intracellular signaling pathway Rho A and its Rho kinase [Rho-associated, coiled-coil containing protein kinase (ROCK)] [7–9]. ROCK plays an important role by mediating several cellular functions such as contraction of vascular smooth muscle cells (VSMCs) [9,10], actin cytoskeleton organization [11–13], cell adhesion and motility [14], cytokinesis [15], and expression of genes involved in vascular remodeling [8,16]. The ROCK signaling pathway participates in pathological cardiovascular and renal remodeling and also in blood pressure regulation. This pathway is activated by receptor agonists coupled to small G membrane proteins, such as Ang II, endothelin-1, or noradrenaline and produces smooth muscle cell contraction and hypertension, by activating phospholipase C, increasing protein kinase C, and by phosphorylation of the myosin II regulatory chain [17]. The RhoA/ROCK pathway activation increases calcium sensitivity in the contraction of the VSMC and modulates the phosphorylation of the myosin light chain (MLC) by inhibiting myosin phosphatase. Furthermore, ROCK activation also produces a chain of intracellular events such as endothelial nitric oxidase synthase (eNOS) deregulation (by reducing its gene expression), NADPH oxidase activation with higher oxidative stress that increases gene expression of profibrotic, procoagulant, and proinflammatory genes, and also nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation promoting increased PAI-1 expression and PI3K-Akt inhibition [18]. Currently, there are specific ROCK inhibitors available, such as fasudil and its active metabolite hydroxifasudil [19–22].

Increased ACE and Ang II levels are associated with higher aortic ROCK activity [23]. However, there are no available data on the relationship between the RhoA/ROCK signaling pathway activation and ACE2/Ang-(1–9) in hypertension. We hypothesized here that in experimental hypertension, ROCK stimulates the ACE2/Ang-(1–9) arm both in plasma and in the arterial wall. Accordingly, we investigated the effects of the ROCK inhibitor fasudil on both enzymatic activity and gene expression of ACE, ACE2, Ang II, Ang-(1–7) and Ang-(1–9) levels in the plasma and arterial wall from hypertensive rats.

## Methods

This investigation complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication N°85 to 23, revised 1996), and it was approved by the Research Commission from the School of Medicine, Pontifical Catholic University of Chile. The deoxycorticosterone acetate (DOCA)-salt hypertensive model was used in

Sprague–Dawley male rats, aged 5–6 weeks ( $150 \pm 10$  g). Under anesthesia with ketamine HCl and xylazine [35/7 mg/kg intraperitoneally (i.p.), respectively], a left nephrectomy was performed. Afterward, DOCA (100 mg/kg, subcutaneous; Steraloids Inc., Newport, Rhode Island, USA) once a week was administered starting immediately after recovery of surgery. All animals were hypertensive 2 weeks after surgery (data not shown). The animals received 1% NaCl and 0.4% KCl in the drinking water. As controls, uninephrectomized rats were used (Sham group). The hypertensive DOCA-salt rats were randomized to receive the specific ROCK antagonist fasudil (DOCA-F, 100 mg/kg per day, by gavage, for 3 weeks; LC Laboratories, Woburn, Massachusetts, USA) [23], starting on the third week after surgery. An additional group of uninephrectomized rats received fasudil (100 mg/kg per day, by gavage, for 3 weeks, Sham-F group). All the animals were kept under standard conditions of light, rat chow, and access to drinking water. SBP was measured by the tail-cuff method under mild ether anesthesia once a week [24]. Six weeks after surgery, the rats were killed by deep anaesthesia (ketamine HCl/xylazine 35/7 mg/kg i.p.) and blood was collected by cardiac puncture in heparin-containing tubes. Blood was centrifuged at 3500g for 10 min at 4°C. The plasma was kept at –80°C until processing. The whole aortas and hearts were carefully separated and washed with cold physiological saline solution and stored at –80°C until processing. The degree of left ventricular hypertrophy was quantified as the left ventricular mass index (LVMI = left ventricular weight  $\times$  100/body weight ratio).

## Preparation of aorta extracts

The aorta was homogenized in cold lysis buffer [Tris HCl 50 mmol/l, NP40 1%, NaCl 150 mmol/l, Na-deoxycolate 0.25%, EDTA 1 mmol/l, SDS 0.1%, aprotinin 1  $\mu$ g/ml, leupeptin 1  $\mu$ g/ml, and phenylmethylsulfonyl fluoride (PMSF) 1 mmol/l] [23]. Samples were centrifuged at 4°C and protein content of supernatants was determined by Bradford assay using bovine serum albumin as standard. Soluble fractions were heated at 95°C with 0.33 vol. of 4 $\times$  SDS-PAGE sample buffer for western blot analysis [23].

## Myosin phosphatase target protein-1 determination

The levels of aortic phosphorylated myosin phosphatase target protein-1 (MYPT1), a downstream target of Rho kinase, an index of ROCK activation [25–28], 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, Richmond, California, USA) for 1.5 h at 300 mA. Membranes were blocked with 1% nonfat milk in phosphate-buffered saline (PBS) containing 0.005%. Membranes were blocked with 1% nonfat milk in PBS containing 0.005% Tween-20 [phosphate-buffered saline Tween (PBST)] at room temperature.

Antiphospho-thr696-MYPT1 (Upstate Biotechnology, Millipore, Charlottesville, Virginia, USA) or anti-MYPT1 (BD Transduction Laboratories, Franklin Lakes, New Jersey, USA) 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 (ECL) with exposure to Kodak film. Each blot was quantified by scanning densitometry with the Un-Scan-It software.

#### **Plasma and aortic wall angiotensin-converting enzyme and homologous angiotensin-converting enzymatic activities**

Plasma and aorta ACE enzymatic activity was measured fluorometrically by the hydrolysis of Z-phenyl-L-histidyl-L-leucine (Bachem Bioscience Inc., King of Prussia, Pennsylvania, USA) as described previously [29] and was expressed in U/ml or U/mg protein, respectively (1 U = 1 nmol L-histidyl-L-leucine/min). ACE2 enzymatic activity was measured fluorometrically by following the hydrolysis of 7-Mca-RPPGFSAFK(Dnp)-OH (Bachem Bioscience Inc.) [29] and was expressed in U/ml or U/mg protein, respectively (1 U = 1 pmol 7-Mca-RPPGFSAFK(Dnp)-OH/min).

#### **Vascular angiotensin-converting enzyme and homologous angiotensin-converting enzyme immunohistochemistry**

This analysis was performed on six sequential 5 µm cross-sections of the proximal thoracic aorta from each rat. For ACE and ACE2 immunohistochemistry, cross-sections adhered to glass slides were washed in PBS and incubated overnight (4°C) with rabbit polyclonal antibodies to ACE (mAb 4051; Chemicon International, Temecula, California, USA) and ACE2 (produced in our laboratory) at a dilution of 1:1000 and 1:3000, respectively. Subsequent steps were performed according to the instructions of the Dako-LSAB kit [Dako-LSAB + system HRP kit (Universal), K0679; Dako, Hamburg, Germany]. Briefly, a universal biotinylated link-antibody served as secondary antibody. Subsequently, streptavidin conjugated to horseradish peroxidase was added. Sections were then incubated with diaminobenzidine chromogen substrate. Finally, sections were washed with Mayer's hematoxylin. Controls were performed on tissue sections without the primary antibody. Tissue sections were examined using light microscopy, photographed with a Nikon DS-Fi1 camera, and digitized (images at 20×). Calculation of the immunostaining intensities was based on Photoshop CS (version 8.0; Adobe Systems, San Jose, California, USA). Pixel values corresponding to immunostaining were converted to area (in mm<sup>2</sup>) by dividing the pixel value by mm<sup>2</sup>. Aortic area was obtained by

subtracting the area encompassed by the lumen from the area encompassed by the external elastic lamina. The immunostained to aortic area ratios were calculated and these values were expressed in arbitrary units.

#### **Circulating angiotensin levels**

Plasma Angs were determined by high-performance liquid chromatography (HPLC) followed by radioimmunoassay [5,30]. Blood was collected from the inferior vena cava directly into a syringe containing 5 ml 4 mol/l guanidine thiocyanate. The blood was centrifuged and the plasma was immediately extracted with Sep-Pak C18 cartridges (Waters Chromatography Division, Milford, Massachusetts, USA) and the peptides were acetylated before HPLC [19]. After reconstitution in water, each HPLC fraction was assayed in duplicate with the amino terminal-directed antibody A41, which measures acetyl-Ang II, acetyl-Ang I, acetyl-Ang-(1-7), and acetyl-Ang-(1-9). Acetylation, HPLC, and radioimmunoassay were performed as described by Campbell *et al.* [30].

#### **Aortic wall angiotensin-converting enzyme, homologous angiotensin-converting enzyme, monocyte chemoattractant protein 1, plasminogen activator inhibitor 1, transforming growth factor-β<sub>1</sub>, and endothelial nitric oxide synthase mRNA levels**

DNAase-treated total RNA (1.5 µg), isolated from the thoracic aorta with Trizol reagent, was quantified by ultraviolet spectroscopy. The RT-PCR assay was performed using the primers for ACE, ACE2, and eNOS described previously [5,31]. The RT-PCR assay for TGF-β<sub>1</sub>, PAI-1, and MCP-1 was performed using published primer sequences and amplification conditions [23]. After PCR, the amplification products were fractionated on a 1.5% agarose gel and visualized by staining with ethidium bromide. Band intensities were quantified by computerized densitometry and normalized with respect to 18S RNA.

#### **Transforming growth factor-β<sub>1</sub>, plasminogen activator inhibitor 1, and monocyte chemoattractant protein 1 levels in the aorta wall**

The levels of TGF-β<sub>1</sub>, PAI-1, and MCP-1 were assessed by western blot as previously described [23]. Each blot was quantified by scanning densitometry with the Un-Scan-It software.

#### **Statistical analysis**

Results (mean ± SEM) were compared by one factor analysis of variance (ANOVA) followed by the Student-Newman-Keul test. A *P* value of 0.05 or less was considered statistically significant.

## **Results**

### **SBP and left ventricular mass**

At the end of the experiments, the body weight was lower by one-third both in the treated and untreated DOCA

**Table 1** Body weight, blood pressure, and relative left ventricular mass index

|                      | Sham     | Sham-F                 | DOCA      | DOCA-F    |
|----------------------|----------|------------------------|-----------|-----------|
| N                    | 11       | 11                     | 13        | 10        |
| BW (g)               | 323 ± 8  | 333 ± 13               | 198 ± 11* | 215 ± 12* |
| SBP (mmHg)           | 123 ± 2  | 98 ± 2* <sup>†</sup>   | 153 ± 1*  | 127 ± 4*  |
| LVW (mg)             | 815 ± 30 | 974 ± 33* <sup>†</sup> | 754 ± 40  | 851 ± 50  |
| RLVMI (mg × 100g BW) | 252 ± 5  | 267 ± 6                | 383 ± 9*  | 396 ± 15* |

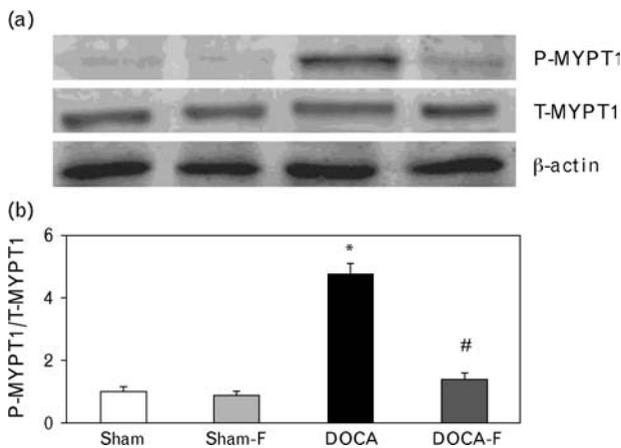
Data are shown as mean ± SEM. BW, body weight; F, fasudil; LVW, left ventricular weight; N, number of animals; RLVMI, relative left ventricular mass index. Symbols: \**P* < 0.05 vs. sham; <sup>†</sup>*P* < 0.05 vs. deoxycorticosterone acetate (DOCA) group; <sup>#</sup>*P* < 0.05 vs. both DOCA and DOCA-F groups, respectively after significant analysis of variance (ANOVA).

animals (Table 1). SBP was higher by 31 mmHg in the DOCA salt rats compared with the control animals and it was significantly reduced by the administration of the ROCK inhibitor fasudil (100 mg/kg per day). A significant degree of left ventricular hypertrophy (by 50%) was observed in the DOCA hypertensive animals as measured by the left ventricular mass index and was not modified by fasudil administered during 3 weeks.

**Phospho-myosin phosphatase target protein-1 aortic levels**

The P-MYPT1/T-MYPT1 ratio, index of ROCK activation [25–28], was higher by 400% (*P* < 0.05) in the aortic wall of the DOCA hypertensive animals as compared with the control sham animals (Fig. 1). Fasudil significantly reduced the aortic P-MYPT1/T-MYPT1 ratio to levels observed in the control sham group.

**Fig. 1**



Effect of Rho-associated, coiled-coil containing protein kinase inhibitor fasudil on aortic wall phospho-myosin phosphatase target protein-1 levels in hypertensive rats. Phospho-myosin phosphatase target protein-1 (MYPT1) (P-MYPT1) and total MYPT1 (T-MYPT1) and β-actin levels were determined in the aortic wall by western blot as described in 'Methods'. Representative photographs of western blots for P-MYPT1, T-MYPT1, and β-actin for the four experimental groups (a). P-MYPT1/T-MYPT1 ratio (b). The values are mean ± SEM; *n* = 10–12 per group. DOCA, deoxycorticosterone acetate; F, fasudil (100 mg/kg). \**P* < 0.05 vs. sham; <sup>#</sup>*P* < 0.05 vs. DOCA group [after analysis of variance (ANOVA)].

**Circulating angiotensin-converting enzyme and homologous angiotensin-converting enzymatic activities**

Plasma enzymatic ACE activity was 107 ± 12 U/ml in the sham animals and it was similar in the DOCA hypertensive rats. Fasudil reduced plasma ACE activity slightly but significantly both in the normotensive and in the hypertensive groups (Fig. 2a).

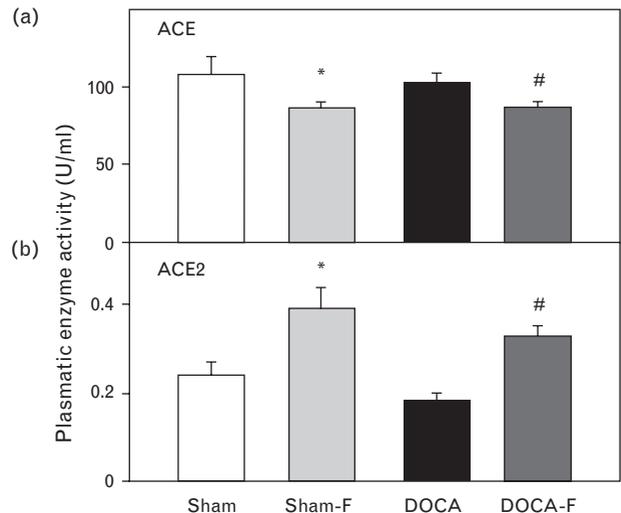
Plasma ACE2 activity was 0.24 ± 0.03 U/ml in the sham animals and similar in the DOCA hypertensive rats. Fasudil administration for 3 weeks significantly increased plasma ACE2 activity by 63% with respect to the sham group and by 83% with respect to the hypertensive DOCA rats (Fig. 2b).

**Aortic angiotensin-converting enzyme and homologous angiotensin-converting enzymatic activities**

In the aorta, the enzymatic ACE activity was 190 ± 12 U/mg protein in the sham animals (and similar in the DOCA hypertensive rats). After 3 weeks of treatment, fasudil significantly reduced aortic ACE activity by 43% in the sham group and by 41% in the DOCA hypertensive rats (Fig. 3a).

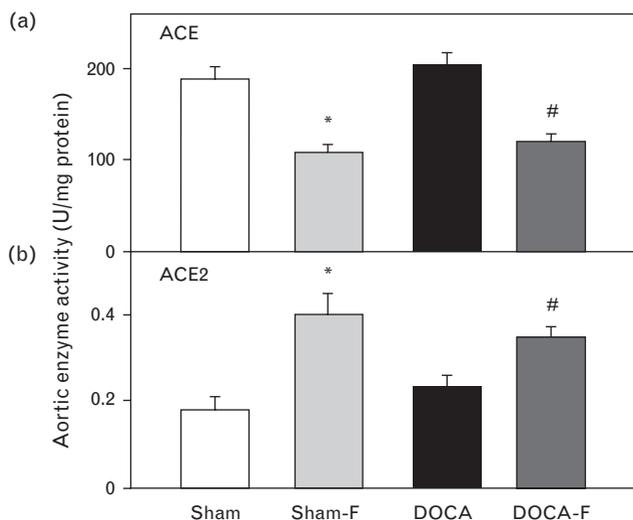
Aortic ACE2 activity was 0.18 ± 0.03 U/mg protein in the control animals and was similar in the DOCA hypertensive rats. After 3 weeks of treatment, fasudil significantly increased aortic ACE2 activity by two-fold in the sham

**Fig. 2**



Effect of the Rho-associated, coiled-coil containing protein kinase inhibitor fasudil on angiotensin-converting enzyme and homologous angiotensin-converting enzyme plasmatic activities. Angiotensin-converting enzyme (ACE) (a) and homologous angiotensin-converting enzyme (ACE2) (b) enzymatic activities were determined as described in 'Methods' in the plasma from hypertensive (DOCA) and sham rats treated with or without fasudil [F (100 mg/kg)]. Values are mean ± SEM; *n* = 7–13 per group. DOCA, deoxycorticosterone acetate; \**P* < 0.05 vs. sham; <sup>#</sup>*P* < 0.05 vs. DOCA group [after analysis of variance (ANOVA)].

Fig. 3



Effect of the Rho-associated, coiled-coil containing protein kinase inhibitor fasudil on aortic angiotensin-converting enzyme and homologous angiotensin-converting enzymatic activities. Angiotensin-converting enzyme (ACE) (a) and homologous angiotensin-converting (ACE2) (b) enzymatic activities were determined as described in 'Methods' in the aortic wall from hypertensive (DOCA) and sham rats treated with or without fasudil (F, 100 mg/kg). Values are mean  $\pm$  SEM;  $n = 7-13$  per group. \* $P < 0.05$  vs. sham; # $P < 0.05$  vs. DOCA group [after analysis of variance (ANOVA)].

group and by 52% in the DOCA hypertensive rats (Fig. 3b).

#### Angiotensin-converting enzyme and homologous angiotensin-converting enzyme immunohistochemistry

As depicted in Fig. 4, aortic ACE and ACE2 immunostainings were similar in sham and DOCA hypertensive rats. After 3 weeks of treatment, fasudil significantly reduced aortic ACE in both groups and increased ACE2 as well.

#### Aortic mRNA levels of angiotensin-converting enzyme and homologous angiotensin-converting enzyme

Aortic mRNA levels of ACE in the aortic wall of the hypertensive DOCA animals were similar to those in the normotensive sham animals ( $1.2 \pm 0.1$  vs.  $1.0 \pm 0.04$ , respectively, Fig. 5). After 3 weeks of treatment, fasudil significantly reduced aortic mRNA ACE levels by 29% in the sham group and by 42% in the DOCA hypertensive rats (Fig. 5).

Aortic ACE2 mRNA levels in the aortic wall of the hypertensive DOCA animals were similar compared to those in the normotensive sham animals ( $1.1 \pm 0.9$  vs.  $1.0 \pm 0.3$ , respectively; Figure 5). The administration of fasudil significantly increased the ACE2 aortic mRNA levels by 66% in the sham group and by 40% in the DOCA hypertensive rats (Fig. 5).

#### Angiotensin plasma levels

Ang II levels were similar in the normotensive sham and in the DOCA hypertensive rats and were significantly reduced by fasudil in both groups by 29% (Table 2). Ang-(1-7) levels were similar in the four experimental groups. Ang-(1-9) plasma levels were similar in the sham and in the DOCA hypertensive animals and were significantly increased by fasudil in both groups by 51 and 43%, respectively.

#### Aortic endothelial nitric oxide synthase mRNA levels

The gene expression of eNOS in the aorta was similar in the DOCA hypertensive rats compared to the normotensive sham animals (Fig. 5). Fasudil significantly increased the eNOS aortic mRNA levels by 3.8-fold in the sham group and by 3.4-fold in the DOCA hypertensive rats.

#### Expression of genes associated with aortic wall remodeling

As depicted in Table 3, mRNA gene expression in the arterial wall of TGF- $\beta_1$ , PAI-1, and MCP-1 was significantly increased in the aortic wall of the untreated hypertensive DOCA rats by seven-fold, five-fold, and 4.7-fold, respectively. Fasudil administration to DOCA hypertensive rats for 3 weeks significantly reduced the levels of these mRNAs to levels observed in the sham animals.

#### Protein levels of transforming growth factor- $\beta_1$ , plasminogen activator inhibitor 1, and monocyte chemoattractant protein 1 in the aorta

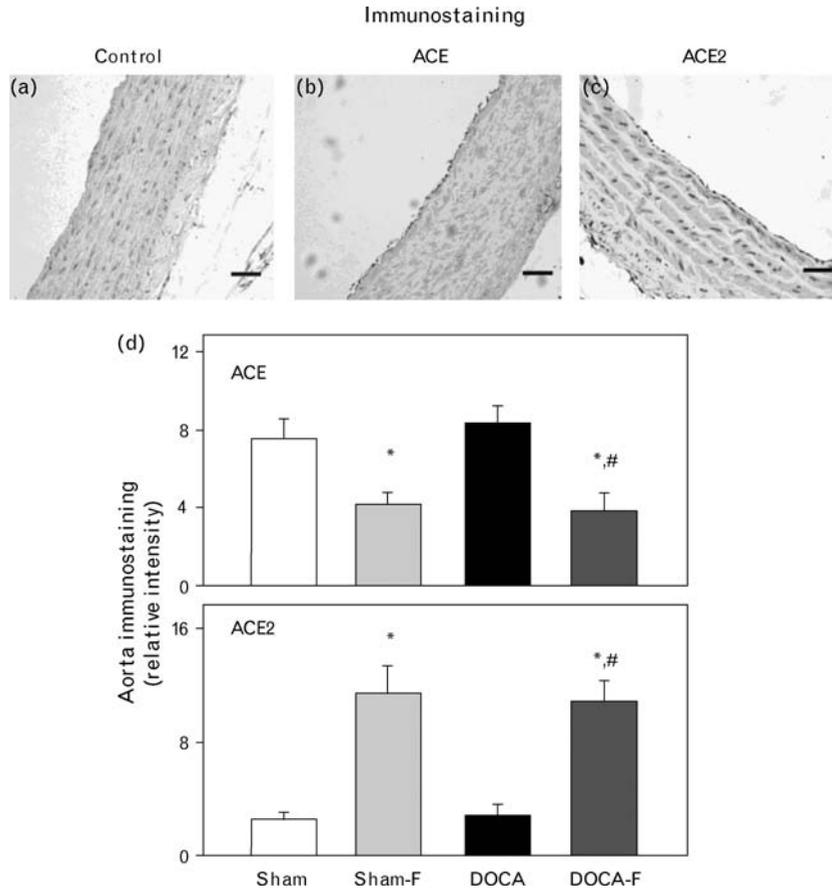
Protein levels of TGF- $\beta_1$ , PAI-1, and MCP-1 were significantly higher in DOCA compared with sham rats by 59, 92, and 66%, respectively ( $P < 0.05$ ; Fig. 6). In the hypertensive animals, fasudil significantly reduced the protein levels of TGF- $\beta_1$ , PAI-1, and MCP-1 to levels similar to those observed in sham rats.

#### Discussion

The main results in this study were that both in experimental hypertension (DOCA salt model) and in normotensive sham animals, ROCK inhibition using the specific inhibitor fasudil reduced SBP and aortic mRNA levels and ACE enzymatic activity and increased aortic mRNA as well as plasma and aortic ACE2 enzymatic activity, eNOS mRNA aortic levels, and Ang-(1-9) plasma levels. In the hypertensive rats, fasudil also decreased aortic mRNA levels of TGF- $\beta_1$ , PAI-1, and MCP-1.

Activation of the RhoA/ROCK pathway is a novel and main mechanism of vasoconstriction and cardiovascular remodeling in hypertension, with a therapeutic and preventive potential [32]. The role of this signaling pathway in hypertension was observed for the first time *in vivo* in 1997 [33]. By using a specific RhoA/ROCK inhibitor for 24 h, a reduction in blood pressure was observed in three

Fig. 4



Immunodetection of angiotensin-converting enzyme and homologous angiotensin-converting enzyme in the aortic wall from rats. Representative immunostaining cross-sections in the endothelium and media of the thoracic aorta depict (a) negative control (without the first antibody), (b) angiotensin-converting enzyme (ACE) in a deoxycorticosterone acetate (DOCA) rat and (c) homologous angiotensin-converting enzyme (ACE2) immunoreactivity of fasudil-treated deoxycorticosterone acetate (DOCA-F), respectively. Bar, 50  $\mu$ m. (d) Quantification of immunostaining in the endothelium and media of thoracic aortas in the four experimental groups. Values are mean  $\pm$  SEM;  $n = 7$  per group. \* $P < 0.05$  vs. Sham; # $P < 0.05$  vs. DOCA group [after analysis of variance (ANOVA)].

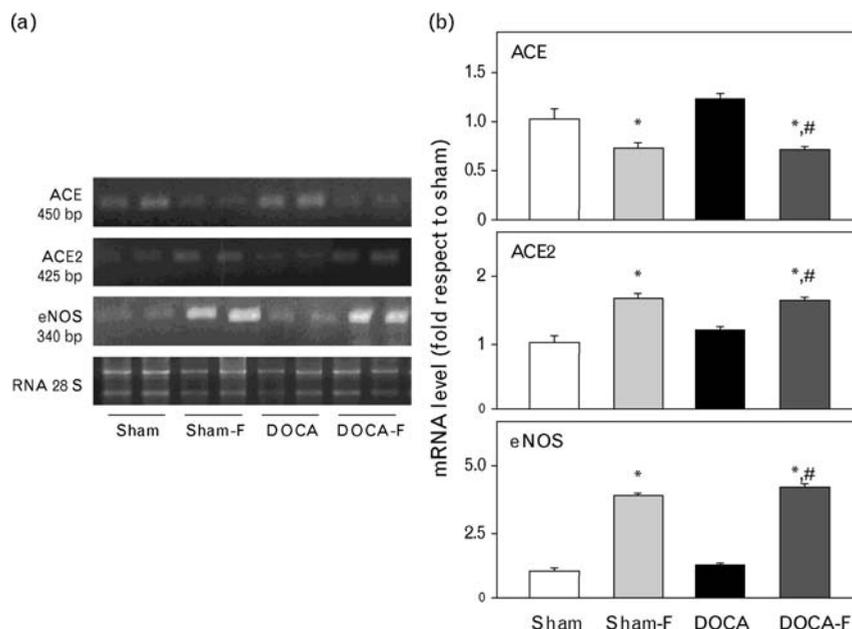
experimental models of hypertension [33]. RhoA/ROCK activation regulates VSMC contraction through MLC phosphorylation, sensitivity of contractile proteins to calcium, and assembling of stress fibers [9,34]. Apart from this, Rho kinase could also be regulated by different elements from the cytoskeleton with a key role in flow mechanotransduction and pressure in the blood vessels. Rho kinase participates in the actin cytoskeleton organization, cell adhesion and motility, cytokinesis, gene expression [34], as well as in the pathogenesis of atherosclerosis [35].

In experimental hypertension induced by nitric oxide synthase (NOS) inhibition with N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) in the rat, the ROCK inhibitor Y-27632 lowers SBP in a dose-dependent manner [36]. In different experimental models of hypertension, RhoA levels are increased [25], which is consistent with our current findings in the DOCA hypertensive rats.

Ang II activates the Rho kinase pathway even without hypertension [23]. In this respect, ROCK is significantly activated in the aortic wall in normotensive Brown-Norway rats with genetically high ACE and Ang II levels and it activates genes that promote vascular remodeling and also increases vascular oxidative stress [23]. In this normotensive experimental model, ROCK inhibition with fasudil normalizes higher aortic mRNA levels of TGF- $\beta$ <sub>1</sub>, PAI-1, and MCP-1 as well as the increased aortic NADPH oxidase activity and  $\bullet$ O<sub>2</sub><sup>-</sup> production [23], in a very similar way as was here observed in the DOCA hypertensive rats.

Recently, a parallel pathway of the RAS has been discovered [1,37]. In this new pathway, ACE2 seems to be a new therapeutic target for the treatment of hypertension and its complications by participating in the metabolism of Angs I and II and by producing Ang-(1-9) and Ang-(1-7).

Fig. 5



Effect of the Rho-associated, coiled-coil containing protein kinase inhibitor fasudil on aortic angiotensin-converting enzyme, homologous angiotensin-converting enzyme, and endothelial nitric oxide synthase mRNA levels. Angiotensin-converting enzyme (ACE), homologous angiotensin-converting enzyme (ACE2), and endothelial nitric oxide synthase (eNOS) mRNA levels were determined by RT-PCR as described in 'Methods' in the aortic wall from hypertensive [deoxycorticosterone acetate (DOCA)] and sham rats treated with or without fasudil (F, 100 mg/kg). Representative photographs are shown in (a) and quantitative data in (b). Values are mean  $\pm$  SEM;  $n = 7-13$  per group. \* $P < 0.05$  vs. sham; # $P < 0.05$  vs. DOCA group [after analysis of variance (ANOVA)].

Ang-(1-7) promotes vasodilatation and apoptosis and has antiproliferative effects [38]. This peptide, through its receptor, the Mas oncogen product [39], stimulates the NOS and counteracts the vasopressor effects of Ang II. Ang-(1-7) also potentiates bradykinin actions through the bradykinin receptor B2 [40]. Therefore, ACE2 should be involved in the regulation of vascular tone, counteracting ACE effects [41]. No differences were observed here in the Ang-(1-7) levels despite increased ACE2 levels with ROCK inhibition. It is possible to hypothesize that ROCK inhibition, by increasing eNOS and/or by reducing both ACE and Ang II, does not activate the Ang-(1-7) path.

Ang-(1-9) is present in human and rat plasma and its circulating levels increase in animals treated with ACE

inhibitors [42]. Some studies have shown that plasma Ang-(1-9) levels are higher than plasma Ang II levels both in humans and rats [43]. Ang-(1-9) also favors the bradykinin binding to its B<sub>2</sub> receptor, possibly by inducing changes in the structure of the ACE-B<sub>2</sub> receptor complex [44]. In the rat, reduction in ACE2 and circulating Ang-(1-9) levels after myocardial infarction is prevented with enalapril [5]. Additionally, administration of Ang-(1-9) to rats with myocardial infarct inhibited ACE activity, reduced plasma Ang II levels, and prevented cardiac myocyte hypertrophy, an effect that was independent of the Mas receptor [45]. Although it is now generally accepted that ACE2 plays a role in hypertension and cardiovascular remodeling, the exact pathway by which ACE2 activity protects the cardiovascular system is

Table 2 Effect of fasudil on circulating angiotensin levels

|                     | Sham          | Sham-F         | DOCA           | DOCA-F          |
|---------------------|---------------|----------------|----------------|-----------------|
| N                   | 11            | 12             | 10             | 8               |
| Ang I (fmol/ml)     | 23 $\pm$ 3    | 29 $\pm$ 2*    | 17 $\pm$ 1*    | 17. $\pm$ 1*#   |
| Ang-(1-7) (fmol/ml) | 12 $\pm$ 1    | 12 $\pm$ 1     | 13 $\pm$ 1     | 10 $\pm$ 1      |
| Ang-(1-9) (fmol/ml) | 14 $\pm$ 1    | 22 $\pm$ 1*    | 17 $\pm$ 1     | 24 $\pm$ 1*#    |
| Ang II (fmol/ml)    | 17 $\pm$ 1.0  | 12 $\pm$ 1*    | 14 $\pm$ 2     | 10 $\pm$ 1*#    |
| Ang-(1-7)/Ang II    | 0.7 $\pm$ 0.1 | 0.9 $\pm$ 0.2  | 0.9 $\pm$ 0.1  | 0.9 $\pm$ 0.1   |
| Ang-(1-9)/Ang I     | 0.6 $\pm$ 0.1 | 1.8 $\pm$ 0.1* | 1.1 $\pm$ 0.1* | 1.5 $\pm$ 0.1*# |
| Ang II/Ang I        | 0.7 $\pm$ 0.1 | 0.4 $\pm$ 0.1* | 0.8 $\pm$ 0.1  | 0.6 $\pm$ 0.1#  |

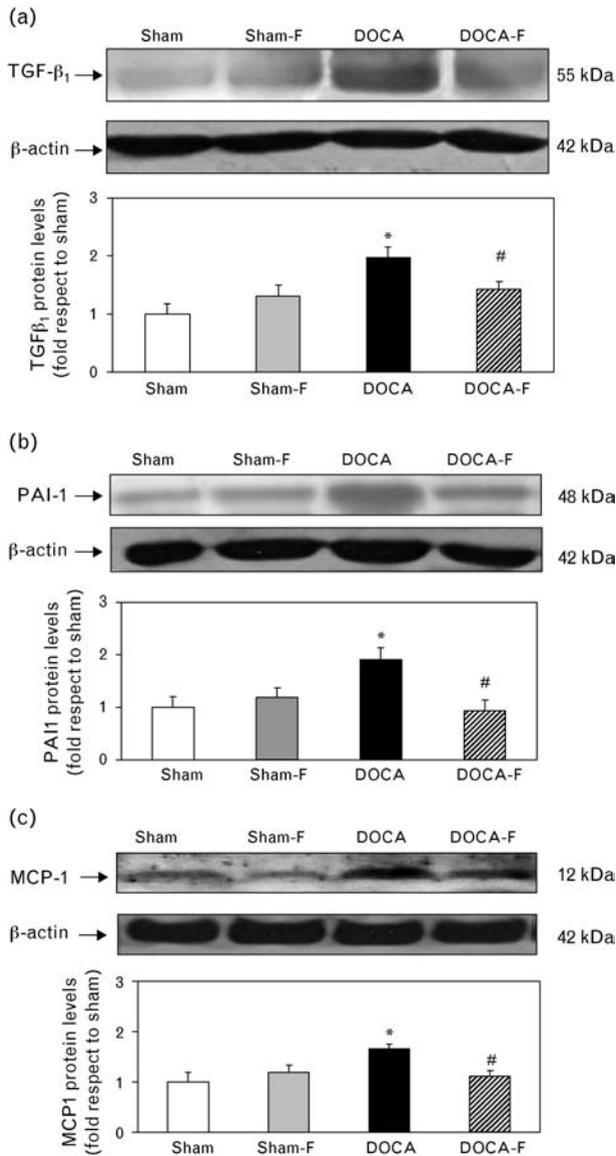
Values as mean  $\pm$  SEM. Ang, angiotensin; F, fasudil (100 mg/kg); N, number of animals. Values as mean  $\pm$  SEM. \* $P < 0.05$  vs. sham (S); # $P < 0.05$  vs. deoxycorticosterone acetate [DOCA; after significant analysis of variance (ANOVA)].

Table 3 Transforming growth factor- $\beta_1$ , plasminogen activator inhibitor 1, and monocyte chemoattractant protein 1 mRNA relative levels in the aortic wall

|                           | Sham          | Sham-F        | DOCA           | DOCA-F         |
|---------------------------|---------------|---------------|----------------|----------------|
| N                         | 10            | 12            | 12             | 7              |
| TGF- $\beta_1$ mRNA (UOD) | 1.0 $\pm$ 0.2 | 1.1 $\pm$ 0.3 | 7.6 $\pm$ 2.5* | 1.2 $\pm$ 0.1# |
| PAI-1 mRNA (UOD)          | 1.0 $\pm$ 0.2 | 1.4 $\pm$ 0.1 | 5 $\pm$ 1.7*   | 1.7 $\pm$ 0.2# |
| MCP-1 mRNA (UOD)          | 1.0 $\pm$ 0.2 | 1.2 $\pm$ 0.3 | 4.7 $\pm$ 1.9* | 1 $\pm$ 0.2#   |

Data are shown as mean  $\pm$  SEM. MCP-1, monocyte chemoattractant protein-1; N, number of animals; PAI-1, plasminogen activator inhibitor 1; TGF- $\beta_1$ , transforming growth factor  $\beta_1$ ; UOD, units of optical density. Symbols: \* $P < 0.05$  vs. sham, # $P < 0.05$  vs. deoxycorticosterone acetate [DOCA; after significant analysis of variance (ANOVA)].

Fig. 6



Effect of fasudil on aortic transforming growth factor- $\beta_1$ , plasminogen activator inhibitor 1, and monocyte chemoattractant protein 1 levels. Transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), plasminogen activator inhibitor 1 (PAI-1), monocyte chemoattractant protein 1 (MCP-1), and  $\beta$ -actin protein levels were determined by western blot as described in 'Methods' in the aortic wall from hypertensive [deoxycorticosterone acetate (DOCA)] and sham rats treated with or without fasudil (F, 100 mg/kg). Representative photographs and quantitative data are shown. Values are mean  $\pm$  SEM;  $n = 7-13$  per group. \* $P < 0.05$  vs. sham; # $P < 0.05$  vs. DOCA group [after analysis of variance (ANOVA)].

still unclear. Potential mechanisms include increased Ang II degradation and increased formation of Ang-(1-9).

In humans, ACE2 enzymatic activity is lower in stable advanced atherosclerotic lesions, compared to early and ruptured carotid atherosclerotic lesions [46], suggesting a differential regulation of ACE2 activity during the progression of atherosclerosis. This novel molecule of the

RAS may play a role in the pathogenesis of cardiovascular remodeling and atherosclerosis.

There are no data in the literature on the relationship between the vasodilator ACE2 pathway and the vasoconstrictor ROCK cascade in the arterial wall. Our results show that by inhibiting the ROCK pathway with fasudil, gene expression and enzymatic ACE activity and plasma levels of Ang II were reduced, whereas gene expression and ACE2 activity were importantly increased, simultaneously with increased mRNA eNOS aortic levels and increased plasma levels of Ang-(1-9) as a consequence of direct ROCK inhibition. This is the first observation concerning a pharmacologic ACE2 activator, both in normotensive and in hypertensive animals, one of the most interesting findings of the study. Additionally, in experimental hypertension, direct ROCK inhibition also normalizes overexpression of genes that promote vascular remodeling. Interestingly, the observed changes in ACE/ACE2 and in Ang-(1-9) levels were present only during fasudil treatment both in sham and in the DOCA hypertensive rats. On the other side, p-MYPT1 was not reduced by fasudil treatment in the sham rats, which suggests that fasudil may have effects on ACE and ACE2 that are independent of ROCK inhibition (or at least by a pathway that is divergent from p-MYPT1).

Clinical studies on the role of the ROCK signaling pathway in hypertension are scanty and most of them are studies on patients with pulmonary hypertension [47-49]. In hypertensive patients, fasudil induced a larger vasodilator response in the arm compared to controls, whereas the vasodilator response to nitroprusside was similar in both groups [50], the first clinical evidence about the role of the RhoA/ROCK pathway in the pathogenesis of increased systemic vascular resistance in hypertensive patients. In hypertensive kidney transplant recipients, increased ROCK mediates VSMC constrictor tone and attenuates basal nitric oxide-mediated tone [51]. In patients with coronary heart disease, fasudil enhanced vasodilation and reduced Rho kinase activity in circulating leukocytes [52].

In conclusion, direct ROCK inhibition reduces blood pressure and increases vascular and plasma ACE2 enzymatic activity. At the same time, ROCK inhibition reduces Ang II and increases Ang-(1-9) plasma levels. Apart from this, fasudil increases vascular eNOS mRNA levels and reduces aortic overexpression of the remodeling promotion proteins TGF- $\beta_1$ , PAI-1, and MCP-1. This novel effect of ROCK inhibition on both ACE2 gene expression and enzymatic activity might additionally contribute to the antihypertensive and antiremodeling effects of ROCK inhibition in hypertension, atherosclerosis, and in cardiovascular and renal pathologic remodeling.

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