Fasudil, a Rho-Kinase Inhibitor, Attenuates Angiotensin II–Induced Abdominal Aortic Aneurysm in Apolipoprotein E–Deficient Mice by Inhibiting Apoptosis and Proteolysis

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Background—Angiotensin II (Ang II) accelerates atherosclerosis and induces abdominal aortic aneurysm (AAA) in an experimental mouse model. Agonism of a G protein–coupled receptor by Ang II activates Rho-kinase and other signaling pathways and results in activation of proteolysis and apoptosis. Enhanced proteolysis and smooth muscle cell apoptosis are important mechanisms associated with AAA. In this study, we tested the hypothesis that fasudil, a Rho-kinase inhibitor, could attenuate Ang II–induced AAA formation by inhibiting vascular wall apoptosis and extracellular matrix proteolysis.

Methods and Results—Six-month-old apolipoprotein E–deficient mice were infused with Ang II (1.44 mg · kg^{-1} · d^{-1}) for 1 month. Animals were randomly assigned to treatment with fasudil (136 or 213 mg · kg^{-1} · d^{-1} in drinking water) or tap water. Ang II infusion induced AAA formation in 75% of the mice, which was accompanied by an increase in proteolysis detected by zymographic analysis and quantified by active matrix metalloproteinase-2 activity, as well as apoptosis detected by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling and quantified by both caspase-3 activity and histone-associated DNA fragmentation. The level of DNA fragmentation in the suprarenal aorta correlated with AAA diameter. Ang II also increased atherosclerotic lesion area and blood pressure. Fasudil treatment resulted in a dose-dependent reduction in both the incidence and severity of AAA. At the higher dose, fasudil decreased AAA by 45% while significantly inhibiting both apoptosis and proteolysis, without affecting atherosclerosis or blood pressure.

Conclusions—These data demonstrate that inhibition of Rho-kinase by fasudil attenuated Ang II–induced AAA through inhibition of both apoptosis and proteolysis pathways. **(*Circulation*. 2005;111:2219-2226.)**

Key Words: angiotensin II ■ aortic aneurysm, abdominal ■ mice, knockout ■ apoptosis ■ metalloproteinases

Angiotensin II (Ang II) promotes vascular inflammation and induces abdominal aortic aneurysm (AAA) formation in apolipoprotein E–deficient (apoE-KO) mice.[1–3] Vascular inflammation is characterized by the increased infiltration of inflammatory cells into the vascular wall. Monocytes and macrophages are a major source of proteases that attack the structural integrity of the vascular wall and degrade components of the extracellular matrix, including elastin and collagen, thus contributing to AAA formation.[4–7] Although much attention has been given to matrix degradation and proteolytic mechanisms of AAA development, accumulating evidence indicates that apoptosis may also play an important role in altering the structural integrity of the vessel wall.[8–18] Macrophages and T lymphocytes produce proapoptotic factors such as perforin, Fas, and Fas ligand.[11] Furthermore, Ang II induces oxidative stress, which can also promote cell death.[19] Indeed, it has been reported that apoptosis of medial smooth muscle cells (SMCs) is increased in aneurysm tissue.[20] Depletion of medial SMC eliminates a cell population capable of synthesizing matrix proteins, which enables tissue repair, thus contributing to AAA formation.[21]

There is evidence that Rho-kinase mediates Ang II–induced vascular inflammation[22] and is involved in the regulation of apoptosis.[23,24] In the present study, we examined the effects of fasudil, a Rho-kinase inhibitor, on Ang II–induced AAA formation and aortic wall apoptosis and proteolysis in apoE-KO mice. Our results indicate that fasudil significantly reduced AAA formation and decreased apoptosis and proteolysis in the aorta, suggesting a role for Rho-kinase in the pathogenesis of AAA.

Received October 11, 2004; revision received December 2, 2004; accepted December 8, 2004.

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Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000163544.17221.BE**

Methods

Animal Preparation

Osmotic minipumps (model 2004, Alzet) containing either PBS or Ang II (1.44 mg · kg^{-1} · d^{-1} in PBS, Calbiochem) were implanted subcutaneously in 6-month-old apoE-KO male mice (Jackson Laboratories). Two days before saline and Ang II infusion, mice were provided with water (Ang II group) or water containing fasudil at a concentration of 0.5 mg/mL (low-dose group) or 1.0 mg/mL (high-dose group). Age-matched apoE-KO mice without any treatment were used as naïve controls. At the end of the 30-day treatment period, mice were euthanized, and the hearts were perfused with DEPC in saline. The arterial tree was rapidly dissected from fat and connective tissue and snap-frozen in liquid nitrogen. Blood cholesterol levels were profiled by IDEXX Veterinary Services using a standard cholesterol enzymatic assay kit (Equal Diagnostics). HDL was quantified after selective solubilization of HDL particles (HDL direct liquid select). LDL level, including both VLDL and LDL cholesterol, was then calculated from the following formula: total cholesterol minus HDL minus triglycerides divided by 5. The Institutional Animal Care and Use Committee approved all animal protocols.

Morphological Examination

Quantification of Aneurysm Formation

After the aorta was dissected free from the surrounding connective tissue, images were recorded with a digital camera and later used to measure the outer diameter of the suprarenal aorta at the midpoint between the diaphragm and right renal artery. A commonly used clinic standard to diagnose abdominal aortic aneurysm is an increase in aortic diameter of ≈50%.[25] The average diameter of the normal suprarenal aorta in naïve control mice is ≈0.8 mm. We therefore set a threshold of 1.22 mm as evidence of an incidence of aneurysm formation. Aneurysm severity was assessed with a scoring system described by Daugherty et al[26]: type 0, no aneurysm (the suprarenal region of the aorta was not obviously different from naïve apoE-KO mice without Ang II treatment); type I, a dilated lumen with no thrombus; type II, remodeled tissue often containing thrombus; type III, a pronounced bulbous form of type II containing thrombus; and type IV, multiple aneurysms containing thrombus. To analyze this measurement semiquantitatively, the numerical score assigned to the type of aneurysm for each animal in a group was averaged to generate a pathology score for statistical comparison.

Quantification of Atherosclerotic Lesion Area

The left and right carotid arteries and the aortic arch were dissected, excised, opened longitudinally, and pinned down on wax-coated Petri dishes. Atherosclerotic lesions were visible without staining. Images of the open luminal surface of the vessels were captured with a digital camera (Sony) mounted on a dissecting microscope. The atherosclerotic lesion area was quantified by use of the C-Simple system (Compix) and expressed as a percentage of the total luminal surface area, as described in detail previously.[2,27,28]

Histology and Immunohistochemical Staining

Two representative suprarenal aortas from each group were fixed in formalin, embedded in paraffin, and cut into 5-μm-thick sections. The adjacent sections were stained with hematoxylin and eosin or by the immunohistochemical method.

To identify macrophages in the aortic wall, a purified rat anti-mouse Mac-3 monoclonal antibody (BD Pharmingen) was used for immunochemistry staining (PhenoPath Laboratories).

To localize cells undergoing nuclear DNA fragmentation, in situ terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) was performed using an in situ apoptosis detection kit (Roche Biochemicals) described in detail previously.[29,30] Briefly, paraffin sections were deparaffinized and rehydrated. Sections were then washed with PBS and incubated with proteinase K (20 μg/mL) for 20 minutes. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in methanol at room temperature. TdT, which catalyzes a template-independent addition of deoxyribonucleotide to 3′-OH ends of DNA, was used to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragment in situ. The TUNEL signal was then detected with an anti-digoxigenin antibody conjugated with peroxidase and developed with diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin, dehydrated, and cleared before coverslips were placed. Both positive and negative control slides were processed at the same time in each experiment. The presence of apoptotic cells was scored as nuclear staining, with a distinctive morphological appearance associated with cell shrinkage and chromatin condensation.

Hemodynamic Measurements

Tail-Cuff Measurement of Systolic Blood Pressure and Heart Rate in Conscious Mice

Systolic blood pressure and heart rate were measured in conscious mice with a noninvasive tail-cuff system (Kent Scientific) before (baseline) and 30 days after treatment (end point) by Ang II with or without fasudil. All mice were first trained to stay quietly in a restrainer placed on a warm pad for periods of ≥30 minutes before the study. On the day of the study, the mice were placed in the temperature-controlled restrainer for 15 minutes. Blood pressure was then measured repeatedly and recorded on a data acquisition system (PowerLab, 16/s, ADInstruments). Systolic blood pressure and heart rate were averaged from 5 consecutive measurements.

Noninvasive Measurement of Aortic Pulse-Wave Velocity

A Doppler method was used to measure pulse-wave velocity as described in detail previously.[31] In brief, mice were lightly anesthetized with 1.5% isoflurane (IMPAC 6, VetEquip). A lead II ECG and Doppler signals were recorded simultaneously by a Doppler data acquisition and processing system (Indus Instruments). Blood flow velocities in the ascending aorta and abdominal aorta were obtained with a 2-mm-diameter 20-MHz pulsed Doppler probe. Pulse-wave velocity, calculated from the time delay between the flow velocity forms measured in the ascending aorta and abdominal aorta divided by the distance, was used as an index of aortic stiffness.[28,31]

Enzyme Immunoassay

Active Caspase-3

Caspase-3 is activated and plays a key role in mediating apoptotic signaling events.[32] Detection of activated caspase-3 is a valuable tool in identifying dying cells even before all the features of apoptosis (eg, DNA fragmentation) are present. No activation of the caspase cascade has been found in necrotic cell death.[33] Thus, caspase activation has been widely used as a biomarker for proapoptotic activity.[32] As described in detail previously,[29] caspase-3 activity in aortic tissues was measured with a caspase-3 fluorescent assay kit (BIOMOL Research Laboratories). This assay measures the fluorescence intensity of chromophore 7-amino-4-methylcoumarin (AMC) cleaved from the C terminus of the peptide substrate. Briefly, aortic samples were weighed and homogenized in lysis buffer (50 mmol/L Hepes, pH 7.4; 0.1% CHAPS; 1 mmol/L dithiothreitol; 0.1 mmol/L EDTA). Samples in duplicate were incubated with caspase-3 substrate (Ac-DEVD-AMC) or with substrate plus inhibitor (Ac-DEVD-CHO) at 37°C for 16 hours. The fluorescence intensity of cleaved AMC was quantified with a multilabel reader (excitation, 355 nm; emission, 460 nm; model 1420; Victor 3, Wallac) and normalized with inhibitor-treated samples as background.

Cytoplasmic Histone-Associated DNA Fragments

To quantify aortic wall apoptosis, cytoplasmic histone-associated DNA fragments (mononucleotides and oligonucleotides) were measured with a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche Biochemicals) described in detail previously.[29,30] Briefly, the aorta was homogenized in 1 mmol/L EDTA in PBS and centrifuged at 1000*g* for 5 minutes at 4°C. The protein content of the supernatant was determined by Bradford assay.[34] The supernatant was incubated with 0.2% Tween 20 and 1% BSA on ice for 30 minutes and centrifuged at 20 000*g* for 15 minutes at 4°C. The cytoplasmic histone-associated DNA fragments in the supernatant

were measured. A set amount of protein (20 μg) was diluted to 100 μL for each sample and then added in duplicate to wells of a microtiter plate coated with an anti-histone antibody. After 90 minutes of incubation, the samples were washed, and an anti-DNA antibody conjugated to peroxidase was added to each well before another 90 minutes of incubation. The plate was washed again, and 2,2′-azino-di-3-ethylbenzthiazoline sulfonate was added for color development. Absorbance was measured at 405 nm.

Active Matrix Metalloproteinase-2

Endogenous levels of free active matrix metalloproteinase-2 (MMP-2) activity in the suprarenal aortic tissue were measured with an MMP-2 Biotrak calorimetric assay kit (Amersham Biosciences). This assay uses the proenzyme form of a detection enzyme that is specifically activated by a single proteolytic event after capture of active MMP-2. The MMP-activated detection enzyme is then quantified with a specific chromogenic peptide substrate. The homogenized samples from the above caspase-3 assay, along with the standards, were incubated overnight in duplicate in 96-well microtiter plates precoated with anti–MMP-2 antibody. After washing to remove other components of the samples, active MMP-2 remained bound to the wells. Detection enzyme and substrate were added to wells containing standards and samples and incubated at 37°C for 6 hours. The resultant color was read at 405 nm in a microplate spectrophotometer. The concentration of active MMP-2 in the sample was determined by interpolation from the standard curve.

Zymographic Analysis of Metalloproteinase Activities

As described in detail previously,[2,6] protein extracts were prepared from the suprarenal aortas of control mice and mice treated with Ang II or Ang II plus fasudil (H). A pool of 5 samples from the treatment group was resolved under nonreducing conditions on a 10% polyacrylamide gel containing 0.1% gelatin as the substrate for MMP activity. After electrophoresis, proteins were renatured by washing in Triton X-100. The gels were then incubated overnight at 37°C in a solution containing 50 mmol/L Tris HCl, 200 mmol/L NaCl, and 5 mmol/L $CaCl_2$, pH 8.0. Zones of lysis were visualized by staining with 0.5% Coomassie blue R-250.

Measurement of Plasma Concentrations of Fasudil and Hydroxyfasudil

Before an animal was euthanized at the end of the experiment, blood was taken randomly during the daytime for determination of plasma concentrations of fasudil and hydroxyfasudil (active metabolite). Because mice drink more at night, the plasma drug concentrations were considered an average level with a tendency toward underestimation. A protein precipitate method was used to remove proteins in the plasma by addition of methanol. Plasma drug concentrations were measured by LC-MS/MS–based analytical method in multiple reaction monitor–positive mode. A Shimadzu high-performance liquid chromatography system (Shimadzu), in tandem with a PE/SCIEX API 3000 triple-quadruple mass spectrometer equipped with a TurboIonSpray source (Applied Biosystems), was used to determine plasma drug levels.

Statistical Analysis

Results are presented as mean±SE for the number of animals used. Statistic comparison for the incidence of AAA was performed by χ^2 analysis. Multiple comparison of mean values was performed by ANOVA, followed by a subsequent Student-Newman-Keuls test for repeated measures. Differences were considered statistically significant at a value of $P<0.05$.

Results

Infusion of Ang II in apoE-KO mice increased daily water consumption, which was not affected by fasudil (Table). The calculated average daily dose of fasudil based on measured daily water consumption was 136±12 and 213±10 mg/kg for the low- and high-dose groups, respectively. In mice treated with high-dose fasudil, the average plasma drug concentrations were 0.7±0.4 μmol/L for fasudil and 4.2±0.7 μmol/L for the active metabolite hydroxyfasudil. Infusion of Ang II increased blood pressure (change in mean arterial pressure [ΔMAP], 31±11 mm Hg), with no significant effect on heart rate, which was not affected by fasudil (ΔMAP, 32±10 mm Hg). Ang II also significantly increased carotid artery and aortic arch atherosclerotic lesion area and aortic stiffness measured by pulse-wave velocity, which were accompanied by an increase in serum cholesterol levels, which were not affected by fasudil (Table). Neither Ang II nor Ang II plus fasudil (H) treatment significantly affected body weight (Table).

General Effects of Fasudil in ApoE-KO Mice Infused With Ang II

	Control	Ang II	Ang II + Fasudil (H)
Daily water consumption, n	15	6	7
Baseline, mL/mouse	3.8±0.1	4.8±0.5	4.1±0.2
End, mL/mouse	4.0±0.2	7.5±0.6*†	7.2±0.5*†
Body weight, n	10	24	28
Baseline, g	29±1	31±0.5	30±0.5
End, g	29±1	29±0.6	30±0.5
Cholesterol, n	12	18	20
Total, mg/mL	549±22	821±60*	832±27*
Triglycerides, mg/mL	213±26	363±36*	376±25*
HDL, mg/mL	59±3	91±7*	71±7‡
VLDL/LDL, mg/mL	447±17	658±57*	685±25*
Doppler measurement, n	12	6	9
PWV, cm/s	3.6±0.1	4.8±0.26*	4.4±0.27*
Heart rate, bpm	524±9	498±14	545±21
Coronary artery lesion area, n	10	18	20
%	10±1.2	27±1.8*	25±2.6*
Aortic arch lesion, n	7	8	8
%	21±1.5	32±2.7*	35±2.8*

Baseline was measured before any treatment. End-point measurements were obtained 28 days after administration of Ang II with or without fasudil. Cholesterol profile, pulse-wave velocity (PWV), heart rate, and atherosclerotic lesions were measured at the end point.

*$P<0.05$ vs control; †$P<0.05$ vs baseline in the same group; ‡$P<0.05$ vs Ang II group.

Infusion of Ang II for 1 month induced AAA formation in 75% (18 of 24) of the apoE-KO mice; the average diameter of the suprarenal aorta increased ≈3-fold compared with the control mice (Figure 1). The AAAs observed in the Ang II group were mostly type III (Figure 2, top). Treatment with fasudil dose-dependently decreased the incidence of AAA and the average diameter of the suprarenal aorta. In the high-dose fasudil group, the incidence of AAA was reduced from 75% (Ang II group) to 41% (12 of 29). The diameter of the suprarenal aorta was reduced from 2.1±0.2 mm (Ang II group) to 1.5±0.1 mm ($P<0.05$). The severity of AAA was also reduced dose-dependently by fasudil (Figure 2, bottom).

Histopathological examination of Ang II–induced AAA revealed a thickening of the abdominal aortic wall, inflam-

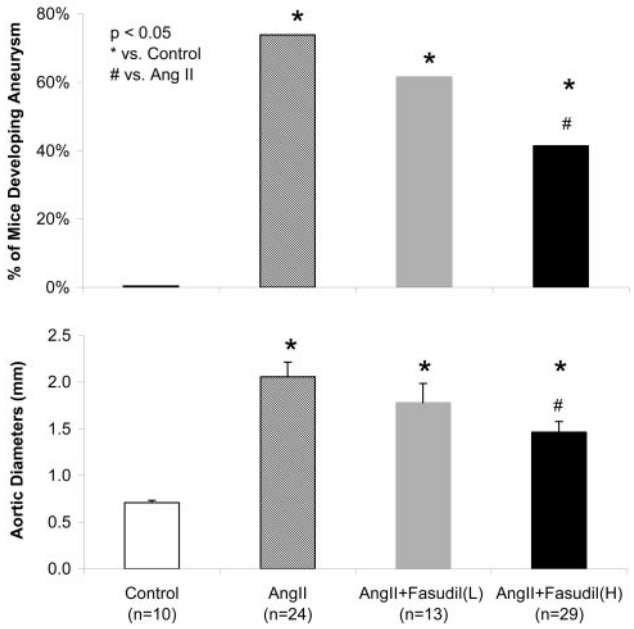


Figure 1. Fasudil attenuated Ang II–induced aneurysm formation in apoE-KO mice measured by percent of mice that developed aneurysm (top) and average diameters of suprarenal aorta (bottom).

matory cell infiltration, medial SMC loss, elastin destruction, and adventitial fibrosis (Figure 3). Mac-3–positive macrophages were detected abundantly in the adventitia and modestly in the media of the suprarenal aortic walls harvested from the Ang II–treated mice. As shown by TUNEL staining, Ang II treatment was associated with apoptosis in the media

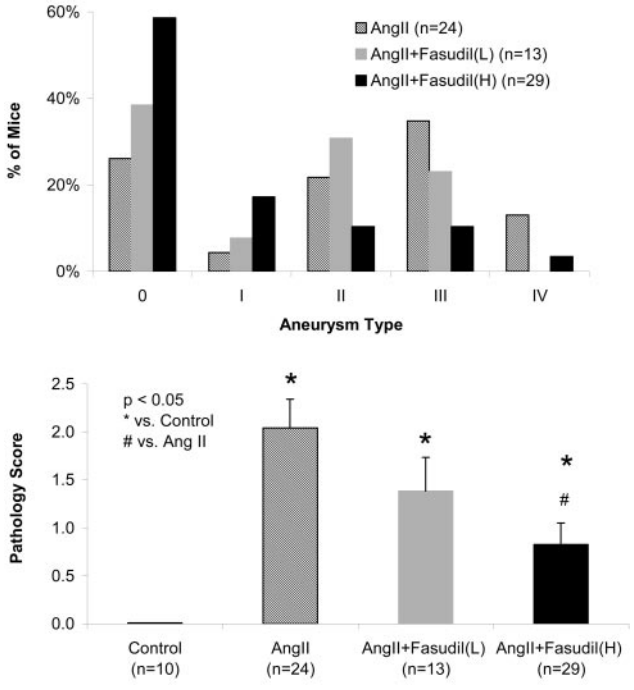
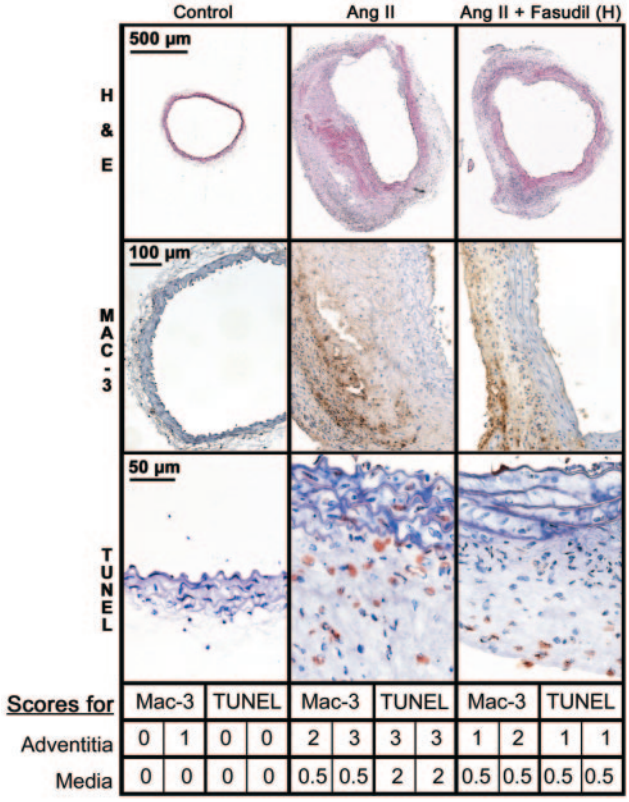


Figure 2. Fasudil reduced severity of aneurysm induced by Ang II in apoE-KO mice measured by pathology categorization (top) and average pathology score (bottom).



Scores for	Mac-3		TUNEL		Mac-3		TUNEL		Mac-3		TUNEL	
Adventitia	0	1	0	0	2	3	3	3	1	2	1	1
Media	0	0	0	0	0.5	0.5	2	2	0.5	0.5	0.5	0.5

Figure 3. Histology of suprarenal aorta stained by hematoxylin and eosin (HE), MAC-3, and TUNEL. Table indicates pathological scores grading "density" of labeled cell nuclei on scale of 0 to 3, representing absent, mild (≈25%), moderate (≈50%), and extensive (≈75%) degrees of Mac-3– or TUNEL-positive cells in adventitia and media from 2 representative samples for each treatment group.

and more markedly in the adventitia of the suprarenal aortic wall. Aortas from the control mice not treated with Ang II showed no Mac-3– or TUNEL-positive staining in either location. These histological changes induced by Ang II were ameliorated by fasudil treatment characterized by attenuated aortic wall thickening, inflammatory cell infiltration, medial SMC loss, elastin destruction, adventitial fibrosis, Mac-3–positive macrophage infiltration (especially in the adventitia), and apoptotic cells (TUNEL-positive staining diminished in the medial layers and scattered in the adventitia).

Ang II infusion also significantly increased the proapoptotic activity as quantified by an ≈40-fold elevation of active caspase-3 in the suprarenal aortas compared with those in control group, which was significantly attenuated by cotreatment with fasudil (Figure 4, top). To further verify the degree of apoptosis in the aortic tissue, a cell death detection ELISA for cytoplasmic histone-associated DNA fragments was performed. Consistent with the TUNEL assay and caspase-3 activity, cytosolic DNA fragments were significantly increased in Ang II–treated apoE-KO mice compared with controls ($P<0.05$) (Figure 4, middle). The increased cytosolic DNA fragmentation by Ang II was markedly reduced by fasudil treatment to the level below that in control mice ($P<0.05$). Levels of cytosolic DAN fragments were positively correlated with diameters of the suprarenal aorta (Figure 4, bottom).

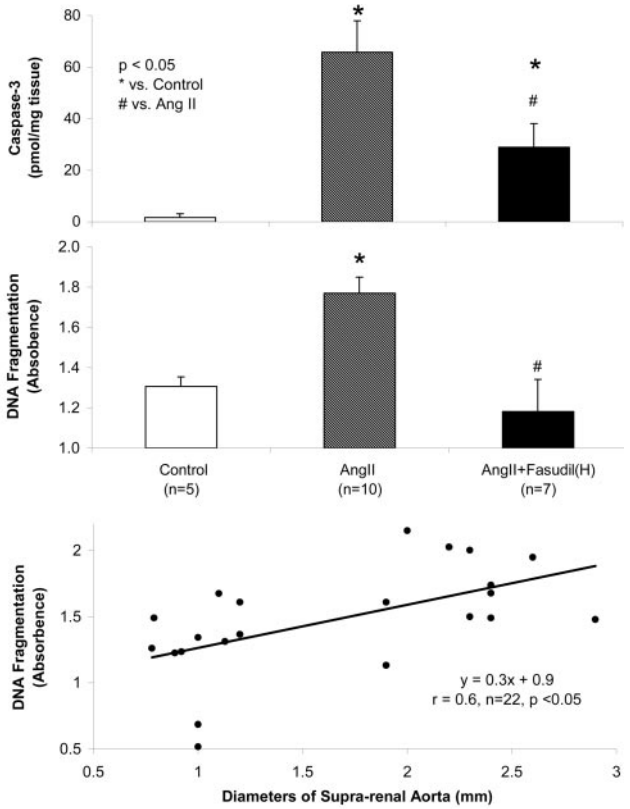


Figure 4. Fasudil attenuated Ang II–induced caspase-3 expression (top) and cytoplasmic histone-associated DNA fragmentation measured by cell death detection ELISA (middle) in suprarenal aortic tissue. Level of cytoplasmic histone-associated DNA fragments is correlated to diameters of suprarenal aorta (bottom).

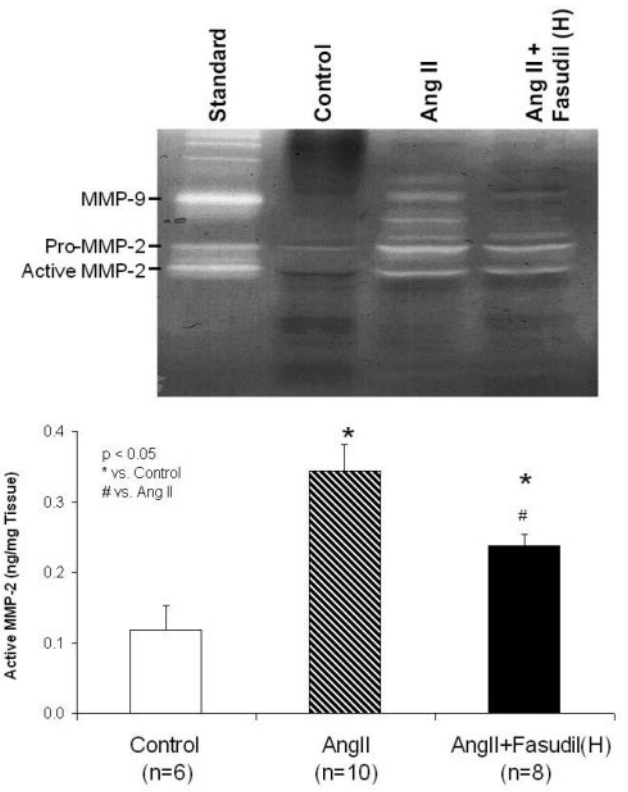


Figure 5. Fasudil attenuated Ang II–induced metalloproteinases expression in suprarenal aortic tissue detected by gelatin zymographic analysis of MMP-2 (72 and 59 kDa) and MMP-9 (92 kDa) activities (top) and quantified by active MMP-2 activity (bottom).

Zymographic analysis of MMP activities showed no detectable signals of either MMP-2 or MMP-9 in the suprarenal aortic tissues of control animals, but both of these MMPs were dramatically enhanced by Ang II treatment (Figure 5, top). Cotreatment with fasudil markedly reduced MMP-9 activity and appeared to reduce the intensity of both pro- and active MMP-2. Furthermore, the active MMP-2 activity from the suprarenal aorta was quantified by an enzymatic assay. Consistent with zymographic analysis, Ang II treatment significantly increased active MMP-2 activity, which was also significantly reduced by cotreatment with fasudil (Figure 5, bottom).

Discussion

Our results demonstrated that both the incidence and severity of Ang II–induced AAA in apoE-KO mice were dose-dependently reduced by fasudil, a Rho-kinase inhibitor. The data further demonstrated that increased proteolytic (MMPs) and proapoptotic (caspase-3) activities are prominent features of AAA in this model, which were also significantly attenuated by fasudil treatment.

It has been shown in vivo that Ang II significantly increased Rho-kinase activity, which can be inhibited by long-term treatment with fasudil.[35] Furthermore, Hattori et al[36] reported that administration of fasudil to mice in drinking water at a daily dose of 100 mg/kg resulted in a plasma concentration of hydroxyfasudil of ≈0.4 μmol/L and significantly inhibited the tissue level of Rho-kinase. However, in the present study, only the high-dose fasudil, which resulted in an average plasma concentration of hydroxyfasudil of ≈4 μmol/L, significantly attenuated Ang II–induced AAA. The probable reason is that hydroxyfasudil cannot easily diffuse into the relatively thick aortic wall to inhibit the tissue level of Rho-kinase. Definite proof requires the measurement of tissue drug concentration, which we were unable to detect in the present study because of technical limitations. After oral administration, fasudil metabolizes to a more selective and potent Rho-kinase inhibitor, hydroxyfasudil,[37] which has been shown to inhibit Rho-kinase ≈100- and 1000-fold more potently than protein kinase C and myosin light-chain kinase, respectively.[35] Thus, attenuation of Ang II–induced AAA after oral administration of fasudil in the present study is likely related to the specific inhibition of Rho-kinase.

Ang II increased blood pressure modestly, but it is unlikely that this small increase in blood pressure contributed to AAA formation in this model.[2] In support of this view, it has been reported that urokinase-type plasminogen activator deficiency[6] or other pharmacological interventions reduced AAA formation without affecting blood pressure in the Ang II model.[27,38] On the other hand, lowering blood pressure by hydralazine treatment did not affect the incidence of spontaneous AAA development in apoE and endothelial nitric oxide double-deficient mice.[39] Previous publications have demon-

strated that activation of Rho-kinase is involved in the contractile response to Ang II,[40] but the present data did not show that fasudil affected Ang II–induced hypertension, a finding that is in agreement with another report that fasudil suppressed Ang II–induced cardiac hypertrophy without affecting blood pressure.[35] Furthermore, Mukai et al[41] reported that fasudil selectively lowered blood pressure only in spontaneously hypertensive rats, not in normotensive controls. Thus, attenuation of AAA by fasudil is independent of blood pressure.

Ang II promotes vascular inflammation characterized by macrophage infiltration in the vascular wall.[1–4,7,27] Macrophages are a major source of proteolytic enzymes, which can degrade components of the extracellular matrix and impair the structural integrity of the vascular wall, leading to AAA formation.[2–4,6] Consistent with previous findings,[2,6] the present data confirmed that the activities of both MMP-2 and MMP-9 were significantly increased by Ang II. It has been reported that activated macrophages produce a number of proapoptotic mediators such as perforin, Fas, and Fas ligand.[11,16] Accumulating evidence indicates that apoptosis is another important pathological change associated with human atherosclerotic AAA.[8–18] Microarray analysis of human AAA and normal aortic tissue revealed that apoptosis-related genes were differentially expressed in AAA and associated with apoptosis of SMCs.[42] Indeed, in the present study, we have detected increased macrophage infiltration, strong TUNEL-positive staining, enhanced activities of caspase-3, and increased cytoplasmic histone-associated DNA fragments in the suprarenal aortas from the mice treated with Ang II. Although the exact cell type that undergoes apoptosis in the aorta cannot be identified in the present AAA model, Satta et al[20] reported that increased media TUNEL-positive apoptotic bodies in human AAA specimens are associated with the loss of SMCs during evolution of AAA. A reduction in SMC would impair matrix synthesis.[21] Thus, vascular inflammation, which triggers both vascular wall proteolysis and SMC apoptosis, could result in an imbalance between matrix degradation and synthesis, favoring tissue destruction and AAA formation. Rho-kinase has been shown to mediate Ang II–induced macrophage migration,[35] which can be inhibited by fasudil.[43,44] This antiinflammatory effect of fasudil could inhibit both the proteolytic and proapoptotic pathways, thus restoring the balance between matrix synthesis and degradation favoring tissue repair. Indeed, in the present study, fasudil treatment inhibited MMP activities and reduced apoptosis, in support of the assertion that both mechanisms contribute to the reduction in AAA formation. Furthermore, Ang II–induced oxidative stress can also contribute to cell death.[19] There is evidence that fasudil inhibits Ang II–induced oxidative stress.[35] This can also indirectly contribute to the antiapoptotic effects of fasudil. Activation of T lymphocytes has been implicated in all stages of human atherosclerotic lesion and AAA formation.[45,46] It has been recently reported that Rho-kinase plays a major role in T-cell activation and inhibition of Rho-kinase–reduced T-cell activation.[47,48] Thus, inhibition of T-cell activation by fasudil may also contribute to attenuated AAA formation.

Rho-kinase may play different roles of pathogenesis at different stages of Ang II–induced AAA development. Saraff et al[49] reported that Ang II initially caused medial accumulation of macrophages and subsequently medial dissection associated with luminal dilation. At the early stage, Ang II initiates vascular inflammation; at the late stage, Ang II could play a greater role through activation of apoptosis and proteases. Our data demonstrated that fasudil reduced macrophage infiltration and attenuated apoptotic and proteolytic activities, thus reducing not only the incidence but also the severity of AAA. These results indicate that Rho-kinase mediate both the early-stage vascular inflammation and late-stage apoptotic and proteolytic activation. However, an additional time-course study could further define the relative contribution of Rho-kinase at different stages of the disease process.

In contrast to our findings, it has been reported that after vascular injury, the Rho-kinase inhibitor Y27632 reduced neointimal formation, which was accompanied by enhanced SMC apoptosis.[24] However, reduced neointimal formation is not due to direct inhibition of cell proliferation but rather to inhibition of SMC migration and enhancement of SMC loss in the media.[50] Abe et al[51] reported that long-term treatment with fasudil attenuated pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular lesions. These effects of fasudil were accompanied by suppressed vascular SMC proliferation and macrophage infiltration and enhanced vascular SMC apoptosis. Thus, it is possible that the role of apoptosis in pathogenesis of AAA may be different from that of other diseases in which links between Rho-kinase and apoptosis may be regulated differently.

Atherosclerosis and AAA are both associated with vascular inflammation. Our finding that fasudil attenuated AAA formation but had no effect on atherosclerotic plaque area in both the carotid artery and aortic arch suggests that there are differences in the pathophysiological role of Rho-kinase. Early atherosclerosis is primarily an intimal disease, whereas AAA is a medial disease. We have demonstrated that Ang II–induced AAA does not require the presence of atherosclerosis because Ang II can also induce AAA in C57 BL/6J wild-type mice in the absence of atherosclerosis.[6] However, preexisting atherosclerosis may promote AAA formation because the incidence of AAA is much higher in apoE-KO compared with wild-type mice. Fasudil may selectively inhibit the signaling pathway(s) activated by Ang II that contribute to AAA formation (eg, proapoptotic and proteolytic pathways) but not directly affect the pathways that promote early atherosclerotic lesions. In support of this view, Manning et al[38] recently reported that doxycycline, an inhibitor of MMPs, attenuated Ang II–induced AAA but did not affect atherosclerosis development.

In summary, as in human AAA tissue, Ang II–induced AAA in apoE-KO mice is associated with a significant increase in apoptosis and protease activation in the aortic wall. Inhibition of Rho-kinase by fasudil attenuated Ang II–induced AAA and aortic wall apoptosis and proteolysis. These findings, consistent with a role of Rho-kinase in the pathogenesis of AAA formation, suggest that targeting Rho-

kinase may be a novel therapeutic strategy for preventing expansion and rupture of AAA.

Acknowledgment

We thank Jian-Ming Gu and Jianhuan Zhang for technical help in the zymography experiments.

References

1. Tham DM, Martin-McNulty B, Wang YX, Wilson DW, Vergona R, Sullivan ME, Dole W, Rutledge JC. Angiotensin II is associated with activation of NF-kappaB–mediated genes and downregulation of PPARs. *Physiol Genomics*. 2002;11:21–30.
2. Wang YX, Martin-McNulty B, Freay AD, Sukovich DA, Halks-Miller M, Li WW, Vergona R, Sullivan ME, Morser J, Dole WP, Deng GG. Angiotensin II increases urokinase-type plasminogen activator expression and induces aneurysm in the abdominal aorta of apolipoprotein E–deficient mice. *Am J Pathol*. 2001;159:1455–1464.
3. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E–deficient mice. *J Clin Invest*. 2000;105:1605–1612.
4. Daugherty A, Cassis LA. Mechanisms of abdominal aortic aneurysm formation. *Curr Atheroscler Rep*. 2002;4:222–227.
5. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet*. 1997;17:439–444.
6. Deng GG, Martin-McNulty B, Sukovich DA, Freay A, Halks-Miller M, Thinnes T, Loskutoff DJ, Carmeliet P, Dole WP, Wang YX. Urokinase-type plasminogen activator plays a critical role in angiotensin II–induced abdominal aortic aneurysm. *Circ Res*. 2003;92:510–517.
7. Thompson RW. Reflections on the pathogenesis of abdominal aortic aneurysms. *Cardiovasc Surg*. 2002;10:389–394.
8. Hara A, Yoshimi N, Mori H. Evidence for apoptosis in human intracranial aneurysms. *Neurol Res*. 1998;20:127–130.
9. Jacob T, Ascher E, Hingorani A, Gunduz Y, Kallakuri S. Initial steps in the unifying theory of the pathogenesis of artery aneurysms. *J Surg Res*. 2001;101:37–43.
10. Holmes DR, Lopez-Candales A, Liao S, Thompson RW. Smooth muscle cell apoptosis and p53 expression in human abdominal aortic aneurysms. *Ann N Y Acad Sci*. 1996;800:286–287.
11. Jacob T, Hingorani A, Ascher E. Examination of the apoptotic pathway and proteolysis in the pathogenesis of popliteal artery aneurysms. *Eur J Vasc Endovasc Surg*. 2001;22:77–85.
12. Kondo S, Hashimoto N, Kikuchi H, Hazama F, Nagata I, Kataoka H. Apoptosis of medial smooth muscle cells in the development of saccular cerebral aneurysms in rats. *Stroke*. 1998;29:181–188; discussion 189.
13. Lopez-Candales A, Holmes DR, Liao S, Scott MJ, Wickline SA, Thompson RW. Decreased vascular smooth muscle cell density in medial degeneration of human abdominal aortic aneurysms. *Am J Pathol*. 1997;150:993–1007.
14. Nataatmadja M, West M, West J, Summers K, Walker P, Nagata M, Watanabe T. Abnormal extracellular matrix protein transport associated with increased apoptosis of vascular smooth muscle cells in Marfan syndrome and bicuspid aortic valve thoracic aortic aneurysm. *Circulation*. 2003;108(suppl II):II-329–II-34.
15. Stehbens WE. Apoptosis and matrix vesicles in the genesis of arterial aneurysms of cerebral arteries. *Stroke*. 1998;29:1478–1480.
16. Henderson EL, Geng YJ, Sukhova GK, Whittemore AD, Knox J, Libby P. Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms. *Circulation*. 1999;99:96–104.
17. Rowe VL, Stevens SL, Reddick TT, Freeman MB, Donnell R, Carroll RC, Goldman MH. Vascular smooth muscle cell apoptosis in aneurysmal, occlusive, and normal human aortas. *J Vasc Surg*. 2000;31:567–576.
18. Sakaki T, Kohmura E, Kishiguchi T, Yuguchi T, Yamashita T, Hayakawa T. Loss and apoptosis of smooth muscle cells in intracranial aneurysms: studies with in situ DNA end labeling and antibody against single-stranded DNA. *Acta Neurochir (Wien)*. 1997;139:469–474; discussion 474–45.
19. Miller FJ Jr, Sharp WJ, Fang X, Oberley LW, Oberley TD, Weintraub NL. Oxidative stress in human abdominal aortic aneurysms: a potential mediator of aneurysmal remodeling. *Arterioscler Thromb Vasc Biol*. 2002;22:560–565.
20. Satta J, Mennander A, Soini Y, Holmes DR, Lopez-Candales A, Liao S, Thompson RW. Increased medial TUNEL-positive staining associated with apoptotic bodies is linked to smooth muscle cell diminution during evolution of abdominal aortic aneurysms. *Ann Vasc Surg*. 2002;16:462–466.
21. Thompson RW, Liao S, Curci JA. Vascular smooth muscle cell apoptosis in abdominal aortic aneurysms. *Coron Artery Dis*. 1997;8:623–631.
22. Funakoshi Y, Ichiki T, Shimokawa H, Egashira K, Takeda K, Kaibuchi K, Takeya M, Yoshimura T, Takeshita A. Rho-kinase mediates angiotensin II–induced monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cells. *Hypertension*. 2001;38:100–104.
23. Matsumoto Y, Uwatoku T, Oi K, Abe K, Hattori T, Morishige K, Eto Y, Fukumoto Y, Nakamura K, Shibata Y, Matsuda T, Takeshita A, Shimokawa H. Long-term inhibition of Rho-kinase suppresses neointimal formation after stent implantation in porcine coronary arteries: involvement of multiple mechanisms. *Arterioscler Thromb Vasc Biol*. 2004;24:181–186.
24. Shibata R, Kai H, Seki Y, Kato S, Morimatsu M, Kaibuchi K, Imaizumi T. Role of Rho-associated kinase in neointima formation after vascular injury. *Circulation*. 2001;103:284–289.
25. Johnston KW, Rutherford RB, Tilson MD, Shah DM, Hollier L, Stanley JC. Suggested standards for reporting on arterial aneurysms: Subcommittee on Reporting Standards for Arterial Aneurysms, Ad Hoc Committee on Reporting Standards, Society for Vascular Surgery and North American Chapter, International Society for Cardiovascular Surgery. *J Vasc Surg*. 1991;13:452–458.
26. Daugherty A, Manning MW, Cassis LA. Antagonism of AT2 receptors augments angiotensin II–induced abdominal aortic aneurysms and atherosclerosis. *Br J Pharmacol*. 2001;134:865–870.
27. Martin-McNulty B, Tham DM, da Cunha V, Ho JJ, Wilson DW, Rutledge JC, Deng GG, Vergona R, Sullivan ME, Wang YX. 17β-Estradiol attenuates development of angiotensin II–induced aortic abdominal aneurysm in apolipoprotein E–deficient mice. *Arterioscler Thromb Vasc Biol*. 2003;23:1627–32.
28. Wang YX, Halks-Miller M, Vergona R, Sullivan ME, Fitch R, Mallari C, Martin-McNulty B, da Cunha V, Freay A, Rubanyi GM, Kauser K. Increased aortic stiffness assessed by pulse wave velocity in apolipoprotein E–deficient mice. *Am J Physiol Heart Circ Physiol*. 2000;278:H428–H434.
29. Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, Yee SP. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. *Circulation*. 2002;106:873–879.
30. Song W, Lu X, Feng Q. Tumor necrosis factor-alpha induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. *Cardiovasc Res*. 2000;45:595–602.
31. Hartley CJ, Reddy AK, Madala S, Martin-McNulty B, Vergona R, Sullivan ME, Halks-Miller M, Taffet GE, Michael LH, Entman ML, Wang YX. Hemodynamic changes in apolipoprotein E–knockout mice. *Am J Physiol Heart Circ Physiol*. 2000;279:H2326–H2334.
32. Stadelmann C, Lassmann H. Detection of apoptosis in tissue sections. *Cell Tissue Res*. 2000;301:19–31.
33. Armstrong RC, Aja TJ, Hoang KD, Gaur S, Bai X, Alnemri ES, Litwack G, Karanewsky DS, Fritz LC, Tomaselli KJ. Activation of the CED3/ICE-related protease CPP32 in cerebellar granule neurons undergoing apoptosis but not necrosis. *J Neurosci*. 1997;17:553–562.
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254.
35. Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, Ichiki T, Takahashi S, Takeshita A. Long-term inhibition of Rho-kinase suppresses angiotensin II–induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. *Circ Res*. 2003;93:767–775.
36. Hattori T, Shimokawa H, Higashi M, Hiroki J, Mukai Y, Tsutsui H, Kaibuchi K, Takeshita A. Long-term inhibition of Rho-kinase suppresses left ventricular remodeling after myocardial infarction in mice. *Circulation*. 2004;109:2234–2239.
37. Shimokawa H. Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. *J Cardiovasc Pharmacol*. 2002;39:319–327.
38. Manning MW, Cassis LA, Daugherty A. Differential effects of doxycycline, a broad-spectrum matrix metalloproteinase inhibitor, on angiotensin II–induced atherosclerosis and abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2003;23:483–488.
39. Chen J, Kuhlencordt PJ, Astern J, Gyurko R, Huang PL. Hypertension does not account for the accelerated atherosclerosis and development of

aneurysms in male apolipoprotein e/endothelial nitric oxide synthase double knockout mice. *Circulation*. 2001;104:2391–2394.
40. Rattan S, Puri RN, Fan YP. Involvement of Rho and Rho-associated kinase in sphincteric smooth muscle contraction by angiotensin II. *Exp Biol Med (Maywood)*. 2003;228:972–981.
41. Mukai Y, Shimokawa H, Matoba T, Kandabashi T, Satoh S, Hiroki J, Kaibuchi K, Takeshita A. Involvement of Rho-kinase in hypertensive vascular disease: a novel therapeutic target in hypertension. *FASEB J*. 2001;15:1062–1064.
42. Zheng Y, Guan H, Li Y, Liu C, Liu B, Sheng Q, Miao S. Microarray in screening for differentially expressed genes of cellular cycle and apoptosis in abdominal aortic aneurysms [in Chinese]. *Zhonghua Wai Ke Za Zhi*. 2002;40:817–819.
43. Miyata K, Shimokawa H, Kandabashi T, Higo T, Morishige K, Eto Y, Egashira K, Kaibuchi K, Takeshita A. Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. *Arterioscler Thromb Vasc Biol*. 2000;20:2351–2358.
44. Ikegaki I, Hattori T, Yamaguchi T, Sasaki Y, Satoh SI, Asano T, Shimokawa H. Involvement of Rho-kinase in vascular remodeling caused by long-term inhibition of nitric oxide synthesis in rats. *Eur J Pharmacol*. 2001;427:69–75.
45. Pinderski LJ, Fischbein MP, Subbanagounder G, Fishbein MC, Kubo N, Cheroutre H, Curtiss LK, Berliner JA, Boisvert WA. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor–deficient mice by altering lymphocyte and macrophage phenotypes. *Circ Res*. 2002;90:1064–1071.
46. Daugherty A, Rateri DL. T Lymphocytes in atherosclerosis: the yin-yang of Th1 and Th2 influence on lesion formation. *Circ Res*. 2002;90:1039–1040.
47. Mallat Z, Gojova A, Sauzeau V, Brun V, Silvestre J-S, Esposito B, Merval R, Groux H, Loirand G, Tedgui A. Rho-associated protein kinase contributes to early atherosclerotic lesion formation in mice. *Circ Res*. 2003;93:884–888.
48. Tharaux PL, Bukoski RC, Rocha PN, Crowley SD, Ruiz P, Nataraj C, Howell DN, Kaibuchi K, Spurney RF, Coffman TM. Rho kinase promotes alloimmune responses by regulating the proliferation and structure of T cells. *J Immunol*. 2003;171:96–105.
49. Saraff K, Babamusta F, Cassis LA, Daugherty A. Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II–infused, apolipoprotein E–deficient mice. *Arterioscler Thromb Vasc Biol*. 2003;23:1621–1626.
50. Negoro N, Hoshiga M, Seto M, Kohbayashi E, Ii M, Fukui R, Shibata N, Nakakoji T, Nishiguchi F, Sasaki Y, Ishihara T, Ohsawa N. The kinase inhibitor fasudil (HA-1077) reduces intimal hyperplasia through inhibiting migration and enhancing cell loss of vascular smooth muscle cells. *Biochem Biophys Res Commun*. 1999;262:211–215.
51. Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, Hattori T, Nakashima Y, Kaibuchi K, Sueishi K, Takeshita A. Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ Res*. 2004;94:385–393.

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Circulation. 2005;111:2219-2226; originally published online April 25, 2005;
doi: 10.1161/01.CIR.0000163544.17221.BE
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539