Effects of a Rho kinase inhibitor on pressure overload induced cardiac hypertrophy and associated diastolic dysfunction

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1Department of Epidemiology and Preventive Medicine, NHMRC Centre of Clinical Research Excellence in Therapeutics, 2Department of Medicine, Monash University, Melbourne; 3Department of Medicine, University of Melbourne, St. Vincent’s Hospital, Melbourne, Australia; and 3Faculty of Medicine, Department of Internal Medicine, Chiang Mai University, Chiang Mai, Thailand

Submitted 17 September 2007; accepted in final form 30 January 2008

Phrommintikul A, Tran L, Kompa A, Wang B, Adrahtas A, Cantwell D, Kelly DJ, Krum H. Effects of a Rho kinase inhibitor on pressure overload induced cardiac hypertrophy and associated diastolic dysfunction. Am J Physiol Heart Circ Physiol 294: H1804–H1814, 2008. First published February 1, 2008; doi:10.1152/ajpheart.01078.2007.—The RhoA-Rho kinase (ROCK) signaling pathway has an important role in cardiovascular diseases. However, the effect of Rho kinase inhibition on pressure overload-induced cardiac hypertrophy (POH) and associated diastolic dysfunction has not been evaluated. This study examined the effect of a selective ROCK inhibitor (GSK-576371) in a POH model, induced by suprarenal abdominal aortic constriction. POH rats were divided into the following four groups: 1 (GSK 1, n = 9) or 3 (GSK 3, n = 10) mg/kg bid GSK-576371, 1 mg·kg−1·day−1 ramipril (n = 10) or vehicle (n = 11) treatment for 4 wk. Sham animals (n = 11) underwent surgery without banding. Echocardiograms were performed before surgery and posttreatment, and hemodynamic data were obtained at completion of the study. Echocardiography showed an increase in relative wall thickness of the left ventricle (LV) following POH + vehicle treatment compared with sham animals. This was attenuated by both doses of GSK-576371 and ramipril. Vehicle treatment demonstrated abnormal diastolic parameters, including mitral valve (MV) inflow E wave deceleration time, isovolumic relaxation time, and MV annular velocity, which were dose dependently restored toward sham values by GSK-576371. LV end diastolic pressure was increased following POH + vehicle treatment compared with sham (6.9 ± 0.7 vs. 3.2 ± 0.7 mmHg, P = 0.008) and was reduced with GSK 3 and ramipril treatment (1.7 ± 0.7, P < 0.01 and 2.9 ± 0.6 mmHg, P < 0.01, respectively). Collagen I deposition in the LV was increased following POH + vehicle treatment (32.2%; P < 0.01) compared with sham animals and was significantly attenuated with GSK 1 (21.7%; P < 0.05), GSK 3 (23.8%; P < 0.01), and ramipril (35.5%; P < 0.01) treatment. These results suggest that ROCK inhibition improves LV geometry and reduces collagen deposition accompanied by improved diastolic function in POH.

Rho kinase; hypertrophy; fibrosis; pressure overload; hypertension; diastolic function; myocardium

Heart failure is a major health problem. Cardiac hypertrophy with or without hypertension is one of the major antecedent pathological changes associated with heart failure (2, 7, 10, 24). In turn, attenuation of cardiac hypertrophy has been a major focus of intervention to prevent both systolic and dia-

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hypertrophy (POH) and its associated diastolic dysfunction has not been previously evaluated. Therefore, this study aimed to determine the effect of a selective ROCK inhibitor (GSK-576371) on POH and diastolic function, as carefully assessed using echocardiographic, hemodynamic, immunohistochemical, and molecular methodologies.

MATERIALS AND METHODS

Animal Preparation

POH was induced by suprarenal abdominal aortic constriction using male Sprague-Dawley (SD) rats (180–230 grams). The abdominal aorta was constricted with a 4–0 silk tie around a blunted 21-gauge probe (0.8 mm outer diameter) just above the right renal artery. The probe was promptly removed after constriction. Sham operation was performed in the same manner, but the abdominal aorta was not constricted. The POH rats received a highly selective Rho kinase inhibitor, GSK-576371, at 1 mg/kg (GSK 1) and 3 mg/kg (GSK 3) two times daily or vehicle treatment. Ramipril (1 mg/kg) one time daily serves as a positive control. All treatments were given by oral gavage and commenced 24 h postsurgery for a period of 4 wk. All animal procedures outlined in this study were approved for use by the St. Vincent’s Hospital Animal Ethics Committee in accordance with the National Health and Medical Research Council of Australia guidelines.

Drugs

Rho kinase inhibitor GSK-576371 was a gift from GlaxoSmithKline (King of Prussia, PA). Ramipril (Aventis, Lane Cove, NSW), a commercially available and widely used angiotensin-converting enzyme inhibitor was purchased from the Alfred Hospital Pharmacy. Both GSK-576371 and ramipril were administered by oral gavage in 0.5% methylcellulose (vehicle) solution.

Assessment of Cardiac Hypertrophy and Function by Echocardiography

Echocardiography was performed at baseline (1 day before surgery) and at the end of study under light anesthesia with ketamine and xylazine (40 and 5 mg/kg ip, respectively) using a Vivid 7 (GE Vingmed, Horton, Norway) echocardiographic machine with a 10-MHz phased array probe. The procedure was performed as per published standard protocol (6). Parasternal short-axis views of the heart at the midpapillary level were used to obtain measures of the interventricular septal (IVSd) and posterior wall thickness (LVPWd) in diastole, and left ventricular internal dimension in diastole and systole. Percent fractional shortening (%FS) and relative wall thickness (RWT) were calculated according to standard formulas (31). Doppler images were obtained from the apical four-chamber view of the heart as previously described in the standard protocol (6). Early and late transmitral peak diastolic flow velocity (E and A waves), mitral valve (MV) inflow E wave deceleration time (DT), and isovolumic relaxation time (IVRT) were measured. Tissue Doppler imaging was performed from the apical four-chamber view and used to assess peak early and late (E’ and A’) diastolic tissue velocity at the septal side of the mitral annulus. The data were obtained for all rats using a dedicated rodent software package. Trans-stenotic pressure gradient was recorded at 1 wk and at the end of the study in the POH group. POH animals with trans-stenotic pressure gradient >50 mmHg were used in the analysis. All parameters were assessed using an average of three beats, and calculations were made in accordance with the American Society of Echocardiography guidelines (31). All data were acquired and analyzed by a single blinded observer using Echo PAC (GE Vingmed) offline processing.

Hemodynamic Parameters

Hemodynamic data were obtained at the end of the study under anesthesia with Nembutal (60 mg/kg) as per published standard protocol (6). Animals were ventilated, and a 2-Fr miniaturized combined catheter-micromanometer (Model SPR-838; Millar instruments, Houston, TX) was inserted in the right common carotid artery to obtain aortic blood pressure and then advanced in the LV to obtain left ventricular pressure-volume (PV) loops. PV loops were recorded at steady state and during transient preload reduction, achieved by transient occlusion of the inferior vena cava and portal vein with the ventilator turned off and the animal anephic. The following validated parameters were assessed using Millar conductance data acquisition and analysis software PVAN 3.2: left ventricular end systolic pressure, left ventricular end diastolic pressure (LVEDP), the slope of the end systolic pressure volume relationship, the slope of the end diastolic pressure volume relationship, maximum and minimum dP/dt (mmHg/s), Tau (τ Logistic), and the slope of the preload recruitable stroke work relationship.

Histological Analysis

At the end of the study, the heart and lungs were harvested, and dried weights were recorded. Transverse sections of the heart were fixed in Tissue-Tek optimum-cutting temperature (OCT)-embedding compound (Sakura Finetek, Chuo-ku, Tokyo, Japan) for

**Table 1. Baseline echocardiographic data**

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 11)</th>
<th>POH + Vehicle (n = 11)</th>
<th>POH + GSK 1 (n = 9)</th>
<th>POH + GSK 3 (n = 10)</th>
<th>POH + Ramipril (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd, mm</td>
<td>1.02 ± 0.03</td>
<td>0.97 ± 0.03</td>
<td>1.03 ± 0.03</td>
<td>0.94 ± 0.03</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>LVPWd, mm</td>
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<td>1.05 ± 0.04</td>
<td>1.12 ± 0.04</td>
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<td>1.07 ± 0.03</td>
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<td>LV mass, g</td>
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<td>0.96 ± 0.01</td>
<td>0.91 ± 0.02</td>
<td>0.98 ± 0.02</td>
</tr>
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<td>LVIDd, mm</td>
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<td>7.56 ± 0.20</td>
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<td>LVIDs, mm</td>
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<td>4.48 ± 0.12</td>
<td>3.73 ± 0.33</td>
<td>4.49 ± 0.23</td>
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<td>FS, %</td>
<td>46.18 ± 3.30</td>
<td>40.26 ± 2.10</td>
<td>37.22 ± 1.05</td>
<td>36.50 ± 3.37</td>
<td>41.1 ± 2.12</td>
</tr>
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<td>IVRT, ms</td>
<td>17.6 ± 0.90</td>
<td>18.2 ± 1.10</td>
<td>18.7 ± 0.90</td>
<td>16.5 ± 1.30</td>
<td>18.4 ± 1.10</td>
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<td>MVDt, ms</td>
<td>37.5 ± 2.90</td>
<td>37.7 ± 2.2</td>
<td>41.1 ± 2.8</td>
<td>33.1 ± 2.70</td>
<td>36.7 ± 2.40</td>
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<td>MV E/A ratio</td>
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<td>1.75 ± 0.08</td>
<td>1.65 ± 0.13</td>
<td>1.93 ± 0.11</td>
<td>1.61 ± 0.11</td>
</tr>
<tr>
<td>E’/A’ ratio</td>
<td>1.31 ± 0.18</td>
<td>1.39 ± 0.19</td>
<td>1.69 ± 0.14</td>
<td>1.31 ± 0.12</td>
<td>1.94 ± 0.32</td>
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<tr>
<td>E/A’ ratio</td>
<td>0.19 ± 0.01</td>
<td>0.21 ± 0.03</td>
<td>0.20 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>302 ± 19</td>
<td>319 ± 12</td>
<td>293 ± 4.0</td>
<td>356 ± 36</td>
<td>329 ± 18</td>
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</tbody>
</table>

Results expressed as mean ± SE; n, no. of experiments. POH, pressure overload-induced cardiac hypertrophy; GKS 1 and 3, GSK-1 and 3 mg/kg bid GSK-576371; IVSd, interventricular septal thickness at diastole; LVPWd, posterior wall thickness at diastole; LV, left ventricular; LVIDs, left ventricular internal dimension in systole; FS, fractional shortening; IVRT, isovolumic relaxation time; MVDt, mitral valve deceleration time; E and A, early and late transmitral peak diastolic flow velocity, respectively; E’ and A’, peak early and late diastolic tissue velocity, respectively; HR, heart rate. P > 0.05 for all comparisons.
histology or frozen with liquid nitrogen for subsequent molecular analyses.

Measurement of Myocyte Cross-sectional Area

Tissue-Tek OCT-embedded tissues were stained with hematoxylin/eosin. The perimeters of 50 myocytes from each animal were traced and analyzed as previously described (32). These data were used to calculate the myocyte mean cross-sectional area in each animal.

Immunoreactivity Analyses for Collagen Type I and III in Left Ventricular Tissue

Tissue-Tek OCT-embedded tissues were cut and mounted on poly-l-lysine (Sigma Diagnostics, St. Louis, MO)-coated slides. Serial sections were stained with a three-layer immunoperoxidase technique for collagen type I and collagen type III and quantitatively assessed by a single blinded researcher as previously described (37). Results were expressed as average percentage area of 10 random fields for each section.

Quantitative mRNA Expression in Left Ventricular Tissue

Total RNA was extracted from frozen cardiac tissues using RNAeasy columns (Qiagen, Hilden, Germany) and reverse transcribed to cDNA. Triplicate cDNA aliquots (0.5 ng each) were amplified using sequence-specific primers (Geneworks, Adelaide, SA, Australia) and a TaqMan fluorogenic probe (Applied Biosystems, Foster City, CA) using an ABI prism 7900HT sequence Detection System (Applied Biosystems). RT-PCR was used to quantify cardiac gene expression of procollagen α1(I) and connective tissue growth factor (CTGF). The primer pairs and probes were designed using Primer Express 2.0 software (Applied Biosystems) based on published sequences (http://www.ncbi.nlm.nih.gov) as previously described (22). 18S rRNA was used as an endogenous control in all experiments to correct for the expression of each gene.

In Vitro Studies

Neonatal rat cardiomyocytes and fibroblast cultures. Neonatal SD rat cardiac myocytes (NCMs) and fibroblasts (NCFs) were isolated from 1-day-old pups with enzymatic digestion as described in detail previously (34, 40). NCFs were seeded and maintained in high-glucose (25 mmol/l) DMEM (Invitrogen Mount Waverley, Vic, Australia) in the presence of 1% antibiotic/antimycotic and 10% FBS (JRH biosciences). NCFs were used at passage 2 (32). Purified NCMs were seeded (1,000 cells/mm²) in six-well plates and maintained in serum-free DMEM (Invitrogen) supplemented with insulin and transferrin as described previously (40). Bromodeoxyuridine was included for the first 3 days. KCl (50 mmol/l) was added to the medium to prevent spontaneous contraction characteristic of the plated NCMs (35).

Measurement of neonatal rat cardiomyocyte hypertrophy. NCM hypertrophy studies were performed as previously described (35). Four hours after treatment with the Rho kinase inhibitor (10⁻⁷ to 10⁻⁵ mol/l GSK-576371), ANG II (10⁻⁷ mol/l) was used to stimulate hypertrophy. After 60 h of stimulation, cells were harvested, and hypertrophy was defined as a significant increase in protein content (Bradford assay) in the absence of any significant change in DNA content (Burton assay; see Ref. 35). Control cells were not preincubated with GSK and were not stimulated with ANG II.

Measurement of collagen synthesis, proliferation, and cell viability in neonatal rat cardiac fibroblasts. NCF collagen synthesis assays were performed as described previously (32). NCFs were plated at a density of 25,000 cells/well in 12-well plates and incubated (5% CO₂) overnight. NCFs were then serum starved for 48 h in

<table>
<thead>
<tr>
<th>Table 2. Echocardiographic data at 4 wk</th>
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<tbody>
<tr>
<td><strong>IVSd, mm</strong></td>
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<tr>
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<tr>
<td>Sham (n = 11)</td>
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<tr>
<td>POH (n = 11)</td>
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<tr>
<td>POH + Ramipril (n = 10)</td>
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</table>

Results expressed as means ± SE; n, no. of experiments. *P < 0.05 vs. sham. **P < 0.01 vs. sham. ***P < 0.05 vs. POH + vehicle. ****P < 0.01 vs. POH + vehicle. **P < 0.05 by trend test.

AJP-Heart Circ Physiol • VOL. 294 • APRIL 2008 • www.ajpheart.org
high-glucose DMEM. The cells were then preincubated for 30 min in the presence or absence of GSK-576371 (10^{-7} to 10^{-5} mol/l) in fresh DMEM-F1–2 before stimulation with 2 x 10^{-10} mol/l of transforming growth factor (TGF)-β1 or 10^{-7} mol/l of ANG II.

For collagen synthesis, 1 μCi of [3H]proline was added to each well and incubated for a further 48 h before harvest. Cells were harvested by precipitation with 10% TCA on ice for 30 min before solubilization with 0.75 ml of 1 mol/l NaOH overnight at 4°C. The samples were then neutralized with 1 mol/l HCl, and ³H levels were counted with 10 ml scintillation fluid on a beta counter to determine the levels of [³H]proline incorporation.

For proliferation studies, NCFs were treated with 1 μCi of [³H]thymidine to each well 2 h before harvesting. Cells were harvested by

Fig. 2. M-mode images representing left ventricle (LV) dimensions. A: 2-dimensional M-mode echocardiography image defining the following LV parameters: interventricular septum in diastole (IVSd), left ventricular posterior wall in diastole (LVPWd), left ventricular internal dimension at diastole (LVIDd), and left ventricular internal dimension in systole (LVIDs). B-F: representative echocardiograms from each group demonstrating changes in wall thickness.

POH + GSK 1

POH + GSK 3

POH + Ramipril
TCA precipitation as described for collagen synthesis above to determine the levels of $[^{3}H]$thymidine incorporation.

**Statistical Analysis**

Data are reported as means ± SE. Comparisons between groups were analyzed by one-way ANOVA followed by Bonferroni’s post hoc t-test as appropriate. Two-sided $P < 0.05$ was considered statistically significant. Dose-dependent effects of Rho kinase inhibition were determined by Tukey trend test. This test is applicable when considering the same drug in the same setting at three or more different dosages (i.e., 0, 1, and 3 mg GSK-576371; see Ref. 1).

**RESULTS**

From 43 POH rats, two were excluded from the study due to trans-stenotic pressure gradient <50 mmHg.

**Mortality**

Survival rate was 100% in sham animals as well as in POH animals receiving all active treatments and 91.7% in POH with vehicle treatment ($P > 0.05$).

**Echocardiographic Measurements**

Baseline echocardiographic findings were not different between groups (Table 1). At the end of 4 wk, there was a significant increase in diastolic wall thicknesses (IVSd and LVPWd) and RWT in the POH + Vehicle group compared with sham. RWT increased by 61.6% ($P < 0.001$) in the POH + Vehicle group compared with sham. GSK 1 and 3 and ramipril treatment significantly attenuated RWT by 24.7% ($P < 0.01$), 26.0% ($P < 0.001$) and 20.2% ($P < 0.05$), respectively, from POH + Vehicle levels (Fig. 1). Left ventricle (LV) cavity size was not significantly different in all groups. LV mass was significantly increased by 27% ($P < 0.01$) in POH + vehicle animals. There was a dose-dependent decrease with GSK 1 and GSK 3 upon trend test, suggesting an antihypertrophic effect of the agent (Table 2 and Fig. 2).

Systolic function measured by FS was preserved in all groups; however, FS was higher in high-dose GSK-567371 treatment compared with sham animals but not different compared with the other POH groups.

There were significant changes in parameters of diastolic function in POH rats measured at 4 wk. MV DT and IVRT were prolonged in POH rats receiving vehicle treatment compared with sham animals. GSK-567371 reduced these parameters in a dose-dependent manner and restored them to sham values with GSK high-dose therapy (Fig. 3). Ramipril did not have any significant effect on diastolic parameters (Table 2).

**Hemodynamic Measurements**

Systolic blood pressure was elevated by 35 mmHg in vehicle-treated POH rats compared with sham animals ($P = 0.018$) (Table 3). Treatment with GSK-576371 and ramipril did not have any significant effect on systolic blood pressure. Furthermore, diastolic blood pressure and mean arterial blood pressure were not different between groups (Table 3). Pulse pressure was, however, significantly higher in POH + Vehicle rats compared with sham animals, and 3 mg/kg bid GSK-576371 attenuated this pressure toward sham values (Table 3).

LVEDP was more than twofold elevated in POH rats receiving vehicle treatment compared with sham animals (6.9 ± 0.7 vs. 3.2 ± 0.7 mmHg, $P = 0.008$), and GSK 576371 lowered LVEDP dose dependently. Similarly, ramipril significantly reduced LVEDP (Table 3).

There was a significant correlation between diastolic parameters from echocardiogram and LVEDP in all groups. Both IVRT ($r = 0.514$, $P = 0.001$) and MV inflow DT ($r = 0.376$, $P = 0.015$) were positively correlated with LVEDP.

**Heart and Body Weight**

Body weight was not different between groups at the end of the study. Heart weight and the ratio of heart weight to body weight were significantly increased by 20.6 and 19.3%, respec-
Table 3. Hemodynamic data at 4 wk

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 11)</th>
<th>POH + Vehicle (n = 11)</th>
<th>POH + GSK 1 (n = 9)</th>
<th>POH + GSK 3 (n = 10)</th>
<th>POH + Ramipril (n = 10)</th>
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<tr>
<td>SBP, mmHg</td>
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<td>154±11*</td>
<td>151±10</td>
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<td>DBP, mmHg</td>
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<td>90±6</td>
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<tr>
<td>MBP, mmHg</td>
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<td>125±8</td>
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<td>PP, mmHg</td>
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<td>33±12d</td>
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<td>LVE, mmHg</td>
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<td>167±13a</td>
<td>159±13a</td>
<td>152±6</td>
<td>136±6</td>
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<tr>
<td>LVEDP, mmHg</td>
<td>3.2±0.7</td>
<td>6.9±0.7h</td>
<td>4.6±0.7</td>
<td>1.7±0.7d</td>
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<td>+ dp/dt, mmHg/s</td>
<td>9,808±308</td>
<td>11,349±688</td>
<td>10,670±876</td>
<td>1,039±381</td>
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<td>PRSW, mmHg</td>
<td>79.9±9.8</td>
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<td>108.6±10.7</td>
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<td>ESPVR, mmHg/µL</td>
<td>1.1±0.6</td>
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<td>−dp/dt, mmHg/s</td>
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<td>TGF-β1, g/µL</td>
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<td>HR, Beats/min</td>
<td>302±19</td>
<td>319±12</td>
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<td>329±18</td>
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</tbody>
</table>

Results expressed as means ± SE; n, no. of experiments. *P < 0.05 vs. sham. **P < 0.01 vs. sham. ***P < 0.05 vs. POH + vehicle. ****P < 0.01 vs. POH + vehicle.

Myocyte Size

Myocyte cross-sectional area was significantly increased in POH + Vehicle treatment by 22.5% (416.6 ± 28.1 µm²) compared with sham animals (298.4 ± 16.8 µm²) (P < 0.05). GSK 1 and 3 and ramipril treatment did not significantly reduce myocyte size (411.6 ± 27.8, 337.2 ± 8.8, and 386.7 ± 17.7 µm², respectively) compared with POH + Vehicle animals (Fig. 4).

Collagen Expression in Left Ventricular Tissue

Immunohistochemistry. An increase in collagen type I deposition in POH + Vehicle rats by 34.5% (P < 0.01) compared with sham animals was observed; this was reduced with GSK 1 and GSK 3 and ramipril treatment by 21.7% (P < 0.05), 23.8% (P < 0.01), and 35.5% (P < 0.01), respectively (Table 5 and Fig. 5). No change in collagen III was detected among all groups (Table 5).

Tissue levels of collagen I were moderately correlated to LVEDP (r = 0.324, P = 0.054).

Real-time PCR. The increase in procollagen α(I) and CTGF gene expression after 4 wk was not statistically significant in POH + Vehicle-treated rats vs. sham (Fig. 6). Similarly, reductions in expression of these genes with active therapy did not reach statistical significance.

In Vitro Studies

Effects of Rho kinase inhibition on cardiomyocyte hypertrophy in isolated neonatal rat cardiomyocytes. ANG II-stimulated cardiomyocytes produced a 25.8% increase in hypertrophy compared with unstimulated cells (P < 0.001). GSK-576371 reduced ANG II-stimulated cardiomyocyte hypertrophy in a dose-dependent manner (Fig. 7A). NCM were unchanged with GSK alone treatment, indicating GSK did not induce NCM hypertrophy (Fig. 7B).

Effects of Rho Kinase Inhibition on Cell Proliferation and Collagen Synthesis in Isolated Cardiac Fibroblasts

Both ANG II and TGF-β1 increased collagen synthesis in rat cardiac fibroblasts by 109% (P < 0.01) and 116% (P < 0.01), respectively, as determined by [%H]proline incorporation. GSK-576371 reduced both ANG II- and TGF-β1-stimulated collagen synthesis in a dose-dependent manner compared with stimulant alone (Fig. 8, A and B, respectively).

Similarly, ANG II and TGF-β1 increased cell proliferation in rat cardiac fibroblasts by 57% (P < 0.01) and 111% (P < 0.01), respectively, as determined by [%H]thymidine incorporation. GSK-576371 reduced both ANG II- and TGF-β1-stimulated cell proliferation in a dose-dependent manner compared with stimulant alone (Fig. 8, C and D, respectively).

DISCUSSION

Heart failure with preserved systolic function (HFPSE) is highly prevalent in the community and associated with significant morbidity and mortality (15, 38). HFPSE is characterized by prolonged myocardial relaxation and abnormal tissue stiffness (43). The major etiological factor for this condition is systemic hypertension, a comorbid risk factor observed in >25% of the adult population in the Western world (3, 18). Abrogation of these abnormalities has been a major therapeutic target in heart failure management. In the present study, we
used a well-validated animal model of diastolic dysfunction that is the cornerstone of HFPSS to study the contribution of the ROCK signaling pathway to this condition. Diastolic dysfunction is of uncertain clinical relevance however is clearly a harbinger of HFPSS. This is, to our knowledge, the first study to examine the pharmacological effects of Rho kinase blockade in diastolic dysfunction, carefully evaluating cardiac function using echocardiographic (including tissue Doppler imaging), hemodynamic, immunohistochemical, and molecular techniques. These data complement and further extend our knowledge base in this area, as generated initially by genetic models approaches to blockade of the RhoA/Rho kinase system have demonstrated reduced cardiac fibrosis and variable success in uncomplicated hypertension. Hypertensive patients with concentric LV hypertrophy (LVH) exhibit a higher morbidity and mortality than those with eccentric LVH (20). Previous studies have found that RWT along with LV mass are important parameters used to classify concentric and eccentric hypertrophy (9, 20). Treatment with GSK-576371 significantly decreased RWT in POH rats, reflecting a geometric alteration toward eccentric hypertrophy. Altering LV geometry from concentric to eccentric hypertrophy may be an important mechanism underlying improved diastolic function with Rho kinase inhibition. Similar findings have been observed with angiotensin receptor blockers on diastolic function in hypertensive patients (26).

LV geometry is a major predictor of morbidity and mortality in uncomplicated hypertension. Hypertensive patients with concentric LV hypertrophy (LVH) exhibit a higher morbidity and mortality than those with eccentric LVH (20). Previous studies have found that RWT along with LV mass are important parameters used to classify concentric and eccentric hypertrophy (9, 20). Treatment with GSK-576371 significantly decreased RWT in POH rats, reflecting a geometric alteration toward eccentric hypertrophy. Altering LV geometry from concentric to eccentric hypertrophy may be an important mechanism underlying improved diastolic function with Rho kinase inhibition. Similar findings have been observed with angiotensin receptor blockers on diastolic function in hypertensive patients (26).

Extracellular matrix remodeling in POH has been demonstrated to be an important contributor to diastolic and eventual systolic dysfunction (4, 5, 28). There is emerging evidence suggesting that increased myocardial fibrosis is a major factor responsible for myocardial stiffness and may be more important than myocardial hypertrophy (17, 21, 41). The present study demonstrated increased collagen I deposition in the LV of POH rats that had a moderate degree of correlation with LVEDP.

H1810

Rho KINASE INHIBITION AND DIASTOLIC DYSFUNCTION

Fig. 4. Myocyte cross-sectional area increased in POH + Vehicle animals compared with sham animals. This was nonsignificantly attenuated with high-dose GSK-576371 treatment (POH + GSK 3) compared with POH + Vehicle treatment (n = 9–11). **P < 0.01 vs. sham (n = 11).

Table 5. Left ventricular immuno-reactivity data for collagen

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 8)</th>
<th>POH + Vehicle (n = 10)</th>
<th>POH + GSK 1 (n = 9)</th>
<th>POH + GSK 3 (n = 10)</th>
<th>POH + Ramipril (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>8.29±0.50</td>
<td>11.15±0.92*</td>
<td>8.73±0.29</td>
<td>8.50±0.26</td>
<td>7.19±0.35*</td>
</tr>
<tr>
<td>Collagen III</td>
<td>6.78±0.2</td>
<td>7.2±0.29</td>
<td>6.82±0.24</td>
<td>7.3±0.29</td>
<td>7.28±0.36</td>
</tr>
<tr>
<td>Collagen I-III</td>
<td>1.16±0.06</td>
<td>1.52±0.14</td>
<td>1.29±0.07</td>
<td>1.16±0.04</td>
<td>1.02±0.06</td>
</tr>
</tbody>
</table>

Results expressed as means ± SE; n, no. of experiments. *P < 0.05 vs. sham. **P < 0.05 vs. POH + vehicle. ***P < 0.01 vs. POH + vehicle.
Treatment with GSK-576371 had significant beneficial effects on extracellular remodeling, with a reduction in collagen I protein in the LV of POH rats.

Attenuation of myocardial fibrosis by GSK-576371 may be a major mechanism associated with the improvement in diastolic function in POH rats. In our in vitro study, the direct effect of GSK-576371 on cardiac collagen turnover was examined in ANG II- and TGF-β1-stimulated cardiac fibroblasts, with a decrease observed in collagen synthesis and cell proliferation. However, because gene expression of collagen I and CTGF is not significantly increased in vivo in POH + Vehicle animals compared with sham, it is somewhat difficult to further interpret the antifibrotic effect of GSK-576371 from these data.

Rho kinase inhibition with fasudil has been shown to reduce cardiac fibrosis in postmyocardial infarction animals, spontaneously hypertensive rats, and in an ANG II infusion model of cardiac hypertrophy (13, 14, 25). Inhibition of Rho kinase by ROCK1 gene deletion also reduced myocardial fibrosis in animals with transverse aortic banding as well as in ANG II-induced hypertension compared with the wild type (29, 42).

Our study showed that Rho kinase inhibition by GSK-576371 attenuated myocardial fibrosis, accompanied by a significant dose-dependent reduction in LV mass on echocardiography. This was paralleled by a dose-dependent reduction in heart weight with GSK-576371 treatment. Previous studies have reported that Rho kinase inhibition with fasudil attenuated...
both cardiac fibrosis and hypertrophy in ANG II-induced cardiac hypertrophy (14, 39). Treatment with Y-27632 has demonstrated an attenuation of myocardial hypertrophy both with and without beneficial effects on myocardial fibrosis in Dahl salt-sensitive rats (19, 30). In contrast, ROCK1 knockout mice exhibited reduced myocardial fibrosis in transverse aortic-banded animals and ANG II-induced hypertension, without effects on cardiac hypertrophy (29, 42).

Rho kinase exists as two isoforms, ROCK1 and ROCK2. It may be that, as alluded to by Zhang et al. (42), ROCK1 plays an important role in the development of pathological fibrosis, and ROCK2 is involved in the development of hypertrophy. Furthermore, the effects of Rho kinase inhibition on cardiac hypertrophy may depend on the degree of hypertrophy developed in a particular animal model. Attenuation of cardiac hypertrophy with Rho kinase inhibition occurs in hypertensive animals that exhibit a >50% increase in hypertrophy compared with their control group (14, 19, 39). In contrast, the degree of hypertrophy was only 10% in a study that did not show a reduction with Rho kinase inhibition (29). In the present in vivo study, an increase in hypertrophy was observed, which was significantly reduced with GSK treatment. Specifically, there was a significant dose-dependent reduction in LV mass on echocardiography as well as a nonsignificant reduction in the heart rate-to-body weight ratio at high doses of GSK-576371. Furthermore, our in vitro study demonstrated that GSK-576371 significantly inhibited ANG II-induced neonatal rat cardiomyocyte hypertrophy.

Rho Kinase Inhibitor Specificity

The agent used in the present study, GSK-576371, nonselectively inhibits both ROCK1 and ROCK2. GSK-576371 is >50–1,000 fold more selective for ROCK than other serine/
threonine kinases, and it inhibits Rho kinase in the low nanomolar range (personal communication, Robert J Willette, GlaxoSmithKline, King of Prussia, PA). In comparison, other Rho kinase inhibitors (Fasudil and Y-27632) are effective in the micromolar range (33).

In summary, Rho kinase inhibition with GSK-576371 improved LV geometry and reduced collagen deposition in a rat POH model of diastolic dysfunction. Improvements in LV geometry and reduction of myocardial fibrosis may be important mechanisms that underlie the beneficial effects of this agent on diastolic function. Based on the above considerations, Rho kinase inhibition may represent a novel therapeutic approach to the treatment of diastolic dysfunction and accompanying HFPSF.

ACKNOWLEDGMENTS
We thank GlaxoSmithKline for kindly providing the drug GSK 576371.

GRANTS
We acknowledge support from the National Health and Medical Research Council Program Grants 334008 and 11079 and the Heart Foundation of Australia (G 06M 2644).

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