Abstract

p38 mitogen-activated protein kinase (p38 MAPK) inhibition exerts beneficial effects on left ventricular (LV) remodeling and dysfunction. p38 MAPK activity is transiently increased soon after myocardial infarction (MI), suggesting brief inhibition may afford the same benefit as long-term inhibition. We examined chronic 12-week p38 MAPK inhibition compared with short-term (7-day) inhibition, and then we discontinued inhibition after MI. Post-MI rats at day 7 received either vehicle, 4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazo[2,1-b]imidazol-2-yl]-3-butyln-1-ol (RWJ67657; RWJ) for 12 weeks (long term; LT-RWJ), RWJ for 1 week and discontinued for 11 weeks (1-week RWJ), or continuous ramipril for 12 weeks. In separate groups of animals, 24 h after MI, vehicle or RWJ was administered for 7 days. Cardiac function was assessed by echocardiography and hemodynamic measurements. Percentage of fractional shortening improved after LT-RWJ and ramipril, but not after 1-week RWJ treatment. Likewise, LV contractility and maximal first derivative of left ventricular pressure (dp/dtmax) was improved (12.5 and 14.4%) and LV end diastolic pressure (LVEDP) was reduced (49.4 and 54.6%) with both treatments. Functional outcomes were accompanied by regression of interstitial collagen I and α-smooth muscle actin expression in LV noninfarct, border, and infarct regions with LT-RWJ and ramipril treatment. Hypertrophy was reduced in noninfarct (18.3 and 12.2%) and border regions (16.3 and 12.0%) with both treatments, respectively. Animals receiving RWJ 24 h after MI for 7 days showed similar improvements in fractional shortening, dp/dtmax, LVEDP, including reduced fibrosis and hypertrophy. In vitro experiments confirmed a dose-dependent reduction in hypertrophy, with RWJ following tumor necrosis factor-α stimulation. Continuous but not short-term p38 MAPK blockade attenuates post-MI remodeling, which is associated with functional benefits on the myocardium.

Myocardial infarction (MI) results in progressive structural changes to the myocardium that progress to left ventricular (LV) dysfunction. As a compensatory measure to the decrease in cardiac output following MI, angiotensin II (AngII) and norepinephrine are activated to maintain cardiac function. However, long-term activation promotes vasoconstriction and pathological cardiac remodeling, leading to cardiac dysfunction. In addition, the release of proinflammatory cytokines contributes to the pathological process. Post-MI remodeling is characterized by LV dilatation, necrosis, and thinning of the LV wall, infarct expansion, hypertrophy of myocytes in the viable myocardium, and an increased accumulation of extracellular matrix material (fibrosis) within the infarcted and noninfarcted heart, impeding ventricular function (Tzanidis et al., 2001; Remme, 2003; See et al., 2004).

Evidence indicates that p38 mitogen-activated protein kinase (p38 MAPK) may be a common intracellular signaling pathway involved in cardiac remodeling, activated by...
various extracellular stimuli. Myocardial infarction (Shimizu et al., 1998), pressure overload (Wang et al., 1998; Fischer et al., 2001), and ischemia/reperfusion (Ma et al., 1999; Gao et al., 2002) are known to increase p38 MAPK activity within the heart. Furthermore, myocardial inflammation as evidenced by polymorphonuclear leukocyte accumulation in ischemia/reperfusion tissue is markedly attenuated by prior p38 MAPK inhibition (Gao et al., 2002). Cell-based studies have reported that neurohormonal factors AngII, endothelin-1, and phenylephrine (Clerk et al., 1998; van Eickels et al., 1999), as well as ischemia (Mackay and Mochly-Rosen, 1999), are known to increase p38 MAPK activity. Furthermore, in myocytes transfected with mitogen-activated protein kinase kinase (MKK) 6, an upstream mediator of p38 MAPK, inflammatory markers are increased in response to cytokine stimulation, an effect that is attenuated by p38 MAPK inhibition (Degousee et al., 2003). Likewise, overexpression of MKK3bE and MKK6bE in cardiac myocytes results in the development of cellular hypertrophy and apoptosis (Wang et al., 1998).

Our group has reported that 3-week treatment with the p38 MAPK inhibitor RWJ67657 (RWJ) in a rat post-MI model improved ventricular function and hemodynamic parameters in comparison with control animals (See et al., 2004). At the protein level, there was a reduction in collagen deposition and α-smooth muscle actin expression. In vitro studies using cardiac fibroblasts also showed that this compound produced a dose-dependent reduction in transforming growth factor-β-induced collagen synthesis (See et al., 2004).

p38 MAPK activity is increased early after MI within the myocardium, followed by a rapid return to baseline (Shimizu et al., 1998). Therefore, a brief period of p38 MAPK inhibition after MI may be sufficient to produce beneficial long-term effects on cardiac function and remodeling as observed previously (See et al., 2004). Hence, the aim of the current study was to examine the effects of 1-week discontinued p38 MAPK inhibition and to compare it with chronic long-term inhibition in an MI animal model, with treatment commencing 1 week after MI to allow for reparative processes at the site of infarction. Interruption of these processes involving cytokines and growth factors, through inhibition of the p38 MAPK pathway, may compromise the repair process and exacerbate cardiac remodeling. Furthermore, to clarify roles of rapid activation of p38 MAPK in response to MI, and to determine the safety of early intervention after MI, we administered RWJ 24 h after MI for 7 days, and then we examined functional and structural outcomes.

**Materials and Methods**

**Experimental Protocol**

Animal studies were performed in accordance with the Declaration of Helsinki and the *Guide for the Care and Use of Laboratory Animals* as adopted by the National Institutes of Health. Ligation of the left anterior descending coronary artery in outbred Sprague-Dawley rats (180–220 g) was performed as described previously (Kompa and Summers, 2000).

**Thirteen-Week Study.** Animals underwent surgery on day 1 to induce MI. On day 7, baseline echocardiographic images were obtained. Treatment in each group was then commenced to allow for the establishment of reparative fibrosis in the infarct zone within this time. Animals were randomized into the following treatment groups: 1) sham group; 2) MI group receiving vehicle (MI + Veh); 3) MI group receiving the p38 MAPK inhibitor RWJ (MI + LT-RWJ at 50 mg/kg/day) for 12 weeks; 4) MI group receiving the same dose of RWJ for 1 week and then vehicle for the remainder of the treatment period (MI + 1-week RWJ); and 5) MI group receiving ramipril (Ram), the standard angiotensin-converting enzyme (ACE) inhibitor therapy for heart failure (MI + Ram at 1 mg/kg/day), for 12 weeks as a positive control (Fig. 1A). Animals received daily treatment by oral gavage, and vehicle consisted of 0.5% methylcellulose. After the treatment period, a second series of echocardiographic images were obtained, and hemodynamic measurements were made before sacrifice and tissue collection.

**Seven-Day Study.** Separate groups of animals were studied to examine differences after 1-week MI (sham, MI + Veh, and MI + RWJ). MI was induced on day 0, and 24 h post-MI echocardiographic images were obtained to determine baseline cardiac function. Treatment in each group (vehicle or RWJ at 50 mg/kg/day) commenced immediately after echo measurement, and it continued daily until day 7 (Fig. 1B). A second series of echocardiographic images were obtained before hemodynamic measurements and tissue collection.

**Echocardiography and Hemodynamic Analysis**

Transathoracic echocardiography was used to obtain two-dimensional and m-mode images at the midpapillary muscle level using an HP Sonos 5500 with a 12-MHz probe (Aglient Technologies, Palo Alto, CA). LV internal diameters in systole (LVIDs) and diastole (LVIDd) were measured offline, and the percentage of LV fractional shortening was determined (See et al., 2004).

After final echocardiography, hemodynamic measurements of mean arterial pressure (MAP), LV end-diastolic pressure (LVEDP), and the maximal first derivative of left ventricular pressure (dP/dtmax) were obtained using a pressure transducer (model 1050; UFI, Morro Bay, CA) connected to a MacLab system (ADInstruments, Castle Hill, NSW, Australia) (Tranidis et al., 2001; See et al., 2004). On completion of these measurements, hearts were collected and processed for histology and molecular analyses.
Histology and Immunohistochemistry

Sections were stained with hematoxylin and eosin to determine infarct size and myocyte cross-sectional area. Infarct size was calculated from images obtained under a low-magnification microscope, and infarct size was expressed as an averaged percentage of the endocardial and epicardial scarred circumferences of the left ventricle using image analysis software Image-Pro Plus, version 5.5 (Media Cybernetics, Silver Spring, MD) (Tzanidis et al., 2001). For myocyte cross-sectional area, images were photographed at 400× magnification in the same plane as assessed by similar sized nuclei. Cells were outlined, and the area was measured using Image-Pro Plus (See et al., 2004).

Antibody staining of LV tissue sections for collagen I (Southern Biotechnology Associates, Birmingham, AL), collagen III (BioGenex, San Ramon, CA), and α-smooth muscle actin (Sigma-Aldrich, St. Louis, MO) was determined using a three-layer immunoperoxidase technique as described previously (See et al., 2004).

Myocardial Hydroxyproline Content

Lyophilized tissues from infarcted and noninfarcted LV were hydrolyzed with 6 M HCl at 100°C overnight, and then an aliquot of the supernatant (250 μl) was evaporated to dryness. Samples were stored at −20°C until assayed. Standards of hydroxyproline (Sigma-Aldrich) were prepared in 50% isopropanol commencing at a concentration of 5 mg/ml with serial half dilutions. Samples were reconstituted in 50% isopropanol, and duplicate aliquots in borosilicate glass tubes were prepared for assay. To each tube, 300 μl of acetate-citrate buffer, pH 6.0 (0.7 M sodium acetate, 0.2 M trisodium citrate, and 45 mM citric acid), in isopropanol (3 parts buffer to 2 parts isopropanol) and 100 μl of 0.3 M chloramine T (Sigma-Aldrich) were added. The contents were mixed and allowed to stand for 5 min. Then, 1 ml of Erlich’s reagent was added, and samples were incubated at 60°C for 30 min. Samples were allowed to cool to room temperature, and then optical density was read at 558 nm and unknown samples determined from the standard curve.

Western Blot Detection of Proteins

Myocardial tissue protein extracts (20 μg) from infarct and noninfarct regions were resolved by 12% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Glasgow, NSW, Australia), and then they were transferred onto polyvinylidene fluoride membranes (GE Healthcare, Chalfont St. Giles, UK) and incubated with a monoclonal anti-α-smooth muscle actin antibody to detect myofibrillar (1:4000; Sigma-Aldrich) as described previously (Tzanidis et al., 2001). Separate blots transferred from 5-μg gel loadings of LV noninfarct tissue from the 7-day study were incubated with a rabbit anti-mouse polyclonal heat shock protein (HSP) 25 antibody (1:5000; Assay Designs, Ann Arbor, MI). HSP25/27 is known to be activated downstream of p38 MAPK and is a substrate for mitogen-activated protein kinase-activated protein kinase (MAPKAPK) 2.

Measurement of TNF-α Plasma Levels

Before sacrifice, blood was collected from animals in EDTA tubes from the tail vein, and it was centrifuged to obtain plasma. TNF-α levels were measured in the plasma using commercially available Bio-Plex kits (Bio-Rad). In brief, plasma was diluted 1:3 with Bio-Plex sample diluent, and then it was centrifuged for 5 min at 15,000 rpm; 50 μl of the diluted plasma was used per well in a 96-well plate. Samples and standards were performed in duplicate, and the remaining procedures followed the protocol from the manufacturer (Bio-Rad).

Neonatal Rat Cardiac Myocytes Isolation and Myocyte Hypertrophy

Neonatal Sprague-Dawley rat cardiac myocytes were isolated from 1-day-old pups with enzymatic digestion as described in detail previously (Thomas et al., 2002; Woodcock et al., 2002). Purified neonatal cardiac myocytes were seeded at high density (1000 cells/mm²) in six-well plates and maintained in serum-free Dulbecco modified Eagle’s minimum media (Invitrogen, Grand Island, NY) supplemented with 10 μg/ml insulin and 10 μg/ml transferrin as described previously (Woodcock et al., 2002). Bromodeoxyuridine (0.1 mM; Sigma-Aldrich) was included for the first 3 days. KCl (50 mM) was added to the medium to prevent spontaneous contraction characteristic of the plated neonatal cardiomyocytes (Thomas et al., 2002). Four hours after treatment with RWJ (10−5–10−7 M), 10 ng/ml TNF-α (R&D Systems, Minneapolis, MN) and 10−7 M AngII (Auspep, Parkville, VIC, Australia) was added to initiate hypertrophy. After 48 h (TNF-α) and 60 h (AngII) of stimulation, cells were harvested and hypertrophy was defined as a significant increase in protein content (measured by Bradford assay; Bio-Rad) in the absence of any significant change in DNA content. Likewise, neonatal cardiac myocytes stimulated with TNF-α in the presence and absence of increasing RWJ (10−7–10−5 M) were harvested for protein and Western blot analysis of HSP25 as described above.

Statistical Analysis

Results are expressed as mean ± S.E.M. Differences between groups were analyzed using a nonparametric one-way analysis of variance with Kruskal-Wallis between-group comparisons. In vitro data were analyzed using the Newman-Keuls method for pairwise comparisons. A value of P < 0.05 was considered statistically significant.

Results

Mortality Rates. A 27.3% mortality rate was observed in the 13-week study and a 22.2% mortality rate in the 7-day study. All mortalities occurred within the first 24 h after MI, that is, before the commencement of treatment.

Echocardiography Data. Baseline echocardiograms demonstrated comparable degrees of ventricular dilatation as evidenced by a significant reduction in the percentage of fractional shortening in each MI group (Table 1).

In the 13-week study, there was no change in absolute fractional shortening from baseline in the sham group; however, a further 5% reduction was observed in MI vehicle-treated animals. Long-term RWJ and ramipril treatment significantly improved fractional shortening compared with vehicle-treated animals (P < 0.05) as observed by a 2.9 and 2.8% respective increase from baseline measurements. Short-term discontinued RWJ treatment after MI resulted in a 3.3% reduction in fractional shortening (Fig. 2A).

In the 7-day study, both MI groups demonstrated a 28 and 24% reduction (P < 0.05) in fractional shortening at baseline compared with sham animals (Table 1). After 7 days, a further 3.8% reduction in fractional shortening in the vehicle-treated group was observed, compared with a significantly lesser 2.6% reduction (P < 0.05) in the RWJ group (Fig. 2B).

Hemodynamic Measurements. The 13-week study demonstrated a significant 15.4% decrease in the rate LV contractility (dP/dtmax) and a 164% increase in LVEDP in the MI vehicle-treated group compared with sham-operated animals (Table 2). Long-term RWJ and ramipril treatment attenuated the rise in LVEDP (RWJ, 80.9%; Ram, 87.6%), and these treatments prevented the reduction in dP/dtmax (RWJ, 67.2%; Ram, 76.9%) in the MI vehicle-treated group. However, a further 9.6% with Ram treatment. Neither
null significantly attenuated the reduction in $dP/dt_{\text{max}}$ (36.3%) and $\text{mALS}$ compared with sham group. Treatment with RWJ nonsignificantly reduced LVEDP (61%). MAP was significantly increased in all MI groups compared with sham animals with a 62% increase in the vehicle group. Long-term treatment with RWJ attenuated this increase (65%) and a similar effect was observed with Ram (43%; $P = 0.056$) (Table 3). Congestive heart failure in animals, defined as a $>1.5$-fold increase of the mean lung weight-to-body weight ratio of sham animals (Liao et al., 2002), was reduced after treatment with long-term RWJ or ramipril (Table 3). Infarct size between all MI groups was closely matched (Table 3).

In the 7-day study, heart weight-to-body weight and lung weight-to-body weight ratios were increased in both MI-vehicle and MI-RWJ-treated animals (Table 3). Congestive heart failure as defined above was not observed at this time point. Infarct size between both MI groups was similar (Table 3).

**Myocyte Hypertrophy.** Myocyte cross-sectional area in the noninfarct zone and border zone was increased in vehicle-treated animals by $1.57 \pm 0.07$- and $3.2 \pm 0.09$-fold, respectively, compared with sham animals in the 13-week study. Long-term RWJ and Ram treatment significantly reduced this hypertrophy in both the noninfarct zone (RWJ, $1.28 \pm 0.05$; Ram, $1.38 \pm 0.02$; $P < 0.05$) and border zone (RWJ, $2.72 \pm 0.08$; Ram, $2.87 \pm 0.05$; $P < 0.05$). One-week discontinued RWJ treatment did not have any effect on myocyte hypertrophy in any region of the LV compared with the vehicle-treated group.

In the 7-day study, noninfarct zone and border zone myocyte cross-sectional areas were increased in vehicle-treated animals by $1.21 \pm 0.06$- and $1.95 \pm 0.08$-fold, respectively, compared with sham animals. RWJ treatment significantly reduced the hypertrophy in the border zone ($1.60 \pm 0.06$; $P < 0.05$).

In vitro studies in rat neonatal cardiac myocytes stimulated with $10 \text{ng/ml TNF-}\alpha$ and $100 \text{nM AngII}$ demonstrated an increase in protein content without change in DNA. RWJ (10 $\mu$M) reduced the increase in hypertrophy following AngII and TNF-\alpha stimulation, with a dose-dependent decrease observed with TNF-\alpha (Fig. 3).

**Collagen Expression.** In the 13-week study, collagen I immunoreactivity accounted for 6% of the LV cross-sectional area in sham animals (Fig. 4). In all post-MI animals, three regions were measured: 1) noninfarct zone (region remote to the infarct), 2) border zone (region between the infarct and noninfarct zone), and 3) infarct zone. In vehicle-treated ani-

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**Table 1**

Baseline echocardiography measurements obtained before the commencement of treatment in the 13-week and 7-day studies

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>LVIDs</th>
<th>LVIDd</th>
<th>%FS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-Wk post-MI animals at 7 days after MI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>9</td>
<td>4.3 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>36.8 ± 1.5</td>
</tr>
<tr>
<td>MI + Veh</td>
<td>11</td>
<td>7.5 ± 0.2</td>
<td>8.9 ± 0.2</td>
<td>15.6 ± 1.3</td>
</tr>
<tr>
<td>MI + LT-RWJ</td>
<td>10</td>
<td>8.0 ± 0.2</td>
<td>9.0 ± 0.1</td>
<td>11.8 ± 1.2</td>
</tr>
<tr>
<td>MI + Ram</td>
<td>10</td>
<td>7.2 ± 0.2</td>
<td>8.6 ± 0.2</td>
<td>17.1 ± 1.5</td>
</tr>
<tr>
<td>MI + RWJ</td>
<td>9</td>
<td>7.6 ± 0.1</td>
<td>9.1 ± 0.2</td>
<td>15.5 ± 1.2</td>
</tr>
<tr>
<td>7-Day post-MI animals at 24 h after MI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>8</td>
<td>2.5 ± 0.1</td>
<td>5.4 ± 0.4</td>
<td>54.2 ± 1.6</td>
</tr>
<tr>
<td>MI + Veh</td>
<td>9</td>
<td>3.8 ± 0.3</td>
<td>6.3 ± 0.3</td>
<td>39.2 ± 1.7</td>
</tr>
<tr>
<td>MI + RWJ</td>
<td>9</td>
<td>3.6 ± 0.2</td>
<td>6.2 ± 0.6</td>
<td>41.3 ± 2.5</td>
</tr>
</tbody>
</table>

* %FS is percentage of fractional shortening calculated by the following formula: %FS = ($LVIDd - LVIDd)/LVIDd × 100.

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Fig. 2. Echocardiography data. Absolute change in percentage of fractional shortening from baseline (0 on y-axis), determined for each group in the 13-week study (A) and 7-day study (B). Values are expressed as mean ± S.E.M., $n = 8$ to 11/group. *, $P < 0.05$ compared with sham; #, $P < 0.05$ compared with MI + Veh-treated animals.

RWJ treatment group had any effect on MAP compared with the vehicle group, suggesting that RWJ may exert its improved functional effects independent of effects on blood pressure (Table 2).

In the 7-day study there was an 18.7% decrease in $dP/dt_{\text{max}}$ and a 157% increase in LVEDP in the MI vehicle-treated animals compared with sham group. Treatment with RWJ nonsignificantly attenuated the reduction in $dP/dt_{\text{max}}$ (36.3%) and significantly reduced LVEDP (61%). MAP was significantly increased 7 days after MI (9.9%); treatment with RWJ prevented this change (Table 2).

**Organ Weights and Infarct Size.** Heart weight-to-body weight ratio in the 13-week study was increased in MI vehi-

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In the 7-day study, noninfarct zone and border zone myocyte cross-sectional areas were increased in vehicle-treated animals by $1.57 \pm 0.07$- and $3.2 \pm 0.09$-fold, respectively, compared with sham animals in the 13-week study.

Long-term RWJ and Ram treatment significantly reduced this hypertrophy in both the noninfarct zone (RWJ, $1.28 \pm 0.05$; Ram, $1.38 \pm 0.02$; $P < 0.05$) and border zone (RWJ, $2.72 \pm 0.08$; Ram, $2.87 \pm 0.05$; $P < 0.05$). One-week discontinued RWJ treatment did not have any effect on myocyte hypertrophy in any region of the LV compared with the vehicle-treated group.

In the 7-day study, noninfarct zone and border zone myocyte cross-sectional areas were increased in vehicle-treated animals by $1.21 \pm 0.06$- and $1.95 \pm 0.08$-fold, respectively, compared with sham animals. RWJ treatment significantly reduced the hypertrophy in the border zone ($1.60 \pm 0.06$; $P < 0.05$).

In vitro studies in rat neonatal cardiac myocytes stimulated with $10 \text{ng/ml TNF-}\alpha$ and $100 \text{nM AngII}$ demonstrated an increase in protein content without change in DNA. RWJ (10 $\mu$M) reduced the increase in hypertrophy following AngII and TNF-\alpha stimulation, with a dose-dependent decrease observed with TNF-\alpha (Fig. 3).

**Collagen Expression.** In the 13-week study, collagen I immunoreactivity accounted for 6% of the LV cross-sectional area in sham animals (Fig. 4). In all post-MI animals, three regions were measured: 1) noninfarct zone (region remote to the infarct), 2) border zone (region between the infarct and noninfarct zone), and 3) infarct zone. In vehicle-treated ani-
mals, collagen I increased 1.5-fold in the noninfarct zone, 3.5-fold in the border zone, and 4.8-fold in the infarct zone (Figs. 4 and 5). Long-term RWJ and Ram treatment attenuated this increase in all regions of the LV (RWJ: noninfarct zone, 128%; border zone, 64%; infarct zone, 65%; and Ram: noninfarct zone, 109%; border zone, 62%; infarct zone, 58%; P < 0.05) (Figs. 4 and 5). One-week discontinued RWJ treatment did not have any effect on collagen I expression in any region of the LV compared with the vehicle-treated group (Figs. 4 and 5).

Collagen III immunoreactivity accounted for 7% of the LV cross-sectional area in sham animals. In vehicle-treated animals, a 1.3-fold nonsignificant increase in collagen III expression was observed in the noninfarct zone (P = 0.082); however, a significant 3.4-fold increase in the border zone and 3.5-fold increase in the infarct zone was observed. None of the treatments had any significant effect on collagen III expression in any region of the LV compared with the vehicle-treated group (Table 4).

The ratio of collagen I to collagen III in vehicle-treated animals was not significantly different from that of sham animals in all regions of the LV (Table 4). Long-term RWJ treatment reduced the ratio of collagen I to collagen III in all regions of the LV (noninfarct zone, 31%; border zone, 44%; infarct zone, 45%; P < 0.05) (Table 4). Ram and 1-week discontinued RWJ treatment did not have any effect on the ratio of collagen I to collagen III in any region of the LV compared with the vehicle-treated group (Table 4).

In the 7-day study, collagen expression was significantly increased (48%) in the infarct region as determined by hydroxyproline content; RWJ treatment attenuated this increase by 87% (P < 0.05). No difference in collagen expression was observed in the noninfarct region (Fig. 6).

α-Smooth Muscle Actin Expression. α-Smooth muscle actin immunoreactivity in the 13-week study accounted for 1.5% of the LV cross-sectional area in sham animals (Fig. 7). In vehicle-treated MI animals, α-smooth muscle actin increased 2.4-fold in the noninfarct zone, 8.4-fold in the border zone, and 22-fold in the infarct zone. Long-term RWJ and Ram treatment prevented this increased expression in the noninfarct zone (P < 0.05) (Fig. 7A) and significantly attenuated this increase in the border zone (RWJ, 42%; Ram, 28%; P < 0.05) (Fig. 7B) and infarct zone (RWJ, 49%; Ram, 18%; P < 0.05) (Fig. 7C). In contrast, 1-week discontinued RWJ treatment did not have any effect on α-smooth muscle actin accumulation in any region of the LV compared with the vehicle-treated group (Fig. 7).

α-Smooth muscle actin in the 7-day study was increased by 90 and 58% in the infarct (sham, 0.98 ± 0.17; MI + Veh, 1.87 ± 0.31; MI + RWJ, 1.37 ± 0.13) and noninfarct regions (sham, 0.98 ± 0.17; MI + Veh, 1.55 ± 0.14; MI + RWJ, 1.11 ± 0.19) following MI. RWJ treatment significantly attenuated this increase by 56 and 77%, respectively.
TNF-α Plasma Levels. Post-MI plasma samples at day 7, assayed for levels of TNF-α were increased 4-fold (sham, 23.4 ± 11.0 pg/ml; MI + Veh, 98.3 ± 21.3 pg/ml; P < 0.05). RWJ treatment attenuated plasma TNF-α levels by 53% (MI + RWJ, 45.9 ± 19.0 pg/ml; P = 0.07).

Heat Shock Protein Expression. HSP25 expression in the noninfarct region of the LV was significantly increased by 1.4-fold (P < 0.01) at 1 week post-MI, treatment with RWJ significantly attenuated this increase by 29% (P < 0.05) (Fig. 8A). In neonatal cardiac myocytes stimulated with TNF-α, there was a significant 21% increase in HSP25 expression. In the presence of RWJ, a dose-dependent reduction in HSP25 expression was observed at 10⁻⁵ M (P < 0.05) (Fig. 8B).

**Discussion**

The present study demonstrates that long-term daily treatment of rats with the p38 MAPK inhibitor RWJ67657 and the ACE inhibitor ramipril results in a reduction in cardiac fibrosis and hypertrophy accompanied by a beneficial outcome on hemodynamic parameters (LVEDP and dP/dt_max) and ventricular function (percentage of fractional shortening) after MI. In contrast, brief (1 week) inhibition with the p38 MAPK inhibitor after MI (followed by no inhibition for a further 11 weeks; MI + 1-week RWJ) seemed to have no ameliorating effect on the progressive ventricular remodeling process. Furthermore, in the 7-day study, p38 MAPK inhibition commenced 24 h after MI provided early cardioprotection in these animals without adverse remodeling. These data confirm the importance of p38 MAPK activation in the
post-MI setting, as well as the need to continually inhibit this enzyme long-term and maximize the beneficial effect of this approach.

We hypothesized that only a short (1 week) period of p38 MAPK inhibition may be required. However, the present study found that short discontinued p38 MAPK inhibition did not afford any protection on the remodeling process compared with vehicle-treated MI animals. Therefore, it seems that this brief, early p38 MAPK activation triggers downstream processes that continue beyond the observed period of p38 MAPK activation; alternatively, p38 MAPK may be reactivated after MI once RWJ therapy is ceased.

It is noteworthy that, as in our previous study (See et al., 2004) and other studies recently reported in mice and rats, following MI (Liu et al., 2005; Engel et al., 2006), the effects of p38 MAPK inhibition with long-term treatment seemed to be independent of any change in blood pressure that may reduce after-load and thus improve cardiac performance indirectly. In contrast, previous reports, including the current study, have shown that ACE inhibition causes a further reduction in peripheral blood pressure after MI reducing after-load, contributing to an improvement in cardiac function (Mulder et al., 1997; Liu et al., 2005).

The present study also explored specific structural changes that may underlie the beneficial effect of long-term p38 MAPK inhibition on ventricular remodeling. In particular, we demonstrated a favorable impact on cardiac fibrosis in the three different zones of the post-MI left ventricle (noninfarct, border, and infarct zones), with a reduction in both collagen and hydroxyproline expression. Collagen I presents as thick fibers in the myocardium giving rise to increased tensile strength, a contributing factor to diastolic stiffness, whereas collagen III forms a fine fibrillar network that is more elastic than type I collagen (Lijnen et al., 2000), and it may provide additional elasticity in the face of myocyte loss after MI. A resultant ratio in favor of type I collagen may explain the progressive myocardial stiffness and reduced cardiac function observed in patients with idiopathic dilated cardiomyopathy (Marijansonski et al., 1995; Pauschinger et al., 1999).

Hence, a reduced collagen I/III ratio following long-term RWJ treatment after MI would explain, at least in part, the improvement in systolic performance. Likewise, this modulation of collagen, in vivo, was also observed with the active comparator ramipril, an effect previously documented with other ACE inhibitors (Mulder et al., 1997; Liu et al., 2005).

The reduction in α-smooth muscle actin expression observed with continued RWJ and ramipril treatment suggests an inhibition or prevention in fibroblast transformation to myofibroblasts (Singer et al., 1984), the key cell type responsible for the generation of collagen. We have previously shown that neurohormonal and cytokine (See et al., 2004; Cantwell et al., 2005) stimulation of cardiac fibroblasts results in increased collagen synthesis; treatment with RWJ attenuates this increase in a dose-dependent manner (See et al., 2004). Previous long-term studies using p38 MAPK inhibitors in an MI model have described both beneficial (Liu et al., 2005) and nonbeneficial (Frantz et al., 2007) improvements in cardiac function and collagen expression. Both studies used different p38 MAPK inhibitors, which may explain the different outcomes.

Myocyte hypertrophy is another process that occurs in the post-MI remodeled left ventricle. The present study demonstrated a reduction in myocyte cross-sectional area with both continuous RWJ and ramipril groups, as well as a reduction in heart weight-to-body weight ratio and lung weight-to-body weight ratio, which may be contributory to and reflective of the beneficial overall effect on remodeling. Similar improvements have been reported in mice with p38 MAPK and ACE inhibition where myocyte cross-sectional area is reduced (Liu et al., 2005). Both long-term RWJ and ramipril-treated groups also had fewer animals develop congestive heart failure. In vitro studies confirmed direct dose-dependent antihypertrophic effects of RWJ on cardiac myocytes, in response to AngII and TNF-α stimulation.

Recent insight into the mechanisms underlying the beneficial effects of p38 MAPK inhibition suggest that TNF-α and interleukin (IL)-6 may be important downstream targets. In the stroke-prone spontaneously hypertensive rat model, fed a high-salt/high-fat diet, LV hypertrophy, interstitial fibrosis, and a deterioration of cardiac function are observed (Behr et al., 2001). Chronic inhibition of p38 MAPK in these animals reduced LV hypertrophy and preserved cardiac function. Furthermore, cytokine production of TNF-α was inhibited in blood from animals receiving the inhibitor (Behr et al., 2001). Transgenic animal models overexpressing the upstream mediators of p38 MAPK, in particular MKK3bE and MKK6bE, have demonstrated decreased cardiac function, phenotypic changes accompanied by marked interstitial fibrosis and myocyte hypertrophy, and increased plasma levels of TNF-α and IL-6; treatment with a p38 MAPK inhibitor attenuated these changes (Liao et al., 2001; Li et al., 2005). In the current study, we showed that TNF-α plasma levels are...
elevated after MI, and treatment with RWJ attenuated TNF-α by 50%.

In the myocardium from patients with ischemic heart disease, p38 MAPK activity is increased compared with nonfailing hearts (Cook et al., 1999). In healthy human subjects, p38 MAPK inhibition was shown to reduce both plasma levels of TNF-α and IL-6 (Fijen et al., 2001). Furthermore, TNF-α has been reported to stimulate p38 MAPK activation in cultured myocytes, suggesting a positive feedback loop between p38 MAPK and TNF-α (Li et al., 2005), which could exacerbate the pathological process of cardiac remodeling. This suggests that p38 MAPK may be a more broad-based alternative to TNF-α antagonism, which has proven unsuccessful in clinical trials of heart failure, perhaps because of the specificity of its actions (Chung et al., 2003; Mann et al., 2004).

The RWJ compound used in this study is primarily a p38 MAPK inhibitor similar to other well studied p38 MAPK inhibitors, such as SB203580, in that it has high potency and it is specifically selective for p38α and p38β but not p38γ and p38δ isoforms (Wadsworth et al., 1999; Fijen et al., 2001). Both compounds are structurally similar, sharing the same imidazole core and two functional groups; RWJ contains a different third functional group and an additional lipophilic side chain. Unlike SB203580, which has an IC50 value of 5 μM against tyrosine kinases p56lck and c-src, RWJ has no significant activity. RWJ and SB203580 exhibit similar potency in vivo at inhibiting TNF-α release; however, in vitro experiments show that RWJ is 10-fold more potent (Wadsworth et al., 1999). We have previously demonstrated that in myocytes overexpressing the angiotsin type 1 receptor, RWJ inhibited the increase in AngII-stimulated expression of phosphorylated p38 MAPK independently of total p38 MAPK, with no effect on phosphorylated or total extracellular signal-regulated kinase 1/2 expression (See et al., 2004).

Thus, the majority of the effects observed with RWJ can be attributed to specific p38 MAPK inhibition.

We further examined HSP25 expression in tissues from post-MI animals, and in vitro experiments were performed in TNF-α-stimulated cardiac myocytes demonstrating increased expression, which was attenuated with RWJ treatment. HSP25 is involved in signaling further downstream of p38 MAPK, being a selective substrate for MAPKAPK-2 (Vertii et al., 2006). HSPs are essential proteins involved in the formation and maintenance of the correct conformational folding of other proteins within the cell, after injury they facilitate in the restoration of normal cellular function. Previous studies have reported increased HSP27 and HSP72 expression after MI (Tanonaka et al., 2003). Our study is the first to demonstrate in cardiac tissue that HSP25 can be regulated both in vivo (after MI) and in vitro with p38 MAPK inhibition. It has been well described in the literature that they are cardioprotective in the setting of ischemic preconditioning (Sötö et al., 2005); hence, the results from this study are not entirely understood and they require further investigation. We can speculate that in the current study, MI was induced by permanent occlusion of the coronary artery; therefore, no prior preconditioning was undertaken and an increased HSP25 expression was observed. Intervention with RWJ may have reduced the amount of protein damage caused by the remodeling process and hence the amount of HSP25 expressed at the end of the treatment period. Likewise, myocytes were not preconditioned before stimulation with TNF-α or treatment with RWJ, and further studies need to be performed to confirm this explanation.

A limitation of the present study included a lack of tissue availability that prevented us from determining the inhibition of p38 MAPK activity within the same cardiac tissues.

### Table 4

Collagen III and collagen I/collagen III ratio determined from immunohistochemical staining with specific antibodies in the different zones of the left ventricle: noninfarct, border, and infarct zones

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Noninfarct Zone</th>
<th>Border Zone</th>
<th>Infarct Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>9</td>
<td>7.1 ± 0.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MI + Veh</td>
<td>11</td>
<td>9.2 ± 0.7</td>
<td>23.3 ± 1.3*</td>
<td>26.0 ± 2.2*</td>
</tr>
<tr>
<td>MI + LT-RWJ</td>
<td>10</td>
<td>7.7 ± 0.6</td>
<td>23.2 ± 1.1*</td>
<td>24.1 ± 2.1*</td>
</tr>
<tr>
<td>MI + 1-wk RWJ</td>
<td>9</td>
<td>7.8 ± 0.8</td>
<td>19.0 ± 1.3*</td>
<td>22.6 ± 1.8*</td>
</tr>
<tr>
<td>MI + Ram</td>
<td>10</td>
<td>7 ± 0.6</td>
<td>19.4 ± 1.6*</td>
<td>20.8 ± 2.3*</td>
</tr>
</tbody>
</table>

Collagen I/collagen III ratio

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Noninfarct Zone</th>
<th>Border Zone</th>
<th>Infarct Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>9</td>
<td>0.92 ± 0.07</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MI + Veh</td>
<td>11</td>
<td>1.00 ± 0.09</td>
<td>0.89 ± 0.09</td>
<td>1.11 ± 0.10</td>
</tr>
<tr>
<td>MI + LT-RWJ</td>
<td>10</td>
<td>0.68 ± 0.07*</td>
<td>0.50 ± 0.04*</td>
<td>0.61 ± 0.04*</td>
</tr>
<tr>
<td>MI + 1-wk RWJ</td>
<td>9</td>
<td>1.05 ± 0.16</td>
<td>1.15 ± 0.08</td>
<td>1.17 ± 0.21</td>
</tr>
<tr>
<td>MI + Ram</td>
<td>10</td>
<td>0.86 ± 0.09</td>
<td>0.70 ± 0.07</td>
<td>0.91 ± 0.15</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with sham animals.
#P < 0.05 compared with MI + Veh animals.

### Figure 6

Collagen hydroxyproline content in noninfarct (A) and infarct (B) zone at 7 days after MI. Values are expressed as mean ± S.E.M., n = 8 to 11/group. *, P < 0.05 compared with the sham animals. #, P < 0.05 compared with MI + Veh-treated animals.
Previous studies using RWJ have described inhibition of p38 MAPK activity as well as its downstream target MAPKAPK-2 in monocytes and macrophages (Wadsworth et al., 1999; Westra et al., 2004). In addition, as mentioned previously, we have shown inhibition of p38 MAPK phosphorylation following angiotensin II stimulation in cardiac myocytes with RWJ (See et al., 2004).

Nevertheless, despite the above-mentioned limitation, the present study demonstrates that long-term, but not short discontinued p38 MAPK inhibition exerts a beneficial effect on the progressive ventricular remodeling process that follows myocardial infarction without a reduction in blood pressure. These observations support the importance of p38 MAPK in ventricular remodeling and the therapeutic potential of inhibiting this kinase. Further studies will be required to elucidate the pathways involved in p38 MAPK cytokine activation, which from recent evidence seem to be implicated in cardiac remodeling.

Many current therapies indirectly target the treatment of heart failure by inhibiting neurohormonal pathways such as the sympathetic nervous system, the renin-angiotensin system. Inhibition of these pathways, although beneficial on the remodeling process, leads to a further reduction in blood pressure, often placing patients at hypotensive risk. The use of compounds such as the p38 MAPK inhibitors directly attenuate the remodeling process without further reduction in blood pressure; therefore, they could be used in conjunction with or as an alternative to blood pressure-lowering medication. Furthermore, the finding that early administration of the drug does not...
adversely affect the reparative processes that occur in the first few days following MI, but is rather beneficial to the remodeling process, suggests it may potentially be administered early after MI for maximal cardioprotection.

The use of p38 MAPK inhibitors have been studied in humans for several inflammatory conditions, including rheumatoid arthritis (Brown et al., 2004) and asthma (Borchardt, 2004). However, their use has been limited by liver abnormalities, hindering their clinical development. Therefore, there is great interest in additionally identifying targets upstream or downstream of p38 MAPK for these indications, including ventricular remodeling.

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