

Inhibition of Collagen Synthesis With Prolyl 4-Hydroxylase Inhibitor Improves Left Ventricular Function and Alters the Pattern of Left Ventricular Dilatation After Myocardial Infarction

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Background—Left ventricular (LV) remodeling after myocardial infarction (MI) is associated with fibrosis, dilatation, and dysfunction. We postulated that prevention of fibrosis after MI with a prolyl 4-hydroxylase inhibitor (P4HI) would preserve LV function and attenuate LV enlargement.

Methods and Results—Adult female rats (200 to 250 g) had experimental MI and were then randomized to treatment with P4HI (MI-FG041, n=29) or vehicle (MI-control, n=29) 48 hours after MI for 4 weeks in 2 phases. Echocardiograms were performed weekly with a 15-MHz linear transducer, and at 4 weeks, collagen isoform determinations and in vivo hemodynamics were performed. At randomization, the infarct size and LV function and size were similar in MI-FG041 and MI-control but significantly different from shams (n=9). At week 4, the LV function in MI-FG041 was significantly better than in MI-controls (fractional shortening 21% versus 16%, $P=0.01$; fractional area change 30% versus 19%, $P=0.002$; ejection fraction 35% versus 23%, $P=0.001$). In the FG041 group, LV area in systole was less ($P<0.05$), the dP/dt_{max} after isoproterenol was higher ($P<0.05$), and types I and III collagen in noninfarcted LV were less than in MI-control. The hydroxyproline/proline ratio was increased by 64% in MI-control and reduced to the sham value in MI-FG041 rats. In the scar tissue, it was reduced by 24% in MI-FG041.

Conclusions—This study demonstrates that prevention of interstitial fibrosis with a P4H inhibitor alters the pattern of LV enlargement and produces partial recovery of LV function after MI. (*Circulation*. 2001;104:2216-2221.)

Key Words: myocardial infarction ■ collagen ■ remodeling

Left ventricular (LV) remodeling after myocardial infarction (MI) involves disruption of supporting structures, cell slippage, myocyte hypertrophy, and collagen deposition both at the site of infarction and at areas remote from the infarct. This process is adaptive initially but may progress to LV dilatation and dysfunction.¹ Interstitial fibrosis of the viable myocardium is believed to be important in ischemic cardiomyopathy² and may contribute to LV dysfunction after MI in part by causing morphological and functional separation of the myocytes.^{3,4} Interruption of fibrosis of the viable myocardium may produce favorable effects on cardiac function and arrhythmogenesis.⁵

Conversely, it has been suggested that disruption of extracellular matrix collagen may be a primary mechanism for LV dilation,⁶ and inadequate or disrupted fibrillar collagen at the site of infarction may lead to myocardial rupture.⁷ Of note, however, are studies using ACE inhibitors and angiotensin receptor blockers that showed thinning of the infarcted segment, reduction of fibrosis in the noninfarcted myocardi-

um, attenuation of LV cavity dilatation, and improvement in LV function.⁸⁻¹¹ These varied experimental findings suggest the complexity of LV remodeling.

It appears that prevention of fibrosis in the noninfarcted myocardium is feasible, thus allowing an assessment of whether the benefit of preventing fibrosis outweighs the potential adverse effects of collagen scar reduction. Prolyl 4-hydroxylase (P4H) is an essential enzyme in collagen biosynthesis. It catalyzes the hydroxylation of specific proline residues on alpha monomers, resulting in the production of thermally stable triple helical procollagen molecules and their subsequent secretion into the extracellular matrix. Because the final common pathway for collagen formation involves the activity of P4H, inhibition of this enzyme after MI would be expected to prevent interstitial fibrosis.

FG041 is an orally available P4H inhibitor. To test the hypothesis that a P4H inhibitor could prevent cardiac fibrosis and produce favorable effects on LV function and structure after MI, we administered FG041 to rats for 4 weeks

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TABLE 1. Changes in LV FS, LVESD, and LVEDD Over Time

Week	FS, %			LVESD, mm			LVEDD, mm		
	MI-FG041	MI-Control	Sham	MI-FG041	MI-Control	Sham	MI-FG041	MI-Control	Sham
0	10±0.8	12±1	34±3†	6.9±0.1	6.7±0.2	4.3±0.3†	7.7±0.2	7.5±0.1	6.7±0.2†
1	17±1*	13±1	33±3†	6.8±0.2*	7.6±0.2	4.4±0.3†	8.2±0.2*	8.8±0.1	6.5±0.2†
2	20±2*	15±2	33±2†	6.9±0.3*	7.4±0.4	4.5±0.2†	8.5±0.2	8.6±0.3	6.9±0.2†
3	21±2*	12±1	35±2†	6.8±0.4*	7.5±0.3	4.5±0.2†	8.5±0.3	8.6±0.2	6.9±0.2†
4	21±3	16±2	36±1†	7.0±0.4	7.4±0.4	4.4±0.2†	8.7±0.3	8.7±0.3	7.1±0.2†

Values are mean±SEM.

* $P<0.05$ vs MI-Control; † $P<0.001$ vs MI-Control and MI-FG041.

beginning 48 hours after experimental MI and evaluated collagen deposition as well as structural and functional changes in the LV.

Methods

All studies described in this article were performed according to the guidelines of the American Physiological Society and were approved by the Institutional Animal Care Committee of the University of Illinois at Chicago.

Experimental Protocol

One hundred thirty-five female Wistar rats weighing 200 to 250 g at the onset underwent MI, and 9 rats (shams) underwent sham operation. Of the 135 rats, 74 survived after surgery, and at 48 hours after MI, 2D echocardiography (2DE) was performed. All animals with fractional shortening (FS) <20% and regional wall motion abnormalities (58 rats) were randomized to treatment with FG041 (MI-FG041, $n=29$) or vehicle (MI-control, $n=29$), and the rest were excluded from study. Shams were also randomized to FG041 ($n=4$) or vehicle ($n=5$). The study was conducted in 2 phases. In phase 1, 14 of 29 MI-FG041 and 12 of 29 MI-control matched rats underwent weekly echocardiography, and at week 4, in vivo LV function and levels of hydroxyproline (Hyp) and proline were determined. In phase 2, 15 of 29 MI-FG041 and 17 of 29 MI-control matched rats underwent weekly 2DE to assess LV area, systolic function, and mitral E-wave velocity, defined as the early mitral filling wave. In each case, treatment was started immediately after randomization and continued for 4 weeks.

Production of MI

An anterior thoracotomy was performed on anesthetized and intubated rats, and the hearts were exteriorized. The left coronary artery was then ligated proximally with a 6-0 silk suture, and the chest wall was closed in layers as previously described.¹² Shams underwent identical surgery without ligation of the coronary artery.

Echocardiography

Animals lightly anesthetized with ether were placed in a supine or left lateral position with ECG electrodes applied at the paws. A 15-MHz linear transducer and the Acuson Sequoia System (C256) were used to obtain 2DE images. In phase 1, 2DE short-axis images at the mid papillary muscle level were obtained for measurement of LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), FS, and wall thickness.

In phase 2, short-axis images, apical 4-chamber views, and mitral E-wave velocity were obtained. FS, fractional area change (FAC), LV ejection fraction (LVEF), LV area in diastole (LVAD), LV area in systole (LVAS), and infarct size were determined. For infarct size, short-axis digital images were slowed, and the cursor was placed at the dyskinetic points. The images were then frozen at end diastole and traced. The infarcted segment was expressed as a percentage of the LV area. Measurements were made offline by 2 observers blinded to treatment according to the American Society for Echocardiography leading-edge method.

FG041 Treatment

FG041 was administered by oral gavage twice daily at a dose of 50 mg/kg beginning 48 hours after production of MI and was continued for 4 weeks. This approach was taken to allow for all surgical mortality and to achieve maximal collagen inhibition while at the same time limiting possible adverse effects on wound healing. Serum levels of FG041 were measured.

Hemodynamic Measurements

After 4 weeks of treatment, animals were anesthetized and ventilated. The right carotid artery was cannulated with a Millar ultra-miniature pressure transducer (SPR-671). The catheter was then advanced into the LV. After steady state had been established, baseline heart rate, developed pressure, contractile index, systolic and end-diastolic pressures (LVEDP), and maximal rates of pressure rise and fall ($\pm dP/dt$) were recorded. Hemodynamic measurements were repeated after a bolus infusion of isoproterenol (0.2 mL of 10^{-7} mol/L solution over 1 minute) via the femoral vein.

Collagen Analysis

Hearts were excised, weighed, and sliced into 3 transverse sections from the apex to the base. In selected hearts, transverse sections were saved for subsequent histological staining. Otherwise, the scarred tissue was grossly dissected out from the noninfarcted tissue, because the scar was well formed. All the scarred tissue, a section of the right ventricle opposite the septum, and the LV opposite the infarcted region were collected in each of the animals, weighed, and used for Hyp assay. Hyp and proline were determined by the method of Palmerini et al,¹³ except that L-azetidine-2-carboxylic acid (Aldrich) was substituted for 3,4-dehydroproline as the internal standard.

Western blotting was performed by standard methods. Collagen isoforms were detected by use of primary antibodies against types I and III collagen (Santa Cruz). The bound antibodies were detected by chemiluminescence (Amersham), and the immunoblots were exposed to Hyperfilm (Kodak) for 5 to 30 seconds.

Histological Analysis

The noninfarcted portion of the LV was dissected free of the scar and was frozen in liquid nitrogen. Subsequently, 5- μ m cryostat sections were cut from the basal, mid, and apical regions of the heart. The sections were separately stained with Masson's trichrome to distinguish areas that represented connective tissue. For image acquisition, slides containing the histological sections for hearts in the untreated and treated MI groups were scanned in color at a resolution of 72 dots per inch with a Nikon LS-3510 film scanner,¹⁴ resulting in consistent magnification from image to image. Images were enhanced with Adobe Photoshop 6.0. The areas for each section that suggested connective tissue staining were traced, and the total areas (pixels) were calculated for each section.

Statistical Methods

Values are given as mean±SEM except where indicated. Differences in Hyp, proline, and echocardiographic and hemodynamic param-

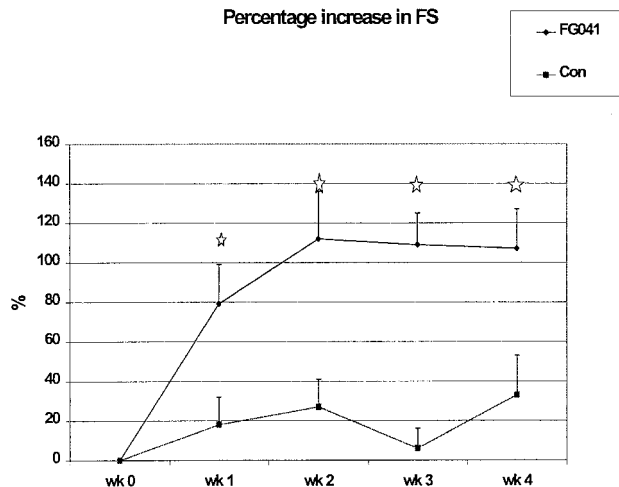


Figure 1. Percentage increase in LV FS over time. Values are mean \pm SEM. FG041 indicates MI-FG041; Con, MI-control. $\star P < 0.05$.

ters between MI-FG041, MI-control, and sham were initially compared by Levene's test for equality of variance followed by *t* test for equality of means with the SPSS statistical program. Values of $P < 0.05$ were considered significant.

Results

At the time of randomization, the MI-FG041 and MI-control animals had similar LV function, internal dimensions, and wall thickness. No differences were detected in the echocardiogram, hemodynamics, and Hyp content between FG041-treated shams and shams that received only the vehicle (sodium methyl cellulose); hence, shams were grouped together in the analysis. Within the period of the study, 7 of 29 MI-control animals (24%) died between days 7 and 28 of MI, and 3 of 29 MI-FG041-treated animals (10%) died between days 14 and 28 of MI. The cause of death was most likely related to progressive heart failure, because autopsy revealed congested lungs, pleural effusions, and markedly dilated right and left ventricles without gross evidence of LV rupture.

Echocardiographic Data

In phase 1 of this study, the baseline (value at time of randomization) FS, LVESD, and LVEDD of the MI-FG041 and MI-control animals were similar but significantly different from those of shams ($P < 0.001$, see Table 1). The FS of MI-FG041-treated animals increased from 10% at baseline to 21% at week 4 and was essentially unchanged in the MI-control and sham animals (see Table 1 and Figure 1).

The LVESD was significantly less in MI-FG041 animals at weeks 1 to 3 ($P < 0.05$, see Table 1) but was no longer significant at week 4, even though the LVESD remained unchanged. The LVEDD, conversely, was significantly less only at week 1 (8% versus 17%, $P = 0.008$, see Table 1). Distortion of the LV as a result of the infarction, however, may affect simple internal dimension measurements.

In phase 2, the FAC, LVEF, LVAS, LVAD, infarct size (52% versus 52%), and mitral E-wave peak velocity at randomization were similar in MI-FG041 and MI-control rats. The LVEF was significantly greater in the MI-FG041

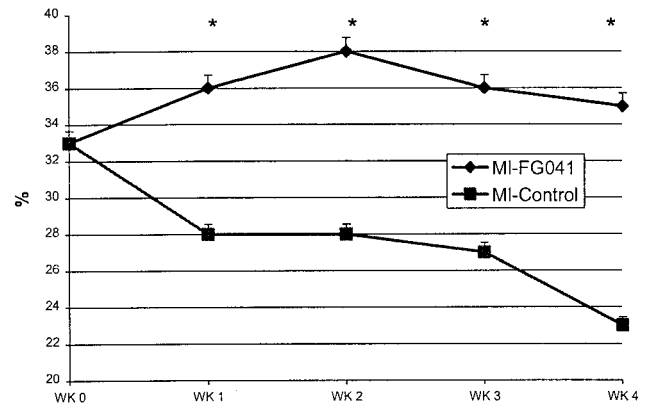


Figure 2. Change in ejection fraction with time. Values are mean \pm SEM. $\star P < 0.01$.

than MI-control at all time points ($P < 0.01$, Figure 2). Similarly, the FAC was significantly better in the MI-FG041 than MI-control ($P < 0.05$, Table 2).

At week 4, the LVAS, a more accurate assessment of LV size, was significantly less in the MI-FG041 animals than MI-control in both the short-axis and 4-chamber views (Table 2). Similarly, the LVAD was significantly less in MI-FG041 than MI-control in the short-axis view but was not statistically different in the 4-chamber view (Table 2). Both MI-FG041 and MI-control showed a restrictive filling pattern in the mitral inflow, with the peak E-wave velocity being similar. Little or no detectable A-wave velocity was present. Significant mitral regurgitation, however, was present in most of the MI animals.

Hemodynamic Data

The baseline (preisoproterenol) heart rate, developed pressure, contractile index, and systolic blood pressure were similar in sham, MI-control, and MI-FG041-treated animals. The $-dp/dt$, however, was significantly higher in the sham and MI-FG041-treated animals than in control animals ($P < 0.05$, see Table 3). After isoproterenol infusion, the $+dp/dt$ was significantly increased in the MI-FG041-treated animals compared with MI-control animals (16 830 versus 13 832 mm Hg/s, $P < 0.05$, Table 3). LVEDP (mm Hg) in the control MI group was 17 ± 6 before isoproterenol infusion and 16 ± 6 after. In contrast, in the MI-FG041 group, LVEDP was 9 ± 4 before and 6 ± 4 after ($P < 0.07$, MI-control versus MI-FG041).

Collagen Expression and Histology

There was a 64% increase in the Hyp-to-proline (Hyp/Pro) ratio of the noninfarcted LV in the MI-control relative to shams (see Table 4), whereas there was no difference between MI-FG041-treated animals and shams. This represents a relative 86% reduction ($P < 0.01$) in MI-FG041 relative to MI-control. In the right ventricle, there was a 25% increase in the Hyp/Pro content of the MI-control animals relative to shams, whereas that of MI-FG041-treated animals was the same as in the shams.

The weights of the scar tissue in the infarcted area of both MI-control and MI-FG041 animals were similar (93 versus

TABLE 2. Detailed Echo Data at Randomization and Week 4

Parameters	MI-FG041	MI-Control	P
Randomization			
LVAD (SA), mm ²	44±3	43±3	NS
LVAD (4C), mm ²	65±2	65±2	NS
LVAS (SA), mm ²	31±2	31±3	NS
LVAS (4C), mm ²	52±2	52±2	NS
FAC, %	29±1	30±1	NS
MV-E peak, m/s	0.80	0.86	NS
Week 4			
LVAD (SA), mm ²	74±5	87±4	0.044
LVAD (4C), mm ²	97±4	106±5	0.07
LVAS (SA), mm ²	53±4	71±4	0.007
LVAS (4C), mm ²	75±4	90±4	0.012
FAC, %	30±1	19±1	0.002
MV-E peak, m/s	1.1	1.1	0.3

SA indicates short axis view; 4C, 4-chamber view; and MV-E, mitral valve E wave. All data are mean±SEM.

91 mg, respectively). There was a significant, 24% reduction of the Hyp/Pro ratio, however, in the scars of the MI-FG041 animals compared with MI-control ($P<0.05$). Both types I and III collagen isoforms were significantly reduced in the noninfarcted portion of the LV compared with MI-control. A representative gel is shown in Figure 3. Representative histological sections of the hearts of animals with similar infarct size at randomization revealed less ventricular cavity dilatation and better myocardial structure preservation in the

TABLE 3. Hemodynamic Data

	MI-FG041	MI-Control	Sham
SBP, mm Hg			
Pre-Isop	143±7	142±3	144±5
Isop	130±9	123±7	197±3†
DP, mm Hg			
Pre-Isop	133±6	133±3	135±6
Isop	121±9	115±8	173±3†
HR, bpm			
Pre-Isop	352±22	379±20	362±15
Isop	399±13	431±26	416±20
+dP/dt, mm Hg/s			
Pre-Isop	9477±581	8642±209	9925±1194
Isop	16 830±1195*	13 832±1097	21 515±1074†
-dP/dt, mm Hg/s			
Pre-Isop	9978±827*	8009±426	11 578±622*
Isop	9234±703	8984±622	11 549±1074
CI			
Pre-Isop	123±3	115±7	123±4
Isop	207±4	186±17	250±7†

SBP indicates systolic blood pressure; DP, developed pressure (difference between SBP and LVEDP); HR, heart rate; ±dP/dt, rates of pressure rise and fall; CI, contractile index (dP/dt_{max}/pressure at that point); Isop, isoproterenol; and Pre-Isop, before isoproterenol infusion. Values are mean±SEM.

* $P<0.05$ vs control; † $P<0.01$ vs control and FG041.

TABLE 4. Hydroxyproline/Proline Content of the Heart

Samples	Hyp/Pro	Hyp, Wet Wt, %	Pro, Wet Wt, %
Normal sham	0.11±0.02	0.10±0.02	0.77±0.05
Normal MI-FG041	0.12±0.06	0.10±0.05	0.74±0.06
Normal MI-Control	0.18±0.05*	0.16±0.05*	0.79±0.05
RV sham	0.15±0.03	0.12±0.04	0.74±0.17
RV MI-FG041	0.13±0.02	0.12±0.02	0.77±0.08
RV MI-Control	0.17±0.03	0.15±0.04	0.80±0.12
Scar MI-FG041	0.34±0.08	0.39±0.09	1.01±0.06
Scar MI-Control	0.45±0.09†	0.53±0.15†	1.04±0.16

Normal indicates noninfarcted myocardium; RV, right ventricle; and scar, scarred myocardium. Values are mean±SD.

* $P<0.05$ vs sham and MI-FG041; † $P<0.05$ vs MI-FG041.

MI-FG041-treated animals (see Figure 4, A and B). In addition, a semiquantitative histological analysis of collagen content in MI-control and MI-FG041 animals with matched infarct size showed a marked reduction in collagen in the drug treatment group (Figure 4, C through E).

Drug Level

The mean peak plasma level of FG041 in MI-FG041 animals determined 2 hours after gavage was 7.9 µg/mL. The trough plasma level determined 8 hours after administration was 3.44 µg/mL. These levels were sufficient to inhibit P4H.

Discussion

This study demonstrates that prevention of interstitial fibrosis in the noninfarcted myocardium alters the pattern of LV enlargement and produces partial recovery of LV function after MI. The effect observed in this study is attributed to reduction of extracellular matrix collagen, because the P4H inhibitor had no afterload-reducing effect.

Collagen Formation After MI

Angiotensin II activation increases mRNA expression of type I and III collagen and synthesis of collagen after myocardial injury.^{15,16} FG041 treatment reduced collagen expression in the noninfarcted LV to the level found in shams but only by 24% at the site of the infarct. This is not unexpected, because diminished blood supply in the area of the infarct should restrict drug delivery. The result was a relatively normal healing process at the site of the infarct combined with prevention of fibrosis of the noninfarcted myocardium. The scar tissue was of similar weight in both groups, even though there was less Hyp in the MI-FG041 (Table 4). This suggests the presence of underhydroxylated collagen or a relative increase of another protein(s) in the scar tissue.

Because matrix metalloproteinases (MMPs), which degrade collagen, have been reported to increase 4.5-fold at day 2 after MI¹⁷ and collagen reportedly increases 3-fold in the first week in rats,¹⁸ it appears that a balance between collagen degradation and deposition must occur to prevent rupture of the ventricle. It is possible that FG041, by decreasing collagen deposition, may also have decreased activation of MMPs by indirect negative feedback, thus maintaining a balance between degradation and deposition

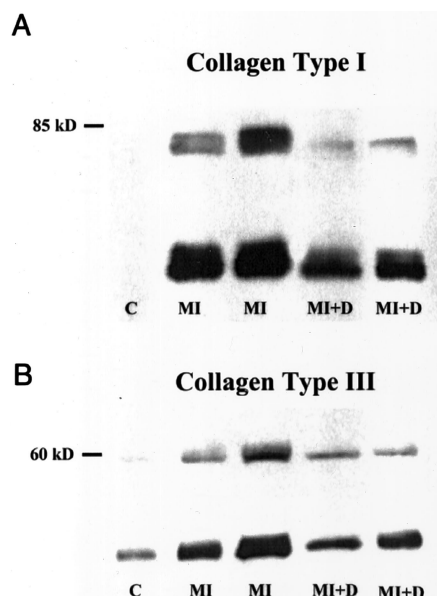


Figure 3. Collagen isoform expression at week 4 showing reduction in collagen isoforms I and III in MI-FG041 relative to MI-Control. C indicates noninfarcted control; MI, MI-control; and MI+D, MI-FG041. Each lane represents a separate animal.

of collagen. To establish this, however, requires direct measurement of MMP activity at the site of infarction in the presence of FG041.

Experimental MI in Rats

After MI in rats, systolic function declines and the LV cavity dilates significantly, and these changes progress over time. We noted improved systolic performance after P4H inhibition. This improvement was quite dramatic and was sustained from week 1 to week 4, suggesting that fibrosis in the noninfarcted myocardium after MI contributes to LV dysfunction.

The early increase in LV cavity dimension in MI-control animals in this study is consistent with previous studies.^{19,20} In contrast, the MI-FG041-treated animals had an altered pattern of LV dilatation with less progressive cavity dilatation. This finding should be contrasted with previously published work. Specifically, Zhao and others^{6,21} suggested that collagen matrix disruption is a primary factor involved in LV dilatation during ischemic cardiomyopathy, whereas Rohde et al²² showed that MMP inhibition, which preserved extracellular collagen, decreased early dilatation of the LV after MI in mice. In addition, a number of studies using ACE inhibitors and angiotensin receptor blockers have also shown reduction of fibrosis at the noninfarcted myocardium, attenuation of LV enlargement, and improved LV function.^{8–11} This underscores the complexity of LV remodeling after MI. In the present study, early LV enlargement was attenuated despite decreased Hyp; hence, prevention of fibrosis in the viable segments allowed for an overall favorable effect on LV function and geometry that counteracted any potential adverse effect of scar-tissue reduction.

Although the LV was not uniform in shape and we did not perform simultaneous echocardiography and hemodynamic

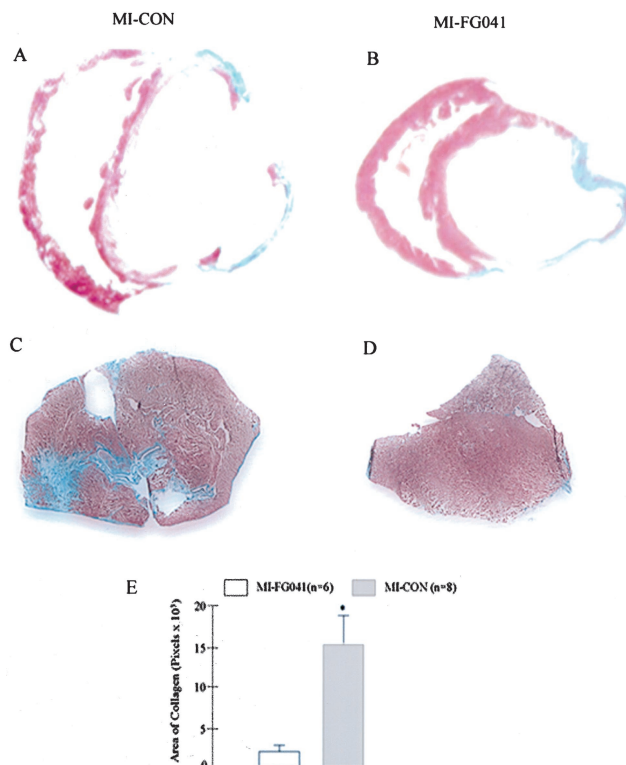


Figure 4. Qualitative and quantitative assessment of collagen deposition in heart. A and B, Cross sections obtained from 2 animals with matched infarct size demonstrating less chamber dilatation in noninfarcted segment of MI-FG041 animal relative to untreated MI-control (MI-CON). C and D, Representative trichrome-stained sections of noninfarcted muscle from MI-FG041 and MI-control. E, Semiquantitative estimate of collagen content in noninfarcted muscle from MI-control and MI-FG041. * $P < 0.01$ MI-CON vs MI-FG041. Sections were obtained from analogous portions of myocardium in animals that had similar infarct sizes at time of randomization (n=number of hearts analyzed).

measurements, the estimated LV end-systolic wall stress at the end of 4 weeks^{23,24} was 35% lower in the MI-FG041-treated animals than the MI-control animals. It is conceivable that the attenuation of LV cavity dimension reduced wall stress and improved LV function.

The systolic thickness of the noninfarcted myocardium was 2.3 mm at week 4 in MI-FG041 compared with 2.0 mm in MI-control, suggesting enhanced contractility in this segment. Enhanced contractility was also evident in the MI-FG041-treated animals after isoproterenol infusion. We postulate that this is a principal mechanism of improved LV function in this setting. By inhibition of fibrosis, myocyte hypertrophy may be unimpeded and there may be less myocyte separation. This could allow for more optimal myocyte performance. This can theoretically be tested by looking at mechanical performance in cardiocytes isolated from treated and untreated cardiac muscle.

Limitations of the Study

Only female rats were used in this study for logistic reasons, and the conclusions should be limited to this sex. Further studies are needed to determine and compare the efficacy of this agent in males, especially because estrogen and proges-

terone have been shown to inhibit cardiac fibroblast activity and collagen synthesis in the aorta.^{25,26} The findings apply to animals with large infarcts, and a larger study is needed to confirm the results in animals with smaller infarcts.

Conclusions

There appears to be potential value in the use of agents that inhibit collagen synthesis in the period immediately after MI. Although the drug effect is, in theory, systemic, the major impact appeared to be restricted to the noninfarcted myocardium. The findings in this study are preliminary, and further studies are needed to address mechanisms, establish safety issues, and define the target population.

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Inhibition of Collagen Synthesis With Prolyl 4-Hydroxylase Inhibitor Improves Left Ventricular Function and Alters the Pattern of Left Ventricular Dilatation After Myocardial Infarction

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