Folic acid mitigated cardiac dysfunction by normalizing the levels of tissue inhibitor of metalloproteinase and homocysteine-metabolizing enzymes postmyocardial infarction in mice

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Qipshidze N, Tyagi N, Sen U, Givvimani S, Metreveli N, Lominadze D, Tyagi SC. Folic acid mitigated cardiac dysfunction by normalizing the levels of tissue inhibitor of metalloproteinase and homocysteine-metabolizing enzymes postmyocardial infarction in mice. Am J Physiol Heart Circ Physiol 299: H1484–H1493, 2010. First published August 27, 2010; doi:10.1152/ajpheart.00577.2010.—Myocardial infarction (MI) results in significant metabolic derangement, causing accumulation of metabolic by product, such as homocysteine (Hcy). Hcy is a nonprotein amino acid generated during nucleic acid methylation and demethylation of methionine. Folic acid (FA) decreases Hcy levels by remethylation of Hcy to methionine, by 5-methylene tetrahydrofolate reductase (5-MTHFR). Although clinical trials were inconclusive regarding the role of Hcy in MI, in animal models, the levels of 5-MTHFR were decreased, and FA mitigated the MI injury. We hypothesized that FA mitigated MI-induced injury, in part, by mitigating cardiac remodeling during chronic heart failure. Thus, MI was induced in 12-wk-old male C57BL/6J mice by ligating the left anterior descending artery, and FA (0.03 g/l in drinking water) was administered for 4 wk after the surgery. Cardiac function was assessed by echocardiography and by a Millar pressure-volume catheter. The levels of Hcy-metabolizing enzymes, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 5-MTHFR, were estimated by Western blot analyses. The results suggest that FA administered post-MI significantly improved cardiac ejection fraction and induced tissue inhibitor of metalloproteinase, CBS, CSE, and 5-MTHFR. We showed that FA supplementation resulted in significant improvement of myocardial function after MI. The study eluted the importance of homocysteine (Hcy) metabolism and FA supplementation in cardiovascular disease.

Methods

Recent studies indicated that FA, through its circulating form 5-MTHF, may have antioxidant properties and exert biological effects in vascular cells not directly related to changes in plasma Hcy level (1). A study (34) reports that, in rats, FA pretreatment blunts myocardial dysfunction during ischemia and ameliorates postreperfusion injury, in part, by high-energy phosphates. Interestingly, the metabolism of methionine to Hcy generates high-energy ATP through the S-adenosyl homocysteine pathway. This suggests that FA mitigates HHcy and improves high-energy phosphates in acute ischemia-reperfusion injury. However, the protective role of FA in MI-induced CHF was unclear. We sought to test the hypothesis that FA treatment post-MI also exerts beneficial effects on cardiac function during CHF. We predicted that an ability of FA to improve arteriogenesis (47) may affect blood circulation by collateralization in the heart and thus improve myocardocyte function, leading to a general improvement in cardiac function.

Methods

Animals. The animals were fed standard chow and water ad libitum. All animal procedures were reviewed and approved by an independent Institutional Animal Care and Use Committee of the University of Louisville School of Medicine, in accord with animal care and use guidelines of the National Institutes of Health. Ten- to 14-wk-old male C57BL/6 mice were anesthetized with pentobarbital sodium (65 mg kg ip). Animals were intubated and ventilated with room air using a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space, and the lungs were retracted to expose the heart. After opening the pericardium, to create MI, the left anterior descending (LAD) coronary artery was ligated with an 8–0 silk suture near its origin between the pulmonary outflow tract and the edge of the atrium. Ligation was deemed successful when the anterior wall of the left ventricle (LV) turned pale. The lungs were inflated by increasing positive end-expiratory pressure, and the thoracotomy side was closed in layers. Another group of mice underwent a sham surgery. They had a similar surgical procedure without tightening the suture around the coronary. The lungs were reexpanded, and the chest was closed. The animals were removed from the ventilator and allowed to recover on a heating pad. FA (0.03 g/l in drinking water) was administered for 4 wk after the surgery. The

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following experimental groups were used: 1) sham (animals underwent a mock surgery); 2) sham + FA (sham animals treated with FA); 3) MI (animals developed MI); and 4) MI + FA (animals with MI treated with FA). It is known that a dose of 2.5 mg/dl leads to ingestion of 8.33 × 10^{-4} mg of FA (17, 44); therefore, we estimated that administration of 0.03 g/l FA in drinking water led to ingestion of 7.5 × 10^{-4} mg of FA.

Echocardiography analysis. Two-dimensional (2-D) echocardiography was performed on mice before and after the surgery using a Hewlett-Packard Son 5500 ultrasonograph with a 15-MHz transducer. The mice were sedated with 2,2,2-tribromethanol (TBE, T48 Hewlett-Packard Sono 5500 ultrasonograph with a 15-MHz transducer). The mice were placed in a custom-made cradle on a heated platform in the supine or the left lateral decubitus position to facilitate echocardiography. For quantification of left ventricular (LV) dimensions and wall thickness, LV short- and long-axis loops and LV 2-D echocardiography was performed on mice before and after the surgery using a 2,2,2-tribromethanol (TBE, T48 Hewlett-Packard Sono 5500 ultrasonograph with a 15-MHz transducer) protocol, after performing a single-plane Simpson’s method, LV volumes at end-diastole and end-systole were derived from long-axis loops (38).

Pressure-volume loop and Hcy measurements. Using a standard Millar (Millar Instrument, Houston, TX) protocol, after performing a two-point calibration, steady-state pressure-volume loops were recorded followed by saline bolus and cuvette calibration for conversion of relative volume units to microliters. LV blood pressure and hemodynamic parameters were measured by a Millar catheter and analyzed by PVAN software (38). Plasma levels of Hcy were measured by HPLC.

Angiography. Barium sulfate has been used as a good contrast for visualizing the gastrointestinal tract in the pediatric population. Barium sulfate is toxic if it enters the systemic circulation and is the reason why mostly iodinated contrasts are used for intravascular imaging. We used barium sulfate for postmortem imaging of mice vasculature (13). The size of barium particles range from 1 to 100 μm, whereas most of the mice microvasculature is <30 μm. Moreover, barium sulfate is insoluble in water. To overcome this problem, we dissolved barium sulfate in acidic pH buffer, and the mixture was used for intravascular infusion. All images were taken with the Kodak 4000 MM image station. Dissected animals were placed in the X-ray chamber, and angiogram images were captured with a high-penetrative phosphorous screen by 31 KVP X-ray exposure for 3 min using Millar (Millar Instrument, Houston, TX) protocol, after performing a single-plane Simpson’s method, LV volumes at end-diastole and end-systole were derived from long-axis loops (38). Ejection fraction (EF) was calculated using volumes for end-diastolic volume (EDV) and end-systolic volume (ESV) as [(EDV − ESV)/EDV] × 100%.

Table 1. Comparison of end-systolic and end-diastolic blood pressure, EF, HR, and PV loop data in sham, MI, and sham and MI mice treated with FA

<table>
<thead>
<tr>
<th></th>
<th>Maximum Volume, μl</th>
<th>Minimum Volume, μl</th>
<th>End-Systolic Volume, μl</th>
<th>End-Diastolic Volume, μl</th>
<th>Stroke Volume, μl</th>
<th>Cardiac Output, μl/min</th>
<th>End-Systolic Pressure, mmHg</th>
<th>End-Diastolic Pressure, mmHg</th>
<th>EF, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>603 ± 10</td>
<td>13 ± 2</td>
<td>6 ± 3</td>
<td>5.84 ± 2</td>
<td>12 ± 1</td>
<td>7.3 ± 4</td>
<td>4,432 ± 45</td>
<td>104 ± 0.67</td>
<td>11.3 ± 0.56</td>
</tr>
<tr>
<td>Sham + FA</td>
<td>605 ± 12</td>
<td>12 ± 3</td>
<td>6 ± 2</td>
<td>5.82 ± 1</td>
<td>12 ± 4</td>
<td>6.5 ± 3</td>
<td>3,986 ± 29</td>
<td>105 ± 0.81</td>
<td>12.3 ± 1.23</td>
</tr>
<tr>
<td>MI</td>
<td>610 ± 7*</td>
<td>83 ± 9*</td>
<td>65 ± 8*</td>
<td>67.23 ± 12*</td>
<td>83 ± 9*</td>
<td>22.2 ± 2*</td>
<td>13,572 ± 56*</td>
<td>73 ± 1.07</td>
<td>20.7 ± 2*</td>
</tr>
<tr>
<td>MI + FA</td>
<td>605 ± 9*†</td>
<td>51 ± 5*†</td>
<td>28 ± 3*†</td>
<td>32.94 ± 8*†</td>
<td>50 ± 7*†</td>
<td>18.1 ± 3*†</td>
<td>10,992 ± 35*</td>
<td>85 ± 1.39†</td>
<td>18.6 ± 1.8*†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 mice for all groups. EF, ejection fraction; HR, heart rate; PV, pressure-volume; MI, myocardial infarction; FA, folic acid. P < 0.05 vs. sham group (*) and vs. MI (†).

Western Blot analysis. Changes in protein content of cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), 5-MTHFR, MMP-2
and -9, and tissue inhibitor of metalloproteinase (TIMP)-1, -2, -3, and -4 in MI were assessed by Western blot analysis (46). Briefly, frozen heart tissue was washed two times with ice-cold PBS and lysed with ice-cold RIPA buffer (containing 5 mM of ethylenediaminetetraacetic acid), which was supplemented with phenylmethylsulfonyl fluoride (1 mM) and protease inhibitor cocktail (1 ml/ml of lysis buffer). Protein content of the lysate was determined using the Bicinchoninic Acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (30 mg) were resolved on 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane as described (29, 42). The blots were incubated with monoclonal anti-mouse respective antibodies for 1 h at room temperature. After incubation, the proteins on blots were detected as described (42). Membranes were stripped and reprobed for β-actin as a loading control. The blots were analyzed with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD) as described earlier (29). The protein expression intensity was assessed by integrated optical density (IOD) of the area of the band in the lane profile. To account for possible differences in the protein load, measurements presented are the IOD of each band under study (protein of interest) divided by the IOD of the respective β-actin band.

Confocal microscopy. Isolated cardiomyocytes were washed two times with the incubation buffer. The cells were fixed in 3.7% paraformaldehyde in PBS for 30 min at room temperature. After fixation, cells were washed and permeabilized with 0.1% Triton X-100 for 20 min. After being washed, the myocytes were incubated with anti-TIMP-1, -2, -3, or -4, MMP-2, or MMP-9 antibodies (1:500 dilution, in 0.02% Tween 20/PBS) overnight at 4°C (Abcam). After additional washes with PBS, the cells were mounted on the glass slides. The images were acquired using a laser-scanning confocal microscope (×60 objective, FluoView 1000; Olympus). To enable the comparison of changes in fluorescence intensity, the images were acquired under the identical set of conditions. FITC fluorescence was imaged using excitation at 488 nm and emission 510–540 nm band pass filters. Myocyte images from four animals in each group were analyzed to determine expression of TIMP-1, -2, -3, and -4, MMP-2, and MMP-9. Total fluorescence (green or red) intensity in five random fields (for each experiment) was measured with image analysis software (Image-Pro PluS; Media Cybernetics). Fluorescence intensity unit values for each experimental group were averaged.

Statistical analysis. Values are reported as means ± SE. Differences between groups were tested by two-way ANOVA. If ANOVA indicated a significant difference (P < 0.05), Tukey’s multiple-comparison test was used to compare group means and were considered significant if P < 0.05.

RESULTS

Gravimetric parameters. LV catheterization and echocardiography were performed to evaluate in vivo cardiac function. Heart rate of animals did not change in any of experimental groups (Table 1). LV systolic blood pressure (LV-SBP) was less in MI mice after 4 wk of surgery compared with sham mice (Table 1). Treatment with FA increased SBP compared with that in MI mice, but it was still less than in the sham group. There was no difference in SBP in sham + FA and sham groups (Table 1).

The results of echocardiography measurements obtained immediately before and 4 wk after the surgery are shown in Table 2. Comparison of %FS and plasma Hcy levels in sham, MI, and sham and MI mice treated with FA

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Baseline, %FS</th>
<th>2 Weeks, %FS</th>
<th>3 Weeks, %FS</th>
<th>4 Weeks, %FS</th>
<th>Hcy, μmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>59</td>
<td>60</td>
<td>62</td>
<td>58</td>
<td>9 ± 2.3</td>
</tr>
<tr>
<td>MI</td>
<td>61</td>
<td>35†</td>
<td>30†</td>
<td>24†</td>
<td>56 ± 3.8*</td>
</tr>
<tr>
<td>Sham + FA</td>
<td>58</td>
<td>60</td>
<td>61</td>
<td>57</td>
<td>7 ± 1.6</td>
</tr>
<tr>
<td>MI + FA</td>
<td>63</td>
<td>45‡§</td>
<td>43‡§</td>
<td>39‡§</td>
<td>25 ± 2.7§</td>
</tr>
</tbody>
</table>

Values for homocysteine (Hcy) are means ± SE; n = 9 for all groups. Presented results are calculated from the following equation [(LVDd – LVDs)/LVDd] × 100% where LVDs and LVDd are left ventricular dimensions at systole and diastole, respectively. P < 0.05 vs. sham and sham + FA (*), vs. respective baseline (before the surgery) (†), vs. respective after 2 wk of the surgery (‡), and vs. MI (§).

Fig. 1. Changes in left ventricle (LV), diastolic and systolic diameters (LVDd and LVDs, respectively) in sham-operated (sham), myocardial infarction-induced (MI), sham-operated and treated with folic acid (sham + FA), and myocardial infarction-induced and treated with folic acid (MI + FA) mice. The red arrow represents diameter in diastole; the white arrow represents diameter in systole. A: examples of M-mode echocardiograms obtained with 2-dimensional echocardiography from a short-axis midventricular view of hearts of the experimental animals. B: bar graphs of changes of LV diameters during diastole (LVDd) and systole (LVDs). Notice increased left ventricular cavity dimensions (LVDd and LVDs) in mice with MI. P < 0.05 vs. sham (*) and vs. MI (#); n = 9 animals for all groups.
Table 2. All variables were similar in animals of all groups immediately before the surgery and remained unaltered in the sham group throughout the study period (Table 2). Supplementation with FA did not alter FS in sham mice (Table 2). Induction of MI resulted in a decrease of FS starting from 2 wk after surgery (Table 2). FS was minimal after 4 wk of surgery and was less than baseline after 2 wk of surgery or baseline in the MI group (Table 2). Similarly, FS was less after 2 wk of surgery than the baseline value in the MI + FA group and was greater than that after 4 wk of surgery (Table 2). However, FA supplementation improves FS in the MI + FA group compared with that in the MI group at each respective time point (2, 3, and 4 wk) after surgery (Table 2). The plasma concentration of Hcy in LAD-ligated animals was increased compared with the sham group. Interestingly, the treatment with FA mitigated the increase in Hcy levels (Table 2).

LV function. LV dimensions (LVDd, LVDs) were increased in mice with MI compared with the sham group (Fig. 1). Supplementation with FA decreased LV dimensions in the MI + FA group compared with the MI group, although they were still lower than in sham animals (Fig. 1). Treatment with FA for 4 wk did not change LV dimensions in sham + FA compared with the sham group (Fig. 1).

As shown in Fig. 2, invasive hemodynamic measurements showed a reduction in EF in MI vs. the sham group (Fig. 2). FA did not change EF in sham (sham + FA) animals (Fig. 2). However, supplementation with FA increased MI-induced lowering of EF in the MI + FA group, although it was still less than in sham animals (Fig. 2).

Myocyte function. MI significantly impaired contractility of isolated cardiomyocytes (Fig. 3). FA supplementation produced at least a 30% increase in the percent shortening of myocyte contractility rate and the rate of myocyte relengthening (\( \pm dL/dt \)) (Fig. 3). These increases were enough to completely prevent MI-induced impaired contractility of myocytes and make it similar to that in the sham group (Fig. 3). Treatment with FA did not change myocyte contractility in sham + FA mice compared with the sham group (Fig. 3).

Histological analysis of collagen level in heart. The intensity of trichrome blue stain demonstrated development of significant fibrotic formations in MI hearts compared with sham (Fig. 4). Interestingly, the treatment with FA mitigated the formation of fibrosis in the MI + FA group (Fig. 4).

Expression of MMP-2, MMP-9, and TIMP-1, -2, -3, and -4. The confocal image analyses indicated induction of intracellular TIMP-1, -2, -3, and -4 expression in myocytes in MI hearts compared with sham (Fig. 5). Supplementation with FA completely abolished this effect in the MI + FA group (Fig. 5). Treatment of sham mice with FA (sham + FA) did not change the expression. MMP-2 and MMP-9 were increased in MI mice compared with the sham group (Fig. 5). Supplementation with FA normalized MMP-2 and MMP-9 production in mice with MI compared with that in mice with MI (Fig. 5). Interestingly, the levels of TIMP-1 and -3 were induced in MI, and treatment with FA mitigated this induction (Fig. 5E). Because the levels of MMP and TIMP were estimated by immunohistochemical staining, the method that is known as semiquantitative at best, we performed Western blot analysis on these samples. We found that the results obtained by Western blot analysis (Fig. 5D) were similar to those obtained by immunohistochemical analysis (Fig. 5D).

Hcy metabolism. Because FA increases the level of 5-MTHFR and thus causes an increase in Hcy metabolism, we measured 5-MTHFR, CBS, and CSE (Hcy-metabolizing enzymes). Induction of MI significantly impaired expression of CBS, CSE, and 5-MTHFR in mice heart tissues (Fig. 6). Supplementation with FA restored expression of CBS, CSE, and 5-MTHFR in...
hearts from mice with MI (Fig. 6). Treatment with FA did not change the expression of CBS, CSE, and 5-MTHFR in sham /H11001 FA mice compared with that in the sham group (Fig. 6).

Collateralization. The angiography data suggested an apparent increase in collateral vessels in the MI hearts after FA treatment (Fig. 7).

DISCUSSION

In the present study, we showed that supplementation with FA at the time of MI limited the extent of MI development in mice. Our study indicated that the decrease in infarct size translated into reduced LV dilatation and improved LV function as measured by echocardiography and LV catheterization. Myocardial injury lowered LV pressure in MI mice, which was improved by FA supplementation. Several studies have indicated that FA and/or its active metabolite 5-MTHF improves endothelial function (10, 36, 49). Effects of FA have been studied in clinical trials (36), particularly to test its potential to lower cardiovascular risk in patients with myocardial vascular disease (3). Such effects may be mediated by the antioxidant properties of FA (10, 49) and are likely to mediate a rapid reduction in arterial stiffness through a reduction in the catabolism of nitric oxide and an enhancement of endothelial-dependent vasodilatation (52).

Postinfarct LV remodeling is a progressive process involving LV chamber dilatation, thinning of the infarcted wall, and compensatory thickening of the noninfarcted heart region (43). The time course of postinfarct LV dilatation can occur in acute, subacute, and later phases (50). Serial echocardiographic studies have shown that LV dilatation can be observed from as early as 2 wk and lasted 30 mo after MI, and the LV dilatation involved both infarcted and noninfarcted heart regions (16, 37). In the early phase of MI, infarct expansion and regional dilatation contribute to the ventricular enlargement (19, 26).

Histological studies have revealed that infarct expansion is due to slippage between muscle bundles, most frequently occurs in transmural infarction (51), and is associated with rupture of the infarcted wall (40, 41). Cardiac rupture could be considered as an extreme form of infarct expansion in which the expanded zone is insufficient to maintain the integrity of the ventricular wall before the deposition of collagen and scar formation (23, 41). In the present study, 20% of infracted mice died of LV rupture, indicating development of a severe form of infarct. However, we found that, in the mice surviving rupture and acute heart failure, LV dimensions increased only modestly at wk 2 after induction of MI. Progressive ventricular dilatation was noted between 2 and 4 wk of observation. This enlargement of LV chamber was associated with lowering of LV-SBP during MI. Based on these results, we choose to observe changes in LV dimensions and cardiac functions after 2 and 4 wk of MI.

We have shown that FA decreases collagen accumulation in hypertensive hearts (32). Others have indicated that FA sup-

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Fig. 3. Myocyte contractility in sham, MI, sham + FA, and MI + FA animals. A: examples of cell shortening traces. B: changes in percent peak shortening presented as changes in baseline percent peak (bl% peak) and in peak height (Peak H). C: rates of contraction (+dL/dt) and relaxation (−dL/dt) of cardiomyocytes. The values are the means of measurements of at least five myocytes from each animal in each experimental group. The mean value of contractility was calculated from at least five contractions of each cardiomyocyte analyzed. P < 0.05 vs. the sham group (*) and vs. MI (#); n = 5 for all groups.

Fig. 4. Heart wall anatomical changes in sham, MI, sham + FA, and MI + FA animals. A: examples of cross-sectional view of the whole hearts at the ventricle level. Note: right and left ventricles are distinctly visible. Magnified areas are the left ventricular walls of sham and sham + FA hearts (left) and MI and MI + FA hearts (right). Note: No visible necroses were found in the left ventricular wall of hearts from sham and sham + FA. B: collagen-associated (blue) intensity changes in hearts from experimental animals. P < 0.05 vs. the sham group (*) and vs. MI (#); n = 4 for all groups.

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Fig. 5. Expression of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of MMP (TIMP)-2, and TIMP-4 in myocytes from sham, MI, sham + FA, and MI + FA mice. A: examples of images indicating expression of MMP-2 (green) and TIMP-4 (red). The last column indicates colocalization of MMP-2 and TIMP-4. B: examples of images indicating expression of MMP-9 (red) and TIMP-2 (green). The last column indicates colocalization of MMP-9 and TIMP-2. Cellular nuclei in all experiments were stained with 4',6-diamidino-2-phenylindole (DAPI). The micrographs were taken under the identical set of conditions for all groups. C: bar graph of changes in integrated optical density (IOD) in expression of MMP-2, MMP-9, TIMP-2, and TIMP-4 in myocytes. The micrographs were taken under the identical set of conditions for all groups. *P < 0.05 vs. sham, sham + FA, and MI + FA. D: Western blots and bar graphs of IOD changes in the levels of MMP-2, MMP-9, TIMP-2, and TIMP-4. *P < 0.05 vs. sham, sham + FA, and MI + FA (*) vs. MI (#). Although we observed variations in the expression of various enzymes, i.e., MMPs and TIMPs, n = 6 in each group. E: Western blots and bar graphs of IOD changes in the levels of TIMP-1 and TIMP-3. *P < 0.05 vs. sham, sham + FA, and MI + FA (*) and vs. MI (#); n = 6 for all groups.
Relative protein expression is reported as a ratio of IOD of each band to the IOD of the respective β-actin band. *P < 0.05 vs. sham, sham + FA, and MI + FA; n = 5 for all groups.

A differential role of MMP-2 vs. MMP-9 has been suggested (13, 15). Targeted detection of MMP-2 amplifies the cardiac failure (21, 31). On the other hand, MMP-9 deletion attenuates LV dilatation after experimental MI (8). Because of this effect on LV remodeling, it was not surprising to find an increased matrix metalloproteinase (MMP)-2 and MMP-9 expression in the 4-wk MI mice hearts compared with that in sham and MI + FA mice. Thus an increase in MMP-9 may result in increased LV size. The data indicate that the level of MMPs was returned to its normal level in MI groups given FA supplementation.

TIMPs are a family of specific protein inhibitors of MMPs, four of which have been demonstrated (12). The data showed that end-stage heart failure, secondary to ischemic dilated cardiomyopathy, is associated with the imbalance in MMP and TIMP expression at both the gene and protein synthesis levels. Myocardial MMP and TIMP are both localized in the interstitium and endocardium (48). It is known that TIMP-1 induces formation of fibrosis (48); TIMP-2 causes cell proliferation (30). TIMP-3 results in apoptosis (2). TIMP-4 acts specifically on cardiac tissue and induces apoptosis in transformed cells without affecting normal cells (45). We found an increase in TIMP-1 and -3 and a decrease in TIMP-2 and -4 in MI cardiomyocytes. Interestingly, the levels of TIMP-1, -2, -3, and -4 were restored in MI animals given FA supplementation, suggesting a direct effect of FA on expression of TIMPs.

In conclusion, we have found novel evidence for FA-induced improvement of myocardial function and reduced infarction during formation of MI. Our study showed that myocardial injury lowered LV-SBP in mice with MI, which was improved by FA supplementation. FA improved infarct size and LV dimensions (FS and LV volume). Treatment with FA restored cardiac myocyte contractility in mice with MI, indicating a direct functional impact of FA on heart. Thus our data suggest that FA supplementation restores expression of TIMPs and MMPs, normalizing the collagen production and preventing the heart wall thinning during development of MI.

**Limitations.** It is tedious to measure epicardial blood flow in a beating heart by laser Doppler flow probe. To determine whether the in vivo effects of FA were mediated through effects on the cardiac myocytes rather than through the effects of FA on vascular function and collateral flow, we measured apparent vascular density by barium-contrast angiography (Fig. 7). The results suggested that, in vivo, effects of FA were also mediated, in part, through its effect on the collateral vessel formation. We found an increase in collateral vessels in the MI + FA hearts compared with MI hearts (Fig. 7). This may equate to the improvement of blood flow in the epimyocardium post-FA treatment in MI hearts. However, the interpretation of improvement of collateral flow in the heart using barium-contrast angiography could be subjective because simple visualization of increased lighted small vessels in one heart does not necessarily mean that...
there is increased collateralization. We observed an apparent increase in vessel numbers in FA-treated hearts. However, Figs. 1 and 2 showed the improvement of cardiac function in the MI + FA group, but still worse than in sham controls. However, in Fig. 3, the single cardiomyocyte contractility from MI + FA mice was completely restored compared with those from sham controls. This may suggest that, during endothelial myocyte coupling, myocyte interaction with...
endothelial cells is partially restored by FA treatment. However, we cannot prove directly that the changes in CBS, CSE, and 5-MTHFR levels are not consequences but the cause for the observed changes. It is known that FA increases the activity of MTHFR and therefore decreases, in part, the levels of Hcy and is responsible for the observed mitigation of cardiac dysfunction post-MI. Although treatment with folate had several effects, it is unclear which (if any) of these account for the differences in cardiac performance. It is known that FA decreases the Hcy level; therefore, our data may in part support the idea that these changes are in part functionally important. FA or its metabolite 5-methyl tetrahydrofolate has been shown to reduce superoxide production (36). In addition, 5-methyl tetrahydrofolate improves nitric oxide production and prevents superoxide generation via uncoupling of nitric oxide synthase by stabilizing tetrahydrobiopterin, a critical cofactor of endothelial nitric oxide synthase, or by regenerating tetrahydrobiopterin from its inactive form dihydrobiopterin. Previously, we demonstrated that FA ameliorates Hcy-induced oxidative stress (32). Here we demonstrate that FA mitigates the cardiac fibrosis and cardiomyocyte dysfunction in MI.

It has been previously reported that CBS is only present in the heart and may play a role in hydrogen sulfide (H2S) generation in the myocardium, since there is expression of both CSE and CBS enzymes restored in response to FA treatment. Therefore, the measurements on the levels of H2S would be very interesting. These experiments are in progress.

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20. Lominadze).

