Heart Failure

High-Dose Folic Acid Pretreatment Blunts Cardiac Dysfunction During Ischemia Coupled to Maintenance of High-Energy Phosphates and Reduces Postreperfusion Injury

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Background—The B vitamin folic acid (FA) is important to mitochondrial protein and nucleic acid synthesis, is an antioxidant, and enhances nitric oxide synthase activity. Here, we tested whether FA reduces myocardial ischemic dysfunction and postreperfusion injury.

Methods and Results—Wistar rats were pretreated with either FA (10 mg/d) or placebo for 1 week and then underwent in vivo transient left coronary artery occlusion for 30 minutes with or without 90 minutes of reperfusion (total n=131; subgroups used for various analyses). FA (4.5×10^{-6} mol/L IC) pretreatment and global ischemia/reperfusion (30 minutes/30 minutes) also were performed in vitro (n=28). After 30 minutes of ischemia, global function declined more in controls than in FA-pretreated rats (ΔdP/dtmax, -878 ± 586 versus -1956 ± 351 mm Hg/s placebo; $P=0.03$), and regional thickening was better preserved ($37.3\pm5.3\%$ versus $5.1\pm0.6\%$ placebo; $P=0.004$). Anterior wall perfusion fell similarly ($-78.4\pm9.3\%$ versus $-71.2\pm13.8\%$ placebo at 30 minutes), yet myocardial high-energy phosphates ATP and ADP reduced by ischemia in controls were better preserved by FA pretreatment (ATP: control, 2740 ± 58 nmol/g; ischemia, 947 ± 55 nmol/g; ischemia plus FA, 1332 ± 101 nmol/g; $P=0.02$). Basal oxypurines (xanthine, hypoxanthine, and urate) rose with FA pretreatment but increased less during ischemia than in controls. Ischemic superoxide generation declined (3124 ± 280 cpm/mg FA versus 5898 ± 474 cpm/mg placebo; $P=0.001$). After reperfusion, FA-treated hearts had smaller infarcts ($3.8\pm1.2\%$ versus $60.3\pm4.1\%$ placebo area at risk; $P<0.002$) and less contraction band necrosis, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling positivity, superoxide, and nitric oxide synthase uncoupling. Infarct size declined similarly with 1 mg/d FA.

Conclusions—FA pretreatment blunts myocardial dysfunction during ischemia and ameliorates postreperfusion injury. This is coupled to preservation of high-energy phosphates, reducing subsequent reactive oxygen species generation, eNOS-uncoupling, and postreperfusion cell death. (***Circulation***. 2008;117:1810-1819.)

Key Words: contractility ■ folic acid ■ ischemia ■ infarction ■ nitric oxide synthase ■ reperfusion ■ superoxide

During acute coronary occlusion, myocardial perfusion and oxygen delivery become insufficient to support active muscle contraction. In the ischemic zone, myocardial high-energy phosphate (HEP) content falls, inorganic phosphate rises, and the tissue is rendered acidotic,[1] resulting in regional dyskinesis and global dysfunction. Coronary reperfusion after ischemia limits tissue damage but confers toxicity from the activation of reactive oxygen species (ROS), calcium-dependent proteases such as calpain, myofilament contracture, microvascular dysfunction, and inflammatory cytokines.[2] ATP depletion during ischemia also may contribute to reperfusion injury.[3] In addition, ROS generated during both ischemia[4] and reperfusion[5] is thought to play a central role, and various antioxidant strategies have been tested to offset this damage.

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Ischemia/reperfusion (IR) damage can be diminished by subjecting hearts to brief ischemia before more prolonged

exposure, a phenomenon called preconditioning. This involves activation of protein kinase C,[6] mitochondrial K_{ATP} channels, and nitric oxide synthesis (NOS),[7] with NOS playing a central role. Infarct size after IR is greater in endothelial NOS (eNOS) −deficient mice[8] and reduced in mice overexpressing eNOS.[9] This role of NOS has led to efforts to enhance its function, including administration of its obligate cofactor, tetrahydrobiopterin (BH_4),[10] to help maintain NOS in a functionally coupled (NO synthesis, little ROS generation) state.[11,12]

A far less costly alternative may be folic acid (FA), a B vitamin that stabilizes BH_4 by augmenting its binding affinity to eNOS[13] and enhances BH_4 regeneration from oxidized and inactive BH_2. FA or its active metabolite is thought to enhance endothelial function by this mechanism.[14,15] FA also is essential for normal mitochondrial protein and purine synthesis[16] and can enhance total HEP levels in chronically hypertrophied right ventricles.[17] In the present study, we tested the hypothesis that high-dose FA pretreatment ameliorates IR injury and explored the mechanisms for such an effect. The data show postreperfusion benefits but more strikingly reveal a marked and surprising effect of FA pretreatment on reducing regional and chamber dysfunction and ROS generation during the period of ischemia itself. This benefit appears to be linked to alterations in purine catabolism and preservation of HEP levels during ischemia.

Methods

Experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH publication 85-23) and approved by the ethics committees of the University of Antwerp and the Johns Hopkins Medical Institutions.

In Vivo Ischemia Model

Adult Wistar rats received FA (10 mg/d unless otherwise stated) or placebo by oral gavage for 7 days before the IR experiment. A total of 131 rats were used, reflecting the multiple assays that could not be performed in each one. Animals were anesthetized (pentobarbital 60 mg/kg), intubated via tracheotomy, and ventilated (Harvard Apparatus, Holliston, Mass). The ECG was monitored, and temperature was maintained at 37.5°C. The left anterior descending coronary artery (LAD) was exposed through the fourth to fifth intercostal space with a suture placed around it, and transient coronary artery ligation performed for 30 minutes with (n=85) or without (n=46) 90 minutes of reperfusion. In 1 subgroup of reperfused animals (n=9), FA was provided 10 minutes after the onset of LAD occlusion (ie, 20 minutes before the onset of reperfusion), delivered as an intravenous bolus.

In Vivo Hemodynamics

Left ventricular (LV) function was assessed in vivo by pressure-volume loops (n=14) both during ischemia and after reperfusion. A 1.4F pressure-volume catheter (SciSense, London, Ontario, Canada) was advanced through the apex, positioned along the longitudinal axis, and attached to a stimulator/analyzer (IOX 1.8.9.19, Emka Technologies, Paris, France). Volume data were calibrated with the hypertonic saline method, assuming a gain of 1. Two animals had catheter dislocation during ischemia; their volume data were not used. Open-chest myocardial anterior wall motion also was measured with a Sequoia Acuson C256 equipped with a 15-MHz linear transducer (Sequoia C256 Echocardiography System, Acuson Corp,

Mountain View, Calif) at the parasternal view of the LV chamber as described.

Assessment of Redox and Energy Metabolism

Snap-frozen samples (n=24) from the anterior wall (with or without FA pretreatment, with or without 30 minutes of ischemia, no reperfusion) were deproteinized and subjected to high-performance liquid chromatography analysis of water-soluble low-molecular-weight compounds reflecting tissue oxidoreductive and energy status. HEPs, oxypurines (hypoxanthine, xanthine, and uric acid), nucleosides (inosine and adenosine), malondialdehyde, and reduced and oxidized glutathione were measured by ion-pairing high-performance liquid chromatography as described.[18]

Myocardial Flow Measurements

Regional myocardial blood flow was assessed (n=12) by nuclear-activated microspheres (15 μmol/L diameter, BioPal, Worcester, Mass) injected into the left atrium (0.3 mL of 2.5×10^6 spheres per 1 mL) at baseline and after 5 and 30 minutes of ischemia.[19] Total counts per minute were normalized to weight, and results from the ischemic zone were normalized to the remote region to provide relative blood flow before and during ischemia.

In Vitro IR Model

Adult Wistar rats (n=28) were anesthetized (pentobarbital 60 mg/kg), and hearts were rapidly excised and mounted onto a retrograde perfusion system (Emka Technologies) with warmed, oxygenated, buffered Krebs-Henseleit solution at a constant perfusion pressure (75 mm Hg). Hearts were paced at 300 bpm and maintained unloaded. Coronary flow was measured by an inline ultrasonic flow probe (Transonic Systems, Ithaca, NY). After 30 minutes of equilibration, bradykinin or sodium nitroprusside was infused by bolus injection (50 μL, 10^{-8} to $10^{-5.5}$ mol/L IC), and coronary flow reserve was assessed at constant perfusion pressure. After baseline was reestablished, hearts received FA (4.5×10^{-6} mol/L IC) or vehicle for 30 minutes and then were subjected to 40 minutes of zero-flow ischemia followed by 40 minutes of reperfusion. Coronary effluent was collected, concentrated (Sartorius-Sipan, Lier, Belgium), and analyzed for lactate dehydrogenase (Vitros 950AT, OCD, Beerse, Belgium). Coronary vasodilator reserve studies were then repeated.

### Infarct Size Analysis and Histology

Infarct size was assessed (n=31) with area at risk (AAR) determined by Evans blue–negative staining and triphenyl tetrazolium chloride staining to detect myocardial necrosis. Regions were planimetered, digitized, and quantified. AAR was not assessed for the in vitro studies because global ischemia was created. Contraction band necrosis was examined in vivo (n=22) by fixation in Carnoy solution and Masson's trichrome staining. Serial adjacent fields were examined throughout the LV to calculate the percent myocardium with contraction bands present. A similar analysis was performed for terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) positivity (Chemicon Intern, Temecula, Calif), also expressed as percent LV area.

ROS Determination

Superoxide was assessed by lucigenin (5 μmol/L) -enhanced chemi-luminescence (Beckman LS6000IC, Beckman Coulter, Fullerton, Calif; n=23)[20] and fluorescent microtopography (dichlorodihydro-fluorescein diacetate, n=16; dihydroethidium, n=24).[18] The direct antioxidant effects of FA were analyzed with an in vitro xanthine/xanthine oxidase system[21] and compared with Tempol.

eNOS Monomer/Dimer Formation and Enzymatic Activity

SDS-resistant eNOS dimers and monomers were assayed on IR tissue (n=16) with low-temperature SDS-PAGE as previously de-

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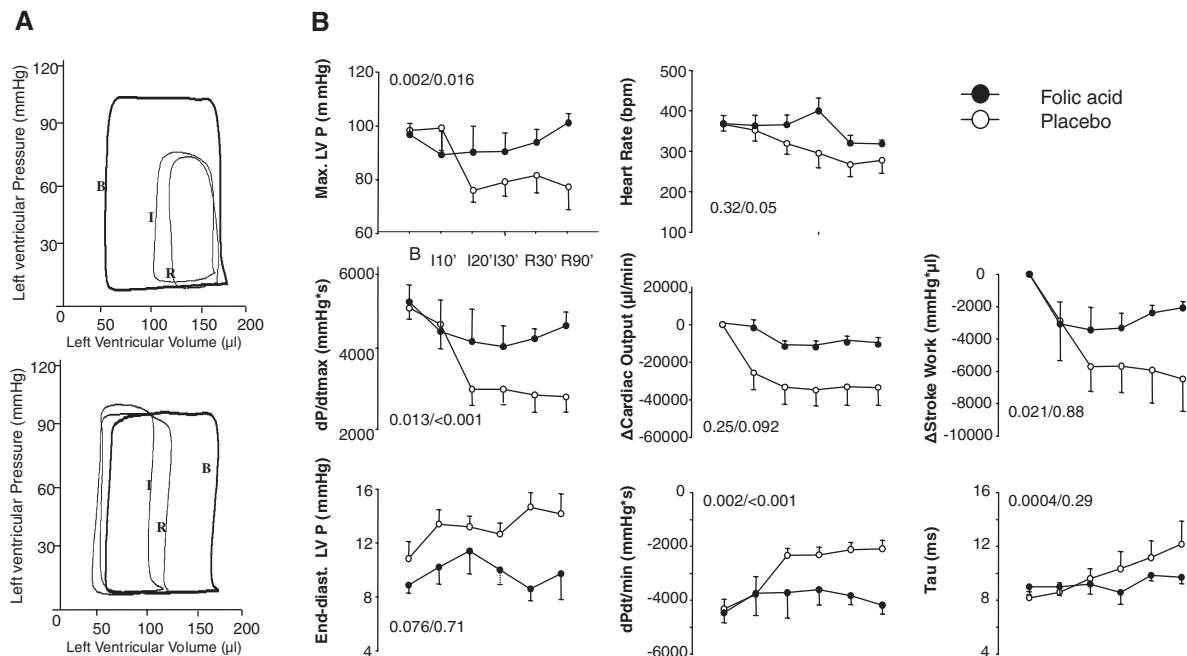
Figure 1. Cardiac function measured by pressure-volume loop analysis. A, Example of pressure-volume loops at baseline (B), at the end of ischemia, and after 90 minutes of reperfusion for a placebo-pretreated (top) and an FA-pretreated (lower) animal. FA pretreatment improved function both during ischemia and after reperfusion. B, Summary hemodynamics for the full IR protocol shows the time course for systolic and diastolic parameters. Probability values are from repeated-measures ANOVA, time× and treatment interaction/treatment effect. LVP indicates LV pressure; End diast., end diastolic; and Tau, time constant of relaxation.

scribed.[18] NOS enzymatic activity was assessed by arginine-to-citrulline conversion assay from extracts obtained from frozen myocardium (n=15).[18]

Data Analysis

Data are presented as mean±SEM, and values of $P<0.05$ were considered statistically significant. Coronary flow reserve and in vivo hemodynamic data were analyzed by repeated-measures ANOVA. Other comparisons used either 1-way ANOVA or Kruskal-Wallis tests to compare multiple independent groups, with a Bonferroni correction for multiple comparisons. Analysis used SPS6 version 11.0 (SPSS Inc, Chicago, Ill). The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

FA pretreatment Improves Cardiac Function With IR

Figure 1A displays example and summary LV pressure-volume data in hearts with or without FA (10 mg/d) pretreatment. Data were measured at initial baseline in open-chest rats, during 30 minutes of coronary occlusion, and after 90 minutes of reperfusion. Control hearts displayed markedly reduced function with a rightward downshift of pressure-volume loops at 30 minutes of ischemia that persisted with reperfusion (Figure 1A, top). With FA pretreatment, cardiac function was better preserved during ischemia and reperfusion (Figure 1A, bottom). Peak systolic pressure changed little despite LAD occlusion in FA-pretreated rats but fell nearly 25% in controls. Similar disparities were observed in dP/dtmax. Cardiac output and stroke work also declined less

in FA-pretreated animals, particularly in late ischemia and reperfusion.

The relative preservation of global function during ischemia was somewhat surprising and suggested less regional dysfunction despite coronary occlusion in FA-pretreated hearts. This was tested by echocardiography measured before and during LAD occlusion (Figure 2). Example M-mode tracings (top left) show marked reductions in anterior wall thickening during ischemia in controls but preserved thickening in FA-treated animals ($5\pm0.6\%$ versus $37\pm5.3\%$; $P=0.004$). Ejection fraction was much higher despite ischemia ($72.8\pm1.2\%$ versus $27.4\pm2.2\%$ placebo; $P<0.001$ at 30 minutes), consistent with the pressure-volume loop data.

FA and Myocardial Flow

Because FA pretreatment improved both regional and global function during LAD occlusion, we tested whether it enhanced myocardial blood flow to reduce the ischemic insult per se. However, after 5 minutes of LAD ligation, the ratio of ischemic to remote zone myocardial perfusion obtained by microsphere analysis declined similarly in placebo- and FA-pretreated groups ($-73.7\pm6.0\%$ and $-77.7\pm5.1\%$, respectively). Flow remained low in both groups at 30 minutes ($-78.4\pm9.3\%$ versus $-71.2\pm13.8\%$ placebo).

FA Preserves Myocardial Levels of HEPs

Because improved perfusion could not explain the FA treatment effect, we next tested whether FA altered HEP metabolism at baseline and/or during ischemia. As shown in Figure 3, FA pretreatment did not alter HEP at baseline but did elevate levels of inositol monophosphate (IMP) and its
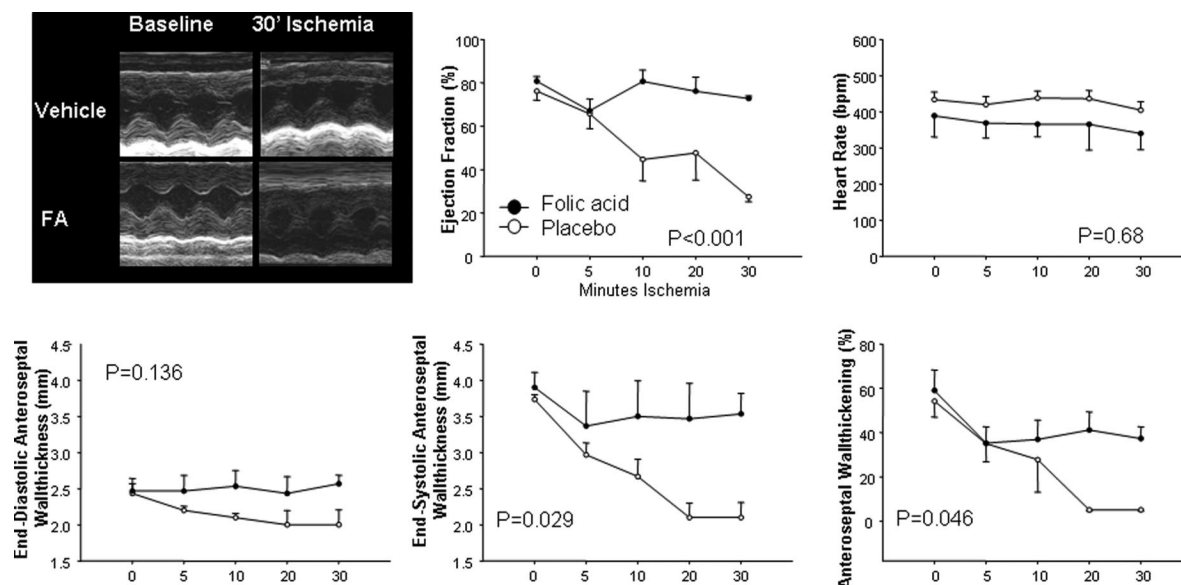
Figure 2. Open-chest echocardiography. Top left, Examples of M-mode echocardiography at baseline and after 30 minutes of ischemia in the placebo- and FA-treated groups. Summary plots show results obtained from these echocardiograms vs time. Ejection fraction and anterior-septal wall thickening declined in the placebo group but were unchanged in the FA group. Probability values are for treatment effect by repeated-measures ANOVA.

catabolites (oxypurines: xanthine, hypoxanthine, uric acid). During ischemia, myocardial ATP and ADP declined >66% in controls, consistent with reported data.[1] However, both were better maintained in FA-pretreated hearts ($P<0.001$ for drug interaction effect). Oxypurines rose markedly during ischemia in controls, consistent with reduced HEP and enhanced AMP catabolism, but changed little or declined in FA-treated hearts. Redox state indexed by malondialdehyde (a marker of lipid peroxidation) and ratio of reduced to oxidized glutathione were little changed by FA pretreatment with or without myocardial ischemia.

FA Pretreatment Reduces Myocardial Infarct Size

A potential consequence of improving both function and HEP metabolism during ischemia is a reduction in infarct size. Infarct size was $60.3\pm4.1\%$ of the AAR in placebo-treated animals versus $3.8\pm1.2\%$ with FA pretreatment ($P<0.002$; Figure 4A). A similar reduction was observed with 40% or 10% of the FA dose (1 or 4 mg/d), although this dose is still fairly high compared with that typically used in humans. Contraction band necrosis was found in $26.7\pm2.6\%$ of the LV in controls versus $4.6\pm1.2\%$ in FA-treated hearts ($P=0.001$; Figure 4B). Similarly, TUNEL-positive myocytes were prevalent ($63.0\pm5.8\%$ of LV fields) in controls but rare ($4.3\pm1.3\%$) with FA treatment ($P=0.001$; Figure 4C). Lethal ventricular arrhythmia was fairly common during ischemia in controls but not FA-treated rats (36.7% versus 8.3%) and was reduced in frequency during reperfusion (6.1% versus 0%; $P<0.01$ for both).

Because postreperfusion infarct size in vivo is related partially to coupling of function with coronary perfusion, we also tested the impact of FA pretreatment in isolated hearts. FA reduced infarct size markedly ($7.7\pm2.8\%$ versus $41.1\pm4.9\%$; $P<0.0001$; Figure 4D) with less lactate dehy-

drogenase in the coronary effluent (Figure 4E), consistent with reduced necrosis.

Folate Pretreatment Versus Acute Folate Administration

In a separate group of 9 animals, FA was administered acutely starting after 10 minutes of coronary occlusion (10 mg IV) when functional responses first appeared to diverge (see Figures 1 and 2) and continued for the remaining 20-minute ischemic period. Infarct size relative to AAR also was reduced (n=5; $3.0\pm2.2\%$; $P<0.001$ versus placebo) with AAR itself similar to placebo ($52.4\pm5.5\%$). Histology (n=4) found reduced TUNEL staining ($4.3\pm1.3\%$ LV) and contraction band necrosis ($5.1\pm0.7\%$ LV; both $P<0.001$ versus placebo). Thus, the effect of FA on infarct reduction did not appear to be a classic preconditioning effect because it could be generated by FA administration after ischemia had begun.

Folate Pretreatment Reduces ROS Generation

Myocardial superoxide (lucigenin chemiluminescence) declined $\approx50\%$ in FA-pretreated animals during ischemia and after 90 minutes of reperfusion (Figure 5A). When extracts were preincubated with 100 μmol/L BH_4, O_2^- generation declined $90.9\pm0.7\%$ in vehicle-controls but less so in FA-pretreated hearts ($52.1\pm11.3\%$; $P<0.03$; Figure 5B). This suggested that an antioxidant pathway targeted by BH_4 (eg, NOS coupling) either was lacking in FA-pretreated hearts or was already ameliorated by FA therapy. Dihydroethidium- and dichlorodihydrofluorescein diacetate–stained myocardial slices also showed marked ROS generation in the placebo group that was reduced with FA pretreatment (Figure 5C through 5E). To test for
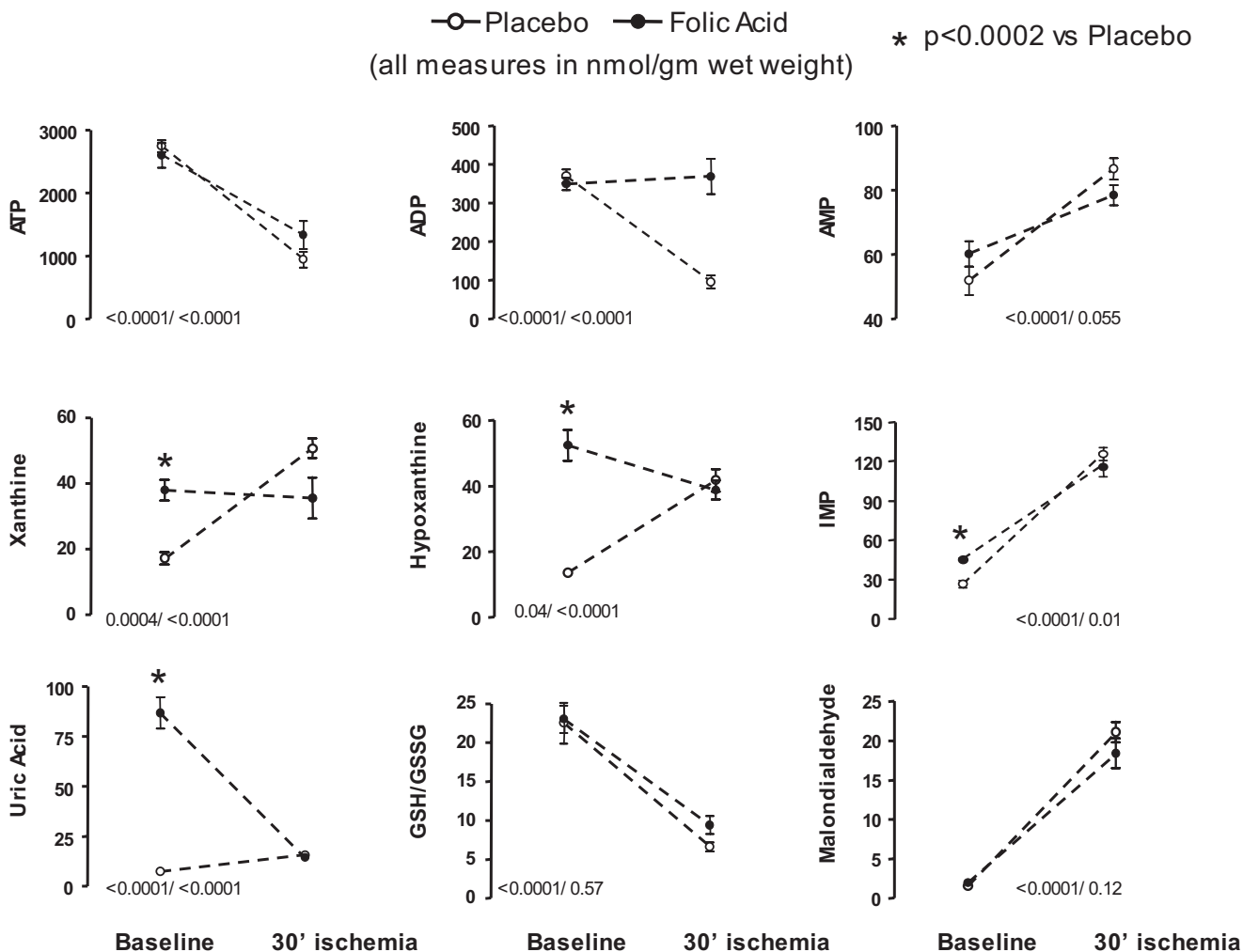
Figure 3. High-performance liquid chromatography analysis of parameters of HEP metabolism and redox state. Under baseline conditions, FA pretreatment did not alter HEP (ATP, ADP, AMP) but did increase IMP and its catabolites (oxypurines: xanthine, hypoxanthine, uric acid). After 30 minutes of ischemia, the levels of HEP were better maintained in the FA-treated hearts vs placebo animals. Although oxypurines rose markedly after ischemia in placebo-treated animals, no rise was observed in the FA-treated rats. Redox state reflected by the reduced/oxidized glutathione ratio (GHS/GSSG) and malondialdehyde changed markedly with ischemia but was unaltered by FA treatment. Probability values are from 2-way ANOVA, with the first value indicating the effect of ischemia and the second showing the interaction between treatment group and ischemia.

direct antioxidant effects of FA, we performed an in vitro assay using a xanthine/xanthine oxidase O_2^- generating system (Figure 5F). FA antioxidant effects were substantial in this assay and were similar to the superoxide dismutase mimetic Tempol.

Folate Pretreatment Improves eNOS Dimerization and Activity and Endothelial Function

Because FA and its metabolites have been linked to the BH_4-mediated improvement in NOS coupling and decline in ROS generation,[14,21] we examined NOS coupling in IR hearts. Immunoblots showed a decline in the ratio of NOS dimer to monomer that was preserved at nearly normal in FA-pretreated hearts (Figure 6A through 6C). Total eNOS was similar among the conditions (Figure 6B). NOS activity (arginine-to-citrulline conversion) was borderline improved by FA pretreatment ($P=0.08$; data not shown).

Coronary endothelial function also improved with FA pretreatment. Bradykinin induced a maximal $108.3\pm9.2\%$

rise in coronary flow at baseline but $67.1\pm8.1\%$ after IR ($P<0.001$; Figure 6D). This decline was absent in FA-pretreated hearts ($122.0\pm11.3\%$). Basal flow before (placebo, 10.2 ± 0.7 mL/min; FA, 11.4 ± 13.8 mL/min; $P=0.5$) and after (placebo, 9.8 ± 0.7 mL/min; FA, 8.9 ± 1.4 mL/min; $P=0.5$) ischemia was similar between groups. Coronary flow rose similarly before and after ischemia with sodium nitroprusside (Figure 6E), supporting the endothelium dependence of the prior effect.

Discussion

This study demonstrates that pretreatment with high doses of oral FA markedly reduces ischemic dysfunction during coronary occlusion and enhances function and diminishes infarct size after reperfusion. These were associated with better-preserved HEPs during ischemia despite a flow reduction, sustaining regional function and reducing oxidative stress, with preserved eNOS coupling after reperfusion. Preserved
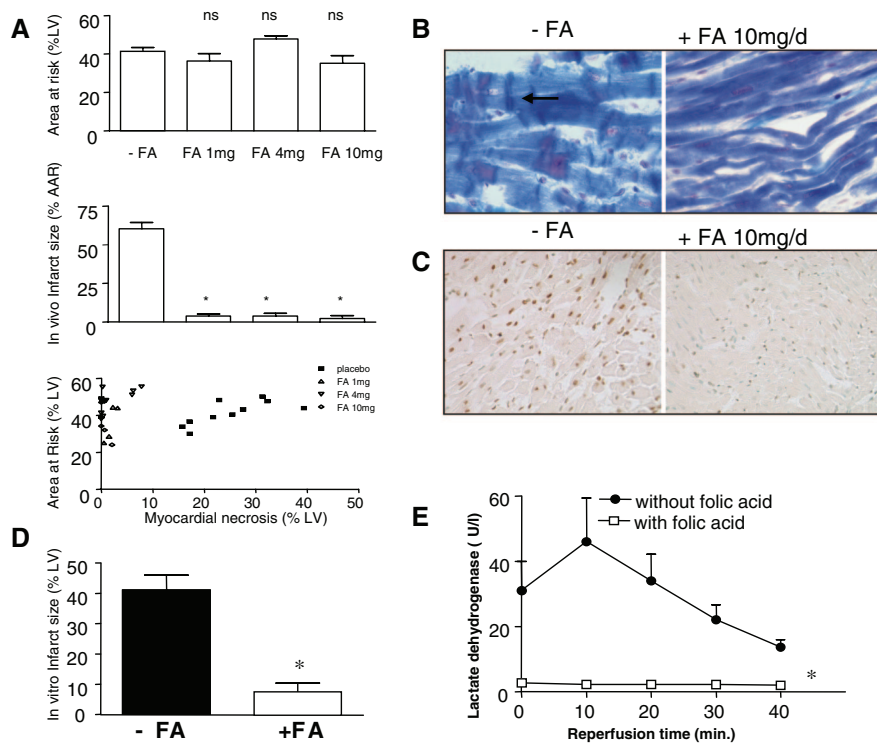
Figure 4. Effect of FA on myocardial necrosis. A, Oral (7 days) FA pretreatment reduced infarct size in vivo (*$P<0.001$, Mann-Whitney test). Top, AAR was comparable between all groups. Middle, Infarct size, expressed as percent AAR, was significantly reduced in all FA groups. Bottom, Individual correlation between myocardial necrosis and AAR. B, FA pretreatment in vivo reduces contraction band necrosis (*$P=0.001$). C, FA pretreatment in vivo reduces apoptosis (TUNEL staining) (*$P=0.005$, $P=0.001$). D, FA pretreatment reduced myocardial necrosis by ≈80% in hearts studied in vitro (*$P<0.0001$). E, Increased lactate dehydrogenase after IR peaked at 10 to 20 minutes, whereas hearts receiving FA pretreatment had minimal lactate dehydrogenase release ($P<0.001$).

function during ischemia is unusual and quite different from the influence of antioxidants and the classic preconditioning agents that typically benefit the heart only after reperfusion. For that matter, the benefit of FA in reducing infarct size even when delivered after ischemia had begun implies a different mechanism.

During normoxia, ATP synthesis is highly regulated and levels are maintained. With ischemia, ATP supplies from anaerobic glycolysis and HEP reserves such as phosphocreatine are insufficient to meet demand, and the net ATP level falls. Theoretically, interventions that could maintain ATP even in ischemic myocardium should delay the advent of lethal injury because low tissue ATP levels (<5 μmol/g dry weight) are associated with depressed HEP resynthesis, failure of cell volume regulation, and irreversible myocardial injury. FA is known to regulate mitochondrial function and plays an important role as a methylation cofactor for the synthesis of thymidylate, purines, and methionine. With regard to purine synthesis, the metabolite N^{10}-formyl tetrahydrofolate contributes 2 carbons (C2 and C8) to the ring structure, with the primary end point being formation of IMP. IMP is then further metabolized to generate AMP or GMP or is catabolized to oxypurines.

The present results support a role of FA in HEP generation. The increased basal level of oxypurines suggests that high doses of FA drove their synthesis (via IMP) by mass action, but because HEP was adequate, IMP catabolites were enhanced with AMP levels unchanged. During ischemia, however, ATP and ADP levels fell, leading to increased AMP and AMP catabolism (IMP, oxypurinols) in controls. In FA-treated hearts, however, purine synthesis would be primed to

help rederive HEP (FA-treated hearts had virtually no decline in ADP during ischemia, and the ATP level decline was about half). Actual ATP generation was probably higher in the FA-treated hearts because the anterior wall was still actively contracted and therefore was using more ATP despite LAD occlusion compared with controls. The concept of FA-enhanced HEP is supported by a recent study by Lamberts et al,[22] who found that chronic high-dose FA restored total HEPs (ATP plus ADP plus AMP) accompanied by improved diastolic function in hypertrophied rat hearts. Our finding that FA administered intravenously 10 minutes into the ischemic period still diminished infarct size also could be explained by such a mass action effect because it may occur quickly. This remains to be confirmed but has intriguing therapeutic implications.

High-dose FA may have potent antioxidant effects both directly and via weak but competitive inhibition of xanthine oxidase,[13] which also could benefit the heart during ischemic and postischemic periods. Although our in vitro data showed similar antioxidant effects for FA and Tempol, in vivo, scavenging effects of FA are thought to be modest even at high doses.[14] Importantly, reduced oxidant stress per se has not previously been shown to enhance HEP; rather, a decline in HEP is linked to mitochondrial damage and ROS generation.[23] Klawitter et al[4] administered 1,2-dihydroxybenzene-3,5-disulfonate (Tiron), a superoxide scavenger, or N-acetyl-L-cysteine before ischemia and found no change in energetic recovery compared with untreated hearts. Tempol reduces postreperfusion infarct size in vivo in rat and rabbit by ≈50% and 33%, respectively,[24] but only modestly improves LV pressure
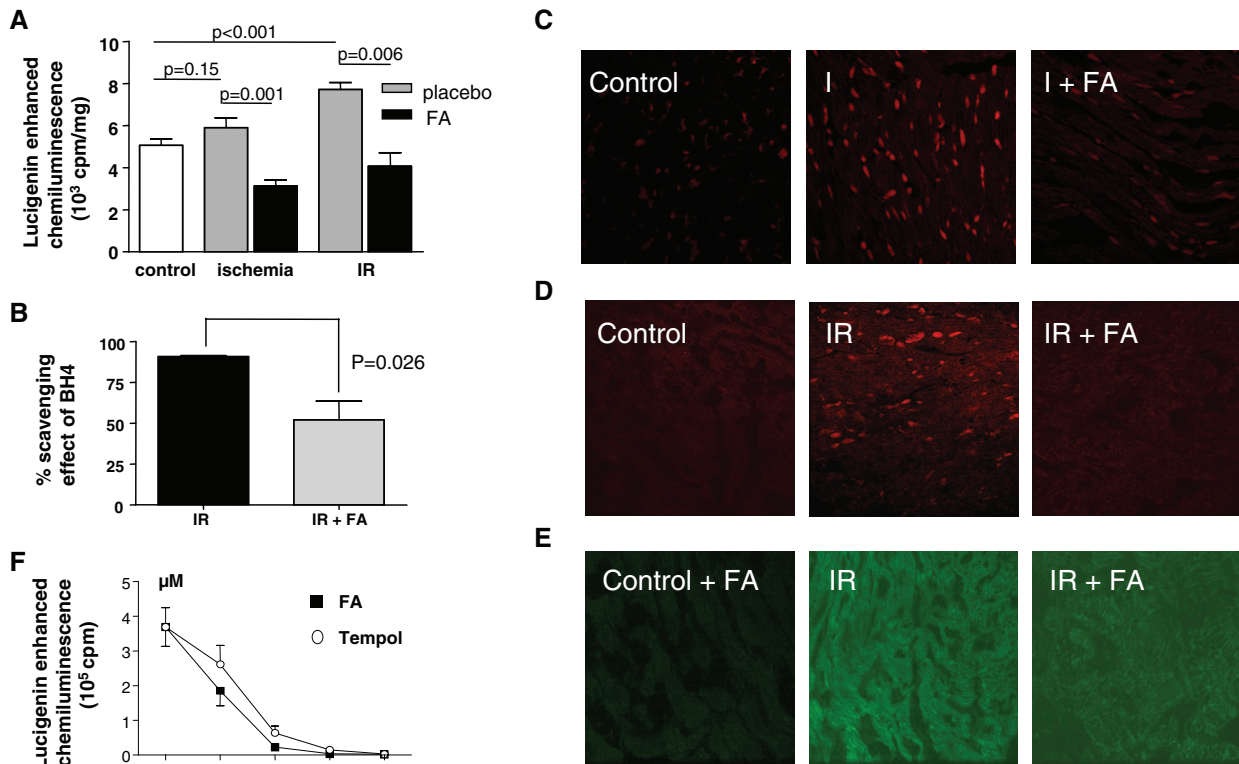
Figure 5. Effects of FA treatment on ROS generation. A, Lucigenin-enhanced superoxide detection shows marked reductions in ischemia and IR-induced superoxide with FA pretreatment. B, O_2^- formation in control vehicle–treated IR hearts was markedly suppressed by the acute addition of BH_4, whereas it was blunted by nearly half in myocardial extracts obtained from FA-pretreated hearts. Dihydroethidium (DHE)-stained (C, D,) and dichlorodihydrofluorescein diacetate (DCF)–stained (E) myocardia reveal increased ROS generation in ischemic or IR myocardium that was substantially reduced in heart receiving FA pretreatment. F, Direct antioxidant effects of FA compared with Tempol. Superoxide was generated by xanthine/xanthine oxidase in vitro and measured by lucigenin-enhanced chemiluminescence at various added concentrations of either FA or Tempol.

recovery after reperfusion.[25] Similar results are reported with other free radical scavengers.[26] Thus, it seems unlikely that the sole or primary mechanism for FA benefit was its antioxidant capacity.

Another potential mechanism is the recoupling of NOS. NOS uncoupling is thought to contribute to the pathophysiology of diseases such as hypertension, atherosclerosis, and cardiac hypertrophy.[18,27–29] Uncoupling involves a decline in the normal electron transfer from the reductase to oxidase domains, leading to reduced NO synthesis and greater ROS generation by NOS.[30] BH_4 plays a key role in maintaining normal eNOS coupling, and FA (or 5-methyltetrahydrofolate) can increase BH_4 levels by facilitating enzymatic reduction of its oxidized forms[31] and are associated with improved endothelial function.[14] The present study supports this mechanism on the basis of the diminished eNOS uncoupling after reperfusion and an in vitro analysis showing that the addition of BH_4 reduced ROS generation far more in untreated than in FA-treated myocardium. Improved NOS coupling also could contribute to improved HEP generation because endogenous NO generation reduces oxygen consumption and improves energetic efficiency[32] partly by enhancing HEP synthesis itself.

This is the first study to test the effects of FA on in vivo ischemia and IR injury. Several recent studies have found that FA and/or its active metabolite 5-methyltetrahydrofolate improve endothelial function,[21,33] although the dose required has been somewhat controversial. Tawakol et al[34] used high-dose FA (30 mg PO) in patients with coronary artery disease and found that it increased both adenosine-stimulated myocardial blood flow and flow reserve in segments with impaired dilator reserve but also acutely lowered arterial pressure. The antinecrotic effect of FA observed in the present study is consistent with earlier in vitro data in which high-dose folate (>1 mmol/L) suppressed apoptosis from oxidant injury in U937 cells.[35] The beneficial effect of FA on in vivo ischemia and reperfusion-induced arrhythmias is similar to the reduction of in vitro reperfusion-induced arrhythmias by FA described by Manning et al.[36] One prior study[10] examined the effects of BH_4 on post-IR infarct size and found more modest changes than we observed with folate. This can be due to dose differences but also to specific influences that folate has that are unrelated to BH_4.

FA has been studied in clinical trials, particularly to test its potential to lower cardiovascular risk in patients with myocardial vascular disease. For example, Oster[37] demonstrated that long-term FA treatment ($\approx$10 years) at a dose far higher than typically used (40 to 80 mg/d) reduced the incidence of myocardial infarction, angina pectoris, and the requirement for nitroglycerin in patients with coronary artery disease. This observation was not confirmed by a placebo-controlled trial, nor was the mechanism explored.
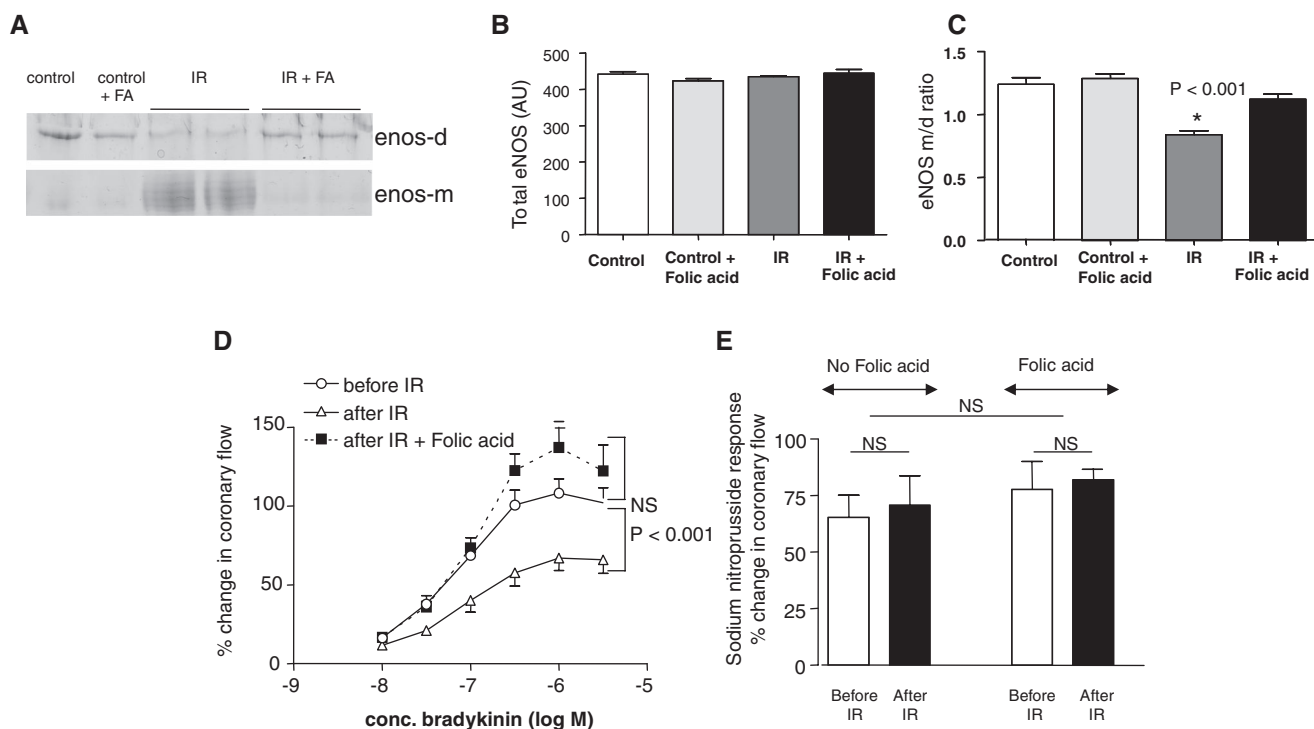
Figure 6. Effect of FA pretreatment on the NO pathway. A, SDS-PAGE gel shows increased eNOS monomer in IR myocardium that was reduced toward control levels by FA pretreatment. B, C, Summary densitometry data of the gel analysis. FA pretreatment reduced the ratio of monomer (m) to dimer but had no effect on total eNOS protein expression. D, Bradykinin administered to hearts in vitro before vs after IR showed a marked decline in endothelium-dependent flow response. This was restored to a normal response by pretreatment with FA. E, FA treatment did not influence the flow response to sodium nitroprusside, supporting the hypothesis that the disparity observed with bradykinin was endothelium dependent.

Other studies focused on the ability of FA to lower homocysteine and found a 3-μmol/L decrease in serum homocysteine (achievable with 0.8 mg/d). Despite these positive results, meta-analyses of multiple FA trials for use in cardiovascular prevention have been unimpressive,[38,39] and it remains unclear whether dose, study duration, target population, or other factors explain this. The doses of FA used in the present study are far above those used in prior cardiovascular interventional studies (5 to 25 mg $\cdot$ 70 kg^{-1} $\cdot$ d^{-1}) or prevention trials (500 μg to 1 mg). Even taking into account that rodents have a higher metabolic turnover, the present dose would likely correlate with between 0.25 and 2.5 g $\cdot$ 70 kg^{-1} $\cdot$ d^{-1} in a human. Although long-term treatment at this level may have adverse off-target effects, short-term dosing in a peri-infarct period should be well tolerated. Admittedly, this remains to be determined.

A few limitations to our study should be noted. Although FA pretreatment improved HEP balance and function during a 30-minute coronary occlusion, it might become less effective if the ischemic period would have been more prolonged. It is also unknown how long after an occlusion we might intervene with FA and still see a benefit. Finally, our data support but do not prove that FA directly helps rederive HEP by mass action effects on purine synthesis. This requires future studies using ^{14}C-labeled folate in which the carbons can be tracked and the direct involvement can be tested.

Conclusions

We have found marked and novel evidence for improved ischemic and post-IR myocardial function and reduced infarction by pretreatment with high-dose FA. The relation to preservation of myocardial HEP during the ischemic period is intriguing and suggests that FA can alter the manner by which HEPs are synthesized despite a decline in myocardial perfusion. This may relate to changes in purine synthesis, enhanced NOS coupling modulated by interactions between folate and BH$_4$, and/or effects of FA on improving mitochondrial function during ischemia. Given that FA is inexpensive, safe even at high doses, and readily available, it is possible that such treatment in high-risk patients could provide a novel means to limit ischemic damage.

Disclosures

None.

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CLINICAL PERSPECTIVE

Cardiac dysfunction/infarction from coronary heart disease is a leading cause of morbidity and mortality. A critical reduction in coronary flow lowers the high-energy supply, and heart function declines as mitochondrial damage and oxidative stress develop. These factors can further amplify damage on coronary reperfusion. Many efforts have been made to ameliorate the consequences of ischemia and reperfusion injury. Here, we report that high-dose folic acid (FA) treatment does both. FA is required for purine biosynthesis and thus formation of high-energy phosphates, is itself an antioxidant, and helps to preserve nitric oxide synthase function. Rats pretreated with FA (10 mg/300 g body weight per day) for 1 week were subjected to 30 minutes of left anterior descending coronary artery occlusion followed by 90 minutes of reperfusion. During the occlusion, FA-treated animals had better global and regional function coupled with enhanced ATP and ADP in the anterior wall. Postreperfusion function was enhanced and myocardial necrosis/apoptosis was reduced by FA treatment, accompanied by reduced oxidant stress (during ischemia and postreperfusion) and preserved nitric oxide synthase function. Reduced necrosis occurred even when FA was administered shortly after the onset of coronary occlusion. FA treatment alone enhanced purine biosynthesis (eg, increased inosine monophosphate and its catabolites), suggesting that by mass action FA may help to maintain high-energy phosphates despite reduced flow and blunt development of irreversible tissue damage coupled to oxidant stress. Clinical translation of these results might pave the way for a novel and inexpensive approach to treat acute coronary syndromes and to potentially reduce postinfarction morbidity and mortality.