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

An L. Moens, MD; Hunter C. Champion, MD, PhD; Marc J. Claeyss, MD, PhD; Barbara Tavazzi, MD, PhD; Pawel M. Kaminski, MD, PhD; Michael S. Wolin, MD, PhD; Dirk J. Borgonjon, MS; Luc Van Nassauw, PhD; Azeb Haile, MS; Muz Zviman, PhD; Djahida Bedja, MS; Floris L. Wuyts, PhD; Rebecca S. Elsaesser, PhD; Paul Cos, PhD; Kathy L. Gabrielson, DVM, PhD; Giuseppe Lazzarino, MD, PhD; Nazareno Paolocci, MD, PhD; Jean-Pierre Timmermans, PhD; Christiaan J. Vrints, MD, PhD; David A. Kass, MD

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 (∆dP/dtmax, −878±586 versus −1956±351 mm Hg/s placebo; P=0.03), and regional thickening was better preserved (37.3±5.3% versus 5.1±0.6% placebo; P=0.004). Anterior wall perfusion fell similarly (−78.4±9.3% versus −71.2±13.8% 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, 2740/55 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±1.2% versus 60.3±4.1% 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
exposure, a phenomenon called preconditioning. This involves activation of protein kinase C, mitochondrial K\textsubscript{ATP} channels, and nitric oxide synthesis (NOS), with NOS playing a central role. Infarct size after IR is greater in endothelial NOS (eNOS)–deficient mice and reduced in mice overexpressing eNOS. This role of NOS has led to efforts to enhance its function, including administration of its obligate cofactor, tetrahydrobiopterin (BH\textsubscript{4}), to help maintain NOS in a functionally coupled (NO synthesis, little ROS generation) state.

A far less costly alternative may be folic acid (FA), a B vitamin that stabilizes BH\textsubscript{4} by augmenting its binding affinity to eNOS and enhances BH\textsubscript{4} regeneration from oxidized and inactive BH\textsubscript{2}. FA or its active metabolite is thought to enhance endothelial function by this mechanism. FA also synthesis and can enhance total HEP levels in chronically hypertrophied right ventricles. 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 spaces and ligated with 7-0 silk. The heart was excised, placed in ice-cold buffer, and incubated at 37°C for 30 minutes and then was subjected to 40 minutes of zero-flow ischemia. Hearts were paced at 300 bpm and maintained unloaded. Coronary flow was measured by an ultrasonic flow probe (Transonic Systems, Ithaca, NY). After 30 minutes of equilibration, bradykinin or sodium nitroprusside was infused by bolus injection (50 μL, 10⁻⁶ to 10⁻⁵ mol/L IC), and coronary flow reserve was assessed at constant perfusion pressure. After baseline was reestablished, hearts received FA (4.5×10⁻⁵ 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 chemiluminescence (Beckman LS6000IC, Beckman Coulter, Fullerton, Calif; n = 23) and fluorescent microtopography (dichlorodihydrofluorescein diacetate, n = 16; dihydroethidium, n = 24). The direct antioxidant effects of FA were analyzed with an in vitro xanthine/xanthine oxidase system 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-
scribed.\textsuperscript{18} NOS enzymatic activity was assessed by arginine-to-citrulline conversion assay from extracts obtained from frozen myocardium (n=15).\textsuperscript{18}

**Data Analysis**

Data are presented as mean±SEM, and values of \textit{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 SPSS 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 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 \(	imes\) and treatment interaction/treatment effect. LVP indicates LV pressure; End diast., end diastolic; and Tau, time constant of relaxation.

**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 (\(-73.7±6.0\%\) versus \(-77.7±5.1\%,\) respectively). Flow remained low in both groups at 30 minutes (\(-78.4±9.3\%\) versus \(-71.2±13.8\%\) placebo). ejection fraction was much higher despite ischemia (72.8±1.2\% versus 27.4±2.2\% placebo; \(P<0.001\) at 30 minutes), consistent with the pressure-volume loop data.

**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 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 \(	imes\) and treatment interaction/treatment effect. LVP indicates LV pressure; End diast., end diastolic; and Tau, time constant of relaxation.
catabolites (oxypurines: xanthine, hypoxanthine, uric acid). During ischemia, myocardial ATP and ADP declined by 66% in controls, consistent with reported data. 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 ± 4.1% of the AAR in placebo-treated animals versus 3.8 ± 1.2% 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 ± 2.6% of the LV in controls versus 4.6 ± 1.2% in FA-treated hearts (P = 0.001; Figure 4B). Similarly, TUNEL-positive myocytes were prevalent (63.0 ± 5.8% of LV fields) in controls but rare (4.3 ± 1.3%) 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 ± 2.8% versus 41.1 ± 4.9%; P < 0.0001; Figure 4D) with less lactate dehydrogenase 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 ± 2.2%; P < 0.001 versus placebo) with AAR itself similar to placebo (52.4 ± 5.5%). Histology (n = 4) found reduced TUNEL staining (4.3 ± 1.3% LV) and contraction band necrosis (5.1 ± 0.7% 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 ~50% in FA-pretreated animals during ischemia and after 90 minutes of reperfusion (Figure 5A). When extracts were preincubated with 100 μmol/L BH₄, O₂⁻ generation declined 90.9 ± 0.7% in vehicle-controls but less so in FA-pretreated hearts (52.1 ± 11.3%; P < 0.03; Figure 5B). This suggested that an antioxidant pathway targeted by BH₄ (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...
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±9.2% rise in coronary flow at baseline but 67.1±8.1% after IR ($P<0.001$; Figure 6D). This decline was absent in FA-pretreated hearts (122.0±11.3%). 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
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 synthesis 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 N5-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 posts ischemic 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 al24 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.
recovery after reperfusion. Similar results are reported with other free radical scavengers. 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. 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. BH₄ plays a key role in maintaining normal eNOS coupling, and FA (or 5-methyltetrahydrofolate) can increase BH₄ levels by facilitating enzymatic reduction of its oxidized forms and are associated with improved endothelial function. 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 BH₄ reduces oxygen consumption and improves energetic efficiency 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, although the dose required has been somewhat controversial. Tawakol et al 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. 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. One prior study examined the effects of BH₄ 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 BH₄.

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 demonstrated that long-term FA treatment (10 years) at a dose far higher than typically used (40 to 80 mg/d) reduced the incidence of myocardial infarction, angi a 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.
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, 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·70 kg⁻¹·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·70 kg⁻¹·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 ¹⁴C-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 BH₄, 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.

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Disclosures

None.

References

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.
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