High dose folic acid pre-treatment blunts cardiac dysfunction during ischemia coupled to maintenance of high energy phosphates and reduces post-reperfusion injury

An L. Moens, MD[1,2], **Hunter C. Champion, MD, PhD**[2], **Marc J. Claeys, MD, PhD**[1], **Barbara Tavazzi, MD, PhD**[3], **Pawel M. Kaminki, MD, PhD**[4], **Michael S. Wolin, MD, PhD**[4], **Dirk Borgonjon, MS**[1], **Luc Van Nassauw, PhD**[5], **Azeb Haile, MS**[2], **Muz Zviman, PhD**[2], **Djahida Bedja, MS**[2], **Floris L. Wuyts, PhD**[6], **Rebecca S. Elsaesser, PhD**[7], **Paul Cos, PhD**[1], **Kathy L. Gabrielson, DVM, PhD**[2], **Giuseppe Lazzarino, MD, PhD**[3], **Nazareno Paolocci, MD, PhD**[2], **Jean-Pierre Timmermans, PhD**[5], **Christiaan J. Vrints, MD, PhD**[1], and **David A. Kass, MD**[2]

1 *University of Antwerp, Department of Cardiology, Antwerp, Belgium*

2 *Johns Hopkins Medical Institutions, Division of Cardiology, Baltimore, MD, USA*

3 *University of Rome, Department of Chemical Science, Rome, Italy*

4 *New York Medical College, Department of Physiology, Valhalla, NY, USA*

5 *University of Antwerp, Department of Cell Biology and Histology, Antwerp, Belgium*

6 *University of Antwerp, Department of Biomedical Statistics, Antwerp, Belgium*

7 *Johns Hopkins Medical Institutions, Division of Pathology, Baltimore, MD, USA*

Abstract

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

Methods—Wistar rats were pretreated with either FA (10mg/d) or placebo for 1-wk, and then underwent *in vivo* transient left coronary artery occlusion for 30min with or without 90min reperfusion (total:n=131; sub-groups used for various analyses). FA ($4.5 \cdot 10^{-6}$M i.c) pretreatment and global ischemia/reperfusion (30 min/30min), was also performed *in vitro* (n=28).

Results—After 30min ischemia, global function declined more in controls than FA-pretreated rats ($\Delta dP/dt_{max}$ -878±586 mmHg/s vs. placebo -1956±351 mmHg/s, p=0.03), and regional thickening was better preserved (37.3±5.3% vs. 5.1±0.6%-placebo, p=0.004). Anterior-wall perfusion fell similarly (-78.4±9.3% vs. -71.2±13.8%-placebo at 30 min); yet myocardial high energy phosphates ATP and ADP reduced by ischemia in controls were better preserved by FA-pretreatment (e.g. ATP: control: 2740±58; ischemia: 947±55; ischemia+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 FA vs. 5898±474 placebo, cpm/mg, p=0.001). After reperfusion, FA-treated hearts had smaller infarcts (3.8±1.2% vs 60.3 ±4.1%-placebo area at risk, p<0.002), less contraction band necrosis, TUNEL-positivity, superoxide, and nitric oxide synthase uncoupling. Infarct size declined similarly with 1 mg/d FA as well.

Corresponding author: David A. Kass, MD, Johns Hopkins Medical Institutions, Division of Cardiology, Ross Research Building Rm. 835, 720 Rutland Ave, Baltimore, MD, 21205, Email: dkass@jhmi.edu, Phone: (410) 955-7153, Fax: (410) 502 2558.

Conclusion—FA-pretreatment blunts myocardial dysfunction during ischemia, and ameliorates post-reperfusion injury. This is coupled to preservation of high energy phosphates, reducing subsequent ROS-generation, eNOS-uncoupling and post-reperfusion cell death.

Keywords

Ischemia; Folic acid; High Energy Phosphates; Superoxide; eNOS-uncoupling

Introduction

During acute coronary occlusion, myocardial perfusion and oxygen delivery become insufficient to support active muscle contraction. In the ischemic zone, myocardial high energy phosphate 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 activation of reactive oxygen species (ROS), calcium-dependent proteases such as calpain, myofilament contracture, microvascular dysfunction, and inflammatory cytokines[2]. ATP depletion during ischemia may also 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 anti-oxidant strategies have been tested to offset this damage.

Ischemia/reperfusion damage can be diminished by subjecting hearts to brief ischemia prior to more prolonged exposure, a phenomenon termed pre-conditioning. This involves activation of protein kinase C[6], mitochondrial K_{ATP} channels, and nitric oxide synthesis[7], with the latter playing a central role. Infarct size after ischemia-reperfusion is greater in eNOS deficient mice[8] and reduced in mice over-expressing eNOS[9]. This role of NOS has led to efforts to enhance its function, including administration of its obligate cofactor, tetrahydrobiopterin (BH4)[10] to help maintain NOS in a functionally coupled (NO synthesis, little ROS generation) state[11,12].

An 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 is also essential for normal mitochondrial protein and purine synthesis[16] and can enhance total high energy phosphate levels in chronically hypertrophied right ventricles[17]. In the present study, we tested the hypothesis that high dose FA pre-treatment ameliorates IR injury and explored the mechanisms for such an effect. The data show post-reperfusion benefits but more strikingly reveal a marked and surprising effect of FA-pre-treatment on reducing regional and chamber dysfunction and ROS generation during the period of ischemia itself. This benefit appears linked to alterations in purine catabolism and preservation of high energy phosphate 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, N° 85-23) and approved by the Ethical Committees of the University of Antwerp and by the Johns Hopkins Medical Institutions.

In vivo ischemia model—Adult Wistar rats received FA (10 mg/day unless otherwise stated) or placebo by oral gavage for 7-days prior to performing the ischemia/reperfusion (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, MA). ECG was monitored and temperature

maintained at 37.5°C. The left anterior artery was exposed through the 4-5th intercostal space and a suture placed around it, and transient coronary artery ligation performed for 30 min with (n=85) or without (n=46) 90 min reperfusion. In one subgroup of reperfused animals (n=9), FA was provided 10 minutes after the onset of LAD occlusion (i.e. 20 minutes before the onset of reperfusion), delivered as an IV-bolus.

In vivo hemodynamics—LV function was assessed *in vivo* by pressure-volume loops (n=14) during both ischemia and following 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 Tech., Paris). Volume data were calibrated using the hypertonic saline method, assuming a gain=1. Two animals had catheter dislocation during ischemia, and their volume data were not used. Open chest myocardial anterior wall motion was also measured using a Sequoia –Acuson C256 equipped with 15 MHz linear transducer (Sequoia C256 Echocardiography System, Acuson Corporation, Mountain View, CA) at the parasternal view of the left ventricle chamber as described.

Assessment of Redox and Energy Metabolism—Snap frozen samples (n=24) from the anterior wall (±FA pre-treatment; ±30 min ischemia, no reperfusion) were de-proteinized and subjected to HPLC analysis of water-soluble low-molecular weight compounds reflecting tissue oxid-oreductive and energy status. High-energy phosphates, oxypurines (hypoxanthine, xanthine, and uric acid), nucleosides (inosine and adenosine), malondialdehyde, and reduced and oxidized glutathione were measured by ion-pairing HPLC as described[18]

Myocardial flow measurements—Regional myocardial blood flow was assessed (n=12) by nuclear-activated microspheres (15μM diameter, BioPal, Worcester, MA) injected in the left atrium (0.3ml of $2.5*10^6$ spheres/ml) at baseline and after 5 and 30 min of ischemia[19]. Total counts per minute (cpm) were normalized to weight and results from the ischemic zone 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 rapidly excised and mounted onto a retrograde perfusion system (Emka, Paris, France) using warmed, oxygenated buffered Krebs-Henseleit solution at constant perfusion pressure (75 mmHg). Hearts were paced at 300 bpm and maintained unloaded. Coronary flow was measured by an in-line ultrasonic flow probe (Transonic Systems, Ithaca, NY). After 30 min equilibration, bradykinin or sodium nitroprusside were infused by bolus injection (50 μl, 10^{-8}-$10^{-5.5}$ M i.c.) and coronary flow reserve assessed at constant perfusion pressure. After re-establishing baseline, hearts received FA ($4.5\ 10^{-6}$M i.c.) or vehicle for 30 min, and then subjected to 40-min zero flow-ischemia followed by 40 min reperfusion. Coronary effluent was collected, concentrated (Sartorius-Sipan, Lier, Belgium) and analysed for lactate dehydrogenase (Vitros 950AT, O.C.D., 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 TTC staining to detect myocardial necrosis. Regions were planimetered and digitized and quantified. AAR was not assessed for the *in vitro* studies since 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. Similar analysis was performed for TUNEL-positivity (Chemicon Intern, Temecula, CA), also expressed as percent LV area.

ROS determination—Superoxide was assessed by lucigenin (5μM)-enhanced chemiluminescence (Beckman LS6000IC, n=23)[20] and fluorescent microtopography (dichlorodihydro-fluorescein diacetate, DCF, n=16) and dihydroethidium, DHE, n=24)[18]. The direct anti-oxidants effects of folic acid were analysed using 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 ischemia/reperfusion-tissue (n=16) using low temperature SDS- PAGE as previously described [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 with p-values<0.05 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 between multiple independent groups, with a Bonferroni correction for multiple comparisons. Analysis utilized SPS6 version 11.0 (Chicago, IL).

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. None of the authors has conflict of interest disclosures.

Results

Folic acid pre-treatment improves cardiac function with I/R

Figure 1A displays example and summary left ventricular PV data in hearts with or without FA (10mg/d) pre-treatment. 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 PV loops at 30 min ischemia that persisted with reperfusion (upper panel). With FA pre-treatment, cardiac function was better preserved during ischemia and reperfusion (lower panel, Fig 1B). Peak systolic pressure changed little despite LAD occlusion in FA pre-treated rats but fell nearly 25% in controls. Similar disparities were observed in dP/dt_{max}. 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 pre-treated hearts. This was tested by echocardiography measured before and during LAD occlusion (Fig 2). Example M-mode tracings (upper left panel) show marked reductions of anterior wall thickening during ischemia in controls but preserved thickening in FA treated animals (5±0.6% versus 37±5.3%, p=0.004). Ejection fraction was much higher despite ischemia (72.8±1.2% vs. placebo: 27.4 ±2.2 %, p<0.001 at 30 min), consistent with the PV-loop data.

Folic acid and myocardial flow

Since FA pre-treatment 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 min of LAD ligation, the ratio of ischemic/remote zone myocardial perfusion obtained by microsphere analysis declined similarly in placebo and FA-pre-treated groups (-73.7±6.0% and -77.7±5.1%, respectively). Flow remained low in both groups at 30 minutes (-78.4±9.3% vs. placebo -71.2±13.8%).

Folic acid preserves myocardial levels of high energy phosphates

Since improved perfusion could not explain the FA-treatment effect, we next tested whether FA altered high energy phosphate (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 catabolites (oxypurines: xanthine, hypoxanthine, uric acid). During ischemia, myocardial ATP and ADP declined more than 66% in controls, consistent with reported data[1]. However, both were better maintained in FA pre-treated 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 reduced/oxidized glutathione ratio was little changed by FA pre-treatment with or without myocardial ischemia.

Folic acid pre-treatment reduces myocardial infarct size

A potential consequence of improving both function and HEP metabolism during ischemia is reduction of infarct size. Infarct size was 60.3±4.1% of the area of risk in placebo-treated animals versus 3.8±1.2% with FA-pre-treatment (Fig 4A, p<0.002). Similar reduction was observed using 40% or 10% of the FA dose (1 or 4 mg/d), though this is still a fairly high dose compared to that typically used in humans. Contraction band necrosis (CBN) was found in 26.7±2.6% of the LV in controls, versus 4.6%±1.2% in FA treated hearts (p=0.001, Fig. 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, Fig 4C). Lethal ventricular arrhythmia was fairly common during ischemia in controls but not FA treated rats (36.7% vs 8.3%) and reduced in frequency during reperfusion as well (6.1 vs 0%, p<0.01 for both).

Since post-reperfusion infarct size *in vivo* is partially related 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% vs. 41.1±4.9%, p<0.0001, Fig 4D) with less lactate dehydrogenase in the coronary effluent (Fig. 4E) consistent with reduced necrosis.

Folate pre-treatment vs. acute folate administration

In a separate group of 9 animals, FA was acutely administered starting *after* 10 min of coronary occlusion (10 mg i.v.) when functional responses first appeared to diverge (c.f. Fig 1, 2), and continued for the remaining 20 min ischemic period. Infarct size relative to AAR was also reduced (n=5; 3.0±2.2%, p<0.001 vs. placebo) with AAR itself similar to placebo (52.4±5.5%). Histology (n=4) found reduced TUNEL staining (4.3±1.3% LV) and CBN (5.1±0.7% LV; both p<0.001 vs. placebo). Thus, the FA effect on infarct reduction did not appear to be a classic preconditioning effect, as it could be generated by FA administration *after* ischemia had commenced.

Folate pre-treatment reduces ROS generation

Myocardial superoxide (lucigenin chemiluminescence) declined ~50% in FA pre-treated animals during ischemia and after 90 minutes reperfusion (Fig 5A). When extracts were pre-incubated with 100 μM BH4, O_2^- generation declined 90.9±0.7% in vehicle-controls, but less so in FA pre-treated hearts (52.1±11.3%, p<0.03, Fig 5B). This suggested that an anti-oxidant pathway targeted by BH4 (e.g. NOS coupling) was either lacking in FA-pre-treated hearts or already ameliorated by FA therapy. DHE and DCF-stained myocardial slices also showed marked ROS generation in the placebo group that was reduced with FA pre-treatment (Fig 5C-E). To test for direct anti-oxidant effects of FA, we performed an *in vitro* assay using a xanthine/xanthine oxidase O_2^- generating system (Fig 5F). FA anti-oxidant effects were substantial in this assay, and similar to the superoxide dismutase mimetic Tempol.

Folate pre-treatment improves eNOS dimerization and activity, and endothelial function

Since FA and its metabolites have been linked to BH4-mediated improvement in NOS coupling and decline in ROS generation[14,21], we examined NOS coupling in I/R hearts. Immunoblots showed a decline in the NOS dimer/monomer ratio that was preserved near normal in FA pre-treated hearts (Fig 6A-C). Total eNOS was similar among the conditions (Fig 6B). NOS activity (arginine-citrulline conversion) was borderline improved by FA pre-treatment (p=0.08, data not shown).

Coronary endothelial function also improved with FA pre-treatment. Bradykinin induced a maximal 108.3±9.2% rise in coronary flow at baseline but 67.1±8.1% after IR, (p<0.001 Fig 6D). This decline was absent in FA pre-treated 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) or after ischemia (placebo: 9.8±0.7 ml/min FA:8.9±1.4 ml/min, p=0.5) was similar between groups. Coronary flow rose similarly pre- and post-ischemia with sodium nitroprusside (Fig 6E), supporting endothelium dependence of the prior effect.

Discussion

This study demonstrates that pretreatment with high doses of oral folic acid markedly reduces ischemic dysfunction *during* coronary occlusion, and enhances function and diminishes infarct size following reperfusion. This was associated with better preserved high energy phosphates during ischemia despite 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 anti-oxidants and classical preconditioning agents which typically benefit the heart only after post-reperfusion. For that matter, the benefit of FA on reducing infarct size even when delivered after ischemia had commenced implies a different mechanism.

During normoxia, ATP synthesis is highly regulated and levels maintained. With ischemia, ATP supply from anaerobic glycolysis and HEP reserves such as phosphocreatine are insufficient to meet demand, and net ATP level fall. Theoretically, interventions that could maintain ATP even in ischemic myocardium should delay the advent of lethal injury because low tissue ATP-levels (< 5 μmoles/g dry weight) are associated with depressed high-energy phosphate re-synthesis, failure of cell volume regulation and irreversible myocardial injury. FA is a known to regulate mitochondrial function plays an important role as a methylation co-factor for the synthesis of thymidylate, purines, and methionine. With regard to purine synthesis, the metabolite N^{10}- formyl tetrahydrofolate contributes two 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 catabolized to oxypurines.

The present results support a role of FA in HEP generation. The increased basal level of oxypurines suggests that high dose of FA drove their synthesis (via IMP) by mass-action, but as HEP was adequate, this enhanced IMP catabolites with AMP levels unchanged. During ischemia, however, ATP and ADP levels fell, leading in controls to increased AMP and AMP catabolism (IMP, oxypurinols). In FA treated hearts, however, purine synthesis would be primed to help re-derive HEP (FA treated hearts had virtually no decline in ADP during ischemia, and ATP levels decline was about half). Actual ATP generation was probably higher since in FA treated hearts, since the anterior wall still actively contracted and was therefore utilizing more ATP despite LAD occlusion versus the controls. The concept of FA-enhanced HEP is supported by a recent study by Lamberts et al[22] who found chronic high dose FA restored total HEP (ATP+ADP+AMP) in hypertrophied rat hearts accompanied by improved diastolic function. Our finding that FA administered i.v. 10 minutes into the ischemic period

still diminished infarct size could also be explained by such a mass-action effect as this may occur quickly. This remains to be confirmed, but has intriguing therapeutic implications.

High dose FA may have potent anti-oxidant effects both directly and via weak but competitive inhibition of xanthine oxidase[13], and this could also benefit the heart during ischemic and post-ischemic periods. While our *in vitro* data showed similar antioxidant effects between 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 been previously shown to enhance HEP, rather, a decline in HEP is linked to mitochondrial damage and ROS generation[23]. Klawitter [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 as compared with untreated hearts. Tempol reduces post-reperfusion infarct size *in vivo* in rat and rabbit by ~50 and 33%, respectively[24], but only modestly improves LV pressure recovery post reperfusion[25], and 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 re-coupling 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]. BH4 plays a key role in maintaining normal eNOS coupling, and FA (or 5-MTHF) can increase BH4 levels by facilitating enzymatic reduction of its oxidized forms[31], and are associated with improved consequentially improved endothelial function[14]. The current study supports this mechanism based on the diminished eNOS uncoupling post-reperfusion, and *in vitro* analysis showing that addition of BH4 reduced ROS generation far more in untreated versus FA-treated myocardium. Improved NOS coupling could also contribute to improved HEP generation, as endogenous NO generation reduces oxygen consumption and improves energetic efficiency[32] partly by enhancing HEP synthesis itself.

This is the first study to test FA effects on *in vivo* ischemia and IR injury. Several recent studies have found FA and/or its active metabolite 5-methyltetrahydrofolate (5-MTHF) improves endothelial function[21,33], though the dose required has been somewhat controversial. Tawakol et al[34] used high dose folic acid (30mg p.o.) in patients with coronary artery disease and found it increased both adenosine-stimulated myocardial blood flow and flow reserve in segments with impaired dilator reserve, but also acutely lowered arterial pressure. The anti-necrotic effect of FA observed in the current study is consistent with earlier *in vitro* data in which high dose folate (>1 mM) 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[36]. One prior study[10] has examined BH4 effects 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 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[37] demonstrated that long-term folic acid treatment (~10 years) at a dose far higher than typically used (40-80 mg/day), reduced the incidence of myocardial infarction, angina pectoris and 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 0.8mg/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 if dose, study duration, target population, or other factors explain this. The doses of FA used in

the present study are far above those employed in prior cardiovascular interventional studies (5-25mg/70kg/d) or prevention trials (500 µg-1mg). Even taking into account that rodents have a higher metabolic turn-over, the present dose would likely correlate with between 0.25-2.5 g/70kg/d in a human. While 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. While FA pre-treatment improved HEP balance and function during a 30 minute coronary occlusion, it might become less effective if the ischemic period were more prolonged. It is also unknown how long after an occlusion can might still intervene with FA and see a benefit. Lastly, our data support but do not prove that FA directly helps re-derive HEP by mass action effects on purine synthesis. This will require future studies employing ^{14}C-labeled folate where the carbons can then be tracked and direct involvement tested.

In conclusion, we have found marked and novel evidence for improved ischemic and post-ischemic/reperfusion myocardial function and reduced infarction by pre-treatment 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 HEP are synthesized despite a decline in myocardial perfusion. This may relate to changes in purine synthesis, enhanced NOS coupling modulated by folate/BH4 interactions, and/or by 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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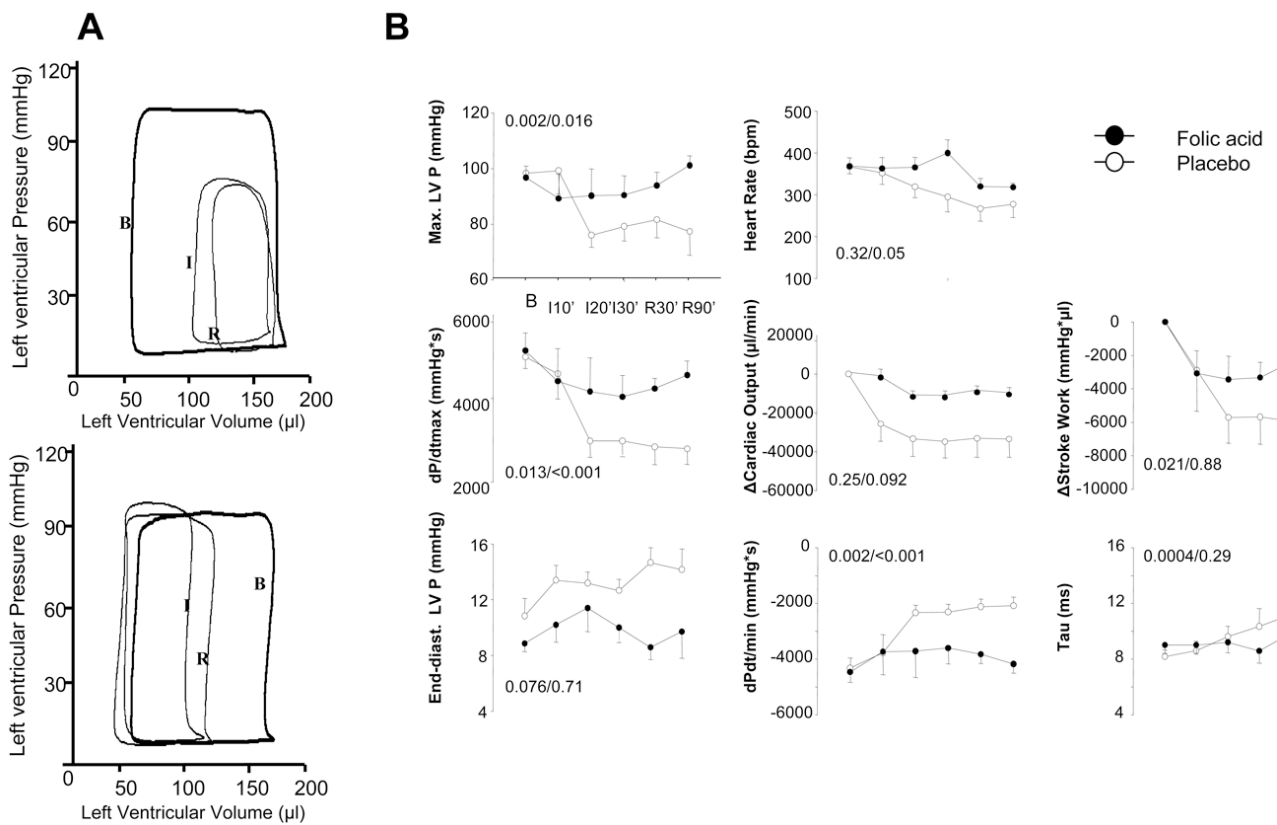
Figure 1. Cardiac function measured by pressure/volume-loop analysis
A] Example of pressure-volume loops at baseline (1), end of ischemia (2), and 90 min reperfusion (3) for a placebo (upper) and FA pre-treated (lower) animal. FA pre-treatment improved function both during ischemia and following reperfusion. **B**] Summary hemodynamics for the full IR protocol shows the time course for systolic and diastolic parameters. (p values are from repeated measures ANOVA, time × treatment interaction/ treatment effect).
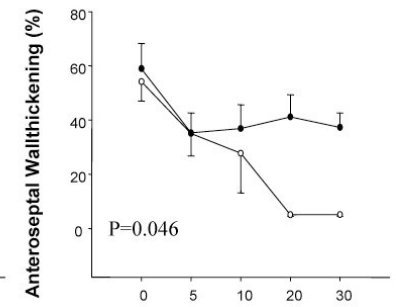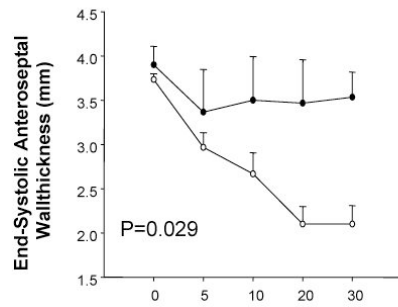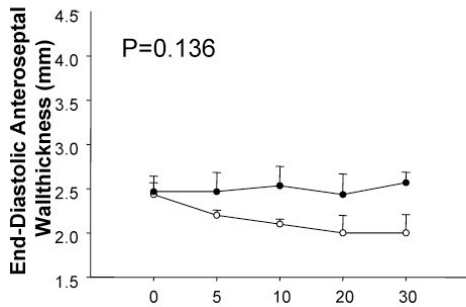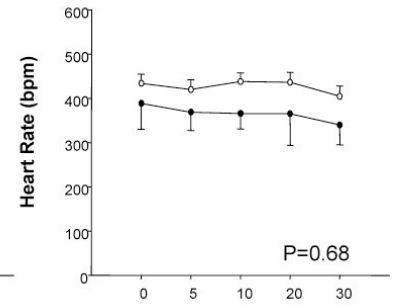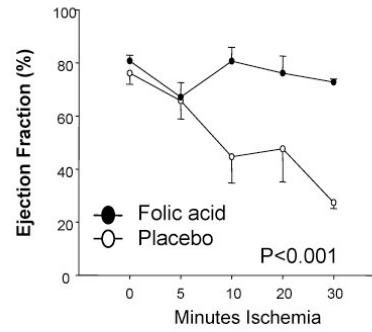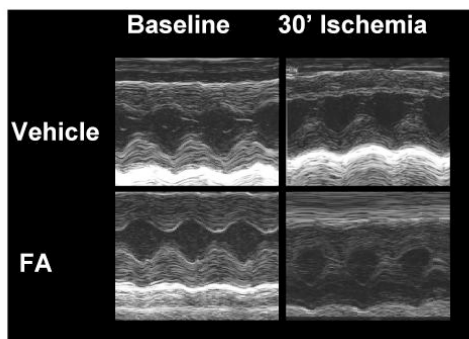
Figure 2. Open chest echocardiography
The upper left panel shows examples of M-mode echocardiography at baseline and after 30 min of ischemia in the placebo and FA-treated group. Summary plots show results obtained from these echocardiograms versus time. Ejection fraction and anterior-septal wall-thickening declined in the placebo group but were unchanged in the FA-group (p-values are for treatment effect by RMANOVA).
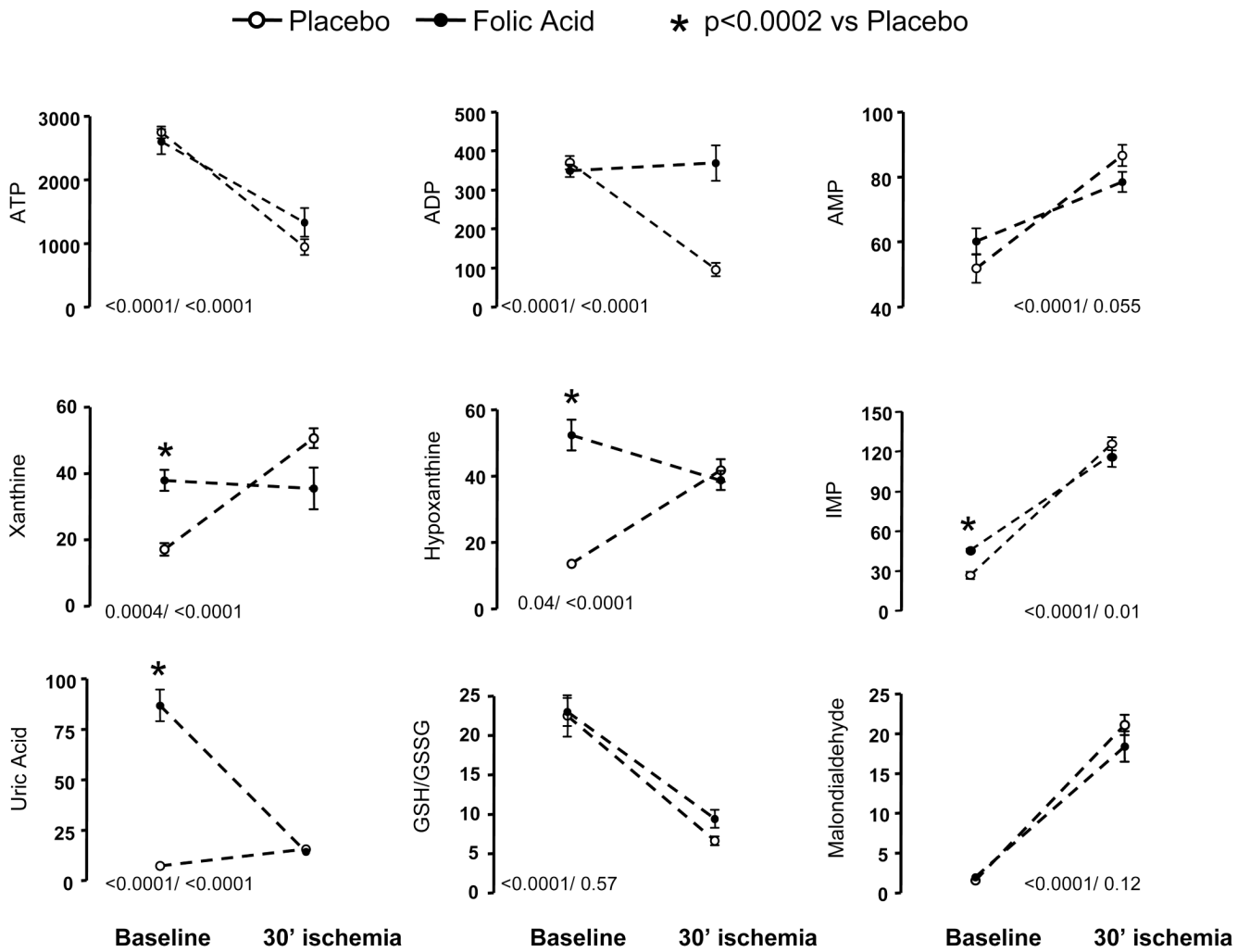
Figure 3. HPLC-analysis of parameters of high energy phosphate (HEP) metabolism and redox-state

Under baseline conditions, FA pre-treatment did not alter HEP (ATP, ADP, AMP), but did increase inositol monophosphate (IMP) and its catabolites (oxypurines: xanthine, hypoxanthine and uric acid). After 30 minutes of ischemia, the levels of HEP were better maintained in the FA-treated hearts compared with placebo animals. While oxypurines rose markedly after ischemia in placebo-treated animals, this was not observed in the FA-treated rats. P-values are from a 2-way ANOVA, with the first value indicating the effect of ischemia, and the second the interaction between treatment group and ischemia.
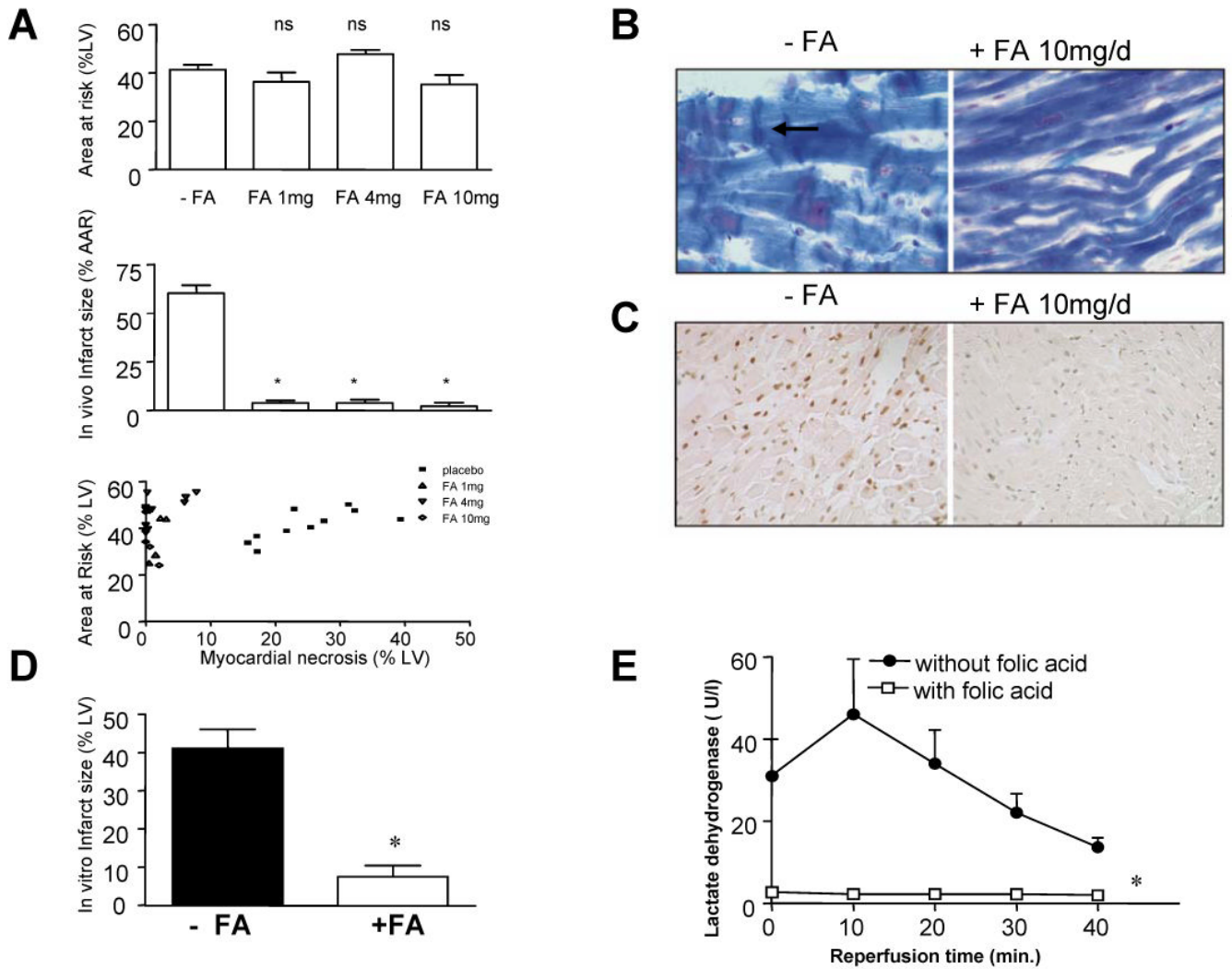
Figure 4. Effect of folic acid on myocardial necrosis
A] Oral (7 days) FA pre-treatment reduced infarct size *in vivo* (*: p<0.001; Mann-Whitney).
Upper panel: Area at risk (AAR) was comparable between all groups. Middle panel: Infarct
size, expressed as % AAR was significantly reduced in all FA-groups. Lower panel: plot-graph
of the individual correlation between myocardial necrosis and AAR. **B]** FA pre-treatment *in
vivo* reduces contraction band necrosis (*p=0.001). **C]** FA pretreatment *in vivo* reduces
apoptosis (TUNEL-staining) (*p=0.005 and p=0.001). **D]** FA pre-treatment reduced
myocardial necrosis by ~80% in hearts studied *in vitro*. (*: p< 0.0001). **E]** Increased lactate
dehydrogenase (LDH) after ischemia-reperfusion peaked at 10 – 20′, whereas hearts receiving
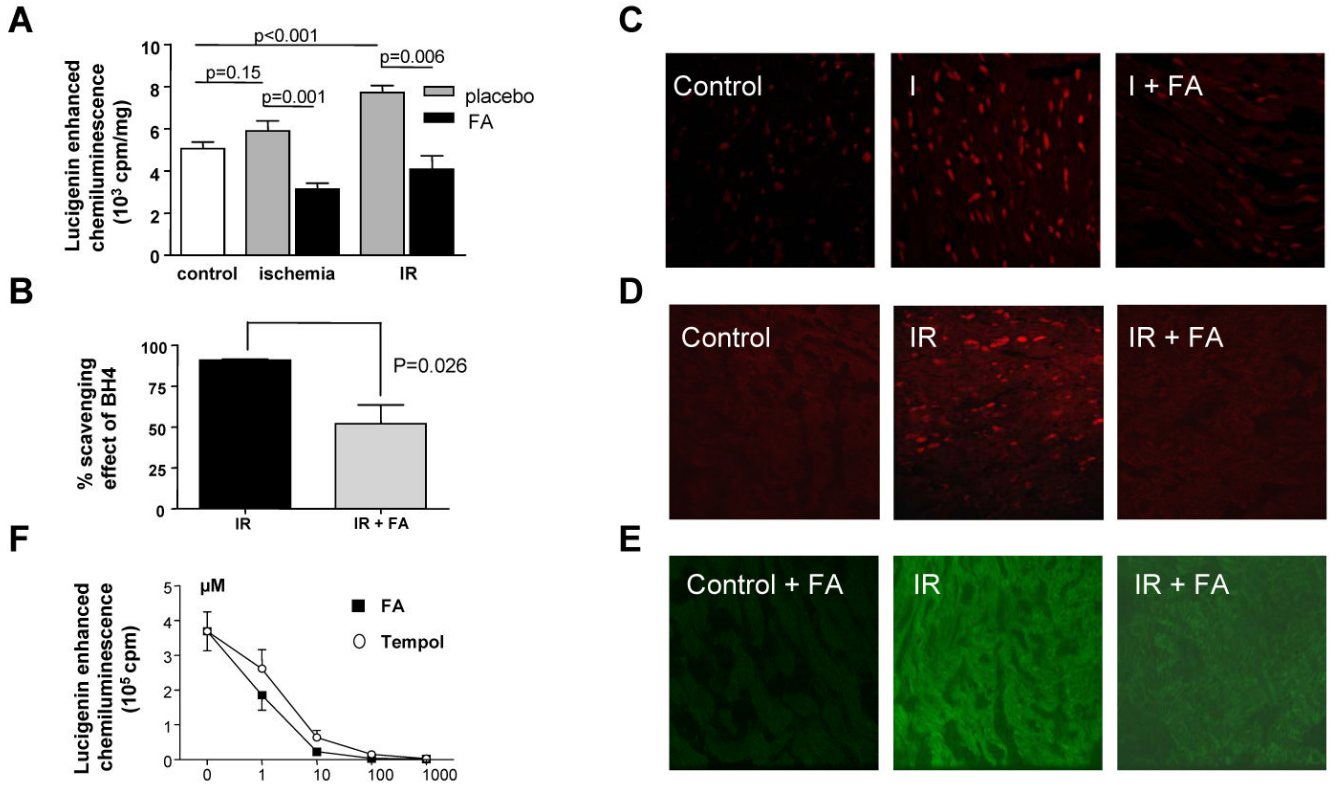FA pre-treatment had minimal LDH release. (p<0.001).

Figure 5. Effects of FA treatment on ROS generation
A] Lucigenin-enhanced superoxide detection shows marked reduction of ischemia and IR-induced superoxide with FA pre-treatment. **B**] O_2^- formation in control-vehicle treated IR hearts was markedly suppressed by acute addition of BH_4, whereas this was blunted by nearly half in myocardial extracts obtained from FA-pre-treated hearts. **C-D**] DHE and **E**] DCF stained myocardium reveal increased ROS generation in ischemic or IR myocardium that was substantially reduced in heart receiving FA pre-treatment. **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 varying added concentrations of either FA or tempol.
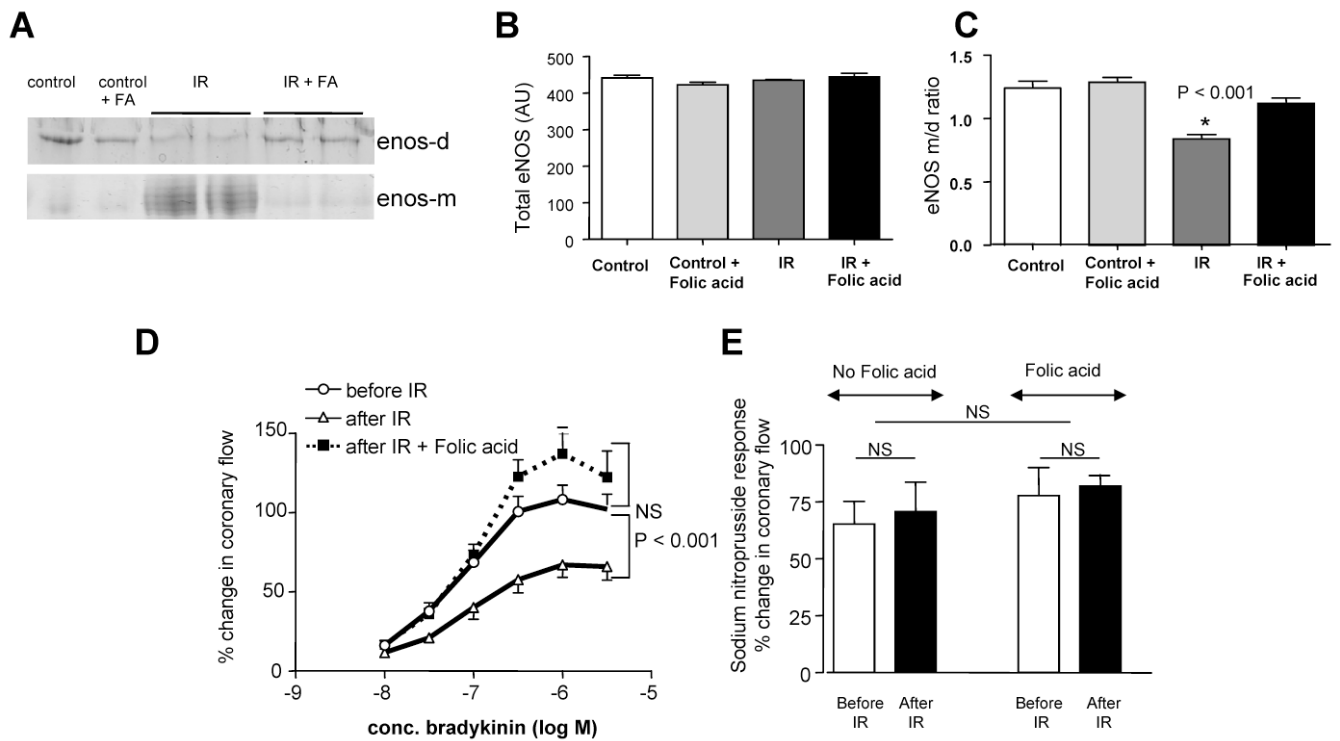
Figure 6. Effect of FA pre-treatment on NO-pathway
A] SDS-Page gel shows increased eNOS monomer in IR myocardium that was reduced towards control levels by FA pre-treatment. **B-C]** Summary densitometry data of gel analysis as shown in panel A. FA pre-treatment reduced the monomer/dimmer ratio, but had no effect on total eNOS protein expression. **D]** Bradykinin administered to hearts in vitro before versus after IR showed a marked decline in endothelium-dependent flow response. This was restored to a normal response by pre-treating with FA. **E]** FA treatment did not influence the flow-response to sodium nitroprusside, supporting that the disparity observed with bradykinin was endothelium-dependent.