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Original article

Mechanistic insights into folic acid-dependent vascular protection: Dihydrofolate reductase (DHFR)-mediated reduction in oxidant stress in endothelial cells and angiotensin II-infused mice: A novel HPLC-based fluorescent assay for DHFR activity

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ABSTRACT

Folate supplementation improves endothelial function in patients with hyperhomocysteinemia. Mechanistic insights into potential benefits of folate on vascular function in general population however, remain mysterious. Expression of dihydrofolate reductase (DHFR) was markedly increased by folic acid (FA, 50 μmol/L, 24 h) treatment in endothelial cells. Tetrahydrofolate (THF) is formed after incubation of purified DHFR or cellular extracts with 50 μmol/L of substrate dihydrofolic acid. THF could then be detected and quantified by high performance liquid chromatography (HPLC) with a fluorescent detector (295/365 nm). Using this novel and sensitive assay, we found that DHFR activity was significantly increased by FA. Furthermore, FA improved redox status of Ang II treated cells by increasing H_4B and $NO^{\bullet}$ bioavailability while decreasing superoxide ($O_2^{\bullet-}$) production. It however failed to restore $NO^{\bullet}$ levels in DHFR siRNA-transfected or methotrexate pre-treated cells, implicating a specific and intermediate role of DHFR. In mice orally administrated with FA (15 mg/kg/day, 16 days), endothelial upregulation of DHFR expression and activity occurred in correspondence to improved $NO^{\bullet}$ and H_4B bioavailability, and this was highly effective in reducing Ang II infusion (0.7 mg/kg/day, 14 days)-stimulated aortic $O_2^{\bullet-}$ production. 5′-methyltetrahydrofolate (5′-MTHF) levels, GTPCH1 expression and activity remained unchanged in response to FA or Ang II treatment *in vitro* and *in vivo*. FA supplementation improves endothelial $NO^{\bullet}$ bioavailability via upregulation of DHFR expression and activity, and protects endothelial cells from Ang II-provoked oxidant stress both *in vitro* and *in vivo*. These observations likely represent a novel mechanism (intermediate role of DHFR) whereby FA induces vascular protection.

1. Introduction

The most widely known therapeutic function of folic acid (FA) is to prevent birth defect (primarily neural tube defects), based on its role in neonatal development [1,2]. Its other major therapeutic function is to treat patients with hyperhomocysteinemia [3]. FA is required for the remethylation of homocysteine to form methionine, thus reducing the homocysteine level in the plasma. Oral supplementation of FA effectively decreases plasma homocysteine levels [3]. Both hyperhomocysteinemia and hyperhomocysteinuria have been shown to be independent risk factors for atherosclerotic vascular diseases [4–10]. One of the molecular mechanisms responsible for hyperhomocysteinemia-induced disease is increased production of reactive oxygen species and consequent endothelial dysfunction [11,12]. It is however unclear whether FA, besides reducing homocysteine levels metabolically, modulates endothelial function directly in subjects with or without hyperhomocysteinemia. It is also unclear whether FA treatment of hyperhomocysteinemic patients is beneficial in disease progression and prognosis.

As a FDA-approved agent, FA or combined vitamin therapy has been investigated for its role in cardiovascular therapeutics in various clinical conditions, and the outcomes (myocardial infarction, stroke, thromboembolic events and mortality) have remained controversial [5,13–15]. However, it is worth noting that the larger trials (i.e. HOST [14], VISP [16], HOPE-2 [17]) were conducted in subjects with advanced atherosclerotic vascular diseases where disease regression might be more difficult. Even though, a significant reduction was observed in stroke morbidity with FA treatment after removal of the HOST trial [14,18] (subjects with renal failure) based on meta-analysis [5,19–21]. Therefore, FA supplementation can potentially benefit patients with vascular diseases, although the role of homocysteine during the course of interventions is unclear [22]. More interestingly, FA supplementation was demonstrated to improve endothelial function in subjects without elevated homocysteine levels, indicating

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an independent effect [22]. Whether this is attributed to direct nitric oxide ($NO^{\bullet}$) production and/or reduced oxidant stress, as well as the potential underlying mechanisms, remains to be fully elucidated.

Accumulating evidence has established that a deficiency in endothelial nitric oxide synthase (eNOS) cofactor tetrahydrobiopterin (H_4B) causes eNOS to produce superoxide ($O_2^{\bullet-}$) rather than $NO^{\bullet}$, resulting in eNOS uncoupling and increased oxidant stress [23,24]. This change in eNOS enzymatic function is independent of gene regulation. Under pathological conditions such as diabetes and ischemic renal dysfunction where angiotensin II (Ang II) levels are elevated [23], we and others have shown that a deficiency in H_4B salvage enzyme dihydrofolate reductase (DHFR) is responsible for reduced H_4B and $NO^{\bullet}$ bioavailability [23,25–27]. Therefore in the present study we examined FA regulation of DHFR protein expression and activity, and DHFR-mediated changes in $NO^{\bullet}$ and $O_2^{\bullet-}$ productions in cultured endothelial cells and Ang II-infused mice aortas.

A sensitive HPLC-based activity assay for DHFR was first established. Endothelial cells were exposed to Ang II in the presence or absence of FA, followed by detection of $NO^{\bullet}$ and $O_2^{\bullet-}$ production specifically and quantitatively using electron spin resonance (ESR). Basal FA regulation of DHFR expression and activity were also analyzed. In Ang II-infused mice, oral administration of FA was examined for its effects on aortic productions of $NO^{\bullet}$ and $O_2^{\bullet-}$. Overall FA potently improved $NO^{\bullet}$ bioavailability, intracellular H_4B content while reducing oxidant stress both *in vitro* and *in vivo*. These responses were found dependent on an upregulation in DHFR. These data demonstrate an innovative mechanism whereby FA may protect against cardiovascular disorders in a general population.

2. Materials and methods

2.1. Materials

Monoclonal antibodies for DHFR and eNOS were purchased from Research Diagnostics (Flanders, NJ) and BD Transduction Laboratories (San Jose, CA) respectively. Tetrahydrofolate, FA, dihydrofolic acid, recombinant DHFR, NADPH and all other reagents were purchased from Sigma-Aldrich in highest purity (St. Louis, USA).

2.2. Cell culture, cell treatments and $NO^{\bullet}$ detection

Bovine aortic endothelial cells (BAECs, Cell Systems, Kirkland, WA) were grown in media 199 containing 10% fetal bovine serum (FBS) until confluence, and quiescent with 5% FBS media. Confluent cells were exposed to FA (50 μmol/L) or Ang II (100 nmol/L) or combination of these two for 24 h, (FA pretreated for 30 min), then rinsed with modified Kreb's/HEPES buffer, incubated with freshly prepared $NO^{\bullet}$-specific spin trap $Fe^{2+}(DETC)_2$ colloid (0.5 mmol/L) for 60 min. Gently collected suspensions of cells were snap-frozen in liquid nitrogen and loaded into a finger Dewar for analysis with an eScan ESR spectrophotometer (Bruker) at the following settings: center field, 3410; field sweep, 100 G; microwave frequency, 9.76 GHz; microwave power, 13.26 mW; modulation amplitude, 9.82 G; 10240 points of resolution; and receiver gain, 356. The aortic $NO^{\bullet}$ production was measured as previously described [25].

2.3. Immunoblot analysis

Cells were lysed with lysis buffer (0.2 mol/L Tris, 1.5 mol/L NaCl, 10 mmol/L EDTA, 10 mmol/L EGTA, 25 mmol/L sodium pyrophosphate, 10 mmol/L β-glycerophosphate, 10 mmol/L Na_3VO_4, 1 mmol/L PMSF, 2 μmol/L leupeptin and 10% Triton, pH 7.4) for 1 h. The samples were then prepared for Western analysis. Forty μg of protein was separated by 10% SDS PAGE, transferred to nitrocellulose membranes, and probed with DHFR (1:1000) or eNOS (1:1000) antibody following standard immunoblotting procedure.

2.4. HPLC-based H_4B measurement

Endothelial cells were lysed using trichloroacetic acid containing 10 mmol/L DTT. Lysates were subjected to differential oxidation in acidic (0.2 mol/L trichloroacetic acid containing 2.5% I_2/10% KI) or alkalytic (0.1 mol/L NaOH containing 0.9% I_2/1.8% KI) solutions as briefly described previously [25]. After centrifugation, 20 μl of supernatant was injected into a HPLC system. Excitation and emission wavelengths of 350 nm and 450 nm were used to detect fluorescent H_4B and its oxidized species.

2.5. Determination of GTPCH1 mRNA expression

Cellular GTPCH1 mRNA was extracted using RNeasy kit from Qiagen (Huntsville, AL) according to manufacturer instruction. The cDNA was synthesized with script III from Invitrogen (Carlsbad CA) and Real-time PCR was performed on a Bio-Rad iCYCLER iQ real-time PCR machine with Cybergreen supermix (Bio-Rad CA) as previously described [28].

2.6. Detection of GTPCH1 activity

Aortic segments were powdered under liquid nitrogen using mortar and pestle. To access GTPCH1 activity, tissue powders or cell pellets were lysed and incubated with GTP (0.75 mmol/L final concentration) at 37 °C for 90 min in the dark. The reaction product was oxidized to neopterin triphosphate by acidic iodine (1% I2 and 2% KI in 2 mol/L TCA). After reduction of excessive iodine by ascorbic acid (2%) and adjustment of pH to 7.0, reaction products were monitored by HPLC with fluorescent detection (365/446 nm). The detailed chromatographic procedure has been described elsewhere [29].

2.7. Determination of 5′-MTHF levels

Tissue powders or cell pellets were dissolved/re-suspended in ascorbic acid (57 mmol/L) solution. Samples were repeatedly frozen and thawed or sonicated to break cell membranes and then vortexed and incubated at 37 °C for 60 min. Samples were diluted with buffer containing 0.2 mol/L potassium phosphate dibasic and 30 mmol/L mercaptoethanol, (pH 8.5) and heated at 100 °C for 10 min. After being vortexed for 30 s and centrifuged at 10,000 *g* for 15 min, the supernatants were collected and analyzed using HPLC as previously described [30]. Briefly, the 5′-MTHF signals were monitored by fluorescent detection (295/360 nm) under the mobile phase of methanol:0.6% acetic acid in water (14:86, v/v) at a flow-rate of 1 ml/min. The quantitative levels of 5′-MTHF were calculated based on a calibration curve established using known concentrations of 5′-MTHF going through the same preparation and detection procedures.

2.8. Oral administration of FA in Ang II-infused mice

The C57BL/6J mice from Jackson Laboratories (6–9 weeks old, total 64) were separated into four groups. Two were put on standard mice chow (Harlan) and two on FA-rich (15 mg/kg/day) chow. On average, mice weighed 25 g and consumed 3.5 g of diet daily. To prepare FA-rich chow, standard mouse chow was grounded, mixed well with FA, and food tablets produced by a customerized press machine. All mice were fed ad libitum and tablets with FA were replaced every two days. Half of mice on each diet were then anesthetized and osmotic pumps (Durect corporation) containing Ang II (0.7 mg/kg/day, Sigma) were implanted subcutaneously after anesthetization. Two weeks later mice aortas were isolated and sectioned. Sections from well-defined regions, 1 mm below subclavian artery, were used for ESR analysis of

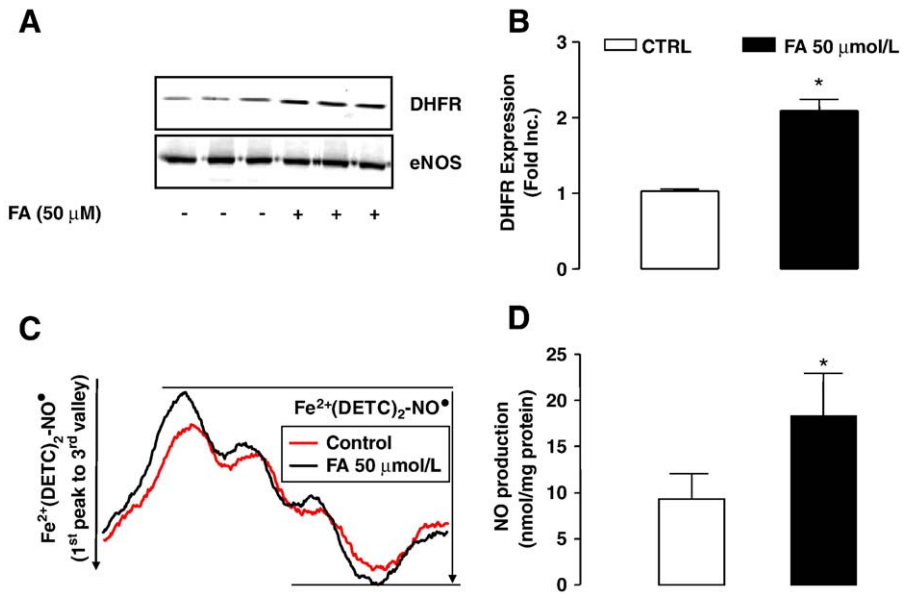

Fig. 1. Effects of folic acid on endothelial DHFR expression and NO• production. (A) Representative Western blot of on DHFR expression in FA (50 μmol/L, 24 h)-treated endothelial cells; (B) Densitometric analysis of the immunoblots from A; (C) Representative spectra of NO• production in FA (50 μmol/L, 24 h)-treated endothelial cells; (D) Grouped densitometric data of NO• production. *$p<0.05$ vs. Ctrl.

$O_2^{\bullet-}$ production. The rest of the aorta was used for ESR measurement of NO• and Western analysis of eNOS and DHFR expression. The procedures were carried out based on protocols approved by the UCLA Institutional Animal Care and Use Committee (IACUC).

2.9. ESR detection of aortic superoxide production

As previously described [25], aortic segments (~3 mm) were mixed with freshly prepared spin trap (CMH, 0.5 mmol/L) in the presence or absence of 100 U/ml polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), and loaded to glass capillaries for immediate kinetic analysis of $O_2^{\bullet-}$ production. The ESR settings were as the followings: biofield, 3494.50; field sweep, 9 G; microwave frequency, 9.75 GHz; microwave power, 21.02 mW; modulation amplitude, 2.47 G; 4096 points of resolution; receiver gain, 1000; and kinetic time, 10 min. The PEG-SOD inhibitable $O_2^{\bullet-}$ signals at 10 min time point, normalized by protein concentrations, were compared among different experimental groups.

2.10. EC-washout and Western blotting

To determine changes in DHFR expression and activity in the mouse endothelium, an additional group of 32 mice were studied as described above. Upon harvest, whole aorta was cut open

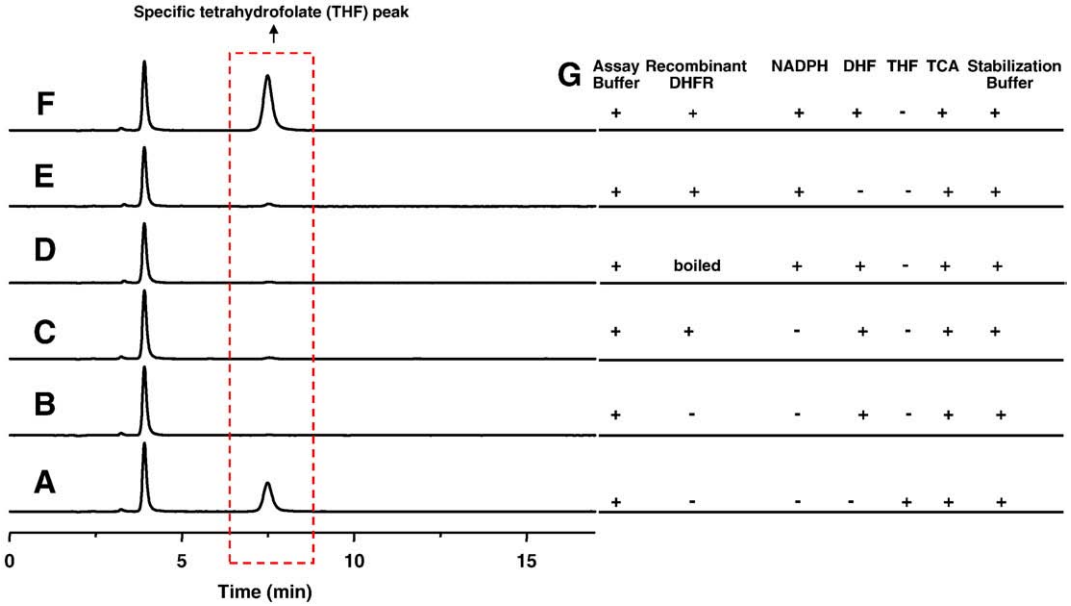

Fig. 2. Specific tetrahydrofolate (THF) peak in the HPLC-based DHFR activity assay. (A) 50 nmol/L of tetrahydrofolate in assay buffer; (B) 50 μmol/L of dihydrofolate in assay buffer (no enzyme and NADPH); (C) recombinant DHFR with 50 μmol/L of dihydrofolate in assay buffer (no NADPH); (D) boiled recombinant DHFR with 50 μmol/L of dihydrofolate and 200 μmol/L of NADPH in assay buffer (inactivated enzyme); (E) recombinant DHFR with 200 μmol/L of NADPH in assay buffer (no substrate); (F) recombinant DHFR with 50 μmol/L of dihydrofolate and 200 μmol/L of NADPH in assay buffer; (G) Sample constituents.

longitudinally and digested with collagenase (0.6 mg/ml) for 20 min at 37 °C. The supernatant was centrifuged and the endothelial cells were collected from the pellet. Cells were lysed and 40 μg of protein separated by 10% SDS/PAGE, transferred to nitrocellulose membranes, and probed with DHFR (1:250) or eNOS (1:1000) antibody respectively. The endothelium-denuded vessels were also homogenated and subjected to Western analysis of eNOS and DHFR following identical procedure.

2.11. Statistics

Data are presented as Means ± SEM from independent experiments. Differences between control and treatment groups were compared by ANOVA and considered significant when $p<0.05$.

3. Results

3.1. Folic acid upregulates endothelial DHFR expression and $NO^{\bullet}$ production

Post confluent bovine aortic endothelial cells were treated with folic acid (FA, 50 μmol/L, 24 h) prior to Western analysis of DHFR and eNOS protein expressions. As shown by representative blot and grouped densitometric data, FA supplementation resulted in a significant upregulation of DHFR while not affecting eNOS expression (Figs. 1A and B). Of note, endothelial cell production of $NO^{\bullet}$ was also increased (Figs. 1C and D).

Table 1
Recovery of tetrahydrofolate in assay buffer with cell lysates.

Concentration (nmol/L)	Recovery (mean ± S.D.)	R.S.D. (%)
30	95.4 ± 1.8	1.9
50	96.1 ± 2.8	2.9
75	98.4 ± 1.8	1.8

3.2. Establishment of a novel HPLC-based assay for DHFR activity

In order to determine specifically DHFR activity from cells, a sensitive HPLC-based assay was established. Purified recombinant DHFR or cell lysates were incubated with dihydrofolate (50 μmol/L) and NADPH (200 μmol/L) for 20 min at 37 °C, in a 0.1 mol/L potassium phosphate assay buffer (pH 7.4) containing 1 mmol/L DTT, 0.5 mmol/L KCl, 1 mmol/L EDTA and 20 mmol/L sodium ascorbate. At the end of the incubation, the reaction was terminated by addition of 0.2 mol/L trichloroacetic acid, and then product was stabilized by addition of stabilization solution (200 mg of sodium ascorbate and 30 mg of DTT in 1 ml of water, 1:10 dilution for the final working solution).

The product, tetrahydrofolate/tetrahydrofolic acid (THF), was measured using a Shimadzu HPLC system consisted of C-18 column (250- 4.6-mm C18 column Alltech, Deerfield) and fluorescent detector, at wavelengths of 295 nm for excitation and 365 nm for emission. The mobile phase was isocratic flow 1 ml/min of mixture of 7% acetonitrile with 5 mmol/L KH_2PO_4; pH 2.3. The retention time of tetrahydrofolic acid was at about 7.5 min. The specific THF peaks, under different assay conditions, were highlighted in dotted frame in

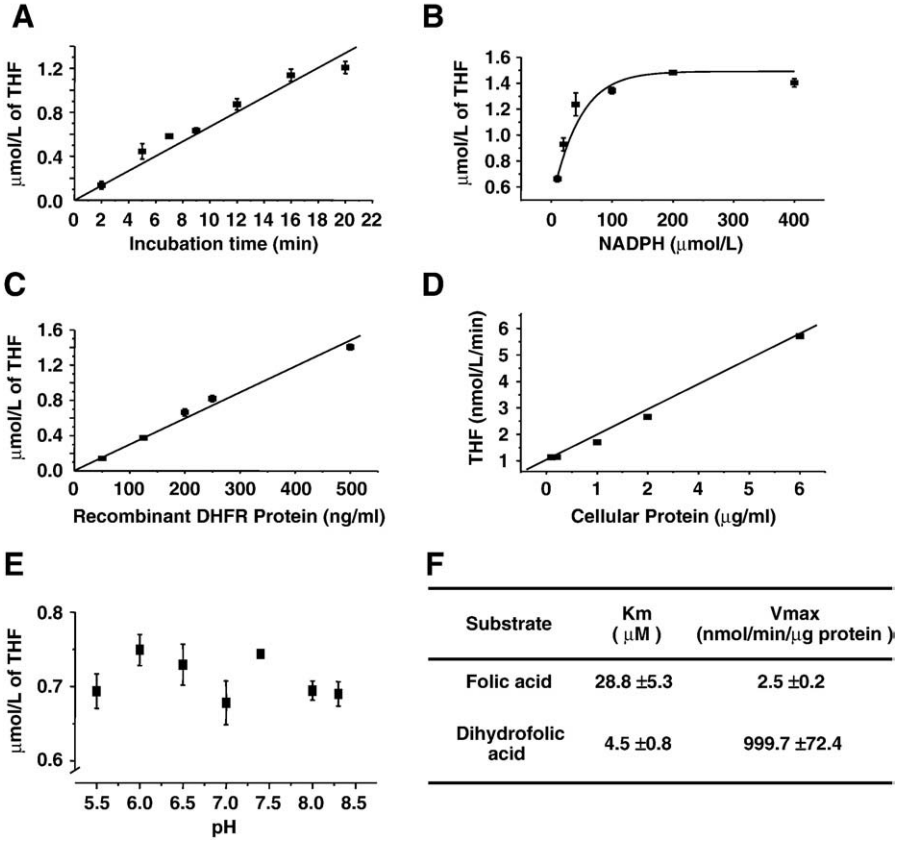

Fig. 3. Optimization of the assay reaction for DHFR activity. DHFR activity is expressed as amount of tetrahydrofolate (THF) produced after 20 min incubation with 200 μmol/L NADPH, 500 ng/ml of recombinant DHFR and 80 μmol/L of dihydrofolic acid at pH 7.4. (A) The THF production was examined after 2, 4, 6, 8, 10, 12, 16 and 20 min incubation while the rest of the assay condition remained the same as above; (B) THF production was examined at different concentrations of NADPH; (C) THF production was examined at different concentrations of recombinant DHFR; (D) THF production was examined at different protein concentration of bovine endothelial cell lysates; (E) THF production was examined at different pH; (F) Km and Vmax of recombinant DHFR from the optimized assay reaction were calculated using Program Hyper 22 for hyperbolic regression analysis of enzyme kinetic data (http://homepage.ntlworld.com/john.easterby/software.html).

Table 2
Intra- and Inter-day precision and accuracy of tetrahydrofolate in assay buffer.

Concentration (nmol/L)	Inter-day precision ($n=9$)		Intra-day precision ($n=3$)	
	R.S.D. (%)	RE (%)	R.S.D. (%)	RE (%)
30	5.4	−3.2	3.5	−1.8
50	1.9	0.9	0.8	2.5
75	2.5	−1.5	2.5	−2.0

Fig. 2. In Fig. 2, tracing A shows a standard peak of THF (50 nmol/L) in assay buffer. Tracing B represents the reaction containing only substrate dihydrofolic acid, indicating that THF peak was unique, without overlapping with the dihydrofolic acid peak. Tracing C and E demonstrate that when NADPH or substrate was missing from the reaction, no THF was produced. Tracing D indicates that an inactive enzyme (boiled to inactivate) also failed to generate a specific THF peak. Only tracing F shows a characteristic THF peak when recombinant enzyme was mixed with proper concentrations of dihydrofolic acid and NADPH in the right assay buffer. The constituents of each tracing are shown on Fig. 2G. The THF content was calculated against a standard curve prepared by using THF solutions in assay buffer. Data are presented as nmol production of THF.

3.3. Optimization of the DHFR activity assay

As shown in Fig. 3A, THF formation was linearly correlated with time within 20 min incubation. A dose-dependent effect of electron donor NADPH (maximized at >200 μmol/L) on THF formation was observed (Fig. 3B). In addition, formation of THF was linearly correlated with protein content either recombinant protein or BAEC lysates (Figs. 3C and D). Using purified DHFR and dihydrofolic acid as the substrate, formation of THF at different pH was compared and pH 7.4–7.5 was found to be the optimal for reaction (Fig. 3E). The Km of the enzyme was calculated to be 4.5 ± 0.8 μmol/L and the Vmax for the enzyme to be 999.7 ± 74.2 nmol/min/μg protein (Fig. 3F). Folic acid, when serving as a much weaker substrate, showed a Km of 28.8 ± 5.3 μmol/L and the Vmax for the enzyme is 2.5 ± 0.2 nmol/min/μg protein.

3.4. Validation of the DHFR activity assay

The validation parameters were specificity, linearity, extraction recovery, precision and accuracy. Blank cell lysates and pure tetrahydrofolate were screened to determine the specificity (see Fig. 2). Linearity was tested on three different sets of calibration curves. The lowest concentration of the linear regression defined the lower limit of qualification (LLOQ). The LLOQ for tetrahydrobiopterin was 25 nmol/L at a signal-to-noise (S/N) ratio of 5 (highest sensitivity, ×16 gain for HPLC setting).

The extraction recovery of tetrahydrofolate was assessed at different concentrations of tetrahydrofolate, and calculated by comparing the peak area of tetrahydrofolate in assay buffer containing cell lysates to the peak area obtained with the same concentration of tetrahydrofolate in assay buffer. The mean recovery rate of tetrahydrobiopterin at low, middle and high concentrations (30, 50, 75 nmol/L) was greater than 95% (Table 1). The mean regression equation weighed by concentration (nM) was $y = (331400 \pm 15010)\,x - (1126000 \pm 853400)$ ($r^2 = 0.9959$).

Intra-day assay accuracy and precision of quality controls were measured using three determinations per concentration at the same day. Inter-day assay accuracy and precision of quality control were measured using three determinations per concentration on three consecutive days. The data of accuracy and precision are represented by relative standard deviation (R.S.D.) and relative error (RE) as shown in Table 2.

3.5. Folic acid improves endothelial nitric oxide bioavailability via DHFR

As shown in Fig. 4A, Ang II decreased DHFR expression in endothelial cells which is consistent with our previous findings [23]. FA supplementation not only increased DHFR expression but also preserved DHFR expression from being attenuated by Ang II (Fig. 4A).

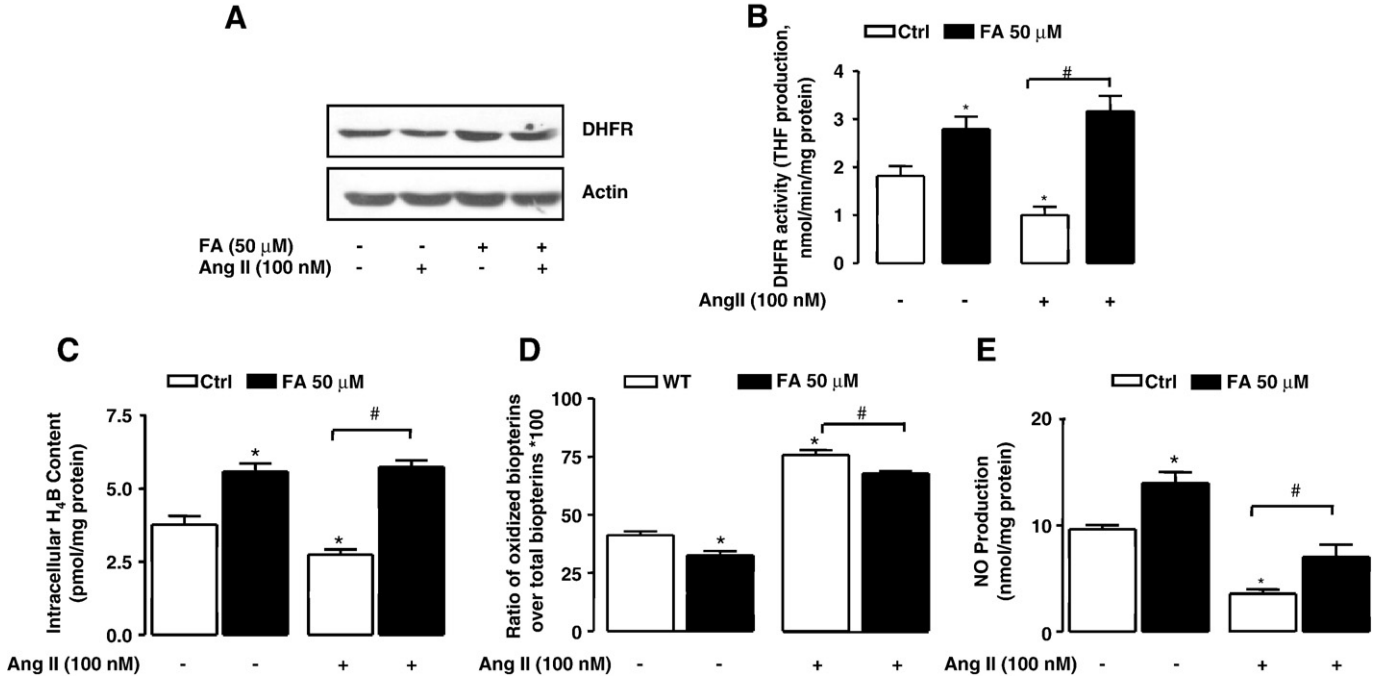

Fig. 4. Effects of folic acid pre-incubation on Ang II induced changes in NO•, H_4B availability and DHFR expression/activity. (A) Effect of FA on Ang II induced changes in DHFR expression; (B) Effect of FA on Ang II induced changes in DHFR activity; (C) Effect of FA on Ang II induced changes in H_4B bioavailability; (D) Effects of FA on the ratio of oxidized biopterins over total pterins; (E) Effects of FA on Ang II induced NO• production. $p<0.05$ vs. control, #$p<0.05$ vs. Ang II alone.

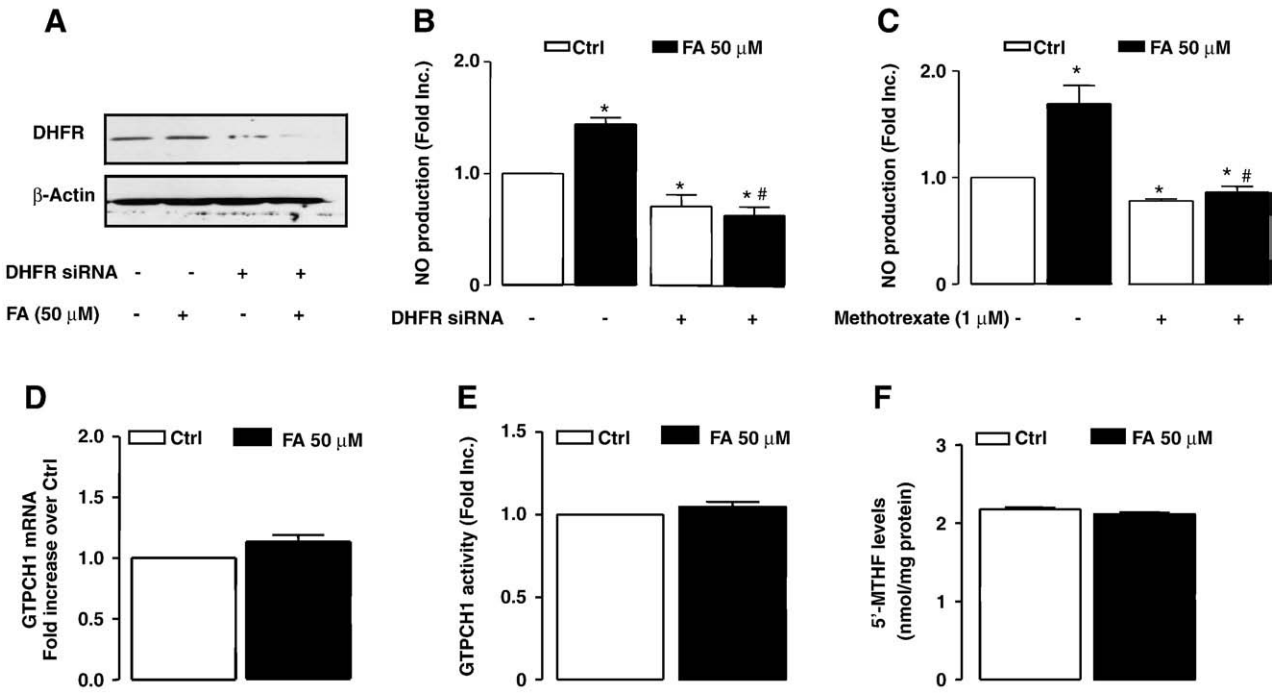

Fig. 5. Effect of DHFR inhibition on folic acid induced NO• production. (A) DHFR siRNA on FA-induced DHFR-upregulation; (B) DHFR siRNA on FA-induced NO• production; (C) Methotrexate on FA-induced NO• production; (D) FA treatment on GTPCH1 mRNA expression; (E) FA treatment on GTPCH1 activity; (F) FA treatment on intracellular 5′-MTHF level. *$p<0.05$ vs. control, #$p<0.05$ vs. FA alone.

Similarly, DHFR activity was downregulated by Ang II but upregulated/restored by FA (Fig. 4B). The upregulation in DHFR expression and activity may increase H_4B level via augmented salvation of H_2B. Indeed, the ratio of oxidized H_4B (H_2B and other intermediates) over total pterins was decreased in FA treated cells. Moreover, FA treatment prevented H_4B from being oxidized by Ang II (Figs. 4C and D). Meanwhile, bioavailable NO• was decreased by Ang II treatment, but markedly improved by FA pre-treatment (Fig. 4E).

In additional experiments, endothelial cells were transfected with control and DHFR siRNA prior to FA stimulation. As shown in Fig. 5A, DHFR siRNA efficiently attenuated DHFR protein expression. Although FA increased NO• production in control siRNA-transfected cells, it failed to provoke any changes in NO• level in DHFR siRNA-transfected cells, indicating a specific and intermediate role of DHFR in FA-induced augmentation of NO• production (Fig. 5B). Moreover, pre-inhibition of DHFR with methotrexate (1 μmol/L) also attenuated the

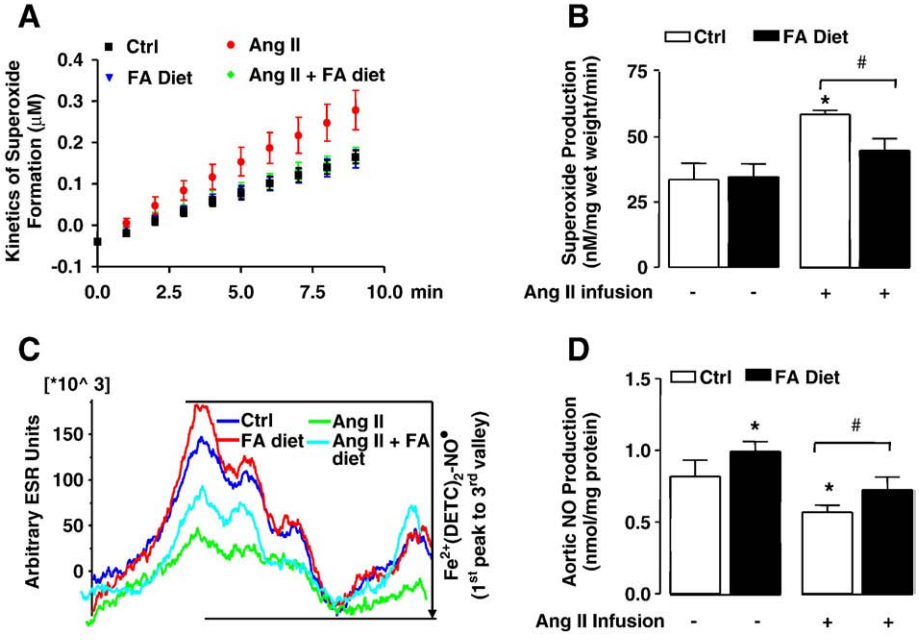

Fig. 6. Effect of oral folic acid supplementation on aortic NO• and $O_2^{\bullet-}$ production in Ang II-infused mice. (A) Kinetic assay of aortic superoxide ($O_2^{\bullet-}$) production in Ang II-infused mice with or without FA supplementation; (B) Group analysis of aortic $O_2^{\bullet-}$ production from A; (C) Representative spectra of NO• production in Ang II-infused mice with or without FA supplementation; (D) Group analysis of NO• production from C. *$p<0.05$ vs. control; #$p<0.05$ vs. Ang II alone.

beneficial effects of FA on endothelial NO• bioavailability following Ang II stimulation (Fig. 5C). GTPCH1, the rate limiting enzyme for H_4B *de novo* synthetic pathway were also examined for its expression and activity. As shown in Figs. 5D and E, both GTPCH1 mRNA level and activity were not changed by FA treatment. 5′-methyltetrahydrofolate (5′-MTHF), a bioactive metabolite of FA which may have similar benefits on eNOS function and mediates FA'S effect on homocysteine [31], also remained unchanged.

3.6. Folic acid increases NO• bioavailability while reducing $O_2^{\bullet-}$ production in angiotensin II-infused mice—role of DHFR

To examine whether FA would exert beneficial effects on vascular endothelial function *in vivo*, control or Ang II-infused mice were on FA-rich diet for 2 weeks. Aortas were then harvested and a 3 mm section of descending aorta was subjected to $O_2^{\bullet-}$ detection immediately using ESR. As shown by representative kinetic curves, and quantitative data for $O_2^{\bullet-}$ production at the end of 10 min, Ang II-dependent $O_2^{\bullet-}$ was completely attenuated by FA treatment (Figs. 6A and B). After $O_2^{\bullet-}$ measurement, the rest of the aortas were subjected to NO• detection by ESR. FA alone increased NO• production by 40% in control mice (Figs. 6C and D). Whereas aortic NO• production was markedly reduced by Ang II infusion, it was restored to near control level by oral administration with FA (Figs. 6C and D).

In additional experiments, DHFR expression was examined using intact or endothelium-denuded aortas. Of note, eNOS was completely absent in the denuded vessels (Fig. 7A) whereas its expression was abundant in the endothelial cells but downregulated by Ang II (Fig. 7A). Oral administration of FA significantly improved endothelial DHFR expression in untreated control mice, as shown by both representative blots and quantitative data (Figs. 7B and C), which is similar to what is observed *in vitro* (Fig. 1). In Ang II-infused animals, endothelial DHFR expression was diminished, which was however significantly restored by FA administration (Figs. 7B and C). Again, this beneficial effect of FA treatment was independent of GTPCH1 activity and intracellular 5′-MTHF level (Figs. 7D–F). These data seem to suggest that the beneficial effects of FA on vascular NO• bioavailability in Ang II-infused mice are mediated by an upregulation of DHFR function.

4. Discussion

The most significant finding of the study is that folic acid (FA) directly improves H_4B and NO• bioavailability while reducing oxidant production both *in vitro* and *vivo*, and that these effects are mediated by an upregulation of dihydrofolate reductase (DHFR) expression and activity. FA treatment of control or Ang II stimulated endothelial cells increased NO• production, which was attenuated by DHFR siRNA or methotrexate. In Ang II-infused mice, aortic $O_2^{\bullet-}$ production was attenuated by FA administration, whereas NO• levels were elevated. Endothelial expression of DHFR was markedly increased by FA, which is corresponding to a similar increase in activity.

As shown in Fig. 1, FA upregulates DHFR expression and NO• production in healthy endothelial cells at baseline. This seems consistent to what we found earlier that endogenous DHFR function is important for basal NO• production [23]. It is thus interesting to speculate that a deficiency in FA, i. e. caused by low dietary supplementation, may induce a DHFR deficiency that predisposes to vascular diseases. In consequent efforts to establish a sensitive and reliable HPLC-based activity assay for DHFR, FA was compared to dihydrofolic acid for substrate capability and found a second titer substrate with much less efficiency (Km 7-fold higher, Vmax 50-fold less). Nonetheless, it seems to implicate that when FA dietary supplementation is sufficient, DHFR activity would be important for its metabolism. Indeed, data from the Framingham Offspring Study demonstrated that a 19-bp polymorphism at intron 1 of *DHFR* gene is associated with increased plasma unmetabolized FA and decreased red blood cells folates [32].

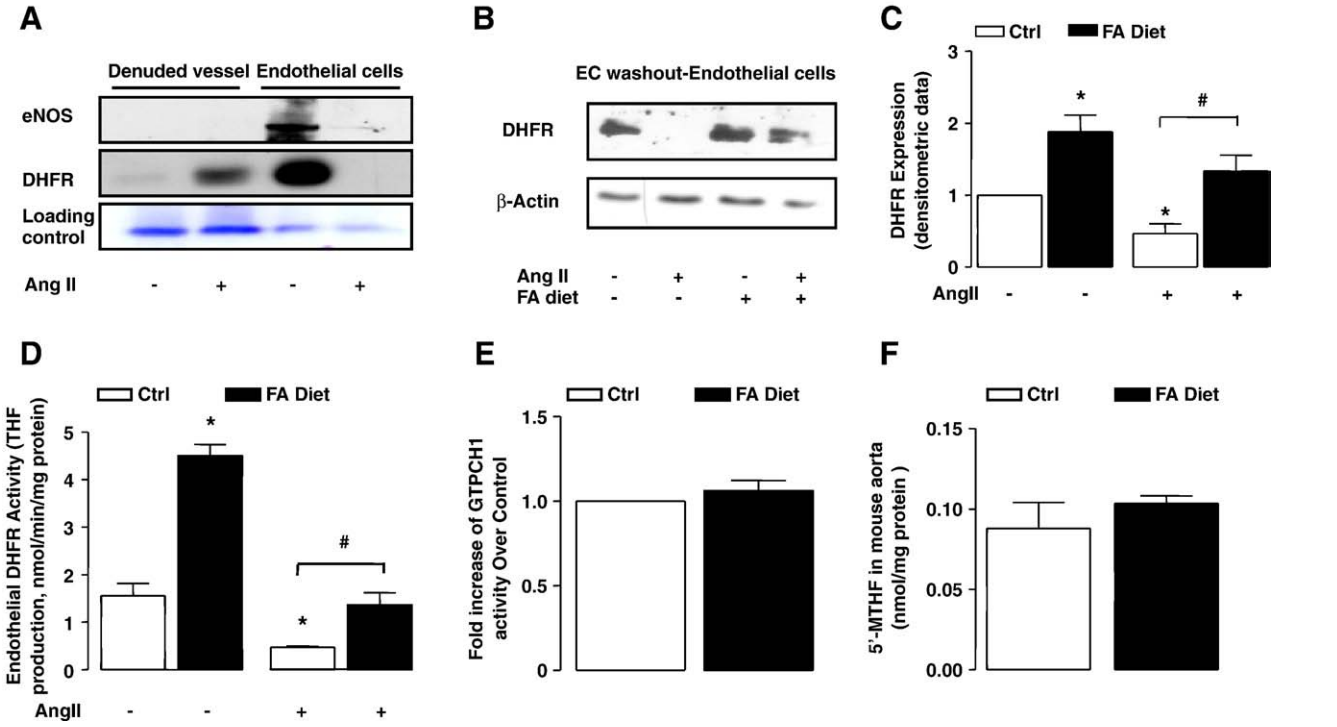

Fig. 7. Endothelium regulation of DHFR expression and activity by Ang II and folic acid in Ang II-infused mice. (A) Differential expression profile of DHFR in endothelium-denuded aortas and endothelial cells of untreated and Ang II-infused mice; (B) Effects of FA on endothelium regulation of DHFR expression in Ang II-infused mice; (C) Grouped densitometric data of B; (D) Effects of FA on endothelium regulation of DHFR activity E) FA diet on endothelium GTPCH1 activity; (F) FA diet on endothelium 5′-MTHF level. *$p<0.05$ vs. control; #$p<0.05$ vs. Ang II alone.

Importantly, we developed a novel and sensitive HPLC-fluorescent based assay to measure DHFR activity. Using cell or tissue lysates, an optimal condition has been selected for maximal detection sensitivity: substrate dihydrofolate at 50 μmol/L, electron donor NADPH at 200 μmol/L, incubation for 20 min at 37 °C, in a 0.1 mol/L potassium phosphate assay buffer (pH 7.4). The stabilization buffer maintains product (tetrahydrofolate, THF) stability at least overnight at 4 °C to allow autosampling performance. The HPLC-coupled electronic detection (ECD) method developed by Aiso et al. [33] has similar sensitivity limit to our assay; but is associated with disadvantages such as handling of radioactivity, cost consumption, and complexity of the assay procedure. DHFR activity has been assayed at various pH and/or ambient temperature ranging from 21 to 28 °C [34,35]. In this study, however, we assayed it at pH 7.4 and 37 °C, which is more physiologically relevant. When dihydrofolic acid was used as a substrate, the reaction was not affected by pH (Fig. 3E), but significantly more sensitive at 37 °C than at ambient temperature. Moreover, the product THF was stable in our assay system even after 20 min incubation at 37 °C, and with the proper stabilizing buffer, can be analyzed by autosampler at 4 °C overnight.

Elevated Ang II levels have been observed in many cardiovascular disorders where endothelial dysfunction occurs [36]. It is an independent risk factor for atherosclerosis and its antagonists have been used clinically to treat patients with hypertension. Thus it would be a good model system to test the effects of FA on endothelial function, which has already been implicated in coronary patients [22,37,38] and patients with hyperhomocysteinemia [39,40]. As expected, Ang II stimulation led to increased $O_2^{\bullet-}$ production and decreased $NO^{\bullet}$ and H_4B bioavailability. These responses were however all reversed significantly by FA pre-treatment. In addition, DHFR expression and activity were increased by FA. Pre-inhibition of DHFR by RNA interference or methotrexate effectively abolished the beneficial effects of FA on endothelial cell functions. Besides these clear-cutting *in-vitro* observations, data collected from Ang II-infused mice also supported a strong impact of FA and an intermediate role of DHFR.

Mice infused with Ang II had markedly increased $O_2^{\bullet-}$ production and reduced $NO^{\bullet}$ bioavailability. This was accompanied by an endothelial downregulation of DHFR. Oral administration of FA significantly improved $NO^{\bullet}$ production, while reducing $O_2^{\bullet-}$ levels. It also reserved DHFR protein abundance from being diminished by Ang II. Changes in DHFR activity mirrored expressional regulations by Ang II and FA. Taken together, these data demonstrate that FA exerts it beneficial effects *in vivo* via regulation of DHFR expression and activity. It is important to note that in the endothelium-denuded aortas DHFR expression was upregulated by Ang II, indicating differential regulation of DHFR in different cell types. This seems logic as Ang II is known to stimulate vascular smooth muscle hypertrophy, and that DHFR in general is important for DNA synthesis and cell growth.

In summary, the data in the present study for the first time established that FA, via modulation of DHFR expression and activity, improves $NO^{\bullet}$ bioavailability while reducing $O_2^{\bullet-}$ production under pathophysiological conditions. These findings may represent a common mechanism whereby FA supplementation exerts beneficial effects on cardiovascular system in populations with and without hyperhomocysteinemia.

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