Folic acid impairs uptake of 5-methyltetrahydrofolate in primary human umbilical vein endothelial cells.

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Submitted
Abstract

Rationale
Although synthetic folic acid (FA) lowers plasma homocysteine levels, clinical trials found no favorable effect on cardiovascular disease.

Objective
We explored the possibility that FA itself may block cellular uptake of the natural circulating plasma folate 5-methyltetrahydrofolate (5MTHF) in endothelial cells.

Methods and results
To investigate short term effects of FA on 5MTHF uptake in primary human umbilical vein endothelial cells (HUVECs), cells were maintained in culture medium containing 200 nmol/L 5MTHF and pre-incubated with 20 nmol/L FA ten minutes prior to the uptake assay (experimental condition A). For the long term effects of FA (experimental condition B), HUVECs were grown for 3 passages on culture media containing either 200 nmol/L 5MTHF or a mixture of 100 nmol/L 5MTHF and 100 nmol/L FA. In both experiments, 5MTHF uptake rate was assessed after exposing cells to 200 nmol/L [13C5]-5MTHF for 10 minutes and quantification of intracellular [13C5]-5MTHF was performed using LC/MS-MS. Experimental condition A revealed a marked and significant inhibition of 5MTHF uptake rate by brief exposure to FA (22±7 versus 51±12 fmol/min/mg protein in control cells; P=0.01). Likewise, experimental condition B showed a marked long-term inhibitory effect of FA on 5MTHF uptake (30±11 versus 51±12 fmol/min/mg protein (P=0.05). These effects may have been attributable to the proton-coupled folate transporter (PCFT), which was expressed on HUVECs.

Conclusion
FA markedly blocked 5MTHF uptake in HUVECs, conceivably involving PCFT. Since FA supplementation is associated with the appearance of unmetabolized FA in plasma, reduced uptake of biologically active 5MTHF in endothelial cells in vivo is a viable possibility which bears important clinical implications.
**Introduction**

Folates have been associated with the pathobiology of cardiovascular disease. Postulated mechanisms involve direct effects of folates on endothelial function, and indirect effects via lowering of homocysteine (Figure 1), an independent predictor of cardiovascular risk.\(^1,2\) However, large intervention trials with folates showed no clinical benefit, the reason for which remains elusive.\(^3-5\)

**Figure 1.** Folate transport routes and metabolism.

Abbreviations: 5,10-CH+THF (5,10-methenyltetrahydrofolate), 5,10-CH2THF (5,10-methylene tetrahydrofolate), 5MTHF (5-methyltetrahydrofolate), DHF (dihydrofolate), DHFR (dihydrofolate reductase), FA (folic acid), FR (folate receptor), 10-FTHF (10-formyltetrahydrofolate), MTHFR (methylenetetrahydrofolate reductase), PCFT (proton-coupled folate transporter), RFC (reduced folate carrier), THF (tetrahydrofolate).

One of several possible explanations for this lack of benefit may include adverse effects of high doses of the synthetic folate derivative folic acid (FA).\(^6\)
FA is an oxidized folate form with no known biological activity of its own. Although natural folate sources are available, FA is inexpensive and therefore widely produced for food fortification and supplements. In humans, conversion of fortified and supplemented FA to a biologically active intermediate can proceed in liver by dihydrofolate reductase (DHFR) which has the capacity to reduce approximately 1 mg FA per 24 hours. Folate in regular food is present as polyglutamate forms and requires (poly)glutamate deconjugation (i.e. gamma glutamyl hydrolase activity), internalization via specific transport routes in the gut wall, and conversion to 5-methyltetrahydrofolate (5MTHF). However, one concern about the use of high-dose FA supplementation is the appearance of unmetabolized FA in the bloodstream. FA appears in the circulation when single doses exceed 300 µg, whereas the doses in clinical trials were often between 0.5-5 mg FA. Plasma concentrations of 10-20 nmol/L FA were measured in FA-fortified populations or individuals following FA supplementation. Little is known about the effects of unreduced FA in the circulation, but some adverse effects, not specifically involving the cardiovascular system, have been suggested. 

For anionic folates to enter cells, specific transporters are required. To date, 3 different folate transport systems are known to accommodate folate uptake. Folate receptors α and β (FRα and FRβ) operate by receptor-mediated endocytosis. The reduced folate carrier (RFC) which operates as an anion exchanger, and the proton coupled folate transporter (PCFT) which acts as proton-folate cotransporter differ in pH optimum (RFC at physiological pH 7.4, PCFT at pH 5.5) and substrate affinity. The latter is an important characteristic as RFC displays a very poor affinity towards FA whereas PCFT displays a high affinity to FA. Moreover, expression patterns of folate transporters differ between cell types. With respect to vascular endothelium, differential expression levels of folate transporters is unknown. Here, we hypothesized that unmetabolized FA might interfere with cellular uptake of the bio-active 5MTHF form in endothelial cells. To this end, we compared intracellular folate content, 5MTHF influx rates, and folate transporter mRNA expression in primary human umbilical vein endothelial cells (HUVECs) in the absence and presence of FA.

**Materials and methods**

**Standards**

FA (purity 98%) was obtained from Sigma (Deiselhofen, Germany).
(6S)-5MTHF (purity 90%), [13C5]-5-formylTHF and [13C5]-5MTHF (isotopic purity >99%) were obtained from Eprova AG (Schaffhausen, Switzerland). [13C5]-5,10-MethenylTHF was prepared using previously described methods.

**HUVEC isolation and cell culture**

Primary HUVECs isolated from six anonymous umbilical cords were grown in FA-free M199 medium (Promocell, Heidelberg, Germany) supplemented with 10% (v/v) heat-inactivated human serum (Brunschiw Chemie, Amsterdam, The Netherlands), 1% (v/v) penicillin-streptomycin (Invitrogen, Carlsbad, California, USA), 10% (v/v) heat-inactivated new born calf serum (BioWhittaker, VWR, Radnor, U.S.A.), 5 U/mL heparin (Leo Pharma, Ballerup, Denmark), 50 µg/mL endothelial cell growth factor (isolated from cow brain) and 1% (v/v) glutamine (BioWhittaker). Selected concentrations of either FA or 5MTHF were freshly added to the medium as indicated. HUVECs were plated at a density of 2x10^5 cells in 1.5 mL medium in gelatin (1% w/v, Merck, Darmstadt, Germany) coated 6 wells plates (Greiner Bio One, Frickenhausen, Germany) and maintained at 37ºC in a 100% humidified atmosphere of 5% CO2. Cell culture media were refreshed every other day, and after reaching confluence (approximately 6 days), cells were detached by trypsinization (Invitrogen) and split 1:3 for renewed passages. Cells reaching passage 3 were used for the various experiments.

**Experimental conditions and analytical methods**

**Experimental condition A (short term exposure)**

Six different primary HUVEC isolates were transferred in 6 wells plates (2x10^5 cells/well containing 1.5 mL FA-free M199 medium supplemented with 200 nmol/L 5MTHF, which allows optimal cell growth. Two days prior to the experiment, the growth medium was replaced with one containing 20 nmol/L 5MTHF. Prior to uptake experiments, medium was aspirated and cells were rinsed twice with 1.5 mL Hanks Buffered Saline Solution (HBSS, pH=7.4) at room temperature. Next, 2x10^5 cells were pre-incubated with either (I) 20 nmol/L of 5MTHF (control) or (II) 20 nmol/L of FA in 1.5 mL HBSS containing 1% (v/v) human serum for 10 minutes at 37ºC. Subsequently, cells were incubated for 10 min with 200 nmol/L [13C5]-5MTHF. Cells were then placed on ice, rinsed twice with 1.5 mL HBSS, and harvested by short trypsinization (1 min, 37ºC). Cells were then washed 3 times with 1 mL ice-cold HBSS. The final cell pellets containing 2x10^5 cells were stored at -80ºC until analysis.
**Experimental condition B (long term exposure)**

Six different primary HUVEC isolates were transferred in 6 wells plates (2x10^5 cells/well) with FA-free M199 medium to which (**I**) 200 nmol/L 5MTHF (control) or (**III**) 100 nmol/L 5MTHF + 100 nmol/L FA were added. Two days prior to the experiment, medium was replaced with a medium containing mol/L 5MTHF (**I**) or FA (**III**). Just prior to the experiment, growth medium was aspirated and cells were rinsed twice with 1.5 mL HBSS at room temperature. Next, cells were incubated with 200 nmol/L $[^{13}C_5]$-5MTHF in HBSS containing 1% (v/v) human serum for 10 min. Processing and storage of cells was performed as described above.

**Sample clean-up and analysis**

A cell pellet of 2x10^5 HUVEC cells was suspended in 500 µL MilliQ and subjected to 3 freeze-thaw cycles, followed by centrifugation for 5 min (4ºC, 8000 g) in an Eppendorf centrifuge. To 400 µL of the supernatant (the remainder was used for a protein determination, bicinechonic acid (Sigma, Deiselhofen, Germany)), 22 pmol of $[^{13}C_5]$-5,10-methenylTHF was added as an internal standard, and the mixture was acidified with 50 µL 6 mmol/L HCl (pH<1). 3-mL Hydrophilic-Lipophilic Balance Oasis cartridges (Waters, Milford, MA, USA) were used for sample clean-up after conditioning with 0.5 mL methanol and 1 mL milliQ. The mixture was introduced to the cartridges, washed with 2 mL of milliQ, and subsequently eluted with 500 µL methanol containing 1 mg/mL ascorbic acid. The samples were then evaporated to almost complete dryness (nitrogen, 40ºC) and dissolved in 100 µL 10 mmol/L formic acid. The $[^{13}C_5]$-5MTHF content was assessed by LC-MS/MS as described previously. Intra-assay CV for $[^{13}C_5]$-5MTHF was < 5.6% and Limit of quantification (LOQ, signal/noise>10) was 0.01 pmol.

**Determination of intracellular 5MTHF concentrations**

Endogenous 5MTHF was measured in 2x10^5 cells from all 6 HUVEC isolates following incubations according to experimental condition B by methods described previously. Intra-assay CV for $[^{13}C_5]$-5MTHF was < 1.2%. LOQ (signal/noise) was 3 fmol.

**Quantification of RFC, PCFT, FRα and FRβ**

Total RNA was isolated from 3 HUVEC isolates from experiment 2 using a RNeasy kit (Qiagen) followed by DNase I treatment to remove contaminating
genomic DNA, according to manufacturer’s protocol. cDNA was synthesized from 1.5 µg of total RNA using random primers and Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was carried out in a total volume of 25 µL containing 5 µL of cDNA, 300 nmol/L of forward and reverse primers, and 1x SybrGreen PCR Mastermix (Applied Biosystems). Primer sets were designed using the Universal Probe Library (Roche Applied Science), according to the GenBank sequences (Supplementary Table 1; human RFC mRNA NM_194255.1, human PCFT mRNA NM_080669.3, human FRα mRNA NM_016729.2, human FRβ mRNA NM_001113536.1 and human β-2-microglobulin (B2M) mRNA NM_004048.2). PCR conditions were as follows: cycling was preceded by a hot start at 50°C for 2 min and denaturation at 95°C for 10 min, followed by 40 cycles of 15s at 95°C and 1 min at 60°C. After amplification, a melting curve was acquired by heating for 15s at 95°C, cooling to 60°C/30s and heating again to 95°C for 15s. Samples were run in triplicate on the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and fluorescence was measured during each annealing step. Expression levels of the genes of interest (GOIs) were normalized to the reference gene B2M by comparative quantification, using the ΔΔCT method. For each sample, ΔC_T was calculated (C_T, GOI - C_T, B2M). The mean relative expression was calculated as 2^-ΔΔCT (ΔΔCT = ΔC_T - ΔC_T, Control).

Table 1. Primer sets for quantitative real-time PCR analysis for folate transporters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>RFC</td>
<td>ACCATCATCACTTTTATTGTCTC</td>
<td>ATGGACAGGATCAGGAAAGTACA</td>
<td>76</td>
</tr>
<tr>
<td>PCFT</td>
<td>CTCATGTTCAAGGATATGGTTG</td>
<td>GCACCCCTGCTCTCTCTCTCTCA</td>
<td>79</td>
</tr>
<tr>
<td>FRα</td>
<td>ACTGGGACTGGATTTAACAAG</td>
<td>GTAGGAGTGAGTCCAGATTTCATTT</td>
<td>86</td>
</tr>
<tr>
<td>FRβ</td>
<td>TATGCAAAGGACTGTCAGC</td>
<td>GGGGAATAGGACTCAAGGTG</td>
<td>127</td>
</tr>
<tr>
<td>B2M</td>
<td>GGCTATCCAGGCTACTCCAAA</td>
<td>CGGCAGGCATCATCATTTTTT</td>
<td>225</td>
</tr>
</tbody>
</table>

Reduced folate carrier (RFC), proton coupled folate transporter (PCFT), folate receptor (FR), β-2-microglobulin (B2M, reference)

Statistics

Paired Student t-tests were used to test for statistical differences between the groups. P-values < 0.05 were considered statistically significant.
Results

mRNA expression of folate transporters in HUVECs

To examine which of the known folate transporters are expressed in HUVECs and at which levels, PCR analysis was performed for quantification of RFC, PCFT, FRα and FRβ mRNA levels relative to B2M as a reference gene. RFC and PCFT appeared to be predominantly expressed; \( \Delta C_T=5 \) and -0.5, respectively. FRα and FRβ were hardly expressed (\( C_T=36 \) and 40, respectively). Using HeLa cells as a reference, HUVEC cells showed comparable expression levels of RFC, whereas PCFT expression levels were approximately 10% of that of HeLa cells data not shown).

![Figure 2](image.png)

**Figure 2.** Folate transporters relative expression in primary HUVECs.

HUVECs were grown in FA-free M199 medium supplemented with 200 nmol/L 5-methyltetrahydrofolate. Cells were harvested at passage 3. \( C_T \) value indicates the fractional cycle number at which the amplified target reaches a fixed threshold, \( \Delta C_T=C_T-C_{\beta-2\text{-microglobulin}} \) (a lower \( \Delta C_T \) represents a higher mRNA expression). Results depict the mean relative expression normalized to B2M (2^-\( \Delta C_T \) ± SD) of 3 separate HUVEC isolates. Abbreviations; RFC, reduced folate carrier; PCFT, proton-coupled folate transporter; FR, folate receptor; n.d., not detectable.
Effect of short- and long term exposure to FA on 5MTHF transport in HUVECs.

The impact of short- and long term exposure to FA on 5MTHF uptake in HUVECs is depicted in Figure 3. Under control conditions (I), influx rates of 5MTHF were 51±12 fmol/min/mg protein. Of note, 5MTHF influx was linear over 10 min and exhibited Michaelis-Menten kinetics between 40 nmol/L and 25 µmol/L 5MTHF with a Km value of 0.25 µmol/L and Vmax of 0.39 pmol 5MTHF/mg protein/min. Short pre-exposure of HUVECs to an equimolar concentration of 20 nmol/L FA (II) significantly reduced 5MTHF influx by 60% (to 22±7 fmol/min/mg protein, p=0.007). A similar reduction was seen when
200 nmol/L FA was used (data not shown). Likewise, long term pre-exposure of HUVECs to FA (III) also reduced 5MTHF influx, now by 41% (to 30±11 fmol/min/mg protein, p=0.05). Under these long term pre-exposure conditions to FA, RFC mRNA expression levels in HUVECs did not change significantly ($2^{-\Delta\Delta CT}$ variation; 1.0-1.1, n=3). PCFT mRNA expression levels, however, were markedly down-regulated to a variable extent ($2^{-\Delta\Delta CT}$ variation: 1.8-8.0 fold, p=0.223, n=3).

**Figure 4.** Effect of folic acid (FA) on intracellular 5-methyltetrahydrofolate (5MTHF) concentration in HUVECs.

Results depict 6 separate HUVEC isolates.

**Effect of long term exposure to FA on intracellular 5MTHF levels in HUVECs**

Consistent with the markedly diminished influx rates of 5MTHF after long term pre-exposure to FA, HUVECs also exhibited significantly lower intracellular...
5-Methyltetrahydrofolate uptake in HUVECs

5MTHF levels when compared to cells maintained under control conditions (Figure 4; p=0.02). For 6 separate HUVEC isolates, the decrease in intracellular 5MTHF levels ranged from 47±21%. For comparison, non-MTHF levels remained unchanged (~4% of total folate), whereas intracellular FA was not detectable in HUVEC cells.

Discussion

The slow metabolic capacity to convert FA to biologically active reduced folate cofactors has been implicated in the emergence of unmetabolized FA in plasma of humans participating in FA supplementation and/or food fortification programs.6, 8, 9 On average, unmetabolized FA concentrations in plasma range from 10-20 nmol/L, but could also peak to higher levels.10 In the present study, we addressed the key question of whether or not FA could interfere with the uptake of the natural circulating reduced folate 5MTHF and thus have unintended deleterious impact on endothelial cell folate pools and homocysteine homeostasis. Interestingly, the present study revealed that beyond the constitutively expressed RFC, HUVECs also expressed the more recently identified PCFT as candidate entry route for 5MTHF. Consistent with studies on blood-brain barrier endothelial cells22, a putative role for either FRα or FRβ in HUVEC folate transport seems unlikely given their poor expression levels. Other than for RFC, which has a very poor affinity for FA over 5MTHF, competitive interaction of 5MTHF uptake by FA may be anticipated from the transport kinetic properties of PCFT, as both of them have comparable Km values.7 Indeed, we observed a marked decrease in 5MTHF uptake and accumulation in HUVECs after preincubation with low FA concentrations similar to those observed following FA supplementation and fortification (>20 nmol/L). This effect could be attributable to direct competition of FA on 5MTHF uptake; moreover, downregulation of PCFT gene expression could also contribute to the significant decrease in uptake and accumulation of 5MTHF in the presence of FA. It should be noted that 5MTHF uptake studies in HUVECs were performed in cell culture media conditions at pH 7.2-7.4. This pH would be more favorable to support 5MTHF uptake via RFC, but does not comply with a competitive/inhibitory effect of low concentrations of FA, for which RFC has a poor affinity. Although PCFT transport activity is optimal at pH 5.5, some substantial residual activity at pH 7.2-7.414,23-25 may still account for inhibitory effects at nanomolar concentrations of FA. Following these initial observations, further studies are warranted to establish the differential contribution of RFC and PCFT in 5MTHF
uptake and accumulation in HUVECs over a broader pH range and assess the impact of FA on this transport. The impairment of 5MTHF uptake by vascular cells could also occur in vivo in patients taking FA supplementation. Firstly, FA concentrations that elicited inhibitory effects (20 nmol/L) are similar to concentrations of unmetabolised FA found in plasma following oral intake of FA. Secondly, although the time frame within which unmetabolized FA emerges in human plasma may be relatively short (1 to 2 hours), unmetabolized FA has slow plasma elimination pharmacokinetics after prolonged exposure to FA. If indeed human endothelial cells would display reduced 5MTHF uptake after brief/intermittent exposure to FA, the consequence could be reduced intracellular folate pools. The importance of adequate folate homeostasis in endothelial cells was underscored in a recent study demonstrating that higher vascular 5MTHF concentrations were associated with lower vascular superoxide radical generation. The fact that plasma homocysteine levels drop after FA supplementation could argue against impaired 5MTHF uptake and diminished intracellular accumulation. However, it is conceivable that different organs and tissues respond differently to FA administration, as components of folate homeostasis (folate transporters, intracellular metabolism and polyglutamylation) will be different. Plasma homocysteine levels are predominantly determined by liver and kidney folate homeostasis, which may not necessarily reflect folate metabolism in endothelial cells. In accordance, a recent study confirmed that the vascular endothelium constitutes a different compartment than blood with respect to its regulation of 5-MTHF homeostasis. In conclusion, FA has the ability to inhibit 5MTHF uptake in primary HUVECs by a mechanism that probably involves interaction with the folate transporter PCFT. As FA supplementation (in doses exceeding 300 µg/day) and food fortification result in the appearance of unmetabolized FA in plasma, this might cause a markedly deleterious effect in terms of decreased uptake of the natural reduced folate cofactor 5MTHF. The subsequent lower intracellular availability of 5MTHF in endothelial cells is of potential concern.

References


