Folic acid, a double-edged sword? Influence of folic acid on intracellular folate and dihydrofolate reductase activity.

Desirée E.C. Smith¹,³, Yvo M. Smulders²,³, Robert M. Kok¹, Henk J. Blom¹,³

¹ Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands. ² Department of Internal Medicine, VU University Medical Center, Amsterdam, The Netherlands. ³ Institute for Cardiovascular Research, ICaR-VU, VU University Medical Center, Amsterdam, The Netherlands.

Submitted
Abstract

Objective
Synthetic folic acid is widely used, amongst others in the prevention of neural tube defects and reducing homocysteine, a risk factor for cardiovascular disease. Its effect on intracellular folate and the activity of essential cellular folate-converting enzymes is poorly understood.

Methods
Six lymphoblast cell lines were cultured for 15 days with medium containing either FA, 5-methyltetrahydrofolate or 5-formyltetrahydrofolate at physiological levels of 20 nmol/L or at supra-physiological concentrations of 2.3 µmol/L (standard concentration in cell culture medium). Subsequently, intracellular concentrations of folate, homocysteine, and activities of dihydrofolate reductase and methylenetetrahydrofolate reductase were measured.

Results
Unmetabolised FA was present inside lymphoblasts cultured on 2.3 µmol/L FA. Under these conditions, methylenetetrahydrofolate reductase activity remained unchanged, but dihydrofolate reductase activity decreased 20% compared to cells cultured on the same amount of 5-methyltetrahydrofolate. For both physiological and supra-physiological folate culture conditions, intracellular total folate concentrations were reduced more than 80% when cells were cultured on FA instead of 5-methyltetrahydrofolate. Intracellular folate concentrations were similar for cells cultured on 5-methyltetrahydrofolate and 5-formyltetrahydrofolate. In addition, FA inhibited the conversion of dihydrobiopterin to tetrahydrobiopterin, which may lead to endothelial nitric oxide synthase uncoupling.

Conclusions
Lymphoblasts cultured on standard medium containing FA show a remarkably lower intracellular folate content and perturbed DHFR activity than cells cultured on 5-methyltetrahydrofolate. Apparently, FA is a much less efficient substrate to boost intracellular folate levels than natural folates, and may even disturb activity of essential enzymes in one-carbon metabolism.
Introduction

The B-vitamin folate serves two major functions in the body. The first is the remethylation of homocysteine (Hcy) and the other the generation of DNA and RNA building blocks (Figure 1). The different forms of folates are structurally related. Differences consist of the oxidation state of the pteridine ring, and one-carbon substitutions at the $N_5$ and $N_{10}$ positions.

![Figure 1. Simplified scheme of the one-carbon metabolism.](image)

DHF (dihydrofolate), DHFR (dihydrofolate reductase), FA (folic acid), MTHFR (methylene tetrahydrofolate reductase), SAH (S-adenosylhomocysteine), SAM (S-adenosylmethionine), THF (tetrahydrofolate).

Folic acid (FA) is a synthetic analogue of folate and contains an oxidized pteridine ring and no one-carbon substitutions. Its structure renders it more stable and cheaper to produce than biological folates. FA has been widely used in the successful reduction of the incidence of neural tube defect and to lower homocysteine in patients with cardiovascular disease.$^{1,2}$ In 1998, FA fortification became mandatory in the US, and many countries followed this example.$^3$ However, systematic exposure to a synthetic analogue may have potential undesired side-effects. Since FA is a synthetic folate form, it is normally not present in blood but previous studies showed that unmetabolized FA appears in the systemic circulation when the oral dose exceeds 300 $\mu$g.$^4$ Many (multi)vitamin and preconception tablets, as well as study doses in
cardiovascular intervention trials, contain more than this amount. In addition, food fortification may lead to higher FA exposure than intended, and unmetabolized FA may occur in plasma of people using a FA fortified diet.\textsuperscript{5} There are still some ambiguities surrounding the complete metabolic fate of FA. For instance, whether FA is reduced and methylated in the small intestine or the liver is still under debate.\textsuperscript{6} The first step in the conversion of FA requires dihydrofolate reductase (DHFR, Figure 1). The low activity of this particular enzyme in humans \textsuperscript{7} may explain why FA appears in systemic circulation even at low doses. When FA enters systemic circulation, it has the potential to reach organs and cells. Indeed, unmetabolized FA has been measured in liver cells from guinea-pigs exposed to radioactive FA.\textsuperscript{8} Since DHFR activity is very low in humans in comparison to rats, extrapolation of animal studies to humans should be done with caution. First, it should be established whether unmetabolized FA is present in human cells, and for how long this persists before all FA is reduced. Prolonged presence of FA in the cell could theoretically interfere with the activity of folate enzymes and transporters, since FA structurally resembles natural folates closely. Indeed, methylenetetrahydrofolate reductase (MTHFR) has been shown to be inhibited by FA.\textsuperscript{9} Inhibition of folate converting enzymes could potentially alter folate distribution in cells. Inhibition of MTHFR shifts the balance in the one-carbon metabolism between the methylation of homocysteine and the generation of DNA building blocks (Figure 1). DHFR stands out as a particularly interesting enzyme, as it is the converter of FA itself, and enhanced FA substrate availability may be expected to either impair or enhance activity. Impairment may occur similar as with methotrexate (MTX), another synthetic folate analogue.\textsuperscript{10} Enhancement may be expected simply as a result of DHFR upregulation as a response to increased demand. Since DHFR also facilitates the conversion of dihydrobiopterin (BH\textsubscript{2}) to tetrabiopterin (BH\textsubscript{4}) other processes could be affected as well. For instance, lowered concentrations of BH\textsubscript{4} could lead to lower NO synthesis.\textsuperscript{11} In this paper we model the long term effect of unmetabolized FA on human cells. For this purpose lymphoblast cells were cultured under different folate and FA conditions for a prolonged time. We determined the presence of FA, folate and homocysteine in these cultured cells, and the effect of FA on folate enzymes such as DHFR and MTHFR. Subsequently, the effect on the different cellular folates was studied.
**Materials and methods**

**Standards**

NADPH, (6R)-5,6,7,8-tetrahydrobiopterin, and 7,8-dihydro-L-biopterin were obtained from Sigma (Deiselhofen, Germany). Folate standards were obtained as described previously.\(^{12}\)

**Culture conditions**

Six human control lymphoblast cell lines were grown in RPMI medium containing no FA (Invitrogen, Carlsbad, California, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen, containing approximately 10 nmol/L 5-methyltetrahydrofolate (5-methylTHF)), and 1% (v/v) penicillin-streptomycin (Invitrogen). Cultures were grown in 175 cm\(^2\) culture flasks (Greiner Bio One, Frickenhausen, Germany) and maintained at 37ºC in an atmosphere of 5% CO\(_2\). Every third day the media was refreshed and the amount of cells was reduced to 1 million cells. Five different folate compositions were used and added to the media every time: 20 nmol/L FA, 20 nmol/L 5-methlyTHF, 2.3 µmol/L FA, 2.3 µmol/L 5-methylTHF, and 2.3 µmol/L 5-formyltetrahydrofolate (5-formylTHF). The cells were harvested after 5 passages (=15 days) and washed twice with Hank’s buffered salt solution (Invitrogen). Cell pellets were stored at -80ºC. Prior to analysis, lymphoblast extracts were obtained by 3 freeze-thaw cycles in 50 mM phosphate buffer (pH=7). Subsequently the cell lysate was centrifuged (8000 g, 10 min., 4ºC) to remove cell membranes and debris.

**Enzyme assays**

MTHFR activity was measured by incubating the lymphoblast cell extract for 30 minutes in 200 µL 250 mmol/L Tris buffer (pH=6.5) containing 2.5 mmol/L ethylenedinitrilotetraacetic acid, 2.5 µmol/L R-5,10-methenylTHF and 1 mmol/L NADPH at 37ºC. To determine whether FA is an inhibitor for MTHFR, a 5 min pre-incubation with different FA concentrations (range 0.04 - 30 µmol/L) was performed in a lymphoblast extract. After the incubation, the cells were placed immediately on ice. Subsequently 125 pmol \(^{13}\)C\(_5\)-5-methylTHF and 2 µmol formic acid was added. The cell suspension was centrifuged at 8000 g at 4ºC using molecular weight cut-off filters (Amicon Ultra, Regenerated cellulose, 10000 MWCO, Merck Millipore, Darmstadt,
Influence of folic acid in lymphoblasts

Germany). Subsequently, 5-methylTHF was measured by LC-MS/MS according to a previously published method.\(^\text{12}\)

DHFR activity was measured by incubating the lymphoblast cell extract for 30 minutes in 100 µL 250 mmol/L Tris buffer (pH=6.5) containing, 12.5 µmol/L dihydrofolate (DHF) and 1.25 mmol/L NADPH at 37ºC. To determine whether FA is an inhibitor for DHFR, a 5 min pre-incubation with different FA concentrations (range 0.04 - 30 µmol/L) was performed in a lymphoblast extract. After the incubation, the cells were placed immediately on ice. Subsequently 125 pmol \(^{13}\text{C_5}\)-5-methylTHF, 2 µmol formic acid and 2 µmol mercaptoethanol was added. The cell suspension was centrifuged at 11,000 rpm at 4ºC using molecular weight cut-off filters (Amicon Ultra, 10000 MWCO). The produced THF was measured by LC-MS/MS according to a previously published method.\(^\text{12}\)

The conversion of BH\(_2\) to BH\(_4\) was measured by incubating the cell extract for 30 minutes in 200 µL 125 mmol/L Tris buffer (pH=6.5) containing 3 mmol/L dithioerythreitol, 250 µmol/L BH\(_2\) and 25 µmol/L NADPH at 37ºC. To determine whether FA is an inhibitor for this reaction, different FA concentrations (range 2.5 - 750 µmol/L) were added to a lymphoblast extract. After the incubation, the cells were placed immediately on ice. The cell suspension was centrifuged at 11,000 rpm at 4ºC using molecular weight cut-off filters (Amicon Ultra, 10000 MWCO). The produced BH\(_4\) was measured by HPLC with electrochemical detection (Coulochem III, Thermo Fisher, Chelmsford, U.S.A.).\(^\text{13}\)

All concentrations and activities were expressed per mg protein. For the protein determination the bicinchononic acid method (Sigma, Deiselhofen, Germany) was used.

**Metabolite concentrations**

For the measurement of folate fractions, the samples were deconjugated, deproteinized, purified using affinity chromatography and subsequently measured by LC-MS/MS (API3000, Applied Biosystems, Foster City, CA, USA). Intra- and interassay CVs for 5-methyltetrahydrofolate were 1.2% and 2.8%, respectively. Intra- and inter-assay CVs for non-methylTHF as a group were 1.6% and 1.5%, respectively. Intra- and inter-assay CVs for FA were 3.2% and 7.1%, respectively.\(^\text{12}\)

For the measurement of Hcy in medium and lymphoblast extracts, the samples were purified using solid phase extractions, and subsequently measured with LC-MS/MS (API3000, Applied Biosystems, Foster City, CA, USA). Intra-assay CVs (n=6) were <9%.\(^\text{14}\)
Western blots

To quantify the protein levels of DHFR in the lymphoblast extracts, 25 µg of total protein was separated on a 4-20% polyacrylamide Criterion TGX Stain-Free gel (Bio-Rad Laboratories, Hercules, U.S.A.). Following electrophoresis (200 V, 40 min), proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot Turbo device (Bio-Rad) according to the manufacturer’s instructions and visualized by immunodetection using rabbit polyclonal anti-DHFR (Sigma, Deiselhofen, Germany) as primary antibody and anti-rabbit IgG-HRP (Dako, Glostrup, Denmark) as secondary antibody. Immunoblots were developed with enhanced chemiluminescent agents (LumiLight Plus Western Blotting Substrate, Roche Applied Science, Indianapolis, IN, USA) and the images were acquired using ChemiDoc MP (Bio-Rad) using the ImageLab 4.1 (Bio-Rad). For quantification, lysates were additionally incubated with rabbit anti-actin (Sigma). DHFR/Actin ratio were used to assess difference in DHFR expression between the groups.

Statistics

All statistical analyses were performed using SPSS 17.0 for Windows. Differences in lymphoblast metabolite concentration and enzyme activity between the different culture conditions were assessed using a non-parametric paired-samples Wilcoxon test.

Results

Presence of unmetabolized FA in cells
The intracellular FA concentrations in lymphoblasts cells cultured on 2.3 µmol/L FA ranged from 0.2 - 25.1 pmol/mg protein (median 3.5, n=6, Table 1). FA was undetectable in the same cell lines cultured on the same concentration of 5-methylTHF or 5-formylTHF. FA was also undetectable in lymphoblast cells cultured on 20 nmol/L FA or 5-methylTHF (Table 1).

When cells on 2.3 µmol/L FA were placed in HBSS for 1 hour at 37ºC, the intracellular FA concentration gradually reduced to approximately 50%.

Alterations in MTHFR activity due to FA
In cultured cells exposed to FA for 5 passages, FA did not have any significant effect on the MTHFR activity (Table 1, Figure 2). The addition of FA to lymphoblast extracts, in-vitro, had only a minimal effect on MTHFR activity.
Figure 2. Methylenetetrahydrofolate reductase (MTHFR) and dihydrofolate reductase (DHFR) activities in lymphoblasts cultured under different folate conditions for 5 passages (15 days).
Influence of folic acid in lymphoblasts

in the enzyme activity assay. As shown in Figure 3, at equimolar ranges of FA and 5,10-methyleneTHF (natural substrate for MTHFR, 2.5 µmol/L in assay mixture), MTHFR activity remains unchanged.

**Alterations in DHFR activity due to FA**

As shown in Figure 3, the addition of FA to lymphoblast extracts reduced DHFR activity by 60% at equimolar concentrations of FA and DHF (natural substrate for DHFR, 12.5 µmol/L in assay mixture). A culture medium of 2.3 µmol/L FA instead of 5-methylTHF or 5-formylTHF lowered DHFR activity significantly (Table 1, Figure 2). However, in western blots the amount of DHFR was not significantly lower (Table 1). There was no difference in DHFR activity observed between cells cultured on 20 nmol/L of FA and 5-methylTHF.

![Figure 3](image-url)  
**Figure 3.** The inhibition of MTHFR and DHFR activity by folic acid in lymphoblast extracts

Lymphoblasts lysates were pre-incubated for 5 min with different amounts of FA prior to performing the enzyme assay. Substrate for MTHFR assay was 2.5 µmol/L 5,10-methyleneTHF. Substrate for DHFR assay was 12.5 µmol/L DHF.
<table>
<thead>
<tr>
<th></th>
<th>5-methylTHF (pmol/mg protein)</th>
<th>Fold Acid</th>
<th>5-formylTHF (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-methylTHF</td>
<td>24.9</td>
<td>5.9-68.5</td>
<td>3.1</td>
</tr>
<tr>
<td>5,10-methenylTHF</td>
<td>17.0</td>
<td>1.0-37.9</td>
<td>3.0</td>
</tr>
<tr>
<td>5-formylTHF</td>
<td>6.6</td>
<td>n.d.-19.0</td>
<td>0.3</td>
</tr>
<tr>
<td>THF (pmol/mg protein)</td>
<td>3.5</td>
<td>0.8-5.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Folic Acid (pmol/mg protein)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Total Folate (pmol/mg protein)</td>
<td>56.4</td>
<td>8.3-116.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Non-methylTHF %</td>
<td>49</td>
<td>19-58</td>
<td>53</td>
</tr>
<tr>
<td>DHFR activity (nmol/h/mg protein)</td>
<td>140</td>
<td>110-230</td>
<td>138</td>
</tr>
<tr>
<td>DHFR/Actin ratio (western blot)</td>
<td>1.15</td>
<td>0.55-1.72</td>
<td>1.11</td>
</tr>
<tr>
<td>MTHFr activity (nmol/h/mg)</td>
<td>29</td>
<td>20-54</td>
<td>28</td>
</tr>
<tr>
<td>Homocysteine (pmol/mg protein)</td>
<td>8.0</td>
<td>7.8-19.8</td>
<td>18.6</td>
</tr>
<tr>
<td>Homocysteine in medium (µmol/L)</td>
<td>1.0</td>
<td>1.0-1.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>5-methylTHF (pmol/mg protein)</th>
<th>Fold Acid</th>
<th>5-formylTHF (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-methylTHF</td>
<td>179.8</td>
<td>101.5-</td>
<td>28.9</td>
</tr>
<tr>
<td>5,10-methenylTHF</td>
<td>73.8</td>
<td>12.5-125.7</td>
<td>18.0</td>
</tr>
<tr>
<td>5-formylTHF</td>
<td>31.2</td>
<td>3.6-48.7</td>
<td>7.6</td>
</tr>
<tr>
<td>THF (pmol/mg protein)</td>
<td>12.1</td>
<td>10.4-17.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Folic Acid (pmol/mg protein)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.5</td>
</tr>
<tr>
<td>Total Folate (pmol/mg protein)</td>
<td>280</td>
<td>141-464</td>
<td>60</td>
</tr>
<tr>
<td>Non-methylTHF %</td>
<td>37</td>
<td>22-50</td>
<td>30</td>
</tr>
<tr>
<td>DHFR activity (nmol/h/mg protein)</td>
<td>147</td>
<td>113-252</td>
<td>119</td>
</tr>
<tr>
<td>DHFR/Actin ratio (western blot)</td>
<td>1.19</td>
<td>0.86-1.74</td>
<td>1.18</td>
</tr>
<tr>
<td>MTHFr activity (nmol/h/mg)</td>
<td>27</td>
<td>19.4-52.2</td>
<td>24</td>
</tr>
<tr>
<td>Homocysteine (pmol/mg protein)</td>
<td>7.4</td>
<td>6.8-10.7</td>
<td>6.9</td>
</tr>
<tr>
<td>Homocysteine in medium (µmol/L)</td>
<td>0.5</td>
<td>0.5-1.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a versus cells cultured on 5-methylTHF, b estimated plasma concentrations, c normal medium concentrations, n.d. (not detected), non-methylTHF (sum of 5-formylTHF, 5,10-methenylTHF and THF)
Alterations in BH$_2$ conversion to BH$_4$ due to FA

The addition of FA to lymphoblast extracts inhibited the conversion of BH$_2$ to BH$_4$ considerably. As is shown in Figure 4, addition of a 100 fold lower amount of FA to the assay mixture in comparison to BH$_2$ (concentration in the assay mixture is 250 µmol/L) already halves the amount of BH$_2$ that is converted to BH$_4$.

![Figure 4](image_url)

The inhibition of the conversion of BH$_2$ to BH$_4$ by folic acid in lymphoblast extracts.

Lymphoblasts lysates were pre-incubated for 5 min with different amounts of FA prior to performing the enzyme assay. BH$_2$ concentration used was 250 µmol/L.

Alterations in folate concentration and distribution in cells due to FA

At physiological folate concentrations in medium (20 nmol/L), lymphoblast cell lines cultured on FA showed significantly lower intracellular total folate levels than cells cultured on the same amount of 5-methylTHF (7.5 versus 56.4 pmol/mg protein, p=0.03). In accordance, the levels of all different folate forms were decreased (Table 1). No FA was measured in lymphoblasts cultured on 20 nmol/L FA. A decrease of intracellular folate content was also
Influence of folic acid in lymphoblasts

seen when supra-physiological concentrations (2.3 µmol/L) of FA were used instead of 5-methylTHF (Table 1, Figure 5). In addition, the percentage of non-methylTHF was significantly reduced. Cells cultured on 2.3 µmol/L 5-formylTHF showed no differences in intracellular folate concentrations compared to cells cultured on 2.3 µmol/L 5-methylTHF but showed a significant increase of the percentage of non-methylTHF (Table 1).

![Culture conditions](image)

**Figure 5.** Intracellular 5-methyltetrahydrofolate (5-methylTHF) and total folate concentrations in lymphoblasts cultured under different folate conditions for 5 passages (15 days).

**Alterations in Hcy concentration due to FA**
At both physiological (20 nmol/L) and supra-physiological folate (2.3 µmol/L) concentrations in medium, Hcy in medium and intracellular Hcy between lymphoblasts cultured on FA did not differ from lymphoblasts cultured on 5-
Influence of folic acid in lymphoblasts

methylTHF (Table 1). Cells cultured on 2.3 µmol/L 5-formylTHF do not differ from cells cultured on 5-methylTHF.

Discussion

Unmetabolized FA has been measured in plasma following FA supplementation and in populations with FA food fortification. Concentrations in the range of 10-20 nmol/L have been measured in plasma. In this study we investigated what the effect of these physiological and higher concentrations could be on intracellular folate concentrations and the activity of specific folate enzymes in cells that are exposed to plasma.

First, we showed that FA is present in substantial amounts in cultured human lymphoblasts that are exposed to FA, and is still present even 1 hour after the substrate has been removed from the medium. For this experiment we used 2.3 µmol/L FA since at 20 nmol/L the amount of intracellular FA was below our quantification limit. Since FA is present in the cell for a prolonged period of time, it could potentially interfere with the activity of the various folate enzymes since it resembles the structure of their natural substrates very closely. After five minutes exposure of lymphoblast extracts, FA inhibited some of the folate enzymes (Figure 3 and 4). MTHFR does not seem to be affected to a large extent but DHFR and the conversion of BH₂ to BH₄ is lowered significantly. In the prolonged FA exposure of cultured lymphoblasts (~15 days), the MTHFR activity remained unchanged due to FA. This is in accordance with previously published data in hens. In cells cultured on 2.3 µM FA the DHFR activity is reduced compared to cells cultured on the same amount of 5-methylTHF. Western blots showed no significant lower amount of DHFR present in the cell. The results do not correspond with previously published data in hens where DHFR activity in liver increased due to FA (10 mg FA/kg for 8 weeks). The discrepancy could be explained by the difference in (species) cell type and their specific metabolic function. The conversion of BH₂ to BH₄ is catalyzed by DHFR as well as dihydropteridine reductase (DHPR). Because both DHFR and DHPR are present in lymphoblasts, it remains unclear whether DHFR or DHPR or both are inhibited by FA. Experiments on purified enzymes could provide further insight on the potential inhibition of these enzymes. Interestingly, the incubation of lymphoblast extracts shows that the overall conversion of BH₂ to BH₄ suffers to a large extent from the presence of FA in the incubation mixture. A 100 fold lower FA concentration than BH₂ already halves the conversion rate of BH₂ to BH₄. Consequences of lowered concentrations of BH₄ could for
instance be endothelial nitric oxide synthase (eNOS) uncoupling and reduced NO production.\textsuperscript{11} Unfortunately, intracellular BH\textsubscript{2} and BH\textsubscript{4} levels of the prolonged lymphoblast experiment were not within the analytical range of our method and could therefore not be obtained.

The predominant folate form in plasma is 5-methylTHF, and this form serves as a folate source for most cells. However, when FA is administered it will also appear in plasma and hence may interfere with folate transport and uptake in various cell types. A remarkable lower intracellular folate content (approximately 80% lower) was found in cultured lymphoblasts using 20 nmol/L synthetic FA compared to cells cultured on the natural substrate 5-methylTHF (Table 1). It has to be kept in mind that basal medium already contains approximately 1 nmol/L 5-methylTHF introduced by foetal bovine serum. Part of our experiments were conducted under physiological folate conditions if we estimated the average human plasma folate concentrations to be 20 nmol/L. Since intracellular folate levels, especially FA, at these physiological culture conditions were near the detection limit of our method, experiments under normal culture media conditions (i.e. 2.3 µmol/L folate) were also performed. These cells showed a comparable decrease in intracellular folate content. To exclude that the increase of 5-methylTHF in the cells cultures on 5-methylTHF was merely a diffusion effect, cells were also cultured on another natural folate, namely 5-formylTHF. These cells showed the same folate concentrations as cells cultured on 5-methylTHF (Table 1).

Taken together, FA appears to be less efficient as a substrate to boost intracellular folate levels. Media levels of 2.3 µmol/L FA result in the same intracellular folates concentrations as is achieved with only 20 nmol/L 5-methylTHF. This complies with previously published in-vivo studies where 5-methylTHF was able to boost red blood cell folate more efficiently than FA\textsuperscript{20,21} and 5-methylTHF showed a higher bioavailability.\textsuperscript{22} Therefore, we recommend that in future studies instead of plasma total folate, the different folate forms are measured, in particular in FA fortified populations or if individuals use vitamin preparations, because this will provide a more accurate picture of the bioavailability of folates in plasma.

Lower intracellular folate concentrations in the FA group could be due to either inhibited DHFR activity (rate limiting step in reduction of FA to THF), less retention in the cell of the monoglutamate FA or less efficient cell membrane transport.\textsuperscript{23} FA can not be transported by the universally expressed reduced folate carrier but can be transported by the folate receptor.\textsuperscript{23} Lymphoblast cell membranes have been shown to contain the folate receptor.\textsuperscript{24} In human umbilical vein endothelial cells, we recently
showed that the presence of FA can lower the uptake of 5-methylTHF (manuscript submitted).
In this study we show that cells cultured on FA show a remarkable lower intracellular folate content than cells cultured on 5-methylTHF or 5-formylTHF. Whether the significantly lower intracellular folate levels are due to inhibition of the already poorly expressed DHFR activity in human cells and/or lowered uptake of FA by cells is unclear. Not only do we show that FA is a less efficient substrate to boost intracellular folate levels than natural folates, but it also is capable to inhibit crucial enzymes of folate metabolism including the conversion of BH2 to BH4. Lower BH4 concentrations may lead to eNOS uncoupling. FA is a synthetic molecule, and we provide evidence that it potentially could have some unwelcome side-effects. It is tempting to compare the effects of FA with for instance another synthetic folate analogue MTX, which inhibition on folate metabolism has been extensively researched, in particular in cancer research. Since FA and MTX share similarities in molecular structure, they could show the same effects on inhibition of enzyme activities and transporter velocities. The effects of FA on folate metabolism are not as severe as MTX since FA is eventually completely metabolized to natural folates, but its potential double-edged sword properties certainly require further investigation.

Acknowledgements

We would like to acknowledge Erwin Jansen for the biopterin determinations.

References