The effect of folic acid on the homocysteine metabolism in human umbilical vein endothelial cells (HUVECs)

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Received 27 March 1995 and in revised form 30 August 1995; accepted 5 September 1995

Abstract. Mild hyperhomocysteinaemia is associated with increased risk for vascular disease. We studied homocysteine export from human umbilical vein endothelial cells (HUVECs) by measuring total homocysteine (tHcy) concentrations in the culture medium. Under standard culture conditions tHcy concentrations in the HUVEC culture medium increased by constant amounts after 24, 48 and 72 h [mean = 2.5 (SD ± 0.7) μmol L⁻¹ homocysteine every 24 h]. As the cells are the only source of homocysteine increase in the culture medium, we designate this as homocysteine export from HUVEC. Folic acid supplementation to the culture medium lowered the homocysteine export in a dose-dependent manner. Methyl-tetrahydrofolate (MeTHF) and folinic acid (a stable precursor of MeTHF) were in this respect about 10 times more effective than folic acid. A 50% reduction in the homocysteine export was seen with 10–30 nmol L⁻¹ MeTHF supplementation; reduction to almost zero was seen with 100–300 nmol L⁻¹ MeTHF. Additions to the culture medium of the other vitamins involved in the homocysteine metabolism, such as vitamin B₁₂, vitamin B₆ or flavin adenine dinucleotide, did not show any effect on homocysteine export. Because homocysteine export reflects an imbalance in the homocysteine metabolism, our observations showed a susceptible dependency of this metabolism on folic acid in endothelial cells.

Keywords: Folic acid, human umbilical vein endothelial cells, methionine/homocysteine metabolism.

Introduction

Severe hyperhomocysteinaemia (homocystinuria) is in most cases caused by an autosomal recessive inherited deficiency of cystathionine synthase (CS) or methylentetrahydrofolate reductase (MTHFR) [1]. Clinically the most life-threatening complication of CS and MTHFR deficiency is severe arteriosclerosis or thrombosis [2,3]. Mild hyperhomocysteinaemia is associated with the development of arteriosclerosis [4-6] and obstetric complications [7 9]. Mild hyperhomocysteinaemia can theoretically be caused by reduced activities of CS, methionine synthase (MS) or MTHFR or by deficiencies of folic acid and vitamin B₁₂. Recently, a common mutation in the MTHFR gene causing reduced enzymatic activity has been described as one of the causes of mild hyperhomocysteinaemia [10] (Fig. 1).

The treatment of hyperhomocysteinaemia (mild or severe) involves administration of pyridoxine, folic acid, vitamin B₁₂ or betaine [11]. In particular, administration of folic acid results in a strong decrease in plasma tHcy concentration in mild hyperhomocysteinaemia and the combination of folic acid and pyridoxine normalizes mild hyperhomocysteinaemia in virtually all cases [12-14].

In vitro high levels of homocysteine can generate H₂O₂ and the vasotoxic properties of homocysteine through lipid peroxidation have been proposed [15]. However, in plasma from patients with severe hyperhomocysteinaemia no increase in lipid peroxidation could be found [16,17]. Several in vitro studies with endothelial cells have shown cell dysfunction when homocysteine is added to the medium. Lentz & Sadler [18] showed that homocysteine reduced the thrombomodulin and von Willebrand factor excretion of HUVECs (human umbilical vein endothelial cells). Also, direct inactivation of protein C by homocysteine has been reported [19]. According to Nishinaga et al. [20] homocysteine reduces the expression of heparan sulphate on the cell membrane, and Hajjar [21] showed that the number of binding sites for tissue plasminogen activator (tPA) on the cell membrane may be reduced by homocysteine. However, the abovementioned studies used very high concentrations of homocysteine in its reduced form (i.e. with free SH groups). This is extremely non-physiological because only 1% of homocysteine is present in blood in its reduced form. A dose of 10 mmol L⁻¹ reduced homocysteine in vitro is about 50,000 times higher than the amount of reduced homocysteine present in the blood of patients with mild hyperhomocysteinaemia [22].

Little is known about the homocysteine metabolism
in endothelial cells. Recent studies with lymphoma cells and fibroblasts described homocysteine export into the extracellular medium [23–25]. The homocysteine export seemed to be dependent on cell proliferation and additions to the culture medium of inhibitors of homocysteine metabolism. It was concluded that homocysteine export reflected an imbalance in homocysteine production and metabolism [11].

In the present paper homocysteine export was studied in human umbilical vein endothelial cells (HUVECs), including the effects of additions to the culture medium of vitamins involved in homocysteine metabolism.

Materials and methods

Cell culture

Endothelial cells from the human umbilical vein (HUVEC) were obtained from umbilical cords of healthy fetuses from uncomplicated pregnancies and deliveries of healthy mothers (non-smoking and without medication). The cord was collected in buffer solution [4 mmol L⁻¹ KCl, 140 mmol L⁻¹ NaCl, 10 mmol L⁻¹ HEPES, 11 mmol L⁻¹ D-glucose H₂O (Merck, Germany), 10⁵ IU of penicillin and 10⁵ IU of streptomycin (Boeringer Mannheim, Germany) with pH 7.3.]

Within 24 h after cord collection endothelial cells were isolated by collagenase (Worthington, NJ, USA) treatment according to the procedure of Jaffe et al. [26]. The endothelial cells were seeded in two wells of a six-well plate, 10 cm² each (all tissue culture plastics used were from Costar, Cambridge, MA, USA) coated with human cryoprecipitate (local bloodbank) and cultured in M199 (Flow laboratories, Irving, UK) supplemented with 20 mmol L⁻¹ Hepes (Merck, Germany), 10% human serum (local blood bank), 10% newborn calf serum (Gibco, Grand Island, NY, USA), 1% 200 mmol L⁻¹ L-glutamine (Flow ICN, USA), 5 IU mL⁻¹ heparin (stock from our own pharmacy) and 150 µg mL⁻¹ crude preparation of endothelial cell growth factor (Macaig et al. [27]) and incubated in 5% CO₂ and 95% air at 37°C. When cells reached confluence, they were detached by trypsin/EDTA sodium salt (Flow ICN, USA) (50 mg/25 mg) diluted in 100 mL of a solution of 136.9 mmol L⁻¹ NaCl, 5.4 mmol L⁻¹ KCl, 4.2 mmol L⁻¹ NaHCO₃, 5 mmol L⁻¹ D-glucose and 0.002% phenol red and seeded in the completely coated six-well plate (split ratio of 1:3). The medium was
renewed every 2 or 3 days. All HUVEC experiments were performed with 3-day confluent cells from the second passage in six-well plates. The standard culture medium was removed and the test medium was applied, then, after incubations of 24, 48 or 72 h, the test medium was collected in separate ampoules and stored at −20°C for further analyses. After removal of the test medium the cells were trypsinized, counted by using the counter chamber (haemocytometer) and checked for viability with trypan blue. All experiments were performed with at least three different HUVEC lines.

**Homocysteine analysis**

Total homocysteine (tHcy) concentrations were measured by an automated high-performance liquid chromatography (HPLC) method with reverse-phase separation and fluorescent detection (Gilson 232-401 sample processor, Spectra Physics 8800 solvent delivery system and Spectra Physics LC 304 fluorimeter) essentially according to Fiskerstrand et al. [28] with some modifications (te Poele-Pothoff et al. [29]) the detection limit was 0.5 ±mol L⁻¹. The intra- and inter run variation coefficient was <5%. The tHcy analysis involved reduction of all disulphide bounds by sodium borohydride (Sigma, USA). After derivatization by monobromobimane (Sigma) the homocysteine–monobromobimane complex was separated by using reverse-phase HPLC.

In a small experiment, reduced homocysteine (with free SH groups) was added to the culture medium, the amount of reduced homocysteine in the culture medium was measured by binding free SH groups with DTNB [5,5'-dithiobis(-nitrobenzoate) or Ellman's reagent]. The solution consisted of 0.1 mmol L⁻¹ DTNB, 0.1 mol L⁻¹ Tris, 0.3 mmol L⁻¹ acetyl CoA and 0.5 mmol L⁻¹ oxaloacetate and absorption was measured at 412 nm [30].

**Vitamin/methionine analysis**

Vitamin B₁₂ and total folic acid concentrations in medium and serum were measured simultaneously with Dualcount (solid-phase boil) SPB radioassay (Diagnostic Products Corporation, Los Angeles, CA, USA) as described previously [31]. According to the manufacturer (Flow Laboratories, Irving, UK), M199 contained 22.7 nmol L⁻¹ (0.01 mg L⁻¹) folic acid. The total folic acid concentration in serum varied from 16.1 to 31.6 nmol L⁻¹, therefore the concentration of total folic acid in the standard culture medium could be calculated to be 21.4 ± 24.5 nmol L⁻¹. Measurements of the total folic acid concentration in the culture medium showed a concentration of 26 nmol L⁻¹. Methionine concentrations were determined using ion-exchange chromatography on an amino acid analyser (Biotronik LC 6001) according to the manufacturer’s instructions.

**Added chemicals**

Folic acid, 5-methyltetrahydrofolate (MeTHF), pyridoxal-5-phosphate (PLP), methylocobalamin (MeCOB), flavin adenine dinucleotide (FAD) and L-homocysteine thiolactone were obtained from Sigma. Hydroxocobalamin and folic acid (leucovorin) were obtained from Byk Nederland.

Stock solution (for direct use) of 0.3 mol L⁻¹ L-homocysteine in its reduced form (with free SH groups) was obtained by incubation of L-homocysteine thiolactone in 200 µL of NaOH (5 mol L⁻¹) at 40°C for 5 min and thereafter neutralized with 400 µL of phosphate buffer pH 7.8 (0.1 mol L⁻¹) and 400 µL of HCl (2.5 mol L⁻¹). Reduced pure L-homocysteine is not commercially available.

**Results**

**Homocysteine concentrations in culture medium**

The mean concentration of tHcy in fresh culture medium before incubation of 23 different experiments was 3.4 ± µmol L⁻¹ (range 3.8 ± 4.3) and originated from the serum.

Addition of freshly prepared reduced homocysteine (i.e. with a free SH group) to the culture medium resulted in a rapid decline in the concentration of reduced homocysteine. The half-life of reduced homocysteine in culture medium was 2.3 h. Free SH groups are reactive and will be rapidly oxidized to disulphides. In human plasma only 1% of the total amount of homocysteine is present in the reduced form [11]. The half-life of reduced homocysteine was the same when no HUVECs or coating of human cryoprecipitate was present and therefore the decline of reduced homocysteine was independent of cell exposure or coating. Incubation with 50 ± 100 µmol L⁻¹ L-homocysteine for 24 h showed no effect on HUVECs (visually and cell viability) when compared with cells incubated in standard medium (data not shown).

**Homocysteine export, methionine and folic acid concentrations in endothelial cell culture medium**

When cells were in the second passage and at least 3 days in monolayer under standard culture conditions, the cells exported a fairly constant amount of homocysteine in the culture medium. Experiments with six different HUVEC lines showed similar results, with a mean homocysteine export after 24 h of 2.4 µmol L⁻¹ (SD ± 0.6) µmol L⁻¹, between 24 and 48 h of 2.7 (SD ± 0.9) µmol L⁻¹ and between 48 and 72 h of 2.3 (SD ± 0.7) µmol L⁻¹ (Fig. 2).

The concentration of methionine decreased from 80 to 69 µmol L⁻¹ in 72 h and the concentration of folic acid from 26 to 23 nmol L⁻¹ in 72 h and the concentration of vitamin B₁₂ from 180 to 160 pmol L⁻¹ in 72 h.
Homocysteine export and effects of vitamin addition

Folic acid. Supplementing the standard culture medium of HUVECs with folic acid reduced homocysteine export in a dose-dependent manner. Addition of 300 nmol L\(^{-1}\) folic acid reduced homocysteine export by about 50%. When 3–10 \(\mu\)mol L\(^{-1}\) folic acid was added, homocysteine export was almost zero. Experiments with two other cell lines showed similar results (Table 1). Addition of folic acid did not affect cell number or viability.

Table 1. Homocysteine concentrations in HUVEC culture medium after incubation minus the homocysteine concentration already present in the culture medium before incubation

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>24 h (±SD)</th>
<th>48 h (±SD)</th>
<th>72 h (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid (nmol L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.0 (±0.4)</td>
<td>5.2 (±1.1)</td>
<td>7.9 (±0.3)</td>
</tr>
<tr>
<td>100</td>
<td>1.9 (±0.4)</td>
<td>4.0 (±0.2)</td>
<td>6.9 (±0.2)</td>
</tr>
<tr>
<td>300</td>
<td>1.3 (±0.3)</td>
<td>3.1 (±0.3)</td>
<td>4.8 (±0.9)</td>
</tr>
<tr>
<td>1000</td>
<td>0.7 (±0.7)</td>
<td>1.4 (±0.2)</td>
<td>2.8 (±1.3)</td>
</tr>
<tr>
<td>3000</td>
<td>0.3 (±0.6)</td>
<td>0.2 (±0.1)</td>
<td>1.7 (±2.1)</td>
</tr>
<tr>
<td>10000</td>
<td>0.1 (±0.6)</td>
<td>0.1 (±0.2)</td>
<td>1.1 (±1.7)</td>
</tr>
</tbody>
</table>

MeTHF and folinic acid. Medium M199 contained only folic acid (i.e. its oxidized form). The serum also contributed to the folic acid level of the standard culture medium (see Materials and methods). Because MeTHF is the main circulating form of folic acid in the body, we considered in our calculations the amount of folic acid in serum as MeTHF. Therefore, the baseline concentration of MeTHF in the standard culture medium was considered to be 4.7 nmol L\(^{-1}\). Addition of various concentrations of MeTHF or folinic acid (the more stable precursor of MeTHF) to the culture medium reduced homocysteine export in a dose-dependent manner. A typical presentation of this dose dependency is shown in Fig. 3. The concentrations of MeTHF or folinic acid were about 10 times lower than that of folic acid required to observe similar homocysteine export reduction (Table 1). Homocysteine export was reduced by 50% by addition of 10–30 nmol L\(^{-1}\) MeTHF or folinic acid, and addition of 100–300 nmol L\(^{-1}\) resulted in almost zero homocysteine export (Table 1). Experiments with two other cell lines showed similar results. Folinic acid seems to be more effective in reducing the homocysteine export than MeTHF, although MeTHF is the direct substrate in the remethylation of homocysteine in methionine.

Hydroxocobalamin/Me-cobalamin, PLP and FAD. Me-cobalamin (active form of vitamin B\(_{12}\)) is a co-factor of methionine synthase in the remethylation of homocysteine to methionine. Medium M199 contained no vitamin B\(_{12}\). The concentration of vitamin B\(_{12}\) in the culture medium was (due to its presence in serum) 180 pmol L\(^{-1}\). Addition of extra vitamin B\(_{12}\) to the culture medium either in the form of hydroxocobalamin or Me-cobalamin had no effect on the homocysteine export (Table 2).

PLP (active form of vitamin B\(_{6}\)) is the co-factor of cystathionine \(\beta\)-synthase (CS), which catalyses the
In the culture medium of methionine, vitamin B6, and the addition of FAD to the culture medium did not affect homocysteine export in a dose-dependent manner, at a constant rate over 72 h under standard culture conditions (Table 2). In all experiments the cell number and the cell viability were not influenced by the additions of these vitamins.

**Discussion**

HUVECs exported homocysteine at a virtually constant rate over 72 h under standard culture conditions (Fig. 2). This effect was highly reproducible in six different HUVEC lines. The cell number and cell viability remained unchanged and the concentrations in the culture medium of methionine, vitamin B12 and total folic acid decreased only slightly over 72 h.

Addition of folic acid to the medium reduced homocysteine export in a dose-dependent manner. MeTHF is the main circulating form of folic acid in blood and is a direct substrate in homocysteine remethylation. MeTHF also reduced homocysteine export in a dose-dependent manner, at a 10 times lower concentration than folic acid (Table 1). Folinic acid, a more stable one-carbon THF precursor of MeTHF, showed similar results. In addition, folinic acid had a greater tendency to reduce homocysteine export than MeTHF, probably because of its higher stability.

In our hospital, normal folate levels in human plasma are considered to be between 7 and 30 nmol L⁻¹. Extrapolating to the *in vitro* situation this means that endothelial cells are exporting a fair amount of homocysteine in the presence of normal folate levels. Even after addition of 10³ 30 nmol L⁻¹ MeTHF, the main form of folate in blood, a homocysteine export of 50% of the initial value was still observed.

Additions of other vitamins involved in homocysteine metabolism, such as Me-cobalamin, PLP and FAD, had no effect on homocysteine export [32]. An explanation is that these compounds are co-factors of the enzymes of homocysteine metabolism, whereas MeTHF is a substrate of homocysteine remethylation.

Rffium *et al.* [23] and Christensen *et al.* [24,25] studied homocysteine export in murine lymphoma cells, rat liver cells and in human fibroblasts, but not in human endothelial cells. In the cell homocysteine is either converted to cysteine or salvaged to methionine through remethylation or exported from the intracellular compartment into the extracellular medium. Ueland *et al.* [11] concluded that homocysteine export reflects an imbalance between homocysteine production and metabolism. In this study with HUVECs, the imbalance in homocysteine metabolism, as reflected by the high homocysteine export, disappeared after addition of folates to the medium.

In the human body the concentration of homocysteine in plasma is probably dependent on cellular homocysteine export and on the capacity for homocysteine degradation, mainly in liver and kidney [33]. Dysfunction of each of these systems can theoretically cause hyperhomocysteinaemia [1,2]. The observed imbalance in the homocysteine metabolism in cultured endothelial cells may reflect the *in vitro* situation but, on the other hand, it may reflect a natural feature of endothelial cells that can contribute to the vulnerability of endothelial cells to mild hyperhomocysteinaemia. Certain conditions are likely to enhance homocysteine export, such as disruption of the endothelial cell layer (resulting in repair, thus enhanced cell proliferation) or of the composition of the extracellular fluid (shortage of vitamins, higher levels of methionine or administration of certain drugs such as methotrexate).

Homocysteine-lowering therapy in patients with severe hyperhomocysteinaemia has proved to be clinically beneficial [3]. In mild hyperhomocysteinaemia, such clinical effects remain to be determined. Folic acid regiments are very effective in lowering plasma homocysteine levels *in vivo* [12,13]. In this study, folic acid proved to be very effective in maintaining low homocysteine levels in the culture medium, probably as a result of the restored balance of the homocysteine metabolism in the cultured endothelial cells.

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**Table 2. Homocysteine concentrations in HUVEC culture medium after incubation minus the homocysteine concentration already present in the culture medium before incubation**

<table>
<thead>
<tr>
<th>tHcy (µmol L⁻¹)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeTHF (µmol L⁻¹)</td>
<td>0</td>
<td>1.3</td>
<td>5.1</td>
</tr>
<tr>
<td>1</td>
<td>1.7</td>
<td>2.7</td>
<td>6.0</td>
</tr>
<tr>
<td>10²</td>
<td>1.6</td>
<td>3.1</td>
<td>5.5</td>
</tr>
<tr>
<td>10⁴</td>
<td>2.0</td>
<td>4.3</td>
<td>6.6</td>
</tr>
<tr>
<td>PLP (µmol L⁻¹)</td>
<td>0</td>
<td>1.6</td>
<td>3.3</td>
</tr>
<tr>
<td>1</td>
<td>1.8</td>
<td>3.5</td>
<td>5.6</td>
</tr>
<tr>
<td>100</td>
<td>1.7</td>
<td>3.5</td>
<td>5.1</td>
</tr>
<tr>
<td>FAD (nmol L⁻¹)</td>
<td>0</td>
<td>2.0</td>
<td>4.9</td>
</tr>
<tr>
<td>120</td>
<td>2.3</td>
<td>4.9</td>
<td>6.7</td>
</tr>
<tr>
<td>300</td>
<td>2.1</td>
<td>6.4</td>
<td>7.7</td>
</tr>
<tr>
<td>750</td>
<td>2.4</td>
<td>4.9</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Incubation times were 24, 48 and 72 h. The culture medium was supplemented with various concentrations of Me-cobalamin (0, 1, 10², 10⁴ nmol L⁻¹), PLP (0, 10, 100 µmol L⁻¹) and FAD (0, 120, 300, 750 nmol L⁻¹).
Acknowledgments

We thank Mrs C. Brouwer and all other personnel of the delivery rooms of the Departments of Obstetrics in the Canisius Wilhelmina Hospital and St. Radboud Hospital for collecting the umbilical cords. We also acknowledge the following technicians for their laboratory analysis of homocysteine and the vitamins: Mrs A. C. de Graaf Hess, D. van Oppenraaij Emmerzel and L. M. F. Geelen. This study was supported by the Dutch Heart Foundation (no. 94.029), The Hague, The Netherlands.

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