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Katharina Kurz, Barbara Frick, Christina Fürhapter, Günter Weiss, Barbara Wirleitner, Norbert Sepp and Dietmar Fuchs*

# Homocysteine metabolism in different human cells

**Abstract:** The effects of cytokine and mitogen stimulation on homocysteine (HCY) metabolism in different cells were investigated: in human dermal microvascular endothelial cells (HDMEC), T lymphocytes, mature and immature dendritic cells, and myelomonocytic (THP-1) and monocytic cell lines (U-937). Furthermore, the influence of supplementation of cells with folate acid, methionine and the combination of both on HCY metabolism was investigated. Unstimulated HDMEC and dendritic cells only produced very little amounts of HCY, and stimulation did not change HCY formation significantly either. However, higher HCY concentrations were detected in HDMEC and dendritic cells under supplementation with methionine and slightly less under supplementation with folate. Proliferating T lymphocytes showed an increase in HCY production on stimulation with increasing doses of mitogens; proliferative activity was associated with HCY formation. THP-1 and U-937 cells produced significantly more HCY than endothelial cells; U-937 cells produced most HCY, which was mainly due to their high proliferation rate. Stimulation of both cell lines with lipopolysaccharide and interferon-γ, respectively, showed a significant effect on HCY production of cells; in THP-1 cells, stimulation with IFN-γ and lipopolysaccharide induced neopterin formation. Methionine supplementation strongly increased and folate supplementation slightly decreased HCY formation in both cell lines. Thus, inflammation may play a role in moderate hyperhomocysteinemia.

**Keywords:** cells; homocysteine; neopterin.

***Corresponding author: Dietmar Fuchs,** Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innrain 80, A-6020 Innsbruck, Austria, E-mail: dietmar.fuchs@i-med.ac.at
**Katharina Kurz:** Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innrain 80, A-6020 Innsbruck, Austria; and Department of Internal Medicine VI, Innsbruck Medical University, Innrain 80, A-6020 Innsbruck, Austria
**Barbara Frick and Barbara Wirleitner:** Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innrain 80, A-6020 Innsbruck, Austria
**Christina Fürhapter and Norbert Sepp:** Department of Dermatology and Venerology, Innsbruck Medical University, Innrain 80, A-6020 Innsbruck, Austria
**Günter Weiss:** Department of Internal Medicine VI, Innsbruck Medical University, Innrain 80, A-6020 Innsbruck, Austria

## Introduction

Moderately elevated homocysteine (HCY) concentrations have been proposed as an independent risk factor for cardiovascular disease, stroke and dementia [1, 2]. The non-proteinogenic amino acid HCY may induce oxidative stress by auto-oxidation, leading to endothelial cell injury [3, 4]. However, data demonstrating a causal role of HCY in the development of cardiovascular disease are missing. Hyperhomocysteinemia is mostly due to deficiency in B-vitamins such as folic acid and vitamin $B_{12}$, which is also reflected by the inverse correlations between blood HCY concentrations and concentrations of B vitamins. Additionally, various other parameters like impaired kidney function, lifestyle, genetic alterations, etc., are also known to influence HCY levels [5].

Interestingly, in patients with cardiovascular disease, elevated HCY and lowered B-vitamin concentrations are found in parallel with elevated neopterin concentrations [6]. Also, in other diseases, e.g., autoimmune diseases like rheumatoid arthritis and neurodegenerative disorders like Parkinson's or Alzheimer's disease, moderate hyperhomocysteinaemia is associated with activation of the immune system [7–9]. In patients after trauma or with sepsis, an increase in HCY concentrations was observed during the stay at the intensive care unit, which was able to discriminate between survivors and non-survivors [10].

B-vitamin supplementation very efficiently lowers HCY concentrations [11]; however, recent meta-analyses confirm that this kind of intervention does not influence the cardiovascular risk of patients [12].

Th1-type immune response and oxidative stress consecutive to immune activation are known to play an important role in atherogenesis, progression of autoimmune diseases and dementia [13–16]. As reactive oxygen species are produced by activated monocytes/macrophages, immune activation rather than HCY may be responsible for oxidative stress and low-density lipoprotein oxidation.

Furthermore, immune activation may contribute importantly to the development of moderate hyperhomocysteinemia; an earlier study of our group could demonstrate that human peripheral blood mononuclear cells (PBMC) release HCY upon mitogen stimulation [17]. We studied the HCY metabolism of different cells stimulated with pro-inflammatory cytokines as an *in vitro* model of immune activation. Furthermore, we examined the influence of folate and methionine on HCY formation of cells.

# Materials and methods

## Cell culture

THP-1 cells and U-937 cells were maintained in RPMI-1640 (PAA-Laboratories, Linz, Austria) supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 2 mmol/L of L-glutamine (Serva, Heidelberg, Germany) and 50 μg/mL of gentamycin (Bio-Whitaker, Walkersville, MD, USA). THP-1 cells were seeded at a density of $1\times10^6$ cells and stimulated with interferon-γ (IFN-γ; 250 U/mL) and lipopolysaccharide (LPS; 1 μg/mL). U-937 cells were seeded at a density of $5\times10^5$ cells and stimulated with LPS (250 and 500 ng/mL). Cells were incubated at 37°C in 5% $CO_2$ for 72 h (THP-1) and 48 h (U-937), respectively. Supernatants were harvested by centrifugation (1500 rpm, 4°C, 8 min) and frozen at –20°C until measurement.

Immature dendritic cells (DC) and T lymphocytes were generated from the peripheral blood of healthy blood donors as described earlier [18, 19]. T lymphocytes were separated from monocytes by incubation of $5\times10^7$ cells of PBMC in Petri dishes coated with γ-globulin for 1 h at 37°C. Afterwards, non-adherent T lymphocytes were removed, seeded at $1\times10^6$ cells/mL and stimulated with mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) for 48 h. Monocytic cells were stimulated with granulocyte-monocyte colony-stimulating factor (GM-CSF; 800 U/mL) and interleukin-4 (IL-4) supernatants of IL-4-producing cells (10%) to differentiate to the DC in Petri dishes. Cultures were fed every 2 day by removing 3 mL of the medium and adding back 4.5 mL of the medium containing GM-CSF (1600 U/mL) and IL-4 supernatants of IL-4-producing cells (20%). After 10 days, non-adherent cells were harvested, analyzed on a fluorescence-activated cell sorter (FACS scan) and plated at a density of $2\times10^6$ cells/mL in 48-well plates in complete RPMI-1640 containing 800 U/mL of GM-CSF and 10% IL-4 supernatants of IL-4-producing cells with or without maturation stimulus [33% monocyte-conditioned medium (MCM)]. At the same time, DC were either stimulated with IFN-γ or supplemented with folate, methionine or the combination of both substances.

Human dermal microvascular endothelial cells (HDMEC) were isolated from surgically removed human foreskins obtained from newborns and children up to 7 years old according to a previously described technique [20]. Cells were cultured in endothelial cell basal medium supplemented with 10% normal human serum, 5 ng of epidermal growth factor per milliliter, 2 mmol/L of L-glutamine, 2 mmol/L of L-arginine, 100 μg of streptomycin per milliliter, 100 U of penicillin per milliliter and 250 ng of amphotericin B per milliliter. HDMEC were grown to confluency and were then stimulated with IFN-γ (500 U/mL), IFN-β (300 U/mL), TNF-α (500 U/mL), LPS (1 μg) or the combination of IFN-γ (250 U/mL) and TNF-α (250 U/mL).

To study the effects of folic acid and methionine (Sigma, Vienna, Austria) and a combination of these two substances on HCY production of cells, the medium was supplemented either with folic acid (FA; final concentration 10 μmol/L) or with methionine (M; final concentration 500 μmol/L) or the combination of both substances (FM; 10 μmol/L folic acid and 500 μmol/L methionine). Experiments were performed for three times with duplicates of controls and stimulated cells.

## Determination of homocysteine

HCY was determined by high-performance liquid chromatography as described previously [21].

## MTT assay

Cells were seeded onto 96-well flat-bottom plastic microtiter plates Nunc 96 well plates (VWR, Radnor, USA, PE) at a density of 10,000 cells per well and cultured in 100 μL RPMI-1640 medium with 10% FCS and 1% L-glutamine. After 2 days, an MTT assay was performed using a commercially available test kit (Biomedica, Vienna, Austria), according to the manufacturer's instructions. In principle, dimethylthiazol-diphenyltetrazolium bromide is reduced by the functional mitochondria of vital cells to an intensely colored formazan derivate. This reaction was quantified by measurement of absorption at 450 nm, indicating the amount of vital and proliferating cells.

## Statistical analysis

For comparisons of grouped data, the Mann-Whitney U-test was applied. p-Values below 0.05 were considered to indicate significant differences.

# Results

Both immunocompetent cell lines, the myelomonocytic cell line THP-1 and the monoblastic cell line U-937, spontaneously produced HCY. In the supernatants of U-937, the highest HCY concentrations of all experiments were determined (mean±SD, 6.1±0.9 μmol/L) (Figure 1). Also, THP-1 cells produced HCY; after 3 days of incubation, mean±SD HCY concentrations were 3.5±0.7 μmol/L (Figure 2). HCY was present in the medium at a concentration of 2.2 μmol/L.

Unstimulated T lymphocytes, which were also seeded in the same medium, were able to metabolize HCY; in supernatants, HCY concentrations were 0.6±0.2 μmol/L ($p<0.01$ compared to medium). Upon stimulation with increasing concentrations of mitogens Con A and PHA, HCY formation of cells increased significantly ($p<0.01$

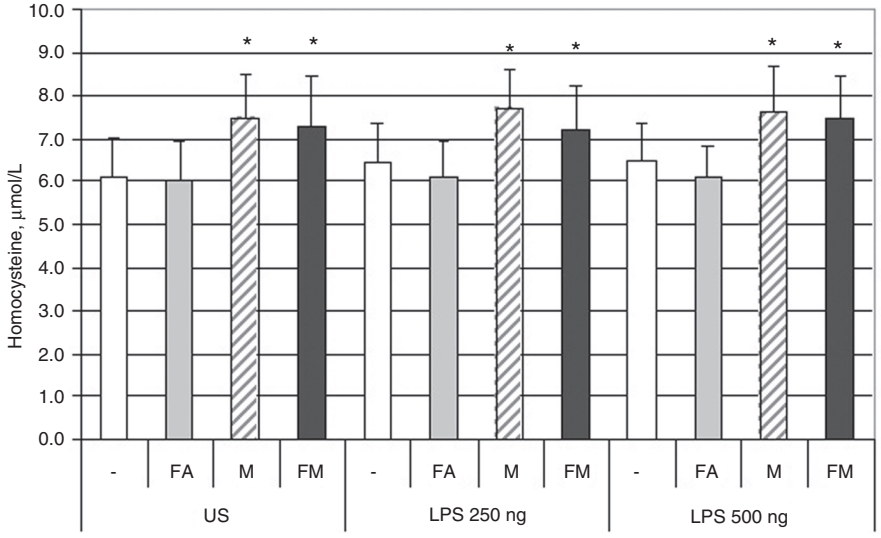


**Figure 1** Homocysteine concentrations determined in unstimulated (US) and stimulated (LPS=lipopolysaccharide) monocytic cells (U-937) that were supplemented with folic acid (FA), methionine (M) and the combination of both compounds (FM). Significant differences are indicated by an asterisk (*p<0.01).

compared to medium and unstimulated cells) up to levels of 4.2±0.5 µmol/L (Figure 3). Proliferation was measured by the MTT test and correlated strongly with the increase in HCY concentrations (p<0.001).

In supernatants of HDMEC, only very small amounts of HCY could be detected (mean±SD, 1.0±0.1 µmol/L), which were mainly due to HCY in the human serum being added to the medium (0.9±0.1 µmol/L). Nearly the same situation was observed in DC: HCY concentrations measured in supernatants differed only slightly from concentrations measured in medium without cells (2.9±0.8 µmol/L in immature DC, 3.2±0.7 µmol/L in mature DC). The medium for mature DC contained 3.0±0.5 µmol/L HCY, while the medium for immature DC contained 4.4±0.3 µmol/L HCY. Both cell populations were able to metabolize HCY, but they did not produce HCY by themselves.

Stimulation of U-937 cells with the stimuli IFN-γ and TNF-α did not influence HCY production, whereas stimulation with LPS induced the production of higher amounts of HCY (p<0.01 for LPS of 250 and 500 ng). When U-937 cells were supplemented with medium containing methionine or the combination of FA and methionine,

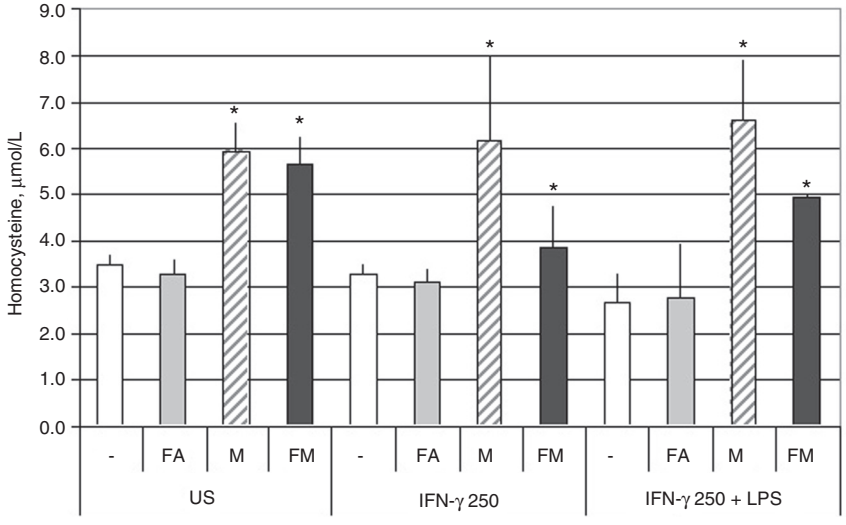


**Figure 2** Homocysteine concentrations measured in the supernatants of unstimulated (US) and stimulated myelomonocytic cells (THP-1) (IFN-γ 250=IFN-γ 250 U/mL) that were supplemented with folic acid (FA), methionine (M) and the combination of both compounds (FM). Significant differences are indicated by an asterisk (*p<0.01).

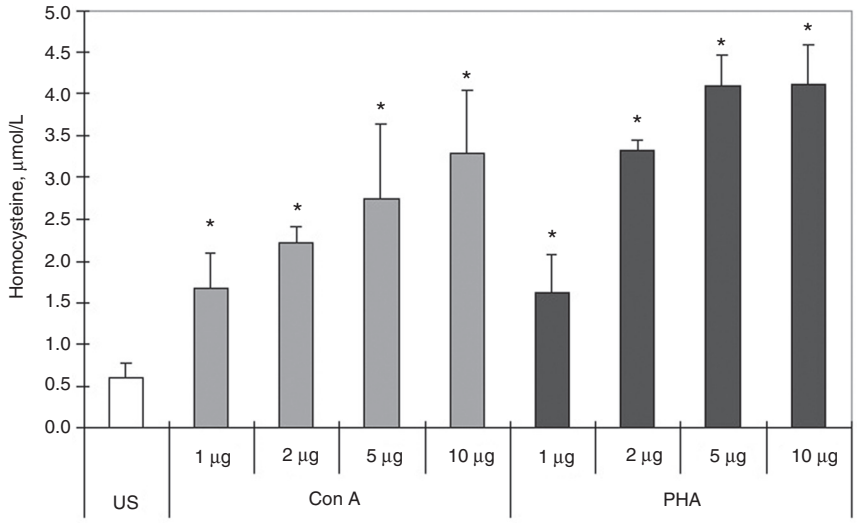


**Figure 3** Homocysteine concentrations determined in the supernatants of unstimulated (US) and mitogen-stimulated T lymphocytes (Con A, concanavalin A; PHA, phytohemagglutinin). Significant differences are indicated by an asterisk (*p<0.01).

HCY production was enhanced significantly (p<0.01); FA supplementation only slightly lowered HCY formation (Figure 1). Cells supplemented with the "combined" medium produced less HCY than cells supplemented with methionine only, showing that FA was able to modify HCY production.

Stimulation of THP-1 cells with the stimulus IFN-γ or the combination of IFN-γ and LPS reduced HCY production of cells (p<0.01 for IFN-γ, p<0.05 for the combination), whereas other stimuli like TNF-α did not have any influence. THP-1 cells produced significantly higher neopterin on stimulation with the proinflammatory cytokine IFN-γ or with the combination of IFN-γ and LPS or TNF-α (p<0.01) in comparison to unstimulated cells. Supplementation of THP-1 with FA slightly decreased HCY production, whereas methionine supplementation or the combination of both substances significantly increased HCY production of cells (p<0.01) (Figure 1).

Stimulation of HDMEC with the stimulus IFN-γ, TNF-α, the combination of IFN-γ and TNF-α or the combination of IFN-γ and TNF-α did not influence HCY production significantly. IFN-γ slightly increased HCY formation; the combination of TNF-α and IFN-γ decreased HCY production. Supplementation of HDMEC with FA and methionine showed similar effects as in cell lines THP-1 and U-937 (Table 1); FA+methionine slightly lowered HCY production; methionine and FA+methionine showed significantly higher HCY production (p<0.05).

In immature and mature DC, stimulation with IFN-γ led to slightly higher HCY concentrations in comparison to unstimulated cells. Furthermore, stimulation induced neopterin formation in both populations, which was more expressed in mature DC. Supplementation with folate did not influence HCY formation; cells supplemented with methionine and FA+methionine released significantly more HCY than control cells; neopterin formation was not influenced by any supplementation. In contrast to immunocompetent cell lines, U-937 and THP-1 and the endothelial cells, in both DC populations supplementation with FA+methionine induced higher HCY levels than supplementation with methionine alone (Figure 4).

## Discussion

Our *in vitro* experiments show that homocysteine (HCY) was produced mainly by quickly proliferating immunocompetent cells, whereas endothelial cells and dendritic cells (DC) did not release HCY. The formation of HCY was

**Table 1** Homocysteine concentrations (µmol/L) measured in the supernatants of unstimulated (US) and stimulated. (IFN-γ, interferon-γ; TNF-α, tumor necrosis factor α) Human dermal microvascular endothelial cells (HDMEC) supplemented with folic acid (FA), methionine (M) and the combination of folic acid and methionine (FM).

| | – | FA | M | FM |
|---|---|---|---|---|
| US | 1.03±0.04 | 0.98±0.07 | 1.71±0.27 | 1.46±0.14 |
| IFN-γ | 1.09±0.02 | 0.95±0.15 | 1.67±0.73 | 0.98±0.37 |
| IFN-γ+TNF-α | 0.98±0.10 | 0.76±0.18 | 1.29±0.13 | 1.24±0.13 |
| TNF-α | 0.91±0.14 | 0.74±0.07 | 1.89±0.85 | 1.27±0.25 |

–, medium with no other supplements. Values are shown as mean±SD.

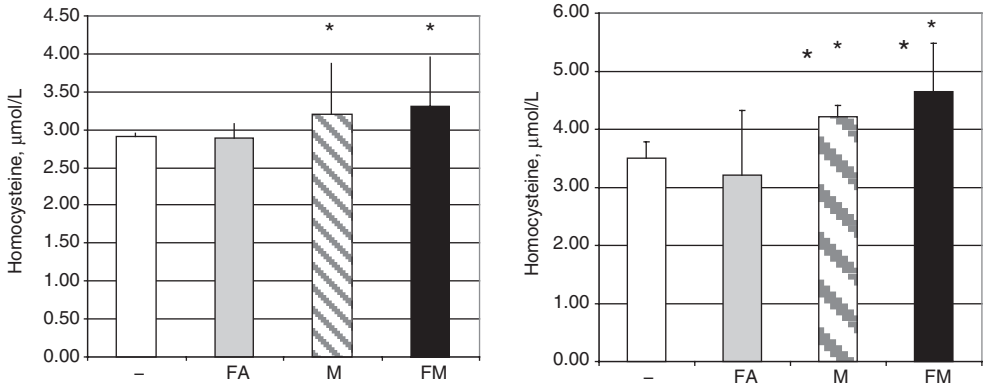


**Figure 4** Homocysteine concentrations measured in the supernatants of immature (right picture) and maturing dendritic cells (left picture) (*p<0.01) supplemented with folic acid (FA), methionine (M) and the combination of both compounds (FM). Significant differences are indicated by an asterisk (*p<0.01).

strongly influenced by the supply of cells with methionine and, to a minor extent, also with folic acid (FA). HCY production was strongly associated with the proliferative activity of cells, which was highest in the monoblastic cell line U-937 (cell number increased three-fold). Stimulation of cells with lipopolysaccharide was able to increase cell proliferation and, thus, HCY production even more in U-937, whereas in the myelomonocytic cell line THP-1 stimulation rather suppressed cell proliferation and, thus, HCY formation.

In tumor cells, enhanced HCY production was demonstrated previously by Sun et al. [22]; serum HCY levels were even suggested to be useful as a tumor marker in patients suffering from cancer [22, 23]. The high spontaneous proliferative activity of tumor cells seems to result in an enhanced demand for methionine and FA, which are both essential in HCY metabolism. Methyl-tetrahydrofolate is essential for the remethylation of HCY to methionine, and decreased availability of folic acid results in HCY accumulation. High methionine concentrations within the medium lead to enhanced HCY formation by cells. Human dermal microvascular endothelial cells (HDMEC) and also DC are both already differentiated cells, and their proliferative capacity is much lower than that of tumor cells. Accordingly, the influence of stimulation with pro-inflammatory cytokines on HDMEC and DC was much lower than in U-937 and THP-1; only slight increases in HCY formation were seen.

Apart from differentiation status, the capability of cells to react to exogenous stimulation also seems to be important for their ability to produce HCY: in earlier experiments, mitogen-stimulated human peripheral blood mononuclear cells (PBMC) were shown to produce HCY dependent on the extent of stimulation [17] and supplementation of FA and methionine. In our consecutive experiments with T lymphocytes, it turned out that the production of HCY is due to the proliferation of T cells, which is also strongly dependent on the extent of mitogen stimulation. HCY formation increased in dependence on the extent of mitogen stimulation.

The fact that immunocompetent tumor cell lines and also "normal" lymphocytes, which serve as essential effector cells of the immune system, show an enhanced HCY methionine metabolism may indicate that proliferating immunocompetent cells could contribute substantially to the development of hyperhomocysteinemia. Incomplete remethylation of HCY to methionine may occur as a consequence of enhanced cell metabolism going along with enhanced consumption of methionine and FA, which are known to be essential for processes like DNA synthesis, DNA repair and methyl group metabolism [24].

Significant associations between elevated concentrations of HCY and the immune activation marker neopterin have been shown in several diseases like coronary heart disease, pre-eclampsia, rheumatoid arthritis and neurodegenerative diseases [25]. Increased amounts of neopterin are produced by human monocytes/macrophages on stimulation with IFN-γ, which is produced by activated T lymphocytes within cellular immune activation. The coincidence of increased neopterin and HCY concentrations in patients indicates that the interaction between activated lymphocytes and macrophages within Th1-type immune response may be involved in the accumulation of HCY. When we tried to study the influence of stimulation with IFN-γ in monocytic cell lines THP-1 and U-937, only THP-1 cells produced neopterin. But although stimulation with IFN-γ significantly induced neopterin production, HCY production was rather suppressed by IFN-γ in THP-1. In mature and immature DC, neopterin formation was also

increased significantly, whereas HCY production was only slightly enhanced.

As the pro-inflammatory cytokine IFN-γ is *in vivo* produced by activated T cells, which also produce HCY when stimulated with mitogens, the association between neopterin and HCY concentrations can probably be monitored better in human PBMC than in other *in vitro* systems. This model system seems to be better suited to reflecting the interaction between T cells and monocytes/macrophages and, thus, also the *in vivo* situation. Within cellular immune activation, especially in chronic disease, there is a high turnover of immunocompetent cells; T-cell activation and proliferation are the main regulators of immune cascades. These results obtained in other immunocompetent and endothelial cells therefore confirm that HCY formation is dependent on the proliferative activity of cells and that immunocompetent cells like T lymphocytes may be responsible for the accumulation of HCY in patients suffering from diseases with chronic immune activation.

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