Is there a role of cyclosporine A on total homocysteine export from human renal proximal tubular epithelial cells?

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Background. Immunosuppressive therapy may influence homocysteine metabolism in allograft recipients. We examined whether cyclosporine A influences the in vitro formation of homocysteine as determined by the measurement of total homocystine (tHcy) concentrations in supernatants of human renal proximal tubule epithelial cells (hRPTEC), an important site of homocysteine metabolism.

Methods. Cells were incubated with and without vitamins in the presence of low or high methionine concentrations at different cyclosporine A concentrations for 24, 48 and 72 hours (N = 7 for each experiment). The concentration of tHcy in culture supernatants was measured by a fluorescence polarization immunosassay. Data were analyzed by four-way ANOVA, three-way ANOVA and t tests.

Results. The Hcy export from hRPTEC (tHcy in the culture supernatant) was 2.69 μmol/L during standard cell culture conditions at time point 24 hours and increased by 28.3% at 48 hours and by 44.6% at 72 hours. Comparisons of tHcy levels in culture supernatants over time by four way ANOVA showed that cyclosporine A at 200 or 800 ng/mL had no influence on tHcy export from hRPTEC (P = 0.67991). In contrast, the presence of vitamins in the medium (P = 0.000001), in vitro methionine loading (P < 0.000001), and prolonged incubation time (P < 0.000001) were associated with an increase of tHcy export from hRPTEC. Significant interactions in this analysis were “vitamins × methionine” (P = 0.001804), “vitamins × time” (P = 0.001478), “methionine × time” (P < 0.000001), and “vitamins × methionine × time” (P = 0.018128), pointing to a combined effect of vitamins in the presence of high methionine concentrations at the later time points.

Conclusion. Our study shows that hRPTEC export Hcy into the cell culture medium, an effect that is enhanced by in vitro methionine loading and modulated by the presence of vitamins. Cyclosporine A had no major influence on Hcy export from tubule cells. Therefore, our findings do not support the assumption that cyclosporine A elevates total homocysteine plasma levels in organ transplant patients.

Total homocysteine (tHcy) plasma concentrations are elevated in a large proportion of renal transplant patients with a prevalence of fasting and post-methionine loading hyperhomocysteinemia of approximately 50 to 60% [1, 2]. One potential explanation for this observation is the influence of the immunosuppressive therapy on Hcy metabolism in transplant patients. Some authors reported an association of cyclosporine A therapy with elevated tHcy levels in kidney or heart allograft recipients [3–6]. This finding, however, has not been confirmed by other studies [7–10].

Because controversy exists regarding the impact of cyclosporine A on Hcy metabolism, we examined whether cyclosporine A influences the in vitro formation of Hcy as determined by the measurement of tHcy export from human renal proximal tubule epithelial cells (hRPTEC), an important metabolic site of Hcy.

METHODS

Cell culture

Human renal proximal tubule epithelial cells (hRPTEC; provided by Bio Whittaker-Clonetics, Walkersville, MD, USA), were obtained in the second passage, subcultured according to the supplier’s specifications, and cryopreserved in the fifth passage. Cryopreserved cells were than thawed and seeded in 175 cm² cell culture flasks. For cell culture until cryopreservation, renal epithelial cell basa medium was enriched with growth factors (REGM singlequots, Bio Whittaker-Clonetics). After thawing cells were cultured in Dulbecco’s modified Eagle medium with 10% fetal calf serum (FCS) and 5 mL penicillin-streptomycin solution.

For the final experiments, 80% confluent cells from one 175 cm² cell culture flask were subcultured in six-well plates using RPMI 1640 medium with 5% FCS, 5 mL of penicillin-streptomycin, 2 mL of fungizone, 5 mL of L-glutamine, 500 μL of gentamycin and 2500 IU heparin (Immuno, Vienna, Austria). The cells were incubated for 24, 48 and 72 hours with vitamin B₁₂ (0.005 mg/L), folic acid (1 mg/L) and pyridoxal-HCl (1 mg/L) or without these three vitamins in the presence of a normal

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methionine concentration (15 mg/L) or in the presence of a tenfold increased methionine concentration (150 mg/L). Cyclosporine A (Sandoz Pharma AG, Basel, Switzerland) was added at a final concentration of 200 or 800 ng/mL, and using the dissolvent in control experiments. Cell culture reagents were obtained from Life Technologies (Vienna, Austria) if not indicated otherwise.

A total number of seven experiments were performed using three different cell batches. By using the trypan blue method, viability of the cells was found to be between 80 and 90% at 24, 48 and 72 hours, respectively, and was independent of the incubation medium used.

**Measurement of total homocysteine in cell supernatants**

The concentration of tHcy in the supernatants was determined in duplicate using a fluorescence polarization immunoassay (IMx analyzer; Abbott Laboratories, Abbott Park, IL, USA). The lower detection limit of the assay was 0.5 μmol/L. The IMx assay revealed comparable results for tHcy concentrations in the cell culture supernatants as compared to the values obtained by high performance liquid chromatography with fluorescence detection [11] (the comparison of both methods for measurement of tHcy in cell culture supernatants will be reported elsewhere).

**Statistics**

Continuous data are given as mean ± SD. Comparisons of tHcy levels in culture supernatants over time were performed by four way ANOVA including the factors cyclosporine A (0, 200, and 800 ng/mL), methionine (low or high), vitamins (yes or no), and time (24, 48 and 72 hours) and the interaction terms for the aforementioned factors. A three-way ANOVA was performed for all measurements for the time points 24, 48, or 72 hours with the factors cyclosporine A, methionine and vitamins. Unpaired comparisons of experiments without or with vitamins were performed at the three different time points for the different cyclosporine A concentrations and low or high concentration of methionine. A P < 0.05 was considered statistically significant. All calculations were performed by Statistica for Windows (release 4.5, Stat Soft Inc. 1993).

**RESULTS**

The Hcy export from hRPTEC as determined by measurement of tHcy in the culture supernatant was 2.69 ± 0.38 μmol/L during standard cell culture conditions at time point 24 hours and increased by 28.3% at 48 hours and by 44.6% at 72 hours. The time course of tHcy export from hRPTEC under the different cell culture conditions is depicted in Figure 1.

Comparisons of tHcy levels in culture supernatants over time by four way ANOVA showed that cyclosporine A at 200 or 800 ng/mL had no independent influence on tHcy export from hRPTEC (P = 0.67991). In contrast, the presence of vitamins in the medium (P = 0.000001), in vitro methionine loading (P < 0.000001) and prolonged incubation time (P < 0.000001) were associated with an increase of tHcy export from hRPTEC. Significant interactions in this analysis were “vitamins × methionine” (P = 0.001804), “vitamins × time” (P = 0.001478), “methionine × time” (P < 0.000001), and “vitamins × methionine × time” (P = 0.018128), pointing to an additive combined effect of vitamins in the presence of high methionine concentrations at the later time points.

Separate analyses of the three different time points by three way ANOVA showed that methionine was the only significant predictor of tHcy export (P < 0.000001) at 24 hours. At 48 hours, vitamins (P = 0.000119), methionine (P < 0.000001) and the interaction “vitamins × methionine” (P = 0.037367) were associated with tHcy export. At the 72-hour time point, the level of significance increased for the three aforementioned terms (P = 0.00004, P < 0.000001, and P = 0.002754, respectively).

Unpaired comparisons of independent experiments with and without vitamins showed no significant differences of tHcy export in low methionine medium with or without cyclosporine A at either time point (Fig. 1). At high methionine concentrations, there was a significant increase of tHcy export in the absence of cyclosporine A at 48 hours (P < 0.015) and at 72 hours (P < 0.0159), and at the low cyclosporine A concentration at 48 hours (P < 0.05) and at 72 hours (P < 0.05) in the presence of vitamins (Fig. 1). There was no difference in Hcy export at the high cyclosporine A concentration.

**DISCUSSION**

Our study shows that cyclosporine A has no influence on export of Hcy from human proximal tubular epithelial cells. By contrast, in vitro methionine loading and the presence of folic acid, vitamin B6, and vitamin B12 in the cell culture medium enhanced Hcy export from tubular cells.

Previous studies demonstrated that the creatinine clearance, the plasma folate status, and the MTHFR 677TT genotype are the most important predictors of tHcy plasma levels in kidney graft recipients [1]. The prevalence of hyperhomocysteinemia is high even in allograft recipients with normal renal function, and one explanation for this observation is the potential influence of the immunosuppressive therapy on Hcy metabolism. Arnadottir et al were the first to suggest that cyclosporine
A, via a yet undefined mechanism, may be associated with higher tHcy plasma levels in kidney graft recipients [3, 4]. This finding, however, has not been confirmed by others [7–10]. Following heart transplantation, an increase of tHcy plasma levels has also been observed [12]. Cole et al examined the effect of several variables on tHcy plasma concentrations in 72 heart transplant recipients. In a multiple linear regression model, only creatinine and trough cyclosporine concentrations were independent positive predictors of tHcy [5]. Comparable results were also obtained in the study of Cook et al [6]. By contrast, azathioprine showed no influence on tHcy plasma levels in renal transplant patients [1].

In the present study, the export of Hcy by human proximal tubule cells was analyzed with regard to the influence of cyclosporine A. Previous studies have shown that non-malignant cells, such as human umbilical cord vein endothelial cells (HUVEC) [13, 14], HeLa cells [15], hematopoietic and bone marrow stromal cells [16], red blood cells [17], liver cells [18], and fibroblasts [18–21] are capable of exporting Hcy into the cell culture medium. Addition of methionine to the culture medium resulted in a two- to threefold increase of Hcy export as has been measured in the culture supernatant of endothelial cells [13], or an even higher export from fibroblasts or hepatocytes [18]. The addition of 5-methyltetrahydrofolate or 5-formyltetrahydrofolate normalized Hcy export from fibroblasts [21]. 5-Formyltetrahydrofolate, but not pyridoxine-HCl or vitamin B<sub>12</sub>, substantially reduced Hcy export from endothelial cells [13]. Supplementation with folic acid also lowered Hcy export from endothelial cells, whereby 5-methyltetrahydrofolate and 5-formyltetrahydrofolate were about ten times more effective [14].

In our study, Hcy export over time from tubule cells was comparable with the Hcy export from endothelial cells in the study of van der Molen et al [13]. The tHcy concentration in the supernatants of tubule cells was 2.69 μmol/L at 24 hours and increased up to 3.89 μmol/L at
72 hours of cell culture. Overall there was a two- to threefold increase of Hcy export in the presence of excess methionine at every time point. Interestingly, the presence of folic acid, vitamin B6 and vitamin B12 increased Hcy export in the presence of excess methionine. Our data are in some contrast to the findings of others who reported on a decrease of Hcy export from endothelial cells or fibroblasts in response to high concentrations of folic acid or its reduced derivatives [14, 21]. However, none of these studies investigated the effect of a multivitamin supplementation consisting of folic acid, vitamin B6 and vitamin B12 on the cellular Hcy export. One explanation for the decreased Hcy export from hRPTEC in the absence of the three vitamins could be that these vitamins are involved in the export mechanism of tubule epithelial cells. By contrast, the addition of cyclosporine A to the culture medium had no effect on Hcy export from tubule cells, regardless of the methionine concentration in the medium. This finding supports the concept that therapy with cyclosporine A does not aggravate hyperhomocysteinemia in transplant patients. However, in experiments without vitamins very high concentrations of cyclosporine A antagonized the decrease of Hcy export in the presence of excess methionine (Fig. 1).

In conclusion, our study shows that hRPTEC export Hcy into the culture medium. This effect is enhanced by in vitro methionine loading and modulated by vitamins. Cyclosporine A has no major influence on Hcy export from tubule cells. Therefore, our findings do not support the concept that cyclosporin A elevates tHcy plasma levels in organ transplant patients.

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