

Long-term exposure of human proximal tubule cells to hydroxycobalamin[c-lactam] as a possible model to study renal disease in methylmalonic acidurias

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Summary Dysfunction of proximal tubules resulting in tubulointerstitial nephritis and chronic renal failure is a frequent long-term complication of methylmalonic acidurias. However, the underlying pathomechanisms have not yet been extensively studied owing to the lack of suitable in vitro and in vivo models. Application of hydroxycobalamin[c-lactam] has been shown to inhibit the metabolism of hydroxycobalamin and, thereby, to induce methylmalonic aciduria in rats, oligodendrocytes, and rat hepatocytes. Our study characterizes the biochemical and bioenergetic effects of long-term exposure of human proximal tubule cells to hydroxycobalamin[c-lactam], aiming to establish a novel in vitro model for the renal pathogenesis of methylmalonic acidurias. Incubation of human proximal tubule cells with hydroxycobalamin[c-lactam] and propionic acid resulted in a strong, time-dependent intra- and extracellular accumulation of methylmalonic acid. Bioenergetic studies of respiratory chain enzyme complexes revealed an increase of complex II–IV activity after 2 weeks and an increase of complex I and IV activity as well as a decrease of complex II and III activity after 3 weeks of incubation. In addition,

human proximal tubule cells displayed reduced glutathione content after the exposure to hydroxycobalamin [c-lactam] and propionic acid.

Abbreviations

| | |
|----------|-----------------------------|
| AdoCbl | 5'-deoxyadenosylcobalamin |
| HCCL | hydroxycobalamin[c-lactam] |
| hPTEC | human proximal tubule cells |
| MCM | methylmalonyl-CoA mutase |
| MMA | methylmalonic acid |
| MMAurias | methylmalonic acidurias |

Introduction

Methylmalonic acidurias (MMAurias) are a group of aetiologically heterogeneous metabolic disorders in the degradation of branched-chain amino acids, odd-numbered fatty acids, and cholesterol. Inherited defects of the mitochondrial enzyme methylmalonyl-CoA mutase (MCM; EC 5.4.99.2, Ledley et al. 1988) or of the synthesis of its cofactor 5'-deoxyadenosylcobalamin (AdoCbl; Hörster et al. 2007; Mahoney et al. 1975) are the most frequent causes of these disorders. MMAurias are biochemically characterized by the accumulation of methylmalonic acid (MMA) (Fenton et al. 2001), propionic acid, 3-hydroxypropionic acid, and 2-methylcitric acid. The disease course is complicated by acute metabolic crises during catabolic states, which can result in multiple organ failure or even death and resemble primary defects of mitochondrial energy metabolism. In addition to acute crises, long-term complications such as chronic renal failure and neurological disease are frequently found in affected patients.

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References to electronic databases: Methylmalonyl-CoA mutase: EC 5.4.99.2. Glutathione reductase: EC 1.8.1.7.

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It has been demonstrated that accumulating organic acids and CoA esters play a central role in the neuropathogenesis of MMAurias. In particular, propionyl-CoA, malonic acid and 2-methylcitric acid synergistically inhibit enzymes of the tricarboxylic acid cycle, pyruvate dehydrogenase complex and respiratory chain (Kolker et al. 2003; Morath et al. 2008; Okun et al. 2002; Schwab et al. 2006), whereas methylmalonic acid is suggested to compete with the mitochondrial import of succinate (Mirandola et al. 2008). In contrast to the neuropathogenesis, mechanisms underlying chronic renal failure have not yet been extensively studied. Clinically, progressive tubulointerstitial nephritis with mononuclear cell infiltration, interstitial fibrosis, and tubular atrophy has been demonstrated (D'Angio et al. 1991). Further, proximal and distal tubules of affected patients display functional abnormalities including decreased resorption of phosphate, impaired acid balance and reduced urinary concentrating ability.

Preliminary results in rats suggested a role of MMA as a possible nephrotoxin causing proteinuria and renal tubular injury (Kashtan et al. 1998). We have recently developed a hypothetical model for the renal pathology of MMA (Morath et al. 2008) postulating that pathophysiologically accumulating metabolites induce in addition to a dysfunction of mitochondrial energy production also a dysfunction of transport processes of the proximal tubule. At the proximal tubule, uptake of organic anions via organic anion transporter (OATs) is coupled with the export of dicarboxylic acids. This drain of dicarboxylic acids—most likely TCA cycle intermediates—is replenished by sodium-coupled dicarboxylate transporters (NaDCs; Burckhardt and Burckhardt 2003) catalyzing the import of these metabolites. We have suggested that MMA might interfere with the uptake of dicarboxylic TCA cycle intermediates via hNaDCs and may induce a depletion of energy substrates in proximal tubule cells (Morath et al. 2008). In analogy to the neuropathogenesis of MMAurias, chronic renal failure is most likely due to a synergism of different pathomechanisms (Kolker and Okun 2005; Morath et al. 2008). Experimental evidence for this hypothesis has yet been missing owing to the lack of suitable *in vitro* and *in vivo* models. A knockout mouse for MMAuria (Peters et al. 2002) turned out to be ineligible since these mice die within 24 h after birth.

An alternative approach to inducing MMAurias *in vivo* or *in vitro* is the application of hydroxycobalamin [c-lactam] (HCCL). Krahenbuhl and colleagues (1990) demonstrated that the administration of HCCL in rats inhibits the metabolism of AdoCbl, the obligatory

cofactor of MCM. Similar results were obtained using loading of oligodendrocytes with HCCL (Sponne et al. 2000) and rat hepatocytes (Brass 1993).

Our study characterizes the biochemical and bioenergetic effects of long-term exposure to HCCL and subsequently induced secondary MCM deficiency in human proximal tubule cells, thereby aiming to establish a novel *in vitro* model to study the renal pathogenesis of MMAurias.

Material and methods

Synthesis of HCCL

HCCL was custom synthesized by Glycoteam GmbH, Hamburg, Germany (95–96% purity, HPLC).

Cell culture

Human proximal tubule cells (hPTEC) were purchased from Clonetics (Lonza, Basel, Switzerland) and cultivated in DMEM/Ham's F-12 (purchased from PAA, Pasching, Austria) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin, 35 ng/ml hydrocortisone, 5 ng/ml hEGF, 6.4 ng/ml T₃, 5 ng/ml selenite, and 3.4 mg/ml NAD. Loading experiments were started when confluency of the hPTEC monolayer reached 50%. For HCCL loading, hPTEC were grown in T75 culture flasks containing standard medium and were incubated with 5 or 10 µg/ml HCCL for up to 28 days. Sister cultures were exposed to 0.1–1 mmol/L propionic acid in addition. For biochemical studies, medium was removed weekly. At days 1, 7, 14, and 21, cells from one flask were harvested using Accutase and centrifuged for 10 min at 1000 *g*_{max} and then washed with PBS. The supernatant was discarded and the pellet was homogenized using a Potter–Elvehjem system.

For bioenergetic studies, cells were harvested and the suspension was centrifuged at 130 *g*_{max} for 10 min at 4°C. The cell pellet was suspended in 500 µl PBS and shock-frozen in liquid nitrogen. Afterwards, cells were thawed by addition of 1 ml cold buffer containing 250 mmol/L sucrose, 50 mmol/L KCl, 5 mmol/L MgCl₂, 20 mmol/L Tris/HCl (pH 7.4, RC-buffer) and 0.01% digitonin. After 10 min incubation on ice, cells were centrifuged at 10 000 *g*_{max} for 5 min at 4°C, and, subsequently, the pellet was suspended in 1 ml cold RC-buffer and centrifuged again at 10 000 *g*_{max} for 5 min at 4°C. After suspension of the pellet in 500 µl RC-buffer, the sample was used for bioenergetic analyses.

Protein content

Protein content of homogenates was determined using Bio-Rad DC Protein Assay. Results of all experiments performed are normalized to protein content.

Quantitative analysis of MMA and 2-methylcitric acid

MMA and 2-methylcitric acid concentrations were quantified according to a method previously described (Okun et al. 2002) using gas chromatography–mass spectrometry and stable-isotope dilution methods.

Quantitative analysis homocysteine

Homocysteine concentrations were determined using the IMx System Homocysteine Assay (Abbott, Diagnostics Divisions, Wiesbaden, Germany).

Spectrophotometric analysis of single respiratory chain complexes I–IV

Steady-state activities of enzyme complexes were recorded using a computer-tuneable spectrophotometer (Spectramax Plus Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) operating in the dual-wavelength mode. Samples were analysed in temperature-controlled 96-well plates in a final volume of 300 μ l. The catalytic activities of respiratory chain complexes I–IV in hPTEC were investigated as previously described for submitochondrial particles from bovine heart (Kolker et al. 2003; Okun et al. 2002; Sauer et al. 2005; complex I, NADH reduction at 340–400 nm; complex II, dichlorophenolindophenol reduction at 610–750 nm; complex III, cytochrome *c* reduction at 540–550 nm; complex IV, cytochrome *c* oxidation at 540–550 nm; complex V, NADH reduction at 340–400 nm). The addition of standard respiratory chain inhibitors (complex I: 2-*n*-decylquinazolin-4-yl-amine [1 μ mol/L]; complex II: thenoyltrifluoroacetone [8 mmol/L]; complex III: antimycin A [1 μ mol/L]; complex IV: NaCN [2 mmol/L]; complex V: oligomycin [80 μ mol/L]) revealed good inhibitory responses (93–100% of control activity, $p < 0.001$ versus controls), confirming a high specific enzyme activity in our assay system.

Glutathione content of tissue homogenates

Glutathione concentrations were determined according to a modification of the method of Sauer et al. (2005). Glutathione reductase (EC 1.8.1.7) activity was assayed in deproteinized cell homogenates in a buffer (adjusted

to pH 7.5; 25°C) containing 180 mmol/L disodium hydrogenphosphate, 6 mmol/L Na-EDTA, 0.3 mmol/L NADPH, 6 mmol/L dithiobis(2-nitrobenzoic acid) and 0.3 U/ml glutathione reductase (Sigma Aldrich, Taufkirchen, Germany). Enzyme activity was determined as dithiobis(2-nitrobenzoic acid) reduction at $\lambda = 412$ nm. Glutathione content was determined by correlating the detected enzyme activities with a calibration curve generated with samples of defined glutathione concentration.

Cell viability assay

Cell viability was tested by Trypan blue staining and cytotoxicity was investigated using the tetrazolium salt 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay was purchased from Sigma Aldrich and carried out according to the manufacturer's manual.

Statistical analysis

Mann–Whitney *U*-test was calculated using SPSS for Windows 16.0 software. A value of $p < 0.05$ was considered as significant.

Results

Incubation with HCCL and propionic acid increases intra- and extracellular MMA concentrations

To inhibit AdoCbl metabolism and, consequently, reduce the AdoCbl-dependent MCM activity, hPTEC were incubated in a medium containing 5 or 10 μ g/ml HCCL for up to 21 days. To further stress the impaired major catabolic pathway of propionic acid through MCM, sister cultures were co-incubated with propionic acid (100–1000 μ mol/L). Exposure of hPTEC to 5 or 10 μ g/ml HCCL alone did not induce a detectable increase of extra- (i.e. medium) or intracellular MMA concentrations (Fig. 1a, b). In contrast, co-incubation of hPTEC with HCCL and 100 μ mol/L propionic acid induced successively increasing MMA concentration during 3 weeks in culture, resulting in a 26-fold elevation of extracellular and a 133-fold elevation of intracellular MMA concentrations (day 21; extracellular 2.6 μ mol/L, intracellular 4 nmol/mg; Fig. 1a, b). Increasing the propionic acid concentration in the medium significantly reduced the cell viability of hPTEC (data not shown) and, therefore, all following studies were performed with 100 μ mol/L propionic acid. Incubation of hPTEC with propionic acid (100 μ mol/L) alone for 3 weeks did not change cell

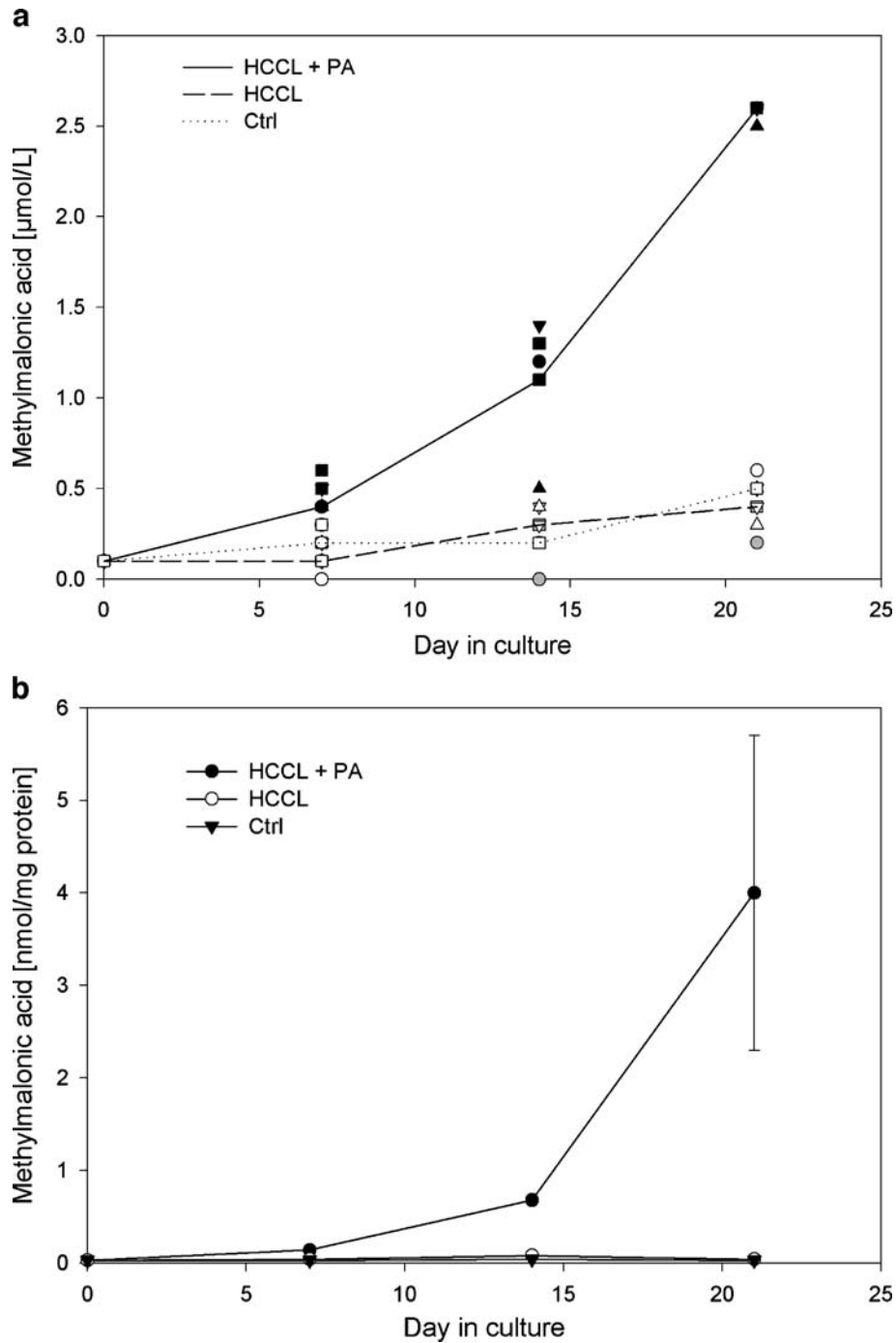


Fig. 1 Extra- and intracellular MMA concentrations. Incubating hPTEC with 10 µg/ml HCCL alone did not increase extracellular (i.e. medium; **a**) or intracellular (**b**) MMA concentrations. Co-incubation of hPTEC with HCCL and 100 µmol/L propionic acid (PA) induced a strong and time dependent elevation of (**a**) extracellular and (**b**) intracellular MMA concentrations. In (**a**), lines indicate the mean of

individual experiments at different time points and symbols represent the corresponding individual values: black symbols, HCCL + PA; grey symbols, HCCL; open symbols, control. (**b**) Corresponding intracellular MMA concentrations of one flask hPTEC at day 1, 7, 14 and three flasks hPTEC at day 21 expressed as median ± range

viability or activities of respiratory chain enzymes (cell viability detected by MTT assay, 95.5% ± 9.1% of control; complex I, 99.0% ± 2.2% of control; complex II, 99.4% ± 4.2% of ctrl; complex III, 95.6% ± 5.9% of control; complex IV, 118.4% ± 28.3% of control; data

are expressed as median ± range, *n*=3). The time course of MMA production did not differ between intra- and extracellular concentrations.

hPTEC reached confluency after 14 days, and after day 21 cell viability started to decrease under control

and loading conditions. Therefore, the experiment was terminated at day 21. Within the whole incubation period, however, cell viability was not affected in cells loaded with HCCL or HCCL plus propionic acid as indicated by Trypan blue staining ($99\% \pm 11\%$ of control; $n=3$) and MTT assay ($95\% \pm 8\%$ of control; $n=3$).

In contrast to the findings with MMA, we have not been able to detect an increase of extra- or intracellular 2-methylcitric acid concentrations. As described previously (Sponne et al. 2000), homocysteine levels were elevated extra- and intracellularly owing to the block of vitamin B₁₂ metabolism (intracellular: control, 0.01 ± 0.05 nmol/mg, HCCL and propionic acid treated cells, 1.86 ± 0.26 μ mol/L; extracellular: control, 12.8 ± 2.54 μ mol/L, HCCL and propionic acid treated cells, 18.3 ± 4.32 μ mol/L; all experiments were performed as triplicates).

Effect of elevated MMA concentration on the activity respiratory chain enzymes

To investigate the hypothesis that bioenergetic impairment plays a role in the pathophysiology of MMAurias, we studied the activities of respiratory chain enzyme complexes at days 14 and 21. We chose these time points

because at day 14 MMA concentrations started to increase significantly, and at day 21 MMA concentrations reached a maximum. Strikingly, enzyme activities were regulated during the loading experiment with HCCL and propionic acid. At day 14, activities of complexes II–IV were elevated compared with the control cells, with the most pronounced increase of complex III activity (complex II 114% of control; complex III 153% of control; complex IV 129% of control; $p < 0.05$; Fig. 2 and Table 1). At day 21, however, activities of complexes I and IV were increased, whereas activities of complexes II and III were slightly reduced (complex I 115% of control; complex II 88% of control; complex III 87% of control; complex IV 117% of control; $p < 0.05$; Fig. 2 and Table 1).

Reduced glutathione content

We studied the glutathione content in hPTEC after incubation with HCCL and propionic acid to evaluate oxidative stress in these cells. Following HCCL exposure, hPTEC showed lower glutathione concentrations than control cells at day 14 and day 21 (80%, 74.6% of control; $p < 0.05$; Fig. 3), suggesting ROS formation.

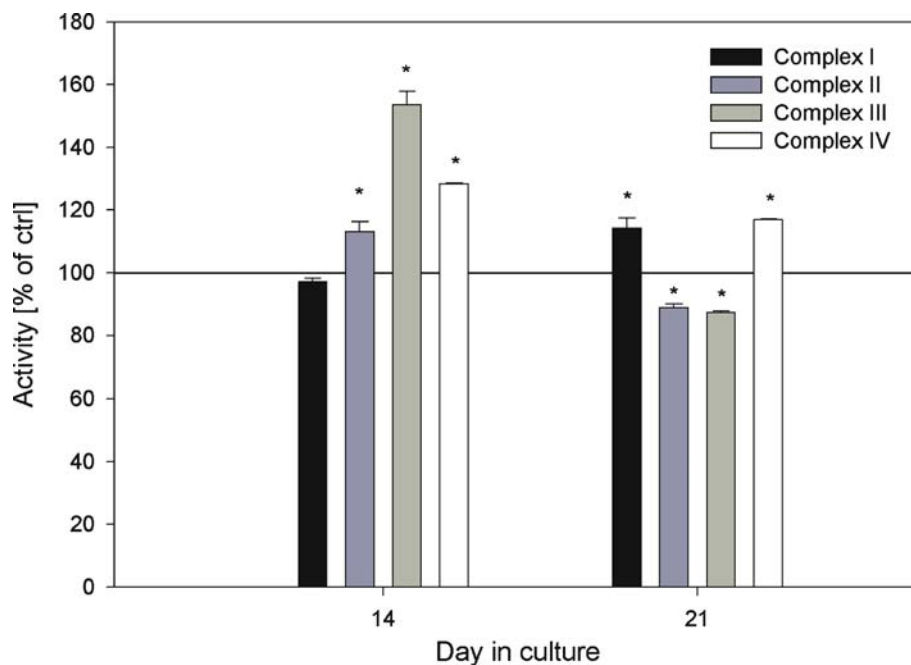


Fig. 2 Activities of respiratory chain complexes I–IV. Activities of respiratory chain single enzyme complexes were measured at days 14 and 21. At day 14, activities of complexes II–IV were elevated compared with the control cells, with the most pronounced increase in complex III activity (complex II, 114% of control; complex III, 153% of control; complex IV, 129% of

control; $p < 0.05$). At day 21, in contrast, activities of complexes I and IV were elevated, whereas activities of complexes II and III were slightly reduced (complex I, 115% of control; complex II, 88% of control; complex III, 87% of control; complex IV, 117% of control; $*p < 0.05$). Data are expressed as median \pm range, $n=3$

Table 1 Activities of respiratory chain complexes I–IV. Activities of respiratory chain single enzyme complexes were measured at days 14 and 21

| | | Complex I | Complex II | Complex III | Complex IV |
|--------|-----------|-----------|------------|-------------|------------|
| Day 14 | Ctrl | 2.61±0.09 | 1.45±0.09 | 4.83±0.13 | 0.14±0.03 |
| | HCCL + PA | 2.53±0.12 | 1.64±0.05 | 7.42±0.21 | 0.18±0.02 |
| Day 21 | Ctrl | 1.32±0.08 | 2.16±0.01 | 6.14±0.17 | 0.22±0.01 |
| | HCCL + PA | 1.50±0.06 | 1.92±0.11 | 5.37±0.02 | 0.26±0.02 |

Data are expressed as median ± range ($n=3$) in mU/mg protein. HCCL, hydroxycobalamin[c-lactam]; PA, propionic acid.

Discussion

Dysfunction of the proximal tubule resulting in tubulointerstitial nephritis is a common long-term complication of MMAurias. However, owing to the lack of suitable in vitro or in vivo models, little is known about the underlying pathomechanisms. Our study therefore aimed to establish and to evaluate an in vitro model for the renal phenotype of affected individuals with MMAurias using hPTEC that were incubated with HCCL. The major result of our study is that co-incubation of hPTEC with HCCL and propionic acid increased the intra- and extracellular concentrations of MMA in a time-dependent way.

It has been shown for rats (2 µg/h; Krahenbuhl et al. 1990) and oligodendrocytes (5, 10 µg/ml; Sponne et al. 2000) that HCCL inhibits cobalamin metabolism and

induces MMAurias. In hPTEC, however, HCCL alone did not induce MMA production, indicating that MCM activity was not or was only mildly affected. Loading of hPTEC with HCCL (10 µg/ml) and propionic acid (100 µmol/L) resulted in a time dependent MMA production detectable in media and in cells peaking at day 21. The finding that only a combination of HCCL and propionic acid induces MMA production in vitro has also been shown by a study of Brass (1993). In their experiments in primary cultures of rat hepatocytes the authors could induce an increase of MMA only when cells were loaded with HCCL (1 mg/L) and propionic acid (2 mmol/L). The MMA concentrations found in our study (extracellular, 2.6 µmol/L; intracellular, 4 nmol/mg) were similar to the concentrations detected by Kolhouse and colleagues (1993) using fibroblasts (extracellular, 1–2 µmol/L), but 20-fold lower

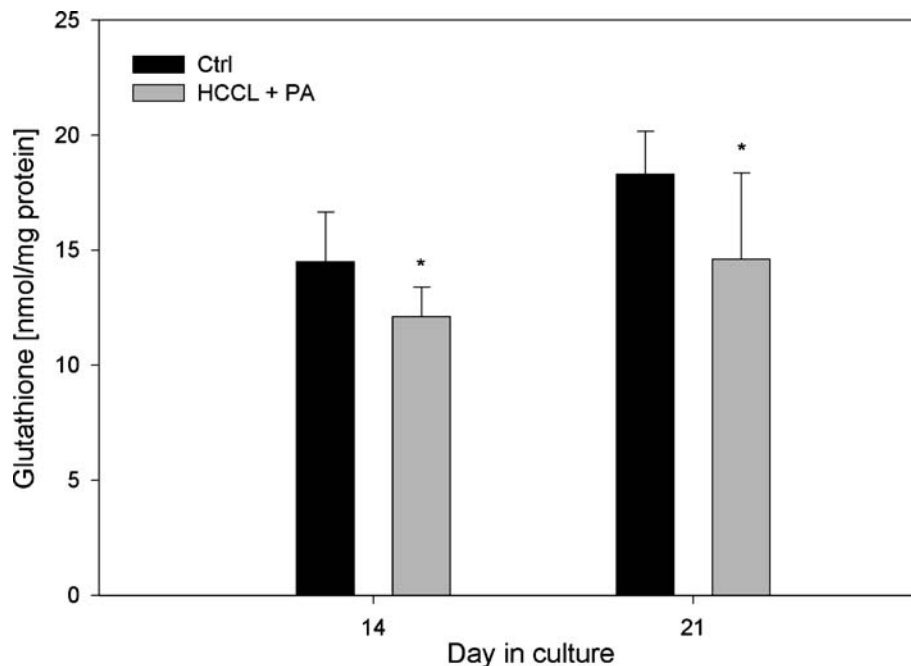


Fig. 3 Glutathione content. hPTEC loaded with HCCL and propionic acid (PA) displayed lower glutathione concentrations than control cells at day 14 and day 21 of incubation. Although

data ranges at day 21 were overlapping, glutathione levels were significantly decreased at day 14 and 21 (80%, 74.6% of control; $*p<0.05$). Data are expressed as median ± range, $n=3$

than described in the study of Sponne and colleagues (2000) in oligodendrocytes (extracellular, 45 $\mu\text{mol/L}$). MMA knockout ($\text{Mut}^{-/-}$) mice displayed kidney MMA concentration of approximately 0.5 nmol/mg tissue (Chandler et al. 2007), which corresponds to a MMA concentration of approximately 2.5 nmol/mg protein assuming a protein content of 20% in the kidney. Our cell culture model mimics the phenotype of MMAurias induced by defects of 5'-deoxyadenosylcobalamin synthesis. Patients with *cbIA* and *cbIB* defects display plasma concentrations of about 22 $\mu\text{mol/L}$ MMA (Hoffmann et al. 1993).

Next, we studied whether the respiratory chain was affected in hPTEC loaded with HCCL and propionic acid. A recent study demonstrated severe OXPHOS dysfunction in tissues rich in mitochondria, namely liver, kidney, heart and skeletal muscle, of patients with methylmalonic and propionic aciduria (de Keyser et al. 2009). Chronic renal failure, however, is a unique feature of MMAuria, underlining the particular vulnerability of proximal tubule cells to pathomechanistic features associated with MMAuria (Morath et al. 2008).

Our bioenergetic studies revealed two intriguing results: (1) increase of complex II–IV enzyme activity after 2 weeks, and (2) increase of complex I and IV activity as well as a decrease of complex II and III activity after 3 weeks of HCCL and propionic acid application. These changes of enzyme activity coincided with the increase and the peak of MMA production, respectively. Interestingly, 5–6 weeks of HCCL administration in rats caused an increase of mitochondrial mRNA and protein content as well as elevated activities of citrate synthetase, succinate dehydrogenase, carnitine palmitoyltransferase, and glutamate dehydrogenase due to increased mitochondrial proliferation in the liver of treated animals (Krahenbuhl et al. 1990). The authors speculated that the increase in mitochondrial capacity may be a compensatory mechanism in response to the mitochondrial toxicity of accumulating metabolites. It has been shown that metabolites accumulating owing to an inhibition of MMA mutase can disturb mitochondrial metabolism at different levels (Cheema-Dhadli et al. 1975; Halperin et al. 1971; Oberholzer et al. 1967; Okun et al. 2002). In contrast, the same authors found that after 5–6 weeks HCCL treatment rats displayed decreased activities of complex III and IV as well as a decreased cytochromes *a*, *b* and *c* content in liver mitochondria (Krahenbuhl et al. 1991). Cytochromes *c* and *b* are integral components of complexes II and III of respiratory chain. Consequently, reduced complex II and III activity found in our study is likely to result from a decreased content of cytochromes. From these findings it can be hypothe-

sized that the biosynthesis of cytochromes may be affected in MMAurias. Porphyrins are the precursor of a variety of haem proteins and, in particular, of cytochromes. The first step of porphyrin synthesis is the condensation of glycine and succinyl-CoA to form 5-aminolevulinate. Thus a reduced mitochondrial succinyl-CoA content resulting from a block of MMA mutase may interfere with porphyrin synthesis. The synthesis of haem itself requires the import of coproporphyrinogen III into the mitochondrial matrix. Interestingly, the mitochondrial 2-oxoglutarate carrier is likely to be involved in this step (Kabe et al. 2006). This carrier works as an exchanger coupling the import of malate to the export of 2-oxoglutarate and is part of the mitochondrial malate shuttle system that has been shown to be inhibited by MMA (Halperin et al. 1971). Thus, accumulating MMA may compete with or interfere with the import of coproporphyrinogen III via this carrier. Summarizing, two different mechanisms are likely to induce the change of enzyme activities found in our study. After 2 weeks the inhibition of MMA mutase by HCCL enhances mitochondrial proliferation, resulting in increased activities of complexes II–IV. The same effect seems to be responsible for the elevated activities of complexes I and IV after 3 weeks of HCCL incubation. However, at this time point accumulating MMA may interfere with the mitochondrial synthesis of cytochromes inducing the reduced activities of complexes II and III apparent in our study. Consistently with the concept of bioenergetic dysfunction in MMAurias, treated cells displayed reduced levels of glutathione, indicating increased formation of ROS.

In conclusion, our study demonstrates that co-incubation of hPTEC with HCCL and propionic acid induces a biochemical phenotype similar to MMAurias. This novel in vitro model may be helpful in specific investigation of the pathomechanism of renal disease in MMAurias.

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