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Organ-specific response to inhibition of mitochondrial metabolism by cyclosporine in the rat

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Abstract To evaluate organ-specific metabolic changes after in vivo cyclosporine (CyA) treatment, male Wistar rats were treated with 10 mg/kg per day CyA orally for 6 days. Blood, kidney, liver, and heart tissues were extracted and analyzed by magnetic resonance spectroscopy (MRS). CyA decreased the energy balance [adenosine triphosphate (ATP)/adenosine diphosphate (ADP)] in all organs (kidney [control]: 50%, liver: 64%, and heart: 62%, all $P < 0.01$) due to decreased activity of the mitochondrial Krebs cycle and oxidative phosphorylation. As a compensatory effect, anaerobic glycolysis (lactate) was increased. This was reflected in the low glucose level in the kidney and heart, but not in the liver where a significant decrease in glycogen was seen. Only in the kidney was mitochondrial inhibition accompanied by decreased polyunsaturated fatty acid (PUFA) concentrations and elevated lipid peroxidation. The metabolic marker for nephrotoxicity, trimethylamine-N-oxide (TMAO), was elevated. While CyA decreased mitochondrial homeostasis in all organ systems, cellular adaptation was different and most efficient in the liver.

Keywords Metabolic toxicity · Glucose metabolism · Energy state · Hypoxia · Magnetic resonance spectroscopy (MRS) · Cyclosporine

Introduction

The calcineurin inhibitor cyclosporine (CyA) has been the basis for most immunosuppressive protocols for more than two decades. Unfortunately, adverse effects of CyA treatment such as nephrotoxicity, neurotoxicity, hypertension, and hyperlipidemia have become major limiting factors in long-term immunosuppressive therapy of transplant patients [9]. This frequently results in the discontinuation of CyA therapy. With regard to CyA-induced neurotoxicity, we have previously demonstrated that CyA, in the rat brain in vitro and in vivo, leads to inhibition of mitochondrial metabolism with subsequent significant changes in high-energy phosphate homeostasis [18, 19, 20]. We have shown that these metabolic changes in the brain may partly be responsi-

ble for the clinical symptoms of CyA neurotoxicity which are very similar to mitochondrial encephalopathies [20]. Based on our metabolic findings in the brain and evidence that depressed energy metabolism may be favorable in preconditioning organs experiencing subsequent ischemia [14], we were recently able to demonstrate that CyA pretreatment decreased cardiac energy metabolism prior to occlusion and protected the rat heart in an in vivo ischemia/reperfusion model [12]. Thus, the effect of CyA on cell metabolism may have entirely different implications in dissimilar experimental and clinical scenarios. Furthermore, organ–specific responses to CyA treatment under the same experimental/clinical conditions in a whole intact animal are, to our knowledge, not available. However, organ-specific metabolic responses to CyA treatment may help to explain why certain organs (e.g., kidney) are at increased risk of CyA toxicity. Previous in vitro studies focused mainly on the effect of CyA on energy production in isolated kidney and liver rat mitochondria [8, 11, 17]. In the last decade, magnetic resonance spectroscopy (MRS) has shown great promise in toxicology, drug discovery, pre-clinical drug development, and hypothesis-driven biomedical research. MRS allows for the simultaneous assessment of all important metabolic pathways in cells including lipid (^{1}H-MRS), glucose (^{13}C–MRS), and high-energy phosphate metabolism (^{31}P-MRS). Thus, we used MRS on tissue extracts to quantify organ-specific metabolites and metabolic pathways and their changes after CyA treatment.

It is unlikely to expect that CyA has a different primary metabolic effect on each single organ. Hence, we hypothesized that the CyA effect per se is of less importance than the cellular adaptation with regard to organ vulnerability. Our goals were (i) to evaluate organ-specific metabolic alterations after CyA treatment in an in vivo rat model and (ii) to assess how these alterations may lead to the development of detrimental side effects of CyA treatment.

Materials and methods

Animals

Ten adult male Wistar rats (6 months old, 280–320 g body weight) from Charles River (Wilmington, Mass.) were used for this study. The animal protocol was reviewed and approved by the Committee on Animal Research of the University of California, San Francisco. Animal care was in agreement with the National Institutes of Health guidelines for ethical animal research (NIH publication No. 80–123, revised 1985). Rats were treated with 10 mg/kg per day CyA or a vehicle solution ($n = 5$ each) for a period of 6 days in order to establish constant drug tissue levels. This oral dose was chosen on the basis of our previous dose-funding studies for the brain [20] and heart [12]. During the study period, the animals were closely monitored, including daily evaluations of appearance, grooming, appetite, behavior, activity, and body weight.

Cyclosporine treatment and study design

Two groups of rats ($n = 5$ each) were used for this study. The control group received 1 ml/kg vehicle solution containing 90% whole milk and 10% olive oil. The CyA group received 10 mg/kg CyA in its Neoral formulation (100 mg CyA per ml, Novartis, Basel, Switzerland) diluted in milk (1:10). The rats were treated once a day by oral gavage. Six days after the treatment and 11 h after the last dose, 250 mg/kg [1-^{13}C]labeled glucose (Cambridge Isotope Laboratories, Andover, Mass.), dissolved in 0.9% sterile NaCl, was injected i.p. to follow up de novo glucose metabolism. One hour after injection and 12 h after the last CyA dose, the rats were anesthetized with 1.8–2.5% isoflurane. Blood samples were collected into heparin-coated tubes for MRS evaluations (1 ml) and in EDTA-coated tubes for LC/MS analysis of CyA levels (0.25 ml). Blood samples were immediately put onto ice for 2 min and subsequently stored at −80°C until analysis. Kidney, liver, and heart tissues were quickly removed and immediately frozen in liquid nitrogen until extraction. Blood and tissue levels of CyA were determined using LC/MS. The absolute concentrations of major cellular metabolites were quantified by multinuclear MRS on the extracts from kidney, liver, heart, and whole blood tissues. Lipid peroxidation (LPO) in the kidney, heart, and liver tissues was measured using the malondialdehyde/thiobarbituric acid (MDA/TBA) test.

LC/MS assay for CyA blood and tissue levels

We used an LC/MS assay with automated online sample extraction (LC/LC-MS, Series 1100 HPLC system with mass selective detector, Hewlett-Packard, Palo Alto, Calif.) for quantification of CyA blood/tissue concentrations based on the previously described method [3]. Briefly, rat tissues (1 g wet weight) were homogenized with 2 ml KH_2PO4 buffer pH 7.4 (1 M) using an electrical homogenizer. For protein precipitation, 200 µl methanol/1 M zinc sulfate (80/20 v/v) were added to 100 µl blood or extracted tissue sample. Cyclosporin D (250 µg/l) was added as an internal standard. After centrifugation, 100 µl of the supernatant was injected onto the extraction column. Samples were washed with a mobile phase of 40% methanol and 60% of 0.1% formic acid supplemented with 1 µmol/l sodium formate. After 0.75 min, the switching valve was activated, and the analytes were eluted in the backflush mode from the extraction into the analytical column. The mass spectrometer was run in the selected ion mode, and positive $[M + Na]^+$ ions were recorded.

Dual extraction of rat tissues

For MRS, we extracted the collected frozen tissue samples using a dual perchloric acid (PCA)/lipid extraction procedure [20]. The powdered frozen tissue was added to 6 ml of ice-cold 12% PCA for protein precipitation and subsequently homogenized using electrical homogenizer. After centrifugation, the aqueous phase was collected, neutralized with KOH, centrifuged again to remove potassium perchlorate, and lyophilized overnight. Then the PCA extracts, which contained the water-soluble tissue metabolites, were dissolved in 0.45 ml D_2O (deuterium oxide) for MRS. All deuterated solvents were obtained from Cambridge Isotope Laboratories (Andover, Mass.). The tissue pellets, remaining after the first centrifugations, were re-dissolved in 4 ml ice–cold water. The re-dissolved pellets, containing tissue lipids, were neutralized with KOH and lyophilized overnight for lipid extracts. The lipid extracts were dissolved in 1 ml deuterated chloroform/methanol ($CDCl_3$/CD_3OD, 2:1 v/v) for MRS.

For MRS analysis of blood samples the dual organic extraction technique was applied. One milliliter whole blood (heparin preservation) was extracted using 2 ml chloroform/methanol (1:1 v/v) and

centrifuged. The supernatant was washed with 1 ml water. The water phase (at the top) was removed and added to the pellet. Two milliliters of water were added, the pellet was centrifuged, and the supernatant was lyophilized overnight. Afterwards the water-soluble part was dissolved in 0.45 ml D_2O and analyzed by MRS. The lipids in the organic phase were evaporated to dryness under a stream of nitrogen at 50°C. The lipid extracts were dissolved in 1 ml of deuterated chloroform/methanol (2:1 v/v) for MRS analysis.

^{1}H-, ^{13}C-, and ^{31}P-MRS evaluations

All one-dimensional MR spectra of tissue PCA and lipid extracts were recorded on a Bruker AMX 360 spectrometer using the WINNMR program (Bruker, Karlsruhe, Germany). A 5-mm ^{1}HX-inverse probe head was used for all nuclei. The MRS parameters were previously described by Serkova et al. [18]. The tissue metabolites in PCA and lipid extracts were identified using a two-dimensional heteronuclear single quantum correlation (2D-HSQC) technique and compared with the chemical shift references reported in the literature [6, 7, 24]. Trimethylsilyl-propionic-2,2,3,3,-d_4 acid (TSP, 0.6 mmol/l for PCA extracts and 6 mmol/l for lipid extracts) was used as an external standard for metabolite quantification. ^{1}H chemical shifts were referenced to TSP at 0 ppm. ^{13}C-MR spectra were subsequently recorded after ^{1}H-MRS. The C1 peak of β-glucose at 96.8 ppm was used as chemical shift reference in ^{13}C-MRS. Before ^{31}P-MR spectra were recorded, 100 mmol/l EDTA was added to each PCA extract for complexation of divalent ions. α–ATP at –9.9 ppm was used as chemical shift reference for ^{31}P-MRS.

Lipid peroxidation measurements in rat tissues

Lipid peroxidation (LPO) was measured in rat tissues using the malondialdehyde/thiobarbituric acid (MDA/TBA) test [12, 22]. Three hundred and fifty microliters of tissue homogenate were added to a solution containing 15 mM sodium dodecylsulfate (SDS), 1 mM EGTA, 2.25 mM butylated hydroxytoluene (BHT), and 20 mM 2-TBA. Twenty percent acetic acid (pH-adjusted with NaOH to 3.5) was added, resulting in a final volume of 2 ml. The reaction mixture was incubated for 60 min at 95°C. After cooling to 0°C, 2 ml butanol/pyridine (1:1 v/v) was added and the solution was centrifuged. The organic phase was used to measure absorption in a photometer at 532 nm or fluorescence with excitation set to 515 nm and emission set to 555 nm. 1,1,3,3-Tetraethoxypropan was used as a standard.

Statistical analysis

Values are expressed as mean ± standard deviation if not otherwise designated. Data from the controls and cyclosporine-treated animals were compared using the unpaired Student's *t*-test. The SPSS software package (version 10.07, SPSS, Chicago, Ill.) was used to calculate distribution statistics and for all statistical analyses. A *P*-value of below 0.05 was considered significant.

Results

Effect of CyA on organ energy and carbohydrate metabolism

^{1}H- and ^{31}P-MRS spectra of PCA extracts allow to quantify major cellular metabolites, of which some were organ-specific (Tables 1 and 2, respectively). The kidney contained high concentrations of various osmolytes and volume regulators (taurine, myo-inositol, betaine, trimethylamine-N-oxide, aspartate) and intracellular amino acids which are intermediates from the Krebs cycle. The liver had the highest concentrations of glucose and glycogen among the three study organs (kidney and heart glycogen levels were below the limit of quantification) as well as the highest level of glutathione. The major metabolites in the heart were the high-energy phosphates phosphocreatine and ATP, and also the glycolysis end-product lactate and the osmolyte taurine. Due to its mechanic activity and the large fraction of muscle tissue, the heart had the highest energy balance [ATP/ADP] as well as the highest adenylate energy charge [(ATP + 0.5ADP)/(ATP + ADP + AMP)]: heart > liver > kidney (Table 2).

Six days after treatment, CyA decreased the high-energy phosphates ATP (kidney: 56%, liver: 67%, heart: 83%, all $P < 0.01$) and phosphocreatine (kidney: 65%, heart: 85%, $P < 0.01$, Fig. 1). As a consequence, the energy balance calculated from the ratios [ATP/ADP] decreased (kidney: 50%, liver: 64%, heart: 62%, all $P < 0.01$). In the CyA-treated kidney (88%) and heart (91%), but not in the liver (94%, n.s.), the energy adenylate charge was significantly lower (both $P < 0.05$) than in the control group. The inhibition of the energy status by CyA was accompanied by decreased concentrations of NAD^+ (kidney: 52%, $P < 0.0001$; liver: 63%, $P < 0.05$; heart: 58%, $P < 0.01$) and amino acids from the Krebs cycle (glutamate, glutamine, aspartate, all $P < 0.05$) in all organ systems (Fig. 1). Simultaneously, the intracellular concentrations of lactate were elevated in all organs (kidney: 147%, $P < 0.05$; liver: 202%, $P < 0.0001$; heart: 139%, $P < 0.01$). This was accompanied by lower glucose levels in the kidney (52%, $P < 0.0001$) and the heart (77%, $P < 0.01$), but not in the liver (98%, n.s., Fig. 1) where a significant decrease of glycogen and UDP-glucose was seen (51% and 69%, $P < 0.001$ and $P < 0.01$, respectively). There were only kidney-specific CyA-induced alterations in the intracellular osmolyte concentrations: taurine and betaine concentrations decreased (50% and 71%, respectively, both $P < 0.01$), while the TMAO concentrations increased (143%, $P < 0.05$, Table 1).

In all organs, the absorbed C1-labeled glucose (two peaks under peak 7 correspond to α- and β-glucose isotopomers, Fig. 2) was metabolized de novo through glycolysis to C3-labeled lactate (peak 1) and through the Krebs cycle to glutamate C2 (peak 5), C3 (peak 2), and C4 (peak 3). In all organ systems de novo glucose metabolism was highly affected by CyA, reflecting stimulation of glycolysis (increase of C3-lactate formation through lactate dehydrogenase) and inhibition of the Krebs cycle (decrease of C4 glutamate formation through pyruvate dehydrogenase) (Fig. 2).

Table 1 Absolute concentrations of water-soluble tissue metabolites in the control group quantified from ^{1}H-MRS of tissue PCA extracts (*ppm* part per million [unit for the chemical shift in NMR], *TMAO* trimethylamine-N-oxide). Creatinine concentration is given for blood, and creatine concentrations are given for kidney, liver, and heart. The values are given as mean ± SD ($n = 5$)

	Chemical shift, ppm	Blood, [μmol/ml]	Kidney, [μmol/g]	Liver, [μmol/g]	Heart, [μmol/g]	Changes by CyA
Valine/Leucine	0.97 (CH_3)	0.68 ± 0.08	2.19 ± 0.62	1.23 ± 0.20	1.21 ± 0.08	
3-Hydroxybutyrate	1.21 (CH_3)	0.14 ± 0.04	0.20 ± 0.15	0.80 ± 0.32	n.d.	↑blood
Lactate	1.32 (CH_3)	1.58 ± 0.93	2.13 ± 0.67	4.49. ± 0.32	10.44 ± 1.77	↑↑↑
Alanine	1.48 (CH_3)	0.31 ± 0.10	0.98 ± 0.22	2.18 ± 0.19	1.33 ± 0.32	
Glutamate	2.36 (CH_2)	0.30 ± 0.09	4.8 ± 0.91	2.22 ± 0.32	5.08 ± 0.33	↓
Succinate	2.38 (CH_2)	0.15 ± 0.07	1.17 ± 0.74	n.d.	2.01 ± 0.22	↑
Glutamine	2.39 (CH_2)	0.31 ± 0.04	2.21 ± 0.21	4.04 ± 0.71	5.89 ± 0.44	↓↓
Citrate	2.52 (CH_2)	0.26 ± 0.12	2.08 ± 1.27	n.d.	n.d.	↓↓kidney
Glutathione	2.57 (CH_2)	n.d.	0.30 ± 0.07	4.95 ± 0.58	n.d.	
Aspartate	2.69 (CH_2)	n.d.	1.02 ± 0.51	1.84 ± 0.29	0.57 ± 0.36	↑
Creatinine/Creatine[a]	3.04 (CH_3)	0.16 ± 0.02	3.05 ± 0.21	n.d.	15.87 ± 3.01	↓↓heart
Cholines	3.20 (CH_3)$_3$	0.36 ± 0.10	2.51 ± 0.89	12.76 ± 0.84	2.33 ± 0.88	
Betaine	3.26 (CH_3)	0.09 ± 0.03	1.35 ± 0.22	6.34 ± 1.25	n.d.	↑kidney
TMAO	3.27 (CH_3)	0.54 ± 0.10	1.22 ± 0.55	2.77 ± 0.33	n.d.	↑kidney
Taurine	3.43 (CH_2)	n.d.	6.71 ± 5.65	n.d.	18.77 ± 3.22	↓↓kidney
Myo-inositol	4.1 (CH)	n.d.	4.73 ± 1.10	n.d.	n.d.	
Glucose	4.65 + 5.24 ($\beta + \alpha$ CH)	3.98 ± 0.41	5.51 ± 0.76	19.85 ± 4.64	2.11 ± 0.98	↓blood ↓kidney, heart
Glycogen	5.40 (CH)	n.d.	n.d.	12.76 ± 3.24	n.d.	↓liver

Table 2 Absolute concentrations of high-energy tissue metabolites in the control group quantified from ^{31}P-MRS of tissue PCA extracts (*ADP* adenosine diphosphate, *AMP* adenosine monophosphate, *ATP* adenosine triphosphate, *NAD*$^+$ nicotinamide adenine dinucleotide, *PDE* phosphodiesters, *PME* phosphomonoesters, *ppm* part per million, *UDPG* UDP-glucose [activated sugar nucleotide]). The values are given as mean ± SD ($n = 5$). The concentrations of phosphate metabolites are given as [μmol/g] tissue, the ratios for high-energy phosphates (two bottom rows) have no units. [ATP/ADP] and [(ATP + 0.5ADP)/(ATP + ADP + AMP)] are the equivalents for renal/hepatic/cardiac energy balance and adenylate energy charge, respectively. Blood samples were not analyzed by ^{31}P-MRS since the high-energy phosphates mostly present in the solid organ tissues and not in the blood cells

	Chemical shift, ppm	Kidney, [μmol/g]	Liver, [μmol/g]	Heart, [μmol/g]	Changes by CyA
PME	5.01 to 3.91	3.91 ± 0.48	7.89 ± 1.82	2.01 ± 0.11	↑kidney
PDE	1.04 to 0.70	1.44 ± 0.17	3.25 ± 1.18	0.23 ± 0.07	
Phosphocreatine	−2.33	1.33 ± 0.22	n.d.	12.33 ± 0.98	↓kidney, heart
NAD^+	−10.42	2.03 ± 0.55	1.00 ± 0.34	1.77 ± 0.34	↓↓
UDPG	−10.75 −11.94	0.99 ± 0.31	0.88 ± 0.22	n.d.	↓liver
AMP	3.21	0.15 ± 0.07	0.24 ± 0.06	n.d.	↑↑kidney, heart
ADP	−5.23 (β-PO_4) −9.59 (α-PO_4)	1.75 ± 0.42	1.22 ± 0.23	1.76 ± 0.39	↑kidney, heart
ATP	−4.85 (γ-PO_4) −9.91 (α-PO_4) −29.28 (β-PO_4)	3.12 ± 0.44	3.77 ± 0.78	7.49 ± 1.22	↓↓
[ATP/ADP]		1.67 ± 0.49	3.24 ± 0.66	4.13 ± 0.79	↓↓
ATP + 0.5 ADP ATP + ADP + AMP		0.74 ± 0.17	0.81 ± 0.27	0.88 ± 0.29	↓

Organ-specific lipid changes after CyA treatment

CyA led to the increased cholesterol concentrations in the rat kidney and heart (129%, $P < 0.05$ and 159%, $P < 0.01$, respectively, Fig. 3). In addition, the kidney showed an elevated triacylglycerol (TAG) concentration (153%, $P < 0.05$). The kidney was the only organ with a decreased concentration of polyunsaturated fatty acids (PUFA, 73%, $P < 0.05$) and the only organ with elevated lipid peroxidation (LPO, 137%, $P < 0.05$) in the CyA-treated group. The liver lipids were not affected by CyA treatment.

Blood metabolite alterations by CyA

CyA blood concentration (12 h after the last dose) was 715 ± 279 ng/ml ($n = 5$, Table 3). The tissue-to-blood partition coefficients for CyA, calculated as [C_{tissue}/C_{blood}] at 12 h after CyA administration for each animal, were: liver 3.81, kidney 1.23, and heart 0.83. Similar to the solid organs, increased blood lactate (119%, $P < 0.05$) and decreased glutamate and glutamine levels (both 80%, $P < 0.05$) were observed in the blood of CyA-treated animals. CyA induced an increase in total blood cholesterol and TAG (140% and 132%, respec-

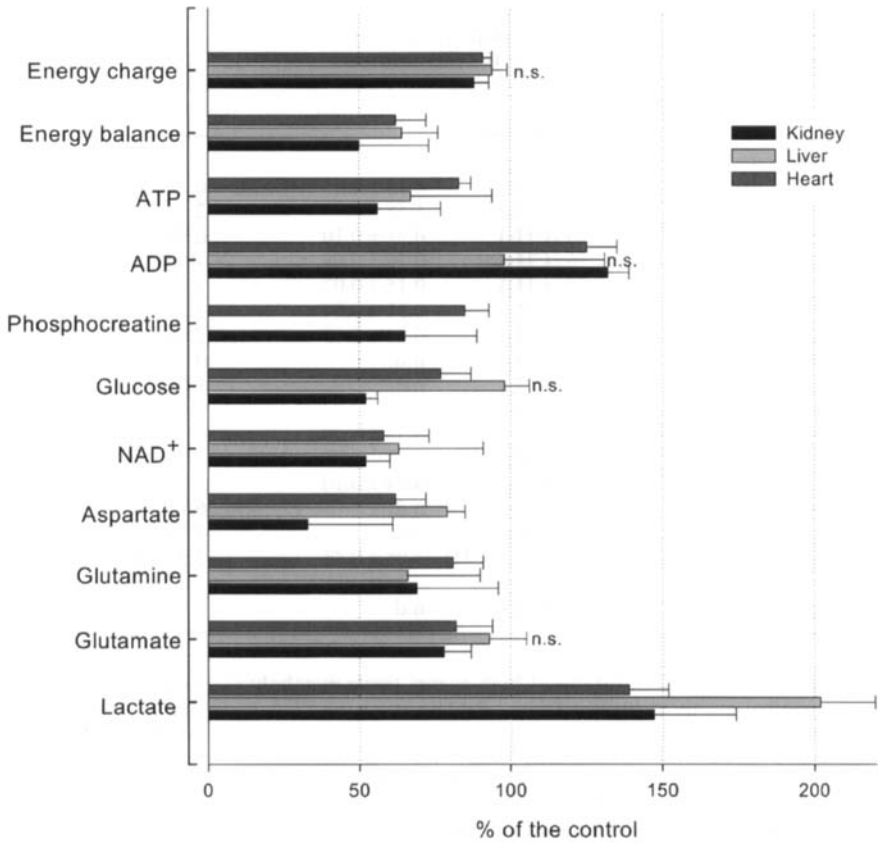

Fig. 1 Changes in metabolite concentrations in rat kidney, liver, and heart after treatment with 10 mg/kg CyA orally for 6 days (*ADP* adenosine diphosphate, *ATP* adenosine triphosphate, NAD^+ nicotinamide adenine dinucleotide). The changes in all metabolites were statistically significant ($P < 0.05$, $n = 5$), with the exception of four liver parameters (glutamate, glucose, ADP concentration, and liver energy charge) that are marked as n.s. (not significant)

tively, both $P < 0.05$, Fig. 3). The blood glucose level was increased to 135% ($P < 0.01$) of the control level. An elevated level of blood creatinine in CyA-treated rats (149%, $P < 0.001$) was also seen. A specific metabolic change in the blood was an increased concentration of 3-hydroxybutyrate (124%, $P < 0.05$, Table 1).

Discussion

In the present study, we report on organ-specific metabolic changes after CyA treatment in the rat kidney, liver, heart, and blood. For the first time we demonstrated in vivo that CyA significantly compromised energy production in major organ systems. The low energy balance caused by CyA can be explained by the decrease in oxidative phosphorylation (NAD^+) and by the direct inhibition of the Krebs cycle (glutamate through private dehydrogenase). Simultaneously, an activation of anaerobic glycolysis was observed. However, the metabolic consequences and adaptation processes to the mitochondrial metabolic arrest were organ-specific. CyA tissue distribution was also different among the study organs, with the liver having the highest CyA tissue partition coefficient: liver > kidney > heart. The daily oral dose of 10 mg/kg CyA resulted in a 12-h blood level of 715 ± 279 ng/ml. This oral dose was chosen on the basis of our previous findings and other studies on the brain and heart [12, 15, 20]. Cylosporine blood concentrations in this study were comparable with therapeutically relevant CyA doses in human recipients [1, 2]. In these clinical studies CyA was administered twice a day, with steady-state CyA concentrations at day 5. The pre-dose level (every 12 h) was 300 ng/ml with a maximum CyA concentration of 1000 ng/ml at 2 h after oral Neoral administration.

In the CyA-treated group, the kidney showed the most dramatic decrease in energy status with the most significant inhibition in oxidative phosphorylation and the Krebs cycle. The very low concentration of glucose in the CyA-treated kidney can be related to increased lactate production [10]. A recent study with renal epithelial $LLCPK_1$ cells [4] has shown that CyA treatment activated GLUT1 gene transcription accompanied by higher glucose consumption and glycolysis rate. In our in vivo study, the kidney seems to compensate for mitochondrial energy inhibition not just through stimulation of glycolysis, but also by elevated oxidation of fatty acids. In our previous study we have shown that the MRS-detectable decrease in polyunsaturated fatty acids (PUFA) is an indirect marker for the increased lipid peroxidation (LPO) in heart tissue [12]. Similarly,

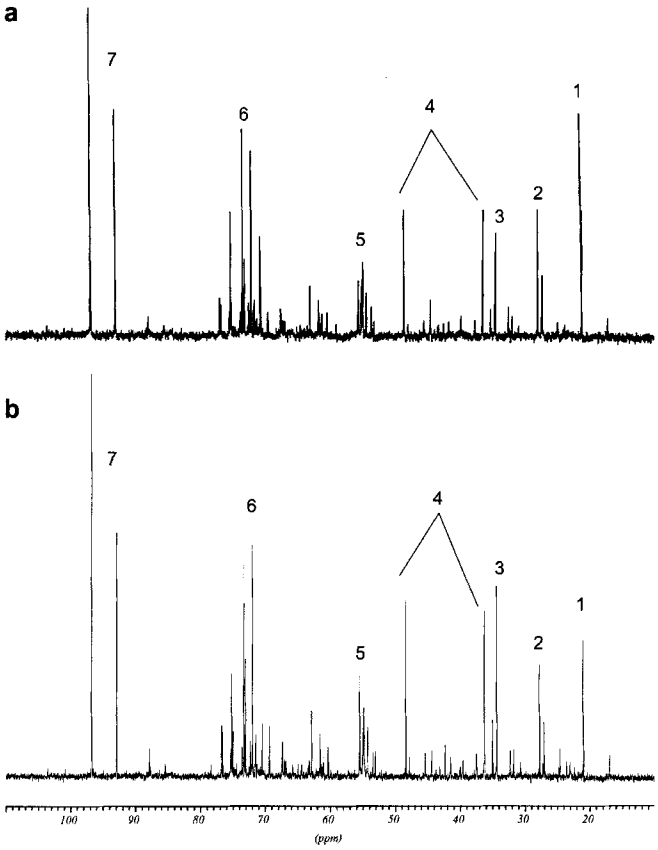

Fig. 2 ^{13}C-MRS spectra of PCA kidney extracts of (**a**) CyA-treated rat and (**b**) control rat. Peak assignment: 1, C3-lactate; 2, C3-glutamate; 3, C4-glutamate; 4, taurine; 5, C2-glutamate; 6, myo-inositol; 7, C1-glucose

the kidney was the only organ with low concentration of PUFA and increased LPO after CyA treatment. The decrease in PUFA can be attributed to the increased stimulation of fatty acid oxidation which is another additional alternative pathway for energy production [10] when the oxidative mitochondrial pathway is inhibited by CyA. However, this adaptive process that results in lipid peroxidation is known to be harmful to the renal cortex and medulla [6]. Indeed, various metabolic markers for nephrotoxicity were found in rat kidney tissue treated with CyA. Betaine is an important osmolyte in renal papilla. Decrease of betaine and taurine, another osmolyte and volume regulator, is related to pharmacologically induced necrosis in the rat papilla [6] and was consistent with our findings. Similarly, the increase of another renal osmolyte, TMAO (trimethylamine-N-oxide), is a specific toxic marker of hypoxic medullary injury [6, 16]. Therefore, the inhibition of mitochondrial homeostasis by CyA and subsequent cellular metabolic adaption may explain the development of nephrotoxicity [13].

In the liver, the glucose and PUFA concentrations were preserved. The preservation of glucose level and fatty acid oxidation is related, at least partly, to the increased utilization of glycogen storage. This was also confirmed by the significantly lower concentrations of UDP–glucose, which is a precursor of glycogen in the liver [10]. This seems to be a more effective way of metabolic compensation, compared to the kidney, since no toxic metabolic markers were found in the liver. In addition, the decrease in energy balance was much smaller in the CyA-treated liver than in the CyA-treated kidney, and the energy charge, a sensitive indicator of

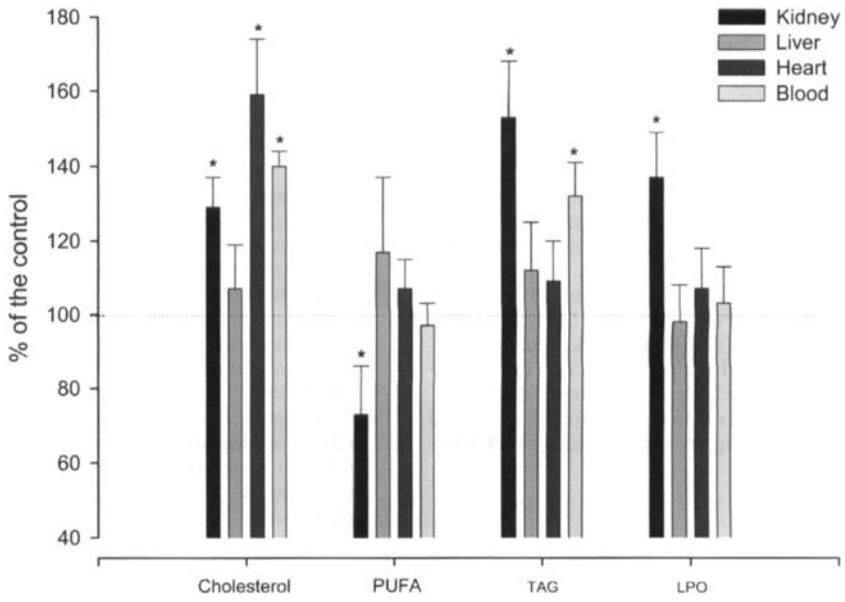

Fig. 3 Changes in lipid profile in rat kidney, liver, and heart after treatment with 10 mg/kg CyA orally for 6 days. (cholesterol = total [unesterified + esterified cholesterol], *PUFA* poly-unsaturated fatty acids, *TAG* triacylglycerol, *LPO* lipid peroxidation). Statistical significance: $*P < 0.05$ ($n = 5$)

Table 3 Blood, kidney, liver, and heart concentrations of CyA after 6 days of treatment and 12 h after the last dose. The values are mean ± SD ($n = 5$). The oral dose was 10 mg/kg CyA per day

Blood, [ng/ml]	Kidney, [ng/g]	Liver, [ng/g]	Heart, [ng/g]
714.7 ± 278.7	859.5 ± 63.5	2684.5 ± 406.7	559.4 ± 72.7

hepatocellular dysfunction [14] was not decreased by CyA in the liver.

In the heart, CyA-related inhibition of mitochondrial energy production led to general non-specific changes in cardiac metabolism: an increase in lactate and a decrease in glucose concentration with no organ-specific adaptive pathways.

The elevated lactate concentration in the blood can be explained by increased anaerobic glycolysis in all organs. This may be related to the occurrence of lactic acidosis in some CyA-treated patients [5, 13]. The increased concentration of β-hydroxybutyrate in the blood of CyA-treated rats indicates that the kidney and heart utilize less ketone bodies due to the inhibition of mitochondrial oxidation by CyA. This is consistent with findings of a recent clinical report of three patients who developed post-transplant diabetic ketoacidosis under CyA treatment [26]. The high level of TAG and total cholesterol can be explained by the deterioration of the Krebs cycle in the organs. The exact mechanisms of the well-known CyA-induced dyslipidemia are not yet fully understood. In an in vivo rat model of CyA-induced hyperlipidemia, the investigators found a significant reduction of hepatic cholesterol 7α-hydroxylase as well as lipoprotein lipase [23]. However, the activity of the major cholesterol-related enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was not affected by CyA. In addition to the reduced activity of hydroxylase and lipase in CyA-treated rats (reduced lipoprotein catabolism), we hypothesized that inhibition of the mitochondrial TCA cycle by CyA may partly contribute to increased lipid synthesis. The decreased activity of pyruvate dehydrogenase leads to an increase of acetyl-CoA, which is an important substrate for both the Krebs cycle as well as lipid synthesis [10]. Since the Krebs cycle is down-regulated by CyA, more acetyl-CoA is able to enter into the lipid metabolism. This increases the production of intracellular cholesterol and triglycerides in the blood and tissues. Clinically, the elevated blood level of cholesterol and TAG constitutes the major problem of CyA therapy [9].

In conclusion, our results indicate that CyA severely decreases energy production in several major organ systems. The mitochondrial metabolic profile is distinctly down-regulated in all three study organs, with subsequent glycolysis stimulation that mimics the metabolic appearance of mild hypoxia. Some recent publications have also reported CyA-induced metabolic arrest and hypoxia-like conditions in the rat liver [27] and kidney (induction of heat shock proteins) [25], which can explain the postulated CyA-related preconditioning [12]. This metabolic preconditioning by CyA might be an alternative mechanism for its cellular protection against hypoxia, in addition to the ability of CyA to inhibit the mitochondrial membrane permeability pore during hypoxic events [12, 21]. While mitochondrial energy metabolism is significantly depressed in all three organs, the extent is distinctly different between them. As a response to the hypoxia-like metabolic profile, the contribution of different compensatory biochemical pathways is organ-specific and may, as in the case of the kidney, be toxic. Our results suggest that the liver (even with the highest CyA tissue level) is more adaptable to CyA–induced mitochondrial depression, in contrast to the kidney.

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