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Low-Salt Diet and Cyclosporine Nephrotoxicity: Changes in Kidney Cell Metabolism

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Abstract

Cyclosporine (CsA) is a highly effective immunosuppressant used in patients after transplantation; however its use is limited by nephrotoxicity. Salt depletion is known to enhance CsA-induced nephrotoxicity in the rat, but the underlying molecular mechanisms are not completely understood.

The goal of our study was to identify the molecular effects of salt depletion alone and in combination with CsA on the kidney using a proteo metabolomic strategy. Rats (n=6) were assigned to four study groups: 1) normal controls, 2) low-salt fed controls, 3) 10 mg/kg/d CsA for 28 days on a normal diet, 4) 10 mg/kg/d CsA for 28 days on low-salt diet.

Low-salt diet redirected kidney energy metabolism towards mitochondria as indicated by a higher energy charge than in normal-fed controls. Low-salt diet alone reduced phospho-AKT and phospho-STAT3 levels, and changed the expression of ion transporters PDZK1 and CLIC1.

CsA induced macro- and microvesicular tubular epithelial vacuolization and reduced energy charge; changes that were more significant in low-salt fed animal, probably because of their more pronounced dependence on mitochondria. Here, CsA increased phospho-JAK2 and phospho-STAT3 levels and reduced the phospho-IKK and p65 proteins, thus activating NF- κ B signaling. Decreased expression of lactate transport regulator CD147 and phospho-AKT was also observed after CsA exposure in low-salt rats, indicating a decrease in glycolysis.

In summary, our study suggests a key role for PDZK1, CD147, JAK/STAT and AKT signaling in CsA-induced nephrotoxicity and proposes mechanistic explanations on why rats fed a low-salt diet have higher sensitivity to CsA.

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Keywords

CsA-induced nephrotoxicity; proteomics; metabolomics; salt depletion

INTRODUCTION

Calcineurin inhibitors (CNIs) cyclosporine (CsA) and tacrolimus (TAC) are potent immunosuppressants currently used in patients as rejection prophylaxis after transplantation. The predisposition of these agents to ultimately damage the very organs they were intended to protect was always recognized but tolerated due to their impressive improvement of short-term outcomes¹. Although recent analyses indicates a moderate increase of renal allograft half-lives, long-term results are still not acceptable². While cardiovascular complications are the major cause of death in kidney transplant patients³, chronic renal allograft injury is the principal cause of late renal allograft loss after the first year^{3, 4}. After approximately 8 years, 50% of renal allograft transplant patients lose their transplant. Surprisingly, while the mechanisms of the immunosuppressive action of CNIs are well understood, knowledge of the basic biochemical mechanisms resulting in CNI nephrotoxicity is incomplete^{5, 6}. One of the major reasons for this is that there are no good animal models: rats and mice are far less sensitive to CNI nephrotoxicity than humans⁷.

At present, the following key mechanisms of CsA toxicity have been identified: oxidative stress, apoptosis, metabolic changes^{8, 9} and an increase of vascular resistance resulting in decreased renal blood flow¹⁰. Our previous proteomic studies evaluating the effects of CNIs alone and in combination with sirolimus in rats on a normal diet showed that several signaling pathways including calcium homeostasis, structural proteins, mitochondrial function and cell metabolism were affected¹¹.

Salt-depletion is known to sensitize the rat kidneys to CNI toxicity. Histological changes develop faster than in normal-fed rats and thus the salt-depleted rat model has extensively been used to study immunosuppressant toxicity¹²⁻¹⁴. However not much is known about the molecular mechanisms responsible for the enhancement of CNI toxicity in this non-physiological model. We believe that a better understanding of the molecular mechanisms enhancing CsA nephrotoxicity in salt-depleted rats will also reveal more in-depth molecular insights into CsA nephrotoxicity itself.

We employed a proteo-metabolomic strategy¹¹ to study the effects of salt depletion on the rat kidney in absence and presence of CsA and correlated the changes in protein expression and metabolite concentrations with tissue histology and established clinical kidney function parameters.

METHODS

Animal protocol

Twenty four male Wistar rats were acclimatized for two weeks and then randomly assigned to four treatment groups (n=6/group) for 28 days:

- I. vehicle controls fed a normal-salt diet,
- II. CsA 10mg/kg/day fed a normal-salt diet,
- III. vehicle controls fed a low-salt diet,
- IV. CsA 10mg/kg/day fed a low-salt diet.

For urine ^1H -NMR analysis urine from all 24 study animals was collected and analyzed. After 27 days of treatment, rats were placed in metabolic cages and their 24h urine was collected. Hereafter the rats were sacrificed and tissue and blood samples were collected. A subset of four animals per study group was randomly selected for proteomics analysis. Salt-depleted animals had been started on a “low salt diet” (obtained from Harlan Laboratories, Madison, WI) one month prior to study initiation. The low salt diet contained 0.07% chloride and 0.01-0.02% background sodium. All animal protocols were approved by the University of Colorado Institutional Animal Care and Use Committee, and animal care was in accordance with the National Institutes of Health guidelines for ethical animal research (NIH publication No. 80-123). All animals were housed in cages in a temperature and light-controlled environment with free access to tap water and food *ad libitum*.

Drugs

Commercially available oral drinking solution of CsA (Neoral, Novartis, East Hanover, NJ) was administered by oral gavage in a constant volume according to group assignments. Neoral formulation was diluted in skim milk to a final concentration of 10 mg/mL and the volume of gavage was adjusted based on the animal weight. The drug doses were based on previous systematic dose-finding studies, which aimed to achieve CsA blood drug concentrations similar to those observed in transplant patients^{11, 15-18}. Vehicle control animals received skim milk only.

Clinical chemistry

Serum analysis for creatinine and plasma blood urea nitrogen (BUN), and urine analysis for creatinine was performed by the University of Colorado Hospital Laboratory.

Histology

Evaluation of kidney histology was carried out in a blinded manner using a semi-quantitative scoring system. Histologies were graded based on their tubular epithelial, glomerular and vascular alterations according to modified Banff classification criteria¹⁹. For a detailed description of the histology scoring procedure, please see the Supplemental Materials. Injury and aggregate injury scores were expressed as median and ranges.

Quantification of high-energy phosphate metabolites in kidney tissues

An average of 600 mg kidney tissue was homogenized in a mortar grinder over liquid nitrogen and extracted with 6 mL ice-cold PCA (12%) as described previously²⁰. Lyophilisates were re-dissolved in 0.5 ml water and adjusted to pH 6.5. An Agilent series 1100 HPLC (Agilent Technologies, Santa Clara, CA) coupled to an ABI Sciex API4000 triple stage quadrupole mass spectrometer (ABI Sciex, Foster City, CA) equipped with an electrospray ionization (ESI) source was employed for quantitation of nucleotide mono-, di-, triphosphates²¹.

Quantification of cyclosporine in blood and kidney tissues

CsA was quantified using a validated online extraction HPLC-MS/MS assay²². Briefly, whole blood samples (500 μL) were collected in heparinized tubes. Flash-frozen renal tissue (100 to 200 mg) was mortared in liquid nitrogen and was homogenized with 2 mL KH_2PO_4 buffer (pH 7.4). For protein precipitation, 800 μL methanol and 0.2 mmol/L ZnSO_4 (80/20, v/v) were added to 200 μL of tissue homogenate or blood. Cyclosporin D (250 $\mu\text{g/L}$, Novartis Pharma AG, Basel, Switzerland) was added as an internal standard for CsA²². After centrifugation (13,000g, 5 min, 4°C), 100 μL of the supernatant was injected onto the extraction column of the LC-MS/MS system and analyzed as described in²².

Sample preparation for 2D-gel electrophoresis and proteomics analysis

Freeze clamped kidneys (approximately 100 mg) were manually homogenized using a mortar and pestle over liquid nitrogen and lysed in 1 mL of chaotropic lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 50 mM DTT, 0.2% carrier ampholytes, 1% protease inhibitor mix). Cells in extracts were lysed on ice for 20 minutes then centrifuged at $100,000 \times g$ for 1 h at 4°C. To determine protein concentrations in the supernatants, a modified Bradford Assay Quick Start (BioRad Laboratories, Hercules, CA) was carried out according to the manufacturer's protocol²³.

Proteomics analysis

A detailed description of the proteomics and mass spectrometry methods is available in the Supplementary Material.

Western blot analysis

Western blot analysis was carried out to verify the proteomics results including the protein identification and quantitation. Aliquots of frozen extracts were thawed and loaded onto a 10% polyacrylamide gel. Proteins were separated using a Thermo EC 135-90 chamber (Thermo Scientific, San Jose, CA) operating for 2h at 60mA and were then transferred (300 mA, 2h) from the gel to an Immobilon-P membrane.

Antibodies used in this study included: oxoglutarate dehydrogenase, NADH-ubiquinone dehydrogenase, fructose-1,6-bisphosphatase (Santa Cruz Biotechnology, Santa Cruz, CA); isocitrate dehydrogenase, succinate dehydrogenase complex subunit A, ATP synthase beta, CD147, aquaporin 1 (Abcam, Cambridge, MA); JAK2, phospho-JAK2, STAT3, phospho-STAT3, Akt/ phospho-Akt, phospho-IKK, phospho-NF- κ B p65 (Cell Signaling, Boston, MA); and superoxide dismutase (Lab Frontier, Seoul, Korea). After incubation with secondary antibody, membranes were subsequently treated with Pierce SuperSignal West Pico Solution (Pierce, Rockford, IL) following the manufacturer's protocol. An UVP BioImaging System (BioImaging Systems, Upland, CA) was used to detect the horseradish peroxidase reaction on the membrane. Densitometry data were normalized to α -actin in each sample.

¹H-NMR-based metabolic profiling in urine

¹H NMR urine analysis was performed using a Varian INOVA NMR 600MHz spectrometer equipped with 5-mm HCN PFG probe. Rat urine (550 μ L) was buffered with 73 μ L 0.2M potassium phosphate buffer in D₂O prior to analysis by NMR spectroscopy. The pH was adjusted to 5.65-5.75 with NaOD and DCl. The external standard compound TMSP (trimethylsilyl propionic-2,2,3,3-d₄ acid dissolved in D₂O to 50 mM in a thin sealed glass capillary) was inserted into the NMR tube. A standard Varian pre-saturation sequence was used to suppress water in urine. ¹H-NMR spectra were obtained at 600 MHz using a spectral width of 7200 Hz and 32K data arrays, and 64 scans with 90° flip angle applied every 14.8 s to allow for full relaxation. Data analysis was performed using MesTrec software version 4.4.1.0 (MesTrecLab Research, Spain). To compensate for differences in urine dilution, all spectra were normalized to the total signal intensity. The integral of each individual metabolite was divided by the total integral of the corresponding urine spectrum. Data is presented as % change in comparison to the control group. Prior to quantitative ¹H-NMR analysis ¹H/¹³C-HSQC experiments had been carried out to verify the integrated signals and metabolite changes.

Statistical analysis

All numerical data are presented as mean±standard deviation. One-way analysis of variance (ANOVA) followed by Tukey *post hoc* analysis was used to determine differences among groups. Significance level was set at $p<0.05$ for all tests. Software used included: SigmaPlot (version 11.0)/ SigmaStat (version 4, both from Systat Software, Point Richmond, CA); and SPSS (version 20.0, IBM/SPSS, Chicago, IL).

RESULTS

Histologies

Blinded histological analysis based on the modified Banff criteria revealed the following mean toxicity grades (Supplemental Figure 1A).

Normal-fed control group—median aggregate score: 0/ range 0. No histological alterations were observed (Figure 1B).

Normal-fed CsA 10 mg/kg/day group—median 2.5/ range (2-4). Histological injury included tubular cell vacuolization (median 1.5 of a maximum score of 3) and tubular atrophy (1.0 of 3.0) (Supplemental Figure 1C).

Salt-depleted control group—median 0/ range 0. No histological damage could be detected (Supplemental Figure 1D).

Salt-depleted CsA 10 mg/kg-day group—median 9/ range (6-12). As expected based on the literature, combination of a salt-restricted diet and CsA resulted in the most extensive histological injuries among the study groups, mainly tubular atrophy (median of 3/maximum score of 3) and interstitial fibrosis (2/3) (Supplemental Figures 1E and 1F). Also, a slight arteriolar hyalinisation (0/3) and morphologic alterations of glomerular damage like glomerular sclerosis (1/3) and mesangial matrix expansion (1/3) were found (Supplemental Figures 1E and 1F).

CsA tissue concentrations and serum chemistry

CsA kidney tissue concentrations were higher in the salt-depleted rats as compared to the CsA-treated rats on a normal-salt diet (Table 1). A statistically significant increase of serum creatinine and blood urea nitrogen (BUN) concentrations was observed after CsA treatment in both rat groups (Table 1).

Kidney high-energy phosphate concentrations^{17, 20}

The differences in the energy balance amongst the study groups are presented in Figure 1. A significant decrease in the kidneys' energy balance was observed after treatment of rats with 10 mg/kg/day CsA while on the low-salt diet (Figure 1). Although there was also a slight decrease in the normal-fed group exposed to CsA (as compared to the normal-fed control), these changes did not reach statistical significance. Interestingly, a higher energy charge was found in the kidneys of control rats on low-salt as compared to those on normal-salt diet.

Protein analysis (Supplemental Table 2)

Metabolic enzymes. Mitochondrial function: TCA cycle activity and oxidative phosphorylation—Kidneys of rats fed a low-salt diet exhibited significantly higher levels of mitochondrial TCA cycle enzymes: isocitrate-, oxoglutarate- and succinate dehydrogenase as compared to those on normal-salt diet (Supplemental Figure 2). Also, while on low-salt diet, rats overexpressed the respiratory chain enzymes ATP synthase-beta

(Supplemental Figure 2). This may be responsible for their increased energy charge (Figure 1).

In contrast, treatment of rats on low-salt diet with CsA reduced expression of isocitrate-, oxoglutarate- and succinate dehydrogenase, as well as the expression of ATP synthase-beta and NADH ubiquinone oxidoreductase (NDUFS1) (Supplemental Figure 2).

Gluconeogenesis and glycolysis—In the cytosol, low-salt diet decreased fructose-1,6-bisphosphatase (gluconeogenesis enzyme, Supplemental Table 1, Figure 2). CsA treatment further reduced its expression in rats in both diet groups. In addition to inhibiting gluconeogenesis, CsA also strongly inhibited glycolysis, repressing several of its enzymes such as alpha-enolase, phosphoglycerate mutase and lactate dehydrogenase (Supplemental Table 2).

Interestingly, CsA also inhibited the expression of cluster of differentiation 147 (CD147) protein (Figure 2), a known regulator of monocarboxylate and lactate transporters²⁴. Since glycolysis and CD147 are closely related with AKT signaling²⁴, we investigated if CsA had any effects on Akt signaling. While CsA did not change expression of pAKT in the kidneys of normal-fed rats, salt depletion alone reduced expression of pAKT more than 5-fold as compared to the normal-salt controls. CsA decreased pAKT expression even further (Figure 2).

In addition to its interaction with AKT²⁵, CD147 is also involved in the regulation of NF- κ B signaling²⁶. Thus we performed Western blot analysis of IKK and NF- κ B p65 proteins. The phosphorylated forms of IKK and NF- κ B p65 were significantly reduced only in the low-salt fed rats treated with CsA, indicating greater NF- κ B activation under these conditions (Figure 2). No changes were detected in normal-salt fed CsA treated rats (Figure 2).

Ion transport—As anticipated, reduced dietary salt intake impacted the expression of several ion transporters in the kidney, including the chloride intracellular channel protein 1 (CLIC1). CLIC1 expression was slightly lower in the rats on low-salt diet than in kidneys of normal-fed rats. Interestingly, CsA induced CLIC1 expression in both, the low-salt and normal-fed rats (Figure 3). In contrast, the expressions of PDZ domain-containing protein in the kidney 1 (PDZK1) and aquaporin 1 slightly increased in the kidneys of rats fed the low-salt diet, but significantly decreased when these were treated with CsA (Figure 3).

Oxidative stress—CsA treatment in combination with low-salt diet affected expression of antioxidant proteins of the peroxiredoxin family. Interestingly, there seemed to be a differential regulation of these proteins, since expression of peroxiredoxin 6 decreased and peroxiredoxin 2 increased (Supplemental Table 2). In addition, the expression of mitochondrial superoxide dismutase 2 (SOD2), a superoxide detoxifying enzyme, was reduced as well (Figure 4).

JAK2/STAT3 proteins: Our results showed that the kidneys of normal-fed rats treated with CsA did not show significant changes in the expression of phospho-JAK2 and phospho-STAT3 proteins (Figure 4). However, when rats were fed a low-salt diet, the expression of these two proteins that are implicated in protection of renal cells from reactive oxygen species (ROS)²⁷ significantly increased (Figure 4). This is of interest since we recently showed that oxidative stress plays a key role in CsA nephrotoxicity in normal-fed rats¹¹.

Metabolic profiling

A principal component (PC) analysis of urinary metabolite patterns revealed separation of all study groups (Supplemental Figure 3A). The separation of the CsA groups from their respective controls was based on PC1 and explained 52% of cumulative variance. The clustering in PC1 was mainly influenced by the spectral regions containing the signals for acetate, taurine, creatinine, lactate and 2-oxoglutarate as evident from the loadings plot in Supplemental Figure 3. Despite the increased expression of isocitrate-, succinate- and oxoglutarate dehydrogenase enzymes in the kidneys of low-salt fed control animals, the concentrations of corresponding Krebs cycle intermediates excreted in urine were significantly lower than those in urine of normal-fed controls. A similar observation was also made for the glycolysis product lactate, which despite the reduced expression of glycolytical enzymes in the kidney, was excreted in a higher amount in urine of low-salt fed control. In CsA-treated animals, concentrations of glycolysis product lactate as well as Krebs cycle metabolites succinate, citrate and 2-oxoglutarate were decreased (Supplemental Figure 3B).

DISCUSSION

CNIs are major immunosuppressive agents used in transplantation. Their use however is complicated by renal and vascular toxicity. In our previous studies^{11, 16}, we showed that after 27 days, rats treated with CsA while on normal diet exhibited detectable impairment of renal function (as demonstrated by a significant reduction of glomerular filtration rates) and histomorphologic alterations (tubular vacuolization, tubular epithelial damage) that are considered typical for CsA toxicity. These results confirmed that we were able to create an animal model for CNI nephrotoxicity based on normal fed rats with drug blood concentrations within the therapeutic target range in transplant patients. However, the majority of the published studies assessing immunosuppressant nephrotoxicity are based on the salt-depleted rat model^{13, 14, 28}, in which the rats undergo renal injury faster than rats on normal-salt diet and show morphological and pathological changes similar to those occurring in renal transplant patients. But, as aforementioned, surprisingly little is known about the molecular mechanisms underlying the enhancement of immunosuppressant nephrotoxicity caused by a low-salt diet. The investigation of molecular mechanisms underlying the effects of salt depletion on the rat kidney in absence and presence of CsA, were the main aims of the present study.

In our study, rats in normal-fed and salt-depleted control groups showed no changes in kidney histology, whereas treatment with CsA led to histological changes mainly focused on the tubules. The highest toxicity scores, as expected, were found in CsA-treated rats on low-salt diet. Here, the tubular cell morphology and architectural structure of the kidney cortex were markedly affected. Besides tubular epithelial atrophy and vacuolization, glomerulopathy including glomerulosclerosis and mesangial matrix expansion were found. This was in accordance with previously published observations^{12, 28-30}.

Interestingly, CsA kidney tissue concentrations were significantly increased in salt-depleted rats as compared to those on normal diet. This is in analogy with previously published results³¹, and could possibly be responsible for the enhanced negative effects of CsA on the kidney of low-salt fed rats.

CsA treatment decreased the kidney's energy charge, especially in animals on low-salt diet. This supports our previous work, which showed that CsA decreases the energy production in brain cells and slices^{32, 33}, as well as in the kidney^{34, 35}. The more pronounced break-down of energy production observed in animals on low-salt as compared to those on normal-salt diet may at least partly be explained by their increased CsA tissue concentrations.

Energy production: Krebs cycle and glycolysis

The majority of proteins differently expressed in normal-fed and low-salt control rats were of mitochondrial origin, namely oxidative phosphorylation and fatty acid β -oxidation. Interestingly, most of these were upregulated as a consequence of the low-salt diet. This clearly suggested that the rats on low-salt diet have an increased energy demand, possibly while trying to retain normal osmolarity within the cells. With our previous studies showing that CsA mainly targets mitochondrial energy metabolism³⁶, this shift in energy production may be the reason why the kidneys of salt-depleted rats are more vulnerable to CsA than those of normal-fed animals.

Based on the expression levels of glycolytical enzymes, it seems that normal-salt-fed rats exposed to CsA upregulate their glycolysis to compensate for the disruption of mitochondrial energy production, something we have previously observed in rat brain slices and neuronal cells^{8, 37-40}. Interestingly, the activity of Akt^{41, 42} appeared to be connected to this metabolic change, with its phosphorylated form slightly increased in kidneys of rats fed a normal-salt diet. In low-salt fed rats, however, glycolysis and the phospho-Akt expression were already downregulated and underwent a further downregulation by CsA.

Changes in metabolic profiles

The approach for the analysis of proton NMR data used in this study was similar to various previously published NMR studies based on urine and focusing on changes in the metabolic profile as a result of nephrotoxicity⁴³⁻⁴⁷. In various animal studies, proximal tubular injury was associated with increased urine concentrations of acetate, lactate, glucose, amino acids and decreased concentrations of hippurate, creatinine, 2-oxoglutarate (2-OG), succinate and citrate. Changes in urine metabolite patterns observed after 28 days of treatment with CsA in the normal salt diet matched those described for nephrotoxins causing tubular injury^{43, 48}. This corresponds to the histology results that also showed specific tubular damage.

Surprisingly, although no histological damage was detected the metabolic pattern of the low salt diet control group differed significantly from the normal salt treated control group. For several metabolites, the trend for the change in the metabolic pattern from normal salt to the low salt treated animals was analogue to the changes observed after CsA treatment. This was the case for acetate, citrate, succinate and 2-OG. Combination with CsA exposure elevated the concentration of acetate even higher and reduced the concentrations of citrate, succinate and 2-OG lower. The amplification of the effects of CsA by low salt diet was best reflected by this set of metabolites. Serum creatinine concentrations were similar in rats fed normal and low salt diets. The creatinine urine concentrations were higher in rats fed low salt than those fed normal salt diet. In both cases CsA treatment resulted in low urine creatinine and relatively high serum creatinine concentrations indicating reduced glomerular filtration rates after CsA treatment but not due to low salt diet alone.

CD147/NF- κ B signaling

CD147 is another glycolysis regulator⁵⁷, functioning through its binding to cyclophilins and FKBP⁵⁸. Downstream of the cyclophilin/CD147 complex, CD147 regulates the expression of monocarboxylate/ lactate transporters (MCT)⁵⁹. CsA's ability to decrease the CD147 expression, especially in the low-salt fed animals, may be responsible for the down-regulation of MCT transporter proteins and the observed decrease in lactate transport.

Mechanistically, CD147 is also involved in the activation of NF- κ B signaling⁶⁰, which itself is regulating inflammatory and proliferative responses. Therefore it was not surprising that CsA-induced downregulation of CD147 was accompanied by a decrease of activated phospho forms of IKK and p65 proteins. IKK interacts with and regulates IKK, an

inhibitor unit of NF- κ B. It is possible that the downregulation of CD147 leading to a decrease of active phospho-IKK is responsible for the observed activation of NF- κ B signaling after CsA treatment⁶¹. Low-salt diet again aggravated the effects of CsA on this pathway. Interestingly, a recent publication investigating the role of IKK in human heart showed that IKK-depleted cardiomyocytes are more susceptible to stress conditions and are more likely to succumb to cell death⁶². The ability of CsA to regulate the IKK/NF- κ B pathway is possibly one of the pathways through which CsA mediates its negative effects on the kidney and the vascular system.

Ion transport: PDZK1 and CLIC1

In our previous work, we have examined the effects of osmotic stress on renal cells, and have shown that the inner medullary collecting duct cells adapt to the impact of high tonicity by increasing the number of mitochondria and their metabolic activity⁶³. This is necessary to maintain the chloride-dependent expression of Na/K-ATPase gamma subunit and sustain the cellular cation balance over the plasma membrane in a hypertonic environment⁶⁴. A similar mechanism could also be relevant in the kidney of rats fed a low-salt diet, where the increased high-energy demand could be a consequence of the changes in membrane transporter activities necessary to maintain normal osmolality within the cells.

In the animals on low-salt diet, expression of CLIC1 was downregulated. Since CLIC1 transports chloride across the apical membrane of renal proximal tubulus cells⁶⁵, its downregulation may reflect adaptation in response to a low chloride environment. Interestingly, CsA treatment increased the expression of CLIC1, however its levels in the CsA-treated rats on low-salt diet were not significantly different from the normal-salt controls. Given its role as a sensor and an effector of oxidative stress⁶⁶, its upregulation by CsA might be related to CsA's ability to induce oxidative stress in the kidney^{11, 37, 63}. In contrast, two other ion transporters were identified as markedly upregulated in the low-salt versus normal-salt controls: the PDZK1 protein and aquaporin 1. PDZK1 is a major scaffold in brush borders of proximal tubular cells, where it functions as a regulatory cofactor of sodium-hydrogen exchanger 1-3 (NHE-3)⁶⁷ and Npt2a⁶⁸. Aquaporin 1 transports solute-free water across cell membranes and plays a major role in concentrating the urine in the kidney⁶⁹. Interestingly, PDZK1 knock-out mice are known to significantly decrease the expression of aquaporin 1 as compared to their wild-type counterparts⁷⁰. Thus, there seems to be a connection between these transporters, both of which are again upregulated in kidneys of animals on low-salt diet. However, when treated with CsA, as previously described⁷¹, the reduced expression of both indicated an impaired ability of the rats to concentrate urine. This represents another potential mechanistic explanation why CsA effects are more pronounced in rats on low-salt diet and why the injury is focused mostly on the proximal tubule.

JAK/STAT pathway

The primary molecular targets of CsA, the cyclophilins were recently shown to affect JAK2 and STAT3 activation and function⁷². It has been suggested that the JAK2/STAT3 pathway is involved in CsA-induced endothelial and renal toxicity²⁷. Also, it was suggested that JAK/STAT signaling may be linked to the mitochondrial permeability transition pore⁷³, which in turn is known to be inhibited by CsA and its interaction with intra-mitochondrial cyclophilin D⁷⁴. Interestingly, the kidneys of rats fed a low-salt diet had significantly lower levels of phospho-STAT3. However, exposure to CsA led to an increase of phosphorylated JAK2 and STAT3, suggesting a key role for JAK2 inhibition in acute kidney injury and CsA-induced nephrotoxicity.

In summary (Figure 5), kidney metabolism of rats fed a low-salt diet relied more on the mitochondrial than on glycolytic energy production. This decrease of glycolytic activity, as a consequence of salt depletion, was associated with upstream downregulation of CD147 protein and pAKT. Due to their higher dependence on the mitochondria, CsA more strongly affected the kidneys of rats fed a low-salt diet. In addition, their kidneys were also less capable of compensating for the reduction of mitochondrial high-energy phosphates *via* glycolysis. The increased oxidative stress and dysregulation of ion transporter activity may contribute to CsA-induced mitochondrial dysfunction. Overall, our study has provided several novel mechanistic explanations on why rats fed a low-salt diet are more susceptible to renal injury mediated by CsA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Changes in the energy balance as determined by HPLC/MS in the different treatment groups (all values are means $\pm$ standard deviations), n=4/group. * significant compared to untreated controls using ANOVA in combination with *post-hoc* pairwise multiple comparison (Holm-Sidak method).

Figure 2.

Western blot analysis of glucose metabolism (gluconeogenesis and glycolysis) regulating enzymes (A) fructose-1,6-bisphosphatase (FBP), (B) CD147, (C) phospho-AKT and NF- κ B signaling intermediates (D) phospho-IKK and (E) phospho-NF- κ B p65 proteins.

All values are means $\pm$ standard deviations, n=3/group. Significance levels estimated based on ANOVA in combination with *post-hoc* pairwise multiple comparison Holm-Sidak test: *: p<0.05; **: p<0.005; ***: p<0.001.

Figure 3.

Western blot analysis of ion transporters (A) chloride intracellular channel protein 1 (CLIC1), (B) PDZ domain-containing protein in the kidney 1 (PDZK1) and (C) aquaporin 1. All values are means $\pm$ standard deviations, $n=3$ /group. Significance levels estimated based on ANOVA in combination with *post-hoc* pairwise multiple comparison Holm-Sidak test: *: $p<0.05$; **: $p<0.005$; ***: $p<0.001$.

Figure 4.

Western blot analysis of proteins involved in the defense against oxidative stress: (A) superoxide dismutase 2 (SOD2), (B) phospho-JAK2 and (C) phospho-STAT3 proteins. All values are means $\pm$ standard deviations, $n=3$ /group. Significance levels estimated based on ANOVA in combination with *post-hoc* pairwise multiple comparison Holm-Sidak test: *: $p<0.05$; **: $p<0.005$; ***: $p<0.001$.

Figure 5.

Summary of low-salt diet induced changes in the rat kidney and the effects of cyclosporine. (A) While on low-salt diet, animals “redirected” their energy production to the mitochondria away from glycolysis probably at least partially due to a negative effect on AKT. The higher energy demand is associated with a change in the expression of ion transporter proteins CLIC1 and PDZK1 (no cause-effect relationship was established), which are necessary for the maintenance of a normal intracellular kidney salt concentration.

(B) Cyclosporine targeted the mitochondria and induced oxidative stress. The major pathways involved were the regulation of CD147 and IKK proteins (involved in NF- B signaling), Akt and JAK2/STAT3 proteins.

Table 1

Cyclosporine (CsA) tissue concentrations, serum creatinine and blood urea nitrogen levels (BUN). All values are means $\pm$ standard deviations, n=4/group. Significance levels as estimated based on the *post-hoc* pairwise multiple comparison Holm-Sidak method).

	CsA tissue concentration [ng/mg _{tissue}]	serum creatinine [mg/dL]	BUN [mg/dL]
Normal-salt, Controls	-	0.47 $\pm$ 0.06	19.7 $\pm$ 2.3
Normal-salt, CsA 10mg/kg/d	17.7 $\pm$ 3.3	0.70 $\pm$ 0.12 *	34.4 $\pm$ 6.3 *
Low-salt, Controls	-	0.54 $\pm$ 0.11	23.8 $\pm$ 5.2
Low-salt, CsA 10mg/kg/d	31.7 $\pm$ 3.3 *	1.16 $\pm$ 0.23 *	40.6 $\pm$ 8.4 *

*
p<0.05