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Proximal tubule cyclophilin D regulates fatty acid oxidation in cisplatin-induced acute kidney injury

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Abstract

Regardless of the etiology, acute kidney injury involves aspects of mitochondrial dysfunction and ATP depletion. Fatty acid oxidation is the preferred energy source of the kidney and is inhibited during acute kidney injury. A pivotal role for the mitochondrial matrix protein, cyclophilin D in regulating overall cell metabolism is being unraveled. We hypothesize that mitochondrial interaction of proximal tubule cyclophilin D and the transcription factor PPAR α modulate fatty acid β -oxidation in cisplatin-induced acute kidney injury. Cisplatin injury resulted in

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AUTHOR CONTRIBUTIONS

HSJ and BJP conceived and designed the research; HSJ, MRN, EMJ, CG, and KWF performed experiments; HSJ, MRN, EMJ, WYK, KWF, CG, and BJP analyzed data; HSJ, DO, FAF, and BJP interpreted results of experiments; HSJ prepared figures; HSJ drafted the manuscript; HSJ and BJP edited and revised the manuscript; HSJ, MRN, EMJ, WYK, KWF, CG, DO, FAF, and BJP approved the final version of the manuscript.

DISCLOSURE

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histological and functional damage in the kidney with downregulation of fatty acid oxidation genes and increase of intrarenal lipid accumulation. However, proximal tubule-specific deletion of cyclophilin D protected the kidneys from the aforementioned effects. Mitochondrial translocation of PPAR α , its binding to cyclophilin D, and sequestration led to inhibition of its nuclear translocation and transcription of PPAR α -regulated fatty acid oxidation genes during cisplatin-induced acute kidney injury. Genetic or pharmacological inhibition of cyclophilin D preserved nuclear expression and transcriptional activity of PPAR α and prevented the impairment of fatty acid oxidation and intracellular lipid accumulation. Docking analysis identified potential binding sites between PPAR α and cyclophilin D. Thus, our results indicate that proximal tubule cyclophilin D elicits impaired mitochondrial fatty acid oxidation via mitochondrial interaction between cyclophilin D and PPAR α . Hence, targeting their interaction may be a potential therapeutic strategy to prevent energy depletion, lipotoxicity and cell death in cisplatin-induced acute kidney injury.

Keywords

acute kidney injury; ATP depletion; chemotherapy; cisplatin nephrotoxicity; mitochondria; proximal tubule

Acute kidney injury (AKI) is associated with high mortality and morbidity in critically ill patients, characterized by a rapid disruption in renal function and massive acute tubular cell death.^{1–3} Toxin-induced AKI is common in patients who have chemotherapy and contributes to approximately 8% to 60% of hospital-acquired AKI patients,⁴ and particularly, in critically ill patients, it could account for 19% of AKI patients.⁵ Cisplatin injury due to uptake in kidney proximal tubules (PTs) as well as in neurons and ears is a major dose-limiting factor in its use.^{6,7} Cisplatin (dichlorodiamino platinum) is widely used to treat various solid malignant tumors, including head and neck, testicular, ovarian, and bladder cancers,⁶ whereas a single dose of 50- to 100-mg/m² cisplatin could generate nephrotoxicity in one-third of patients with the treatment.⁸

Fatty acid β -oxidation (FAO) constitutes the major source of energy for the renal tissue and is inhibited during AKI, leading to energy deprivation as well as lipotoxicity by intracellular lipid accumulation, tubular cell injury, and death.^{9,10} Reduced DNA binding activity of peroxisome proliferator-activated receptor- α (PPAR α) and decreased expression of its tissue-specific coactivator PPAR- γ (PGC-1) are 2 potential mechanisms that may account for decreased FAO.^{11,12} However, the mechanisms that may lead to inhibition of the PPAR α signaling pathway are not defined.

The mitochondrial matrix protein cyclophilin D (CypD) is an established regulator of mitochondrial permeability transition pore (PTP), and targeting of CypD can attenuate ischemic reperfusion injury in the heart, brain, and kidney.^{13–17} In addition, CypD may play a pivotal role in regulating overall cell metabolism including glucose and fatty acid metabolism,¹⁸ as well as mitochondrial bioenergetics including tricarboxylic acid cycle, electron transporter chain, and oxidative phosphorylation in the cell in a tissue/organ-specific manner.¹⁷ Many of the emerging and newly revealed functions of CypD cannot be attributed exclusively to the modulation of the PTP, suggesting PTP-independent mechanism

for CypD functions. We and others previously demonstrated that CypD knockout (KO) mice are resistant to high fat diet–induced obesity or hepatic steatosis.^{19,20} However, the mechanism by which CypD regulates FAO during AKI remains elusive.

We hypothesized that CypD inhibits PPAR α expression and/or activity and thus blocks transcription of PPAR α -regulated FAO genes to inhibit FAO during cisplatin AKI. Establishing the mechanism by which CypD regulates FAO is significant, as pharmacologic inhibition of CypD has the potential as a therapeutic target in AKI, heart disease, neurodegeneration, and metabolic imbalance.^{21–24}

RESULTS

PT-targeted deletion of CypD prevents cisplatin AKI

To investigate the *in vivo* role of proximal tubule–specific cyclophilin D deletion (PT-CypD KO) in AKI, we generated PT-CypD KO mice by crossing CypD^{fl/fl} mice with Pepck cre–expressing mice (Supplementary Figure S1A). Deletion of CypD in all segments of PT was confirmed by immunohistochemistry (Supplementary Figure S1B). Similar to our previous findings, cisplatin injury resulted in renal functional and histologic damage in wild-type (WT) mice,²⁵ but prevented in PT-CypD KO at 3 and 5 days post-treatment (Figure 1a–c, Supplementary Figure S2). Apoptotic and necroptotic cell deaths were increased in the kidney after cisplatin treatment, but apoptotic, not necroptotic, cell death was suppressed in PT-CypD KO (Figure 1d–g, Supplementary Figure S3). Inflammation and cell cycle G2/M arrest makers were highly increased in the kidney of cisplatin-treated WT, but attenuated in PT-CypD KO mice kidneys (Figure 1h–l, Supplementary Figure S4A). Decreased proliferation of interstitial cells,²⁶ but not tubular cells, was observed in PT-CypD KO compared with WT (Supplementary Figure S4B–D). These data suggest that decreased histopathology, apoptosis, tubular cell cycle arrest, and interstitial inflammation may contribute to reduced cisplatin AKI in PT-CypD KO. Intriguingly, global CypD KO did not protect against cisplatin AKI, compared with those of WT (Supplementary Figure S5). It is plausible that cisplatin may adversely affect other target organs, including liver, heart, and brain, and may contribute to the injury. After ischemic AKI, however, the global CypD KO mice were well protected, most likely because only the kidneys were severely injured in this experimental model.¹⁶

PT-targeted deletion of CypD prevents mitochondrial injury and dysfunction after cisplatin injury

Because CypD is a critical component of mitochondrial PTP and is responsible for the fate of mitochondria following exposure of stimuli,^{27–29} we determined whether PT-CypD KO directly suppresses cisplatin-induced mitochondrial damage in the kidney. By electron microscopy study, we showed that cisplatin treatment results in mitochondrial damage, including mitochondrial swelling and reduced cristae in WT, but was markedly suppressed in PT-CypD KO mice kidneys (Figure 2a–c). Expressions of electron transporter chain, (complexes I through V, but not complex II)–related genes, were better preserved in PT-CypD KO compared with WT, showing maintenance of mitochondrial integrity (Figure 2d). Next, we investigated kidney adenosine triphosphate (ATP) level to confirm whether

prevention of mitochondrial damage indeed results in preservation of kidney ATP level during cisplatin injury. Cisplatin induced decline of ATP level in WT, but ATP level was better preserved in PT-CypD KO kidney (Figure 2e), indicating protection of mitochondrial function in cisplatin AKI. In addition, mitochondrial dynamics evaluated by the expression of fission and fusion genes was blunted in WT, but enhanced in PT-CypD KO (Figure 2f–h), presumably reflecting an improved adaptability to acute injury.

PT-targeted deletion of CypD prevents cisplatin-induced impairment of FAO

Given that cisplatin-induced AKI is associated with insufficient level of energy,⁶ and deficient FAO, we determined whether the protective effect demonstrated in PT-CypD KO mice against cisplatin AKI is due to enhanced FAO. We investigated the consequences of CypD deletion on FA metabolism using quantitative real-time polymerase chain reaction analyses and the RT² Profiler PCR Array (Qiagen, Hilden, Germany). Interestingly, most of the PPAR α -regulated FAO enzymes and regulators in the mitochondrial and peroxisomal FAO, including that in lipid transport, were downregulated in the kidney after cisplatin injury, but were suppressed in those of PT-CypD KO (Figure 3a; see Supplementary Figure S6 and Tables S1 and S2). The reduced gene expression observed in polymerase chain reaction array was confirmed by quantitative polymerase chain reaction (Figure 3e, Supplementary Figure S7A). To evaluate intrarenal lipid deposition as a result of impaired FAO, renal tissues derived from WT and PT-CypD KO were stained with oil red O stain. Data demonstrate that cisplatin induces intrarenal lipid accumulation in WT kidneys, but is prevented in PT-CypD KO mice (Figure 3b, Supplementary Figure S2D). Plasma triglyceride levels were increased in WT mice, but PT-CypD KO rectified the FAO defect (Figure 3c). Ultrastructure analysis also showed increased number of large-size lipid droplets in the cortex of WT, while the size and number of lipid droplets were drastically reduced in those of PT-CypD KO (Figure 3d, Supplementary Figure S7B).

Pharmacological inhibition of CypD prevents cisplatin-induced lipid deposition

To determine whether the effect of genetic deletion of CypD in impaired mitochondrial FAO can be replicated by its pharmacological inhibition, we investigated the effect of sanglifehrin A (SfA), an inhibitor of mitochondrial permeability transition and a potent inhibitor of CypD, which binds to CypD with 20-fold higher affinity than that of cyclosporin A, an immunosuppressant,^{30,31} in cisplatin-induced AKI. SfA suppressed cisplatin-induced damage of mitochondrial structure (Figure 3f). To check the effect of SfA in intrarenal and circulating lipid contents, oil red O staining on kidney section and plasma triglyceride assay were carried out. Lipid levels were suppressed in SfA-treated mice compared with in vehicle-treated mice (Figure 3g–h). Electron microscopy analysis also demonstrated that intracellular lipid accumulation was attenuated in SfA-treated mice (Figure 3f) with more preserved mitochondrial and peroxisomal FAO genes (Supplementary Figure S8A and B). We further showed that SfA inhibited cisplatin-induced tubular damage and functional impairment, as well as increased neutrophil accumulation and tubular cell cycle arrest (Supplementary Figure S8C–F). These data demonstrate that preservation of mitochondrial FAO is among the mechanisms by which CypD prevents cisplatin AKI.

CypD deletion maintains nuclear expression of PPAR α and its transcriptional activity in cisplatin AKI

FA uptake, oxidation, and synthesis are tightly regulated by the transcription factor PPAR α and its coactivator PGC-1. We hypothesized that CypD may regulate the expression and/or activity of PPAR α and PGC-1 to account for decreased FAO^{32–34} in cisplatin AKI. Data in Figure 4a and b demonstrated that PGC-1 α and PPAR α expression are downregulated in WT in cisplatin AKI, but both were maintained in PT-CypD KO mice kidneys. PPAR α was expressed mostly in the nucleus of vehicle-treated WT and PT-CypD KO mice kidneys (Figure 4c). In cisplatin AKI kidneys, nuclear expression of PPAR α was scant and was mostly localized to the cytoplasm (arrowhead in Figure 4c). However, in cisplatin-treated PT-CypD KO mice kidneys, PPAR α localization was localized to the nuclei compared with WT (Figure 4c). Both PPAR α activity and protein expression were decreased in WT kidneys of cisplatin AKI. However, PPAR α activity was preserved, but not the protein expression, in PT-CypD KO at both early and later points of the injury (Figure 4d, Supplementary Figure S9), suggesting the preserved transcriptional activity of PPAR α by PT-CypD KO.

CypD binds PPAR α and reduces PPAR α activity to downregulate FAO in cisplatin AKI

To determine whether CypD interacts with PPAR α , kidney protein samples from WT or PT-CypD KO mice treated with cisplatin or vehicle (control) were immunoprecipitated with anti-CypD antibody followed by immunoblotting using PPAR α antibody. Data demonstrate that CypD and PPAR α are indeed binding partners, but the binding was absent in those of PT-CypD KO (Figure 4e), suggesting mitochondrial translocation of PPAR α and its binding with CypD during cisplatin AKI.

Reduced acetylation of PPAR α in oxidative stress-induced damage was reported,³⁵ suggesting that acetylation of PPAR α might be involved in protection to acute stimuli. To determine whether acetylation of PPAR α , PGC-1 α , and carnityl acyltransferase 1a (Cpt-1a) play a role in cisplatin injury, the acetylation statuses were assessed. Our data demonstrate that acetylation of PPAR α and PGC-1 α were decreased in cisplatin-treated WT mice kidneys, but attenuated in those of PT-CypD KO (Supplementary Figure S10). Collectively, these data suggest that CypD regulates the nuclear versus mitochondrial localization and activity and acetylation of PPAR α in cisplatin AKI.

Next, to define whether activation of PPAR α reverses cisplatin-induced disruption of FAO, we administered a PPAR α agonist, fenofibrate, 30 minutes prior to cisplatin treatment into WT mice. Fenofibrate suppressed intrarenal and plasma lipids and prevented pathological features of cisplatin AKI (Figure 4f–k). Alternatively, blockage of PPAR α with GW6471 in PT-CypD KO showed cisplatin-induced increase of histologic and functional damages, as well as lipid contents in kidney and plasma, reversing the protective effect of PT-CypD KO (Figure 4f–k). These data indicate that PT-CypD inhibits PPAR α -regulated FAO, resulting in lipotoxicity in cisplatin AKI.

Furthermore, we confirmed the role of a PPAR α target, Cpt-1, which is a rate-limiting enzyme in FAO and its downregulation is associated with AKI.^{12,36} Cpt-1 and -2 expressions were downregulated in WT mice kidneys with cisplatin AKI, but it was restored

to normal levels in PT-CypD KO (Figures 3a and e and 5a). Etomoxir, an inhibitor of Cpt-1, reversed beneficial effect on FAO observed in PT-CypD KO in cisplatin AKI (Figure 5b–g). These data further reiterate that PT-CypD KO confers protective effect against cisplatin AKI through preservation of FAO.

Inhibition of CypD preserves FAO and ATP level after cisplatin injury in *in vitro* PT cells

To investigate the role of CypD in cisplatin-induced mitochondrial injury, we checked the effect of SfA on mitochondrial membrane potential, reactive oxygen species level, and intracellular lipid content after cisplatin treatment in a mouse cortical tubular epithelial cell line (MCT cells)³⁷ or a primary PT cell from WT or PT-CypD KO. Similarly with previous reports, high concentration of cisplatin, 800 $\mu\text{mol/l}$, induced cell necrosis⁸ and showed massive cell loss with prolonged treatment (data not shown). A time course of changes of mitochondrial membrane potential, reactive oxygen species, and lipid level as a function of CypD after cisplatin treatment was studied. Cisplatin resulted in loss of mitochondrial membrane potential, as shown by tetramethylrhodamine, methyl ester from very early time (3 minutes) (Figure 6a–c), increased intracellular and mitochondrial reactive oxygen species detected by 2',7'-dichlorodihydrofluorescein diacetate and Mitosox, respectively (Figure 6d and e, Supplementary Figure S11). Consistent with mitochondrial damage, intracellular lipid accumulated rapidly from 3 minutes post-treatment of cisplatin (Figure 6a and b). Inhibition of CypD reversed all of these adverse effects (Figure 6c–f, Supplementary Figures S12 and 13A).

To determine whether mitochondrial respiration may be altered as a function of CypD, we evaluated oxygen consumption rate (OCR) in primary PT cell (PTC) from WT and PT-CypD KO exposed to oxidant injury, hydrogen peroxide (H_2O_2), which is a critical mechanism of acute tubular injury.^{38,39} OCR was measured under basal conditions followed by the sequential addition of oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, as well as rotenone + antimycin A. Basal level of OCR did not differ between cells from WT and PT-CypD KO (Supplementary Figure S13B). On H_2O_2 treatment, OCR level in PTC from WT dramatically declined, but it was significantly attenuated in PT-CypD KO (Supplementary Figure S13C). Similarly with OCR data, H_2O_2 or cisplatin-induced intracellular lipid accumulation and decline of ATP level were relatively preserved in CypD inhibition compared with WT or vehicle-treated cells (Figure 6f and g, Supplementary Figures S12C and 13A). Furthermore, pharmacological inhibition of PPAR α reversed the beneficial effect of PT-CypD KO in mitochondrial respiration (Supplementary Figure S13C), suggesting that CypD-mediated inhibition of FAO via blocking PPAR α is the crucial event that may eventually lead to impaired FAO and energy depletion in PTC.

CypD binds and sequesters PPAR α in mitochondria to prevent PPAR α nuclear expression and FAO

We used a mouse cortical tubular epithelial cell line (MCT cells), a PTC line, to determine whether PPAR α translocates to mitochondria to bind with CypD on cisplatin treatment. Nuclear expression of PPAR α was significantly decreased after cisplatin injury, but pharmacological inhibition restored its expression to the nucleus (Figure 7a and b, Supplementary Figure S14). Next, to confirm whether PPAR α and CypD indeed interact on

cisplatin injury, we used the BioID method (Kyle Roux, North Sioux Falls, SD), a recently developed technique that enables researchers to screen protein-protein interactions with high efficiency and selectivity in live cells⁴⁰ (Supplementary Figure S15). BioID and immunoprecipitation studies indicated that PPAR α binds to CypD in cisplatin-treated PTCs (Figure 7c and d) and its pharmacological inhibition of CypD suppressed the binding (Figure 7d). Moreover, to confirm whether PPAR α interacts with CypD in mitochondria, we isolated mitochondrial fraction from cisplatin-treated PTCs. Mitochondrial PPAR α was increased on cisplatin injury, while nuclear PPAR α was decreased (Supplementary Figure S16). However, these changes were blunted after CypD inhibition (Supplementary Figure S16). Slight levels of coexpression of PPAR α and CypD were observed in nucleus, possibly due to overlaying of cytoplasmic structures on the nucleus, but nuclear expression level of CypD was not changed by cisplatin treatment (Figure 7b, Supplementary Figure S16A). These data suggest that mitochondrial sequestration of PPAR α by its binding to CypD is a mechanism by which impaired FAO occurs in the kidney on acute stress.

Furthermore, we examined the potential amino acid residues that may be involved in the physical interaction between PPAR α and CypD using bioinformatics. Molecular docking analysis was used to identify potential interacting residues based on geometric, electrostatic, and shape complementarities. Interactions between the PPAR α and CypD (peptidylprolyl isomerase D) proteins in the available protein-protein interaction databases have not been reported. However, because crystal structures are available for both PPAR α (5HYK) and peptidylprolyl isomerase D (2BIT) proteins in the Protein Data Bank,^{41,42} we analyzed a potential docking pose between the 2 proteins. Interacting regions in both proteins, corresponding to known domains (identified by InterPro⁴³ domain search) that include the nuclear hormone receptor ligand-binding domain (Pfam: PF00104, <https://pfam.xfam.org>) of PPAR α and the cyclophilin peptidylprolyl cis-trans isomerase signature (ProSite: PR00153, <https://prosite.expasy.org>) of peptidylprolyl isomerase D (CypD) were observed (Figure 7e). Moreover, the isomerase signature is repeated 4 times in the peptidylprolyl isomerase D protein and 3 out of these 4 signatures are shown to participate in the interacting interface with PPAR α (Figure 7f); therefore, further experimental validation of these docking poses is warranted.

DISCUSSION

In the present study, we identified a novel role for PT-CypD in FAO during cisplatin AKI. Deletion of CypD in the PT, which is the primary target of most nephrotoxicants, was sufficient to protect cisplatin-induced FAO impairment in cisplatin AKI. Pharmacological inhibition of CypD also prevented impairment of FAO in cisplatin AKI. Binding studies using the BioID and immunoprecipitation demonstrated that CypD binds PPAR α in the mitochondria and thereby suppresses PPAR α nuclear expression and transcription of PPAR α -regulated FAO genes during cisplatin AKI. Inhibition of CypD prevented impairment of mitochondrial FAO, resulting in suppression of intrarenal lipid accumulation and ATP depletion. Promotion of FAO prevented renal dysfunction and all secondary events in cisplatin AKI. These data for the first time suggest that PT-CypD is the primary cause of disruption of FAO and energy depletion in cisplatin AKI, through mitochondrial sequestration of PPAR α . However, protective effect shown by PT-CypD KO to cisplatin AKI

was not complete, suggesting involvement of other factors affecting ATP level and AKI pathogenesis, such as glycolysis or peroxisomal β -oxidation.

Improvement in mitochondrial structure and dynamics and ATP preservation prompted us to determine whether there is preserved FAO in PT-CypD KO during cisplatin AKI. We discovered that most of the PPAR α -regulated FAO enzymes and regulators in the mitochondrial and peroxisomal FAO were downregulated in kidneys from WT after cisplatin injury, but preserved in CypD KO. FAO constitutes the major source of energy for the renal tissue.^{1,2} However, reduced FAO and persistent increases of tubule cell nonesterified fatty acid levels occur during ischemia and postreperfusion *in vivo* in association with downregulation of mitochondrial and peroxisomal FAO genes.^{1,2,44–46} PTs subjected to hypoxia or reoxygenation or both *in vitro* exhibited decreased ATP, and nonesterified fatty acid overload is the primary cause of energetic failure and impaired recovery of mitochondrial membrane potential during reoxygenation.⁴⁷ Similarly, we showed that intracellular lipid accumulation was attenuated by inhibition of CypD. Basal levels of OCR did not differ between cells from WT and PT-CypD KO. However, on H₂O₂ treatment, OCR level in PT cell from WT dramatically declined, but it was significantly attenuated in those of PT-CypD KO. Furthermore, pharmacological inhibition of PPAR α abolished the protective effect of PT-CypD KO in FAO, suggesting that PT-CypD promotes energy depletion on acute injury through blockage of PPAR α -regulated FAO. These data, however are in contrast to a recent report that loss of CypD in mouse embryonic fibroblasts cell lines induced defects in mitochondrial bioenergetics due to impaired FAO accompanied by transcriptional upregulation of glycolysis mediators and a general switch toward glucose metabolism.¹⁸ CypD-deficient hearts or cardiomyocytes also showed decreased FAO rates, increased oxidation of glucose, and attenuated mitochondrial maximal respiration rate.^{48,49} Menazza *et al.*⁴⁸ reported that acyl-CoA-related pathways were altered in the heart of CypD KO, but mitochondrial respiration or H₂O₂ generation were not affected. Similarly, we showed that many genes of acyl-CoA-related pathways were slightly decreased in naïve kidney of PT-CypD KO compared with WT (Supplementary Table S2), but there are no significant differences in mRNA expression level of Cpt-1 α or mitochondrial respiration evaluated by OCR. Collectively, these data suggest that CypD may regulate both glucose and FA metabolism in the cell, based on tissue or organ and injury context. Whether these confounding results on FAO and glucose metabolism observed in different cell or tissue types is via mitochondrial PTP or mitochondrial PTP-independent mechanism is yet to be established. On the other hand, our data showed that global CypD KO does not protect against cisplatin injury. Global CypD deletion is associated with many aspects of metabolism including alterations in Krebs cycle, branch chain amino acid degradation, pyruvate metabolism, and glycolysis,^{18,48,49} as well as in systemic effect of cisplatin tissue toxicity. Further study is required to define the distinct mechanism between global and PT-specific CypD KO mice.

Based on our finding that PPAR α -regulated FAO enzymes were downregulated in the kidneys from WT, we hypothesized that CypD may regulate the expression or activity of the transcription factor PPAR α . Studies by Portilla *et al.*^{11,12,50–52} have demonstrated persisting disturbances of mitochondrial and peroxisomal FAO by downregulation of PPAR α expression or activity during both ischemic and cisplatin AKI. However, the mechanisms

that may lead to downregulation of PPAR α signaling pathways in AKI are not defined. Our data demonstrate that in cisplatin AKI kidneys, nuclear expression of PPAR α was scant and was mostly localized to the cytoplasm, but PPAR α was localized to the nuclei in PT-CypD KO mice kidneys. Interestingly, the overall expression level of PPAR α was not different between PT-CypD KO mice kidneys. Using BioID and immunoprecipitation, we demonstrated that mitochondrial sequestration of PPAR α by its binding to CypD could be a primary cause by which impaired FAO occurs in cisplatin AKI. These data suggest that CypD regulates the nuclear versus mitochondrial localization of PPAR α and thus may decrease the transcription of FAO genes. In addition, we showed the change of protein acetylation, which could play a role in transcriptional regulation of PPAR α and PGC-1 α . Acetylation of PPAR α and PGC-1 α is reduced in cisplatin-treated WT mice kidneys, but not in PT-CypD KO. In line with our data, Barreto-Torres *et al.*³⁵ reported that oxidative stress results in reduced acetylation of PPAR α , suggesting that post-translational modifications such as acetylation of PPAR α might be involved in protection to acute stimuli, but the molecular mechanisms remain elusive.

Clinical studies reveal that cisplatin induces increase of lipid level in patients, showing the involvement of dysregulated FAO or mitochondrial metabolism in cisplatin nephrotoxicity.^{53–55} PPAR α -targeted drugs, including fenofibrate, are currently being tried in clinical trials,⁵⁶ although no trial has been initiated in AKI. Our findings suggest that targeting CypD would be an option for treatment of AKI that is capable of preventing both tubular cell death and metabolic impairment. Collectively, our data for the first time provide a mechanistic understanding of the CypD-dependent malevolent metabolic consequences that occur in kidneys under acute stress conditions. The result may pave the way to design therapeutic strategies targeting CypD, a key modulator of FAO, and/or intervening in CypD-PPAR α interaction to improve FAO in cisplatin AKI and other types of AKI.

METHODS

Study approval

Mice were cared for before and during the experimental procedures in accordance with the policies of the Institutional Animal Care and Use Committee, University of Nebraska Medical Center, and the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. All protocols had received prior approval from the University of Nebraska Medical Center–Institutional Animal Care and Use Committee.

Statistical analyses

Data are expressed as the mean $\pm$ SD. Differences between 2 groups were assessed by 2-tailed unpaired Student's *t* test. For multiple group comparison, 1-way analysis of variance with Bonferroni analysis was applied (GraphPrism 5.0 software; GraphPad Inc., San Diego, CA). *P* values < 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Statement

Acute kidney injury (AKI) is a major unmet medical need with no effective treatment. Current findings suggest that mitochondrial cyclophilin D (CypD) interacts with peroxisome proliferator-activated receptor- α (PPAR α) to block its nuclear translocation, resulting in impaired fatty acid β -oxidation (FAO), adenosine triphosphate (ATP) depletion and renal dysfunction in cisplatin AKI. Developing strategies to intervene in CypD-PPAR α interactions to promote FAO will have translational potential in patients where cisplatin AKI is expected, as a result of chemotherapy. Furthermore, targeted inhibition of signaling molecules that elicit ATP depletion will have wide implications because this strategy is potentially extensible to viable therapeutic approaches to treat a wide range of other disease states including ischemic AKI, heart failure, and stroke.

Figure 1 | Prevention of cisplatin (Cis)-induced functional impairment and tubular injury after proximal tubule (PT)-specific deletion of cyclophilin D.

Kidney and blood samples were collected at 24 hours or 72 hours post-treatment of Cis. **(a)** Paraffin-embedded kidney section at postinjury day 3 was used for periodic acid–Schiff (PAS) staining. Yellow asterisks indicate tubular cast. **(b)** Histologic damage score was measured in 5 randomly chosen fields per kidney using a PAS-stained kidney section ($n = 5-6$). **(c)** Plasma creatinine (PCr) was used as an index of kidney function ($n = 5-6$). **(d)** Paraffin-embedded kidney section after 72 hours post-treatment of Cis was used for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, and the number of apoptotic cells was evaluated in 5 randomly chosen fields per kidney ($n = 5$). Green and blue fluorescence indicates a TUNEL-positive cell and nuclei, respectively. **(e)** Expression of cleaved caspase-3 (C. caspase-3) and active Bax was examined by Western blot analysis using specific antibodies ($n = 5$). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as a loading control (Con). **(f,g)** Western blot band densities were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD). **(h)** Evaluation of polymorphonuclear neutrophil (PMN)-positive cells was performed in paraffin-embedded kidney sections using immunohistochemistry, and the data were quantified from 5 randomly chosen fields per kidney ($n = 5$). **(i,j)** *Ccl2* and *interleukin-6* (*IL-6*) mRNA levels were evaluated using quantitative real-time polymerase

chain reaction and quantified using the formula described in the Methods section ($n = 5$). (k) Paraffin-embedded kidney section was used to carry out immunohistochemistry for evaluating the number of p-histone H3-positive cells, a marker of G2/M cell cycle arrest ($n = 5$). Arrows indicate p-histone H3-positive cells. (l) Expressions of cell cycle arrest-associated proteins, cyclin B1 and D1 (B1/D1), p21, and p53 were examined by Western blot analysis ($n = 5$). Anti-GAPDH antibody was used as a loading Con. Expression levels were evaluated using ImageJ software. Bars = 50 μ m. Data are expressed as means $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Cx, cortex; G, glomerulus; IM, inner medulla; K, proximal tubule-knockout; KO, knockout; OM, outer medulla; V, vehicle; Veh, vehicle; W, wild type; WT, wild type. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 2 | Effect of proximal tubule (PT)-specific deletion of cyclophilin D on mitochondrial ultrastructure and function in mice kidney after cisplatin (Cis) treatment.

(a) Electron microscopy was used to evaluate mitochondrial ultrastructure. Bars = 20 μ m (low power) and 500 nm (high power). Numbers of (b) mitochondria and (c) cristae were quantified in 5 randomly chosen fields per kidney ($n = 5$). (d) Expression levels of *Ndufa1* (complex I), *Sdha* (complex II), *Uqcrl1* (complex III), *COX IV* (complex IV), and *ATP5o* (complex V) mRNA were evaluated using quantitative real-time polymerase chain reaction and quantified using a formula described in the Methods section ($n = 5$). (e) Adenosine triphosphate (ATP) level was evaluated by using ATP fluorometric assay ($n = 5$). (f) *Drp-1*, (g) *OPA1*, and (h) *Mfn-1* mRNA levels were evaluated using quantitative real-time polymerase chain reaction and calculated by using a formula described in the Methods section ($n = 5$). Bars = 50 μ m. Data are expressed as means $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. BB, brush border; K, knockout; KO, knockout; V, vehicle; Veh, vehicle; W, wild type; WT, wild type. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 3 | Cisplatin (Cis)-induced alteration of fatty acid oxidation-related genes and lipid accumulation, and its prevention by genetic or pharmacologic inhibition of cyclophilin D. Mice received cisplatin i.p. Kidney and blood samples were collected at 72 hours post-treatment of Cis. **(a)** Alteration of fatty acid metabolism-associated genes by Cis treatment was confirmed by polymerase chain reaction array. **(b)** Intrarenal lipid accumulation and **(c)** plasma triglycerides (TGs) were evaluated by oil red O stain or an EnzyChrom TG assay kit ($n = 5-6$; Bioassay Systems, Hayward, CA). Oil red O-positive area was evaluated using ImageJ software (National Institutes of Health, Bethesda, MD) in 5 randomly chosen fields per kidney. Red colors indicate lipid deposition. Bars = 50 μ m. **(d)** Electron microscopy (EM) was used to confirm accumulation of lipid droplets. Numbers of lipid (L) droplets and size were evaluated in 5 randomly chosen fields per kidney ($n = 5$). Bars = 500 nm. **(e)** Most changed genes evaluated by polymerase chain reaction array were confirmed using quantitative real-time polymerase chain reaction and calculated by using a formula described

in the Methods section ($n = 5$). Mice received Cis and also vehicle (Veh) or sanglifehrin A (SfA) 30 minutes prior to Cis treatment. Kidney and blood samples were collected at 72 hours post-treatment of cisplatin. (f) EM was used to evaluate mitochondrial ultrastructure and lipid accumulation ($n = 5$). Bars = 500 nm. Insets are low-powered images. (g) Intrarenal lipid accumulation ($n = 5-6$) and (h) plasma TGs ($n = 5-6$) were evaluated by oil red O stain and an EnzyChrom TG assay kit, respectively. Oil red O-positive area was evaluated using ImageJ software in 5 randomly chosen fields per kidney. Red colors indicate lipid deposition. Bars = 50 μm . Data are expressed as means $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. K, knockout; KO, knockout; PT, proximal tubule; W, wild type; WT, wild type. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 4 | Role of peroxisome proliferator-activated receptor- α (PPAR α) in resistance to cisplatin (Cis) acute kidney injury in proximal tubule (PT)-cyclophilin D (CypD) knockout (KO).

Mice received Cis and either vehicle (Veh) or fenofibrate (Feno; PPAR α receptor agonist) or GW6471 (GW; PPAR α antagonist) 30 minutes prior to Cis treatment. Kidney and blood samples were collected at 72 hours post-treatment of Cis. (a) *Pgc-1 α* and (b) *Ppara* mRNA levels were evaluated using quantitative real-time polymerase chain reaction and calculated by using a formula described in the Methods section ($n = 5$). (c) Paraffin-embedded kidney section was used with immunohistochemistry for evaluating the expression pattern of PPAR α ($n = 5$). Inset images highlight nuclear expression of PPAR α . Arrowheads indicate the expression of PPAR α in the injured tubule. (d) PPAR α activity was evaluated by PPAR α transcription assay ($n = 5$). (e) Interaction between CypD and PPAR α were confirmed by immunoprecipitation (IP) ($n = 5$). (f) Intrarenal lipid accumulation and (g) plasma triglycerides (TGs) were evaluated by oil red O stain ($n = 5-6$) or an EnzyChrom TG assay kit ($n = 5-6$; Bioassay Systems, Hayward, CA). The oil red O-positive area was quantified using ImageJ software (National Institutes of Health, Bethesda, MD) in 5 randomly chosen fields per kidney. Red colors indicate lipid deposition. (h) A paraffin-embedded kidney section was used for periodic acid-Schiff staining, and histopathologic scoring was carried out in 5 randomly chosen fields per kidney ($n = 5$). (i) Plasma creatinine (PCr) was used as an index of kidney function ($n = 5-7$). Evaluation of (j) polymorphonuclear neutrophil (PMN), a marker of neutrophil, and (k) G2/M arrest marker p-histone H3-positive cells was performed by immunohistochemistry with specific antibodies and in 5 randomly chosen fields per kidney ($n = 5$). Bars = 50 μ m. Data are expressed as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N, negative control; W, wild type; WT, wild type. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 5 | Role of peroxisome proliferator-activated receptor- α (PPAR α) target, carnityl acyltransferase 1 (Cpt-1), in preventing cisplatin (Cis) acute kidney injury in proximal tubule (PT)-cyclophilin D knockout (KO) mice.

Mice received Cis i.p. Some mice had vehicle (Veh) or etomoxir (Eto) 30 minutes prior to Cis treatment. Kidney and blood samples were collected at 72 hours post-treatment of Cis.

(a) *Cpt-1a* mRNA levels were evaluated using quantitative real-time polymerase chain reaction and calculated using a formula described in the Methods section ($n = 5$). (b) Intrarenal lipid accumulation and (c) plasma triglycerides (TGs) were evaluated by oil red O stain or an EnzyChrom TG assay kit ($n = 5$). The oil red O-positive area was evaluated using ImageJ software (National Institutes of Health, Bethesda, MD) in 5 randomly chosen fields per kidney. (d) A paraffin-embedded kidney section was used for periodic acid-Schiff (PAS) staining, and histopathologic scoring was evaluated in 5 randomly chosen fields per kidney ($n = 5$). (e) Plasma creatinine (PCr) was used as an index of kidney function ($n = 5$). The evaluation of (f) polymorphonuclear neutrophil (PMN), a marker of neutrophil, and (g) the G2/M arrest marker p-histone H3-positive cell was performed by immunohistochemistry with specific antibodies and in 5 randomly chosen fields per kidney ($n = 5$). Arrowheads indicate PMN-positive cells. Bars = 50 μ m. Data are expressed as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. WT, wild type. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 6 | Mitochondrial injury and function and fatty acid oxidation in cisplatin (C) injury in the proximal tubule cell as a function of cyclophilin D.

(a,b) Seventy percent to 80% confluent of a mouse cortical tubular epithelial cell line (MCT cells) was incubated with tetramethylrhodamine, methyl ester (TMRM) and boron-dipyrromethene (BODIPY) at 37 °C and treated with 800 μmol/l cisplatin at diverse time points and then harvested immediately ($n = 5$). $**P < 0.01$ versus control (0 minutes). Eight hundred μmol/l cisplatin were treated with 70% to 80% confluent MCT cells during 1 hour with or without 500 nmol/l sanglifehrin A (S) 1 hour prior to cisplatin treatment. At 1 hour post-treatment of cisplatin, cells were washed twice with Dulbecco's phosphate-buffered saline and then incubated with (c) TMRM, (d) 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), and (e) Mitosox for measurement of mitochondrial membrane potential, intracellular reactive oxygen species, and mitochondrial reactive oxygen species, respectively ($n = 5$). Evaluation of individual intensity or area was performed in 5 randomly chosen fields per well. (f) The oil red O⁺-positive area was evaluated using ImageJ software (National Institutes of Health, Bethesda, MD) in 5 randomly chosen fields per well ($n = 5-6$). (g) The adenosine triphosphate (ATP) level was evaluated by using ATP fluorometric assay ($n = 5$). Bars = 10 μm. Data are expressed as means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. RFU, radiofrequency unit; V, vehicle. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 7 | Interaction between peroxisome proliferator-activated receptor- α (PPAR α) and cyclophilin D (CypD) in proximal tubule cells on cisplatin (Cis) injury. Seventy percent to 80% confluent of a mouse cortical tubular epithelial cell line (MCT cells) was treated with 800 μ M Cis for 1 hour with or without 500 nM sanglifehrin A (SfA) 1 hour prior to Cis treatment. (a) The cells were fixed with 4% paraformaldehyde and immunostained with anti-PPAR α (green) antibody. Nuclei are indicated by 4',6-diamidino-2-phenylindole (DAPI; blue). Data are representative of at least 5 independent experiments. Bars = 10 μ m. (b) Cells fixed with 4% paraformaldehyde were immunostained

with anti-PPAR α (green) and/or anti-CypD (red) antibodies. DAPI (blue) indicates the nucleus. Yellow dots indicate colocalization of CypD and PPAR α . Data are representative of, at least, 5 independent experiments. Bars = 10 μ m. (c) BioID (Kyle Roux, North Sioux Falls, SD) pull down was used to confirm the interaction between PPAR α and CypD. MCT cells with pretreatment of vehicle (veh) (dimethylsulfoxide) or 1 mmol/l SfA followed by 400 μ mol/l Cis 1 hour post-treatment of SfA were incubated with 50 μ mol/l biotin for 4 hours. Magnetic bead-conjugated streptavidin was used to collect biotinylated protein. Expressions of CypD and hemagglutinin (HA) were examined by Western blot analysis. HA, which is tagged to PPAR α , was used as a loading control. Data are representative of 3 independent experiments. (d) The interaction between CypD and PPAR α was confirmed by immunoprecipitation (IP) ($n = 5$). (e) The best-scoring docking pose generated between PPAR α (blue cloud) and peptidylprolyl isomerase D (PPID; green cloud) with the interacting residues within 1 Å distance is shown in the red cloud. (f) The residues identified in the binding site are listed in the table with their positions and are shown as side chain representations in the figure. Bars = 10 μ m. ALA, alanine; ARG, arginine; ASN, asparagine; ASP, asparaginase; ILE, isoleucine; LEU, leucine; LYS, lysine; MET, methionine; PHE, phenylalanine; PRO, proline; SER, serine; THR, threonine; TRP, tryptophan; TYR, tyrosine; VAL, valine. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.