Renal Circadian Clock Regulates the Dosing-Time Dependency of Cisplatin-Induced Nephrotoxicity in Mice

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Received September 24, 2013; accepted February 24, 2014

ABSTRACT

Cisplatin, cis-diamminedichloro-platinum (CDDP), is a widely used anticancer agent, the clinical applications of which have been limited by severe nephrotoxicity. Although dosing time-dependent differences in CDDP-induced nephrotoxicity have been reported in both humans and laboratory animals, the underlying mechanism remains unknown. In the present study, we investigated the molecular mechanism for the dosing-time dependency of the nephrotoxic effect of CDDP in mice. CDDP-induced nephrotoxicity was significantly attenuated by injecting CDDP at times of the day when its renal clearance was enhanced. The dosing-time dependency of the nephrotoxic effect was parallel to that of CDDP incorporation into renal DNA. Two types of transporters, organic cation transporter 2 (OCT2, encoded by Slc22a2) and multidrug and toxin extrusion 1 (MATE1, encoded by Slc47a1), are responsible for the renal excretion of CDDP. The expression of OCT2, but not MATE1, exhibited a significant time-dependent oscillation in the kidneys of mice. The circadian expression of OCT2 was closely related to the dosing-time dependency of CDDP incorporation into renal DNA. Molecular components of the circadian clock regulated the renal expression of Slc22a2 mRNA by mediating peroxisome proliferator–activated receptor-α, which resulted in rhythmic oscillations in OCT2 protein levels. These findings indicate a molecular link between the circadian clock and renal xenobiotic excretion.

Introduction

Daily variations in biologic functions such as gene expression and protein synthesis are thought to be important factors affecting the efficacy of drugs. Dosing time–dependent differences in the therapeutic effects of drugs are, at least in part, due to circadian-related changes in drug disposition, such as absorption, distribution, metabolism, and elimination (Labrecque and Bélanger, 1991; Bélanger et al., 1997; Ohdo, 2007, 2010). In mammals, the master circadian pacemaker resides in the suprachiasmatic nucleus of the anterior hypothalamus and is responsible for adapting endogenous physiologic functions to daily environmental cues such as light and food (Green et al., 2008; Bass and Takahashi, 2010; Asher and Schibler, 2011; Morf et al., 2012). The mammalian circadian clock constitutes a transcriptional–translational feedback loop, in which CLOCK and BMAL1 activate the transcription of Period (Per) and Cryptochrome (Cry) via E-box enhancer sequences (Green et al., 2008; Bass and Takahashi, 2010; Asher and Schibler, 2011). Once PER and CRY proteins have reached critical concentrations, they attenuate CLOCK/BMAL1 transactivation, thereby generating circadian oscillations in their own transcription. The transcription of orphan nuclear receptor Reverbera and activating transcription factor-4 (Atf4) are also activated by CLOCK/BMAL1 and repressed by PER and CRY, resulting in circadian oscillations in Bmal1 transcript (Preitner et al., 2002; Koyanagi et al., 2011).

The molecular oscillator regulates daily variations in output physiology through clock-controlled output genes (Jin et al., 1999; Maemura et al., 2000; Cheng et al., 2002). Prolin-rich acid–rich basic leucine zipper proteins, hepatic leukemia factor, thyrotroph embryonic factor, and D-site binding protein are examples of such output mediators, because their expression is probably regulated by core oscillator components (Gachon et al., 2004). Circadian-controlled output pathways include xenobiotic detoxification mediated through the circadian expression of many enzymes and transporters (Gachon et al., 2006). Clock genes comprising the core oscillation loop have also been shown to govern the expression of peroxisome proliferator–activated receptor-α (PPARα) (Oishi et al., 2005). PPARα controls the transcription of its target genes through PPARα response elements (PPREs) and participates in the circadian expression of fibroblast growth factor-21 and plasminogen activator inhibitor-1 (Oishi et al.,...
2008). Recent studies have revealed that PPARα regulates the transcription of several types of transporters, including fatty acid transport protein, ATP-binding cassette–transporter family A1, and equilibrative nucleoside transporter 1 (Martin et al., 1997; Chinetti et al., 2001; Montero et al., 2012), suggesting that the function and expression of PPARα-targeted transporters may also exhibit circadian oscillations.

Cisplatin, cis-diaminedichloro-platinum (CDDP), is a platinum (Pt)-based anticancer agent that is widely used in the treatment of solid cancers, such as lung, ovarian, and esophageal cancers. However, CDDP can cause severe nephrotoxicity, which is a dose-limiting factor of CDDP therapy. Recent studies have reported that organic cation transporter 2 (OCT2) and multidrug and toxin extrusion 1 (MATE1) are responsible for CDDP-induced nephrotoxicity (Filipski et al., 2009; Nakamura et al., 2010). OCT2 is expressed in the basolateral membrane of proximal tubule cells in the kidney, whereas MATE1 is located in the brush border membrane. CDDP is incorporated into renal cells by OCT2, and then MATE1 excretes this drug into the urine. The CDDP incorporated into renal cells forms DNA adducts and activates apoptotic signals, resulting in renal cell death. Therefore, much effort has been directed toward attenuating CDDP-induced nephrotoxicity (Pabla and Dong, 2008; Miller et al., 2010; dos Santos et al., 2012). For example, hydration with sodium chloride is frequently given before and during CDDP therapy to prevent its accumulation in renal cells. A previous study showed that hydration decreased CDDP-induced nephrotoxicity by 60–70% in rats (Litterst, 1981).

On the other hand, a significant dosing time–dependent difference in CDDP-induced nephrotoxicity has been reported in both humans and laboratory animals (Hrushesky et al., 1982; Levi et al., 1982a,b, 1990; Boughnertas et al., 1990). CDDP-induced nephrotoxicity was attenuated in humans by administering the drug during the afternoon. Consequently, CDDP has been empirically administered to patients during this time window. However, the mechanism underlying dosing time–dependent changes in CDDP-induced nephrotoxicity remain to be clarified.

In this study, we investigated the mechanism underlying dosing time–dependent differences in CDDP-induced nephrotoxicity in mice. CDDP-induced nephrotoxicity varied according to circadian changes in the renal excretion function. CDDP-induced nephrotoxicity was attenuated by injecting CDDP at times of the day when renal CDDP clearance was increased. Therefore, we focused on the function of OCT2 and MATE1 to investigate the underlying mechanism of dosing time–dependent changes in CDDP-induced nephrotoxicity.

Materials and Methods

Animals and Cells. Male Clock mutant (Clock/Clock) mice (C57BL/6jClock<tm1Zbo>)/ on a ICR background, male PPARα-null mice (crossed 129S4SvJae-PPARα<tmGonz>) with Jcl:ICR), and male wild-type mice of the same strain were kept in a temperature-controlled room (24 ± 1°C) under a 12-hour light/dark cycle with food and water ad libitum. Under the light/dark cycle, Zeitgeber time (ZT) 0 was designated as lights on and ZT12 as lights off. Animals were cared for in accordance with the guidelines established by the Animal Care and Use Committee of Kyushu University.

NIH3T3 cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; AFC Biosciences, Lenexa, KS) at 37°C under a humidified 5% CO2 atmosphere.

Determination of Blood Urea Nitrogen Levels. Mice were injected intravenously with a single dose of 15 mg/kg CDDP (Wako Chemicals, Osaka, Japan) or equivalent volume of saline at ZT2, ZT6, ZT10, ZT14, ZT18, or ZT22, and blood samples were collected 72 hours after the injection. Serum was separated by centrifugation. Serum blood urea nitrogen (BUN) levels were determined using a manufactured kit (Wako Chemicals).

Pharmacokinetic Analysis of CDDP. To estimate dosing time–dependent differences in the pharmacokinetic parameters of CDDP, mice were injected intravenously with a single dose of 15 mg/kg CDDP at ZT2 and ZT14. During the duration of the experiment, mice were kept individually in cages containing paper bedding (Oriental Yeast Co., Ltd., Tokyo, Japan). Blood samples were drawn via the orbital sinus 1 hour and 4 hours after the CDDP injection. At the same time, urinary volume was measured by calculating differences in the weight of paper bedding before and after the experiment. The loss of evaporated urine from paper bedding during the experiment was adjusted for by measuring the evaporation loss of 2.0 ml water from the bedding. Platinum concentrations in the serum and urine were determined by inductively coupled plasma mass spectrometry. The area under the serum Pt concentration-time curve from 1 hour to 4 hours after the CDDP injection (AUC1–4) was calculated according to the trapezium rule. The renal clearance of CDDP during the elimination phase was calculated using the following equation:

$$\text{renal clearance} = \frac{U}{ke} \left( C_1 - C_4 \right)$$

where $U$ is the total amount of urinary Pt excretion; $ke$ is the elimination rate constant; $C_1$ and $C_4$ are serum Pt concentrations 1 hour and 4 hours after the CDDP injection, respectively.

Determination of Pt Incorporation into Renal DNA. After obtaining blood and urine samples for pharmacokinetic analysis, the kidneys were removed from mice to determine the amount of Pt incorporated into renal DNA. Genomic DNA was extracted from the renal cortex using the Wizard Genomic DNA Purification Kit (Promega, San Luis Obispo, CA). Pt concentrations were measured in renal genomic DNA by inductively coupled plasma mass spectrometry as described above, and Pt incorporation into renal DNA was expressed as the Pt amount (pg) per mg of DNA.

Determination of the Glomerular Filtration Rate. The glomerular filtration rate (GFR) was determined by estimating the renal clearance of fluorescein isothiocyanate (FITC)–inulin (Sigma-Aldrich), as described previously (Qi et al., 2004). To remove residual FITC not bound to inulin, FITC–inulin powder was dissolved in saline and dialyzed at room temperature for 24 hours using a 1000-Da cutoff dialysis membrane (Tube-O-DIALYZER; Wako Chemicals). FITC–inulin was continuously infused into mice using an osmotic minipump (Model 1007D; ALZET, Cupertino, CA). A micro-osmotic pump filled with 100 μl of dialyzed 5% FITC–inulin was implanted into the dorsal skin of mice. Mice were transferred into individual cages containing paper bedding. Six days after implantation, blood samples were drawn via the orbital sinus at ZT4 and ZT16. Urine volumes during ZT2–6 or ZT14–18 were measured on the same day, as described above. FITC–inulin concentrations in the serum and urine were determined by spectrofluorometry. Renal clearance of FITC–inulin (GFR) was calculated by the following equation:

$$\text{GFR} = \frac{U}{C_{ss}}$$

where $U$ is the total amount of urine-excreted FITC–inulin per 1 hour; $C_{ss}$ is the serum FITC–inulin concentration at a steady-state.

Western Blot Analysis. To extract renal membrane fractions, the kidneys were homogenized with Krebs-Ringer buffer (11.7 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2, 1.2 mM Na2HPO4, 2H2O, 25 mM NaHCO3, 2.5 mM CaCl2·2H2O, 11 mM d(+)-glucose). Whole lysate was separated...
by centrifugation for 15 minutes at 8000g, 4°C, and the resulting supernatant was ultra centrifuged at 100,000g, 4°C for 1 hour. The obtained pellet was resuspended in 20 mM 4-morpholinepropanesulfonic acid–Tris, pH 7.0, 300 mM sucrose, 5 mM EDTA, protease inhibitor (2 μg/ml leupeptin, 2 μg/ml aprotinin, and 100 μM phenylmethylsulfonyl fluoride), and was further ultra centrifuged at 100,000g, 4°C, for 1 hour. The pellet was resuspended in Tris-EDTA buffer (10 mM Tris-HCl, 5 mM EDTA, pH7.0) containing appropriate protease inhibitors. This suspension was used as the renal membrane fraction. Renal nuclear fractions were prepared using an NE-PER extraction kit (Pierce Biotechnology/Thermo Scientific, Pittsburgh, PA). Renal membrane fractions were denatured at 60°C for 30 minutes with 0.1% Triton X-100, 1% SDS, and 5% 2-mercaptethanol, while renal nuclear fractions were denatured at 99°C for 5 minutes with 1% SDS, and 5% 2-mercaptethanol. Denatured renal membrane or nuclear fraction samples were separated by SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride membrane and reacted against OCT2 (Alpha Diagnostic International, San Antonio, TX), MATE1 (kindly provided by Dr. M. Otsuka, Setsunan University, Osaka, Japan), TATA-binding protein (Abcam, Cambridge, UK), and PPARα and retinoid X receptor-α (RXRα) (Santa Cruz Biotechnology, Dallas, TX) antibodies. Specific antigen–antibody complexes were visualized using horseradish peroxidase–conjugated secondary antibodies and Chemi-Lumi One (Nacalai Tesque Inc., Kyoto, Japan) or visualized using horseradish peroxidase technology) coupled to Dynabeads protein G (Invitrogen) using bis 2-mercaptethanol. Denatured renal membrane or nuclear fraction samples were separated by SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride membrane and reacted against OCT2 (Alpha Diagnostic International, San Antonio, TX), MATE1 (kindly provided by Dr. M. Otsuka, Setsunan University, Osaka, Japan), TATA-binding protein (Abcam, Cambridge, UK), and PPARα and retinoid X receptor-α (RXRα) (Santa Cruz Biotechnology, Dallas, TX) antibodies. Specific antigen–antibody complexes were visualized using horseradish peroxidase–conjugated secondary antibodies and Chemi-Lumi One (Nacalai Tesque Inc., Kyoto, Japan) or Immuno Star LD (Wako Chemicals). Visualized images were scanned by LAS4000.

**Quantitative Reverse Transcription–Polymerase Chain Reaction Analysis.** Total RNA was extracted using RNAiso reagent (Takara Co., Ltd., Osaka, Japan). Complementary DNA was synthesized by reverse transcribing 1 μg of RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Sequences of primer pairs; 5′-TTGATCAATG-TCCCAGATGACCCTCA-3′ and 5′-TGATTCGG-TTT-3′. The quantitative reliability of PCR was evaluated by kinetic analysis of the amplified products to ensure that signals were derived only from the exponential phase of amplification. Chromatin immunoprecipitation assay proceeded in the presence of rabbit IgG as negative controls.

**Simulation of CDDP Accumulation into Renal DNA.** We hypothesized that CDDP would be incorporated into renal cells at a constant rate (k-inc, influx rate constant) and excreted in the urine at a first order rate (k-out, efflux rate constant). To assess an in vivo simulation, the average of OCT2 or MATE1 protein band intensities at ZT2 and ZT6 was expressed as k-inc or k-out during the light phase, and the average at ZT14 and ZT18 was expressed during the dark phase, respectively. The volume of CDDP distribution in renal cells (Vc) was fitted to the actual values of Pt incorporation into renal DNA in Fig. 2C because we were unable to estimate Vc in renal DNA.

**Statistical Analysis.** The significance of the 24-hour variation in each parameter was tested by an analysis of variance. The significance of differences among groups was analyzed by analysis of variance and the Tukey–Kramer post hoc test. A paired t test was used for comparison analysis within the same group, and an unpaired t test for the comparison of data between two groups. A 5% level of probability was considered to be significant.

**Results**

**Dosing-Time Dependency of CDDP-Induced Nephrotoxicity in Mice.** To test whether CDDP-induced nephrotoxicity changed depending on its dosing time, mice were injected with a single intravenous dose of CDDP (15 mg/kg) at six different dosing times. At 72 hours after the CDDP injection, serum BUN levels in wild-type mice were elevated in a dosing time–dependent manner (Fig. 1A). Serum BUN levels were significantly increased after the CDDP injection at ZT6 ($P < 0.05$); a significant elevation in BUN levels was not observed when mice were injected with CDDP at the other dosing times. In contrast, no significant dosing time–dependence differences were observed in serum BUN levels after the CDDP injection in Clock/Clock mice (Fig. 1B). Serum BUN levels in

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**Fig. 1.** Dosing-time dependency of CDDP-induced nephrotoxicity in mice. Wild-type (A) or Clock mutant (Clock/Clock) mice (B) were injected intravenously with a single dose of CDDP (15 mg/kg) or equivalent volume of saline at the indicated times. Blood samples were collected 72 hours after the CDDP injection. BUN levels in the serum were assessed as an index of CDDP-induced nephrotoxicity. Each value is the mean ± S.E. ($n = 3–6$). *$P < 0.05$, significant difference between the groups.
Clock/Clock mice were elevated after the CDDP injection at both dosing times, ZT6 and ZT18. These results indicate that CDDP-induced nephrotoxicity in wild-type mice could be attenuated by changing the dosing time. Dosing time–dependent differences in CDDP-induced nephrotoxicity appear to be under the control of the molecular clockwork.

Dosing-Time Dependency of CDDP Pharmacokinetics in Mice. To elucidate the underlying mechanism of the dosing-time dependency of CDDP-induced nephrotoxicity, we performed pharmacokinetic analysis of CDDP in wild-type and Clock/Clock mice after injecting the drug at ZT2 and ZT14. The area under the curve (AUC1–4) of serum Pt concentrations following the CDDP injection in wild-type mice at ZT14 was significantly lower than that at ZT2 (P < 0.05; Fig. 2A). Although significant dosing time–dependent differences were also observed in AUC1–4 after the CDDP injection in Clock/Clock mice, AUC1–4 values in Clock/Clock mice were higher than those in wild-type mice (Fig. 2A).

CDDP is mainly excreted from the kidney (Lange et al., 1972; Siddik et al., 1987). Since CDDP is retained in the renal tissue for a long period of time, it may readily cause nephrotoxicity (Farris et al., 1988). The renal clearance of CDDP after its injection in wild-type mice varied in a dosing time–dependent manner (Fig. 2B). The renal clearance of CDDP in wild-type mice after its injection at ZT14 was significantly higher than that at ZT2 (P < 0.05). On the other hand, no significant difference in the renal clearance of CDDP was observed in Clock/Clock mice. The renal clearance of CDDP in Clock/Clock mice was low at both dosing times.

Consistent with these results, dosing time–dependent differences were also observed in Pt incorporation into the renal DNA of wild-type mice (Fig. 2C). The incorporation of Pt into renal DNA after the CDDP injection at ZT14 tended to be lower than that at ZT2. On the other hand, no significant dosing time–dependent differences were observed in Pt incorporation into the renal DNA of Clock/Clock mice (Fig. 2C). The amount of incorporated Pt in the renal DNA of Clock/Clock mice was higher than that of wild-type mice. These results indicate that the dosing-time dependency of CDDP-induced nephrotoxicity can be attributed to circadian variations in renal excretion function. The kidney may be exposed to high concentrations of CDDP after its injection at times of the day when renal excretion function is decreased. Because Clock/Clock mice failed to show a significant dosing-time dependency in CDDP pharmacokinetics, the renal function for xenobiotic excretion appears to be under the control of the molecular clockwork.

The renal clearance of CDDP consists of both glomerular filtration and transporter-mediated tubular secretion. The GFR of wild-type mice exhibited significant time-dependent variations (P < 0.05; Fig. 2D), with the filtration rate being facilitated during the dark phase. This may also contribute to dosing time–dependent differences in the renal clearance of CDDP. On the other hand, GFR did not show significant time-dependent variations in Clock/Clock mice; the contribution rate of GFR to renal clearance of CDDP was over 60% at both light and dark phases. Conversely, these results suggest that tubular secretion clearance of CDDP is reduced in Clock/Clock mice.

Circadian Oscillations in the Renal Expression of OCT2. In the kidney, CDDP is taken up into the renal proximal tubule cells mainly via Slc22a2 organic cation transporter 2 and is secreted into the lumen via other transporters including the Slc47a1 multidrug and toxin extrusion 1 (Yonezawa et al., 2006; Filipski et al., 2009; Nakamura et al., 2010). The levels of Slc22a2 mRNA and OCT2 protein in the kidneys of wild-type mice showed circadian oscillations (Fig. 3A), with these levels being higher around the mid–light phase. In contrast, OCT2 protein levels in the kidneys of Clock/Clock mice did not show any clear circadian oscillations; the transporter proteins remained at low levels throughout the day. Similar decreases in the amplitude of the Slc22a2 mRNA oscillation were also observed in the kidneys of Clock/Clock mice (Fig. 3B), suggesting that the CLOCK protein positively regulates OCT2 expression at the transcriptional level.

The protein levels of MATE1 in the kidneys of wild-type mice failed to show any clear circadian oscillations (Fig. 3A), which appeared to be the result of no time-dependent variations in Slc47a1 mRNA levels (Fig. 3B). No clear circadian oscillations in the expression of MATE1 were also found in the kidneys of Clock/Clock mice (Fig. 3A); the transporter proteins remained at low levels throughout the day. Consequently, Slc47a1 mRNA levels in Clock/Clock mice were significantly lower than those in wild-type mice (Fig. 3B). Taken together, these results suggest that the CLOCK protein acts as a positive regulator of Slc47a1 gene expression but does not rhythmically derive its expression.

CLOCK Protein Regulates the Expression of Slc22a2 through the Mediation of PPARs. To examine how CLOCK protein rhythmically drives the expression of Slc22a2, we

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**Fig. 2.** Dosing time–dependent changes in CDDP pharmacokinetics in mice. (A) Dosing time–dependent differences in AUC1–4 after the CDDP injection (15 mg/kg i.v.) in wild-type or Clock mutant (Clock/Clock) mice. (B) Dosing time–dependent differences in the renal clearance (CLR) of CDDP (15 mg/kg i.v.) in wild-type or Clock/Clock mice. (C) Dosing time–dependent differences in the amount of Pt incorporated into the renal DNA of wild-type or Clock/Clock mice at 4 hours after the CDDP (15 mg/kg i.v.) injection. (D) Time-dependent changes in the GFR in wild-type or Clock/Clock mice. Each value is the mean ± S.E. (n = 5–16). **P < 0.01; *P < 0.05, significant difference between the groups.
searched consensus sequences for response elements to circadian transcriptional factors within the promoter region of the mouse Slc22a2 gene. Four nucleotide sequences showing homology with PPREs were found within 2.1 kilobases (kb) of the Slc22a2 gene 5′-flanking region (Fig. 4A). Furthermore, three ROR response elements and two cAMP binding protein (CREB) response elements were also identified in the 5′-flanking region of the Slc22a2 gene. To study the functional importance of these sequences for expression of the Slc22a2 gene, we performed a transient transcriptional assay using Slc22a2 luciferase reporter constructs.

As shown in Fig. 4B, cotransfection of the Slc22a2 (−2101)–Luc reporter with PPARα and RXRα resulted in a 10-fold increase in transactivation activity. Although RORα also enhanced transcription of the Slc22a2 reporter, these enhancement effects were weaker than those by PPARα/RXRα. Therefore, we further assessed the ability of PPARα and RXRα to regulate Slc22a2 transcription. Deleting the sequence of the 5′-flanking region of the Slc22a2 gene from bp −2101 to −1331 caused a marked decrease in the transactivation effect of PPARα/RXRα, by approximately 70% (Fig. 4C). The nucleotide sequence located between bp −2101 to −1331 in the Slc22a2 gene showed homology with the two consensus sequences of PPREs (Fig. 4A), suggesting that these sites are responsible for the transcriptional regulation of Slc22a2 by PPARα/RXRα.

The results of Western blot analysis revealed that PPARα, but not RXRα, were time-dependently expressed in the kidneys of wild-type mice (Fig. 5A). The amount of PPARα in wild-type kidneys increased at the peak time of Slc22a2 mRNA expression (Fig. 3B; see ZT6). In contrast, neither PPARα nor RXRα proteins showed clear time-dependent variations in the kidneys of Clock/Clock mice (Fig. 5A). PPARα protein levels decreased in Clock/Clock mice. In fact, PPARα binding to the promoter region of the mouse Slc22a2 gene in the kidney of wild-type mice also increased at the times of day when Slc22a2 mRNA was abundant (Fig. 5B), but the time-dependent variation in the PPARα binding was undetectable in Clock/Clock mice. Furthermore, the levels of Slc22a2 mRNA in the kidney of PPARα-null mice failed to show significant time-dependent variation (Fig. 5C). These results suggest that the CLOCK-regulated output pathway, including PPARα, contributed to the circadian expression of Slc22a2 in the kidney. This notion is further supported by the facts that the temporal profiles of renal clearance of CDDP and Pt incorporation into renal DNA of PPARα-null mice exhibited a similar phenotype as observed in Clock/Clock mice (Fig. 5, D and E).

**Discussion**

In this study, we used laboratory animals to investigate the mechanism underlying dosing time–dependent differences in CDDP-induced nephrotoxicity from the viewpoint of its pharmacokinetics. Previous studies have suggested that circadian changes in the urinary volume contribute to the dosing-time dependency of CDDP-induced nephrotoxicity because increases...
in the urinary volume accelerate the excretion of CDDP from the body and prevent the accumulation of Pt in renal cells (Levi et al., 1982b). The renal clearance of CDDP in wild-type mice was significantly increased during the dark phase, such that CDDP-induced nephrotoxicity was attenuated by injecting CDDP at times of the day when renal clearance was enhanced. Therefore, renal cells are more likely to be exposed to high concentrations of CDDP after the drug has been administered during the light phase. In fact, the accumulation of Pt in renal DNA was enhanced by injecting CDDP into wild-type mice during the light phase (ZT6). It is thus possible that the ability of renal cells to uptake CDDP vary in a circadian time-dependent manner.

In renal proximal tubule cells, OCT2 and MATE1 function as key molecules for the excretion of CDDP, and they have been implicated in CDDP-induced nephrotoxicity (Filipski et al., 2009; Nakamura et al., 2010). Mice deficient for both

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**Fig. 5.** CLOCK rhythmically drives the renal expression of Slc22a2/OCT2 through the mediation of PPARα. (A) Temporal expression profiles of PPARα and RXRα in nuclear fractions of the kidneys of wild-type or Clock mutant (Clock/Clock) mice. The TATA-binding protein (TBP) indicates the approximately equal loading of nuclear extracts. (B) Temporal profiles of PPARα binding to the promoter region of the mouse Slc22a2 gene in the kidneys of wild-type or Clock/Clock mice. (C) Temporal expression profile of Slc22a2 mRNA in the kidneys of wild-type or PPARα-null mice. (D) Dosing time–dependent differences in the renal clearance (CLR) of CDDP (15 mg/kg i.v.) in wild-type or PPARα-null mice. (E) Dosing time–dependent differences in the amount of Pt incorporated into the renal DNA of wild-type or PPARα-null mice at 4 hours after the CDDP (15 mg/kg i.v.) injection. Each value is the mean ± S.E. (n = 4–6). *P < 0.01; **P < 0.05, significant difference between the groups.

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**Fig. 6.** Schematic representation of the mechanism for dosing time–dependent changes in CDDP-induced nephrotoxicity in mice. In wild-type mice, the amount of glomerulus-filtrated CDDP is increased during the dark phase, resulting in the enhancement of its renal clearance. However, during this time window the renal expression of OCT2 is at a low level, so that incorporation of CDDP into renal cells is decreased by injecting the drug during the dark phase. Therefore, the nephrotoxic effect of CDDP is attenuated by injecting the drug at the times of day when renal expression of OCT2 is decreased. In Clock mutant (Clock/Clock) mice, the renal clearance of CDDP remains at a low level throughout the day. Neither OCT2 nor MATE1 exhibit circadian oscillation in the kidneys of Clock/Clock mice. The expression levels of those transporters are decreased in Clock/Clock mice. Consequently, CDDP is likely accumulated in renal cells after the injection of the drug at both light and dark phases. This may account for severe nephrotoxic effect of CDDP in Clock/Clock mice regardless of its dosing time.
Oct1 and Oct2 were shown to suppress the accumulation of Pt in the kidney following an injection of CDDP (Filipiski et al., 2009). Since the expression of OCT2, and not OCT1, is higher at the basolateral site of the renal proximal tubule cells (Urakami et al., 1998; Karbach et al., 2000; Terada and Inui, 2007), OCT2 appears to promote the incorporation of CDDP into renal cells. On the other hand, mice lacking the Slc47a1 gene have exhibited severe CDDP-induced nephrotoxicity (Nakamura et al., 2010). Both BUN plasma levels and renal Pt accumulation in CDDP-injected Slc47a1 knockout mice were significantly higher than those in wild-type mice. Because MATE1, which is encoded by Slc47a1, is known to be expressed at the apical site of renal proximal tubule cells (Otsuka et al., 2005), this transporter promotes the excretion of CDDP from renal cells. Although MATE1 protein levels in the kidneys of wild-type mice failed to show clear circadian oscillations, OCT2 protein levels were increased during the light phase. The incorporation of Pt into renal DNA was attenuated by an injection of CDDP during the dark phase. Furthermore, CDDP-induced nephrotoxicity was also significantly attenuated by an injection of CDDP during these time windows. These results suggest that oscillations in the expression of OCT2 in renal proximal tubule cells underlie the dosing-time dependency of CDDP-induced nephrotoxicity (Fig. 6). In contrast, Clock/Clock mice did not show significant dosing-time dependency of CDDP-induced nephrotoxicity, accompanied by the arrhythmic expression of OCT2 in renal cells. This may account for the importance of the OCT2 expression rhythm in inducing dosing time–dependent changes in CDDP-induced nephrotoxicity. However, despite the low level of OCT2 expression, Clock/Clock mice showed severe CDDP-induced nephrotoxicity as well as the higher accumulation of Pt in renal DNA.

To interpret this discrepancy, we focused on differences in MATE1 protein levels between wild-type and Clock/Clock mice and attempted a kinetic simulation for Pt incorporation into renal DNA. The results of the simulation analysis revealed that Pt incorporation into renal DNA was enhanced by decreasing both OCT2 and MATE1 protein levels (Fig. 7). Decreases in OCT2 protein levels delayed the incorporation of Pt, whereas low levels of MATE1 expression enhanced the accumulation of Pt in renal cells. Since both OCT2 and MATE1 protein levels were reduced in Clock/Clock mice, decreases in the functions of these transporters appeared to enhance the accumulation of Pt in the renal DNA of Clock/Clock mice, thereby causing severe CDDP-induced nephrotoxicity at both dosing times (Fig. 6).

The CLOCK19 mutant protein, which is produced in Clock/Clock mice, can still interact with BMAL1 but fails to activate transcription (Gekakis et al., 1998; Katada and Sassone-Corsi, 2010); therefore, the amplitude of the rhythm in many circadian genes is reduced in Clock/Clock mice. Oscillations in the renal expression of the PPARα protein in wild-type mice corresponded to the rhythm of Slc22a2 mRNA expression. On the other hand, PPARα protein levels did not show clear time-dependent variations in the kidneys of Clock/Clock mice, which suggested that oscillations in the renal expression of the PPARα protein are also under the control of the function of the CLOCK protein. Considering the ability of PPARα to regulate transcription of the Slc22a2 gene, altered rhythms in the expression of PPARα proteins could account for the blunted rhythm of Slc22a2 expression in the kidneys of Clock/Clock mice. In fact, the time-dependent difference in the PPARα binding to the promoter region of the mouse Slc22a2 gene was undetectable in the kidney of Clock/Clock mice. The blunted rhythm of Slc22a2 mRNA also appeared to decrease the amplitude of OCT2 oscillations. These results suggest that the CLOCK protein controls the rhythm of the renal expression of Slc22a2 mRNA and the OCT2 protein through mediation via PPARα.

PPARα has been shown to function as a nuclear receptor to activate the transcription of its target genes. Previous studies have reported that a number of endogenous substrates, such as polyunsaturated fatty acids, serve as ligand activators of this nuclear receptor (Göttlicher et al., 1992; Keller et al., 1993). Several PPAR agonists were shown to protect against CDDP-induced nephrotoxicity (Li et al., 2004, 2005; Nagothu et al., 2005). The underlying mechanisms were attributed to modulations in the expression of genes, such as proinflammatory cytokine tumor necrosis factor-α, interleukin-6, and proapoptotic Bax (Li et al., 2005; Nagothu et al., 2005). Therefore, the mechanism for the protective actions of PPAR agonists against CDDP-induced nephrotoxicity seems to be distinct from our present findings.

In conclusion, the results of the present study obtained from an animal model suggest a mechanism underlying the dosing-time dependency of CDDP-induced nephrotoxicity and also provide a molecular link connecting the renal circadian clock...
to renal xenobiotic excretion. Identifying the factors that affect the disposition of drugs is important to achieve rational pharmacotherapy in humans. Our present findings may contribute to the optimization of a dosing schedule for CDDP and attenuation of its nephrotoxicity.

Acknowledgments

The authors thank the technical support staff of the Center of Advanced Instrumental Analysis, Kyushu University, for measurement of platinum.

Authorship Contributions

Participated in research design: Oda, Koyanagi, Matsunaga, Ohdo. Conducted experiments: Oda, Koyanagi, Tsurudome, Kanemitsu.

Contributed new reagents or analytic tools: Oda, Tsurudome, Matsunaga.

Performed data analysis: Oda, Koyanagi.

Wrote or contributed to the writing of the manuscript: Oda, Koyanagi, Ohdo.

References


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