The Protective Role of *Per2* Against Carbon Tetrachloride-Induced Hepatotoxicity

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Period 2 (*Per2*) is a key component of the core clock oscillator and is involved in regulating a number of different biological processes and pathways. Here we report that *Per2* plays a protective role in carbon tetrachloride (CCl₄)-induced hepatotoxicity via the modulation of uncoupling protein-2 (*Ucp2*) gene expression in mice. Hepatic injury after acute CCl₄ injection was monitored in both wild-type and *Per2*-null mice. At the 12-hour time point after CCl₄ treatment, many more vacuolations were observed in the liver tissues of *Per2*-null mice whereas fatty tissue degeneration primarily occurred in the liver tissues of wide-type mice. Serum alanine and aspartate aminotransferase activities were elevated in *Per2*-null mice compared with wide-type mice at 24 hours after CCl₄ treatment, which was in agreement with the observation of significantly larger areas of centrilobular necrosis in the livers of *Per2*-null mice. A deficit of the *Per2* gene enhanced *Ucp2* gene expression levels in the liver. As a consequence, intracellular levels of ATP markedly decreased in the liver, allowing increased production of toxic CCl₄ derivatives. The absence of *Per2* expression caused a dramatic elevation of *Clock* expression and influenced *Ucp2* through a mechanism that involved a *Clock*-controlled PPAR- α signal transduction pathway. Our studies suggest that the *Per2* gene functions in hepatocyte protection from chemical toxicants via the regulation of hepatic *Ucp2* gene expression levels. (*Am J Pathol* 2009, 174:63–70; DOI: 10.2353/ajpath.2009.080430)

Circadian rhythms in mammals are endogenous oscillators that drive the daily oscillations of multiple biological processes.^{1–3} Disruption in circadian timing, either by mutation in mice or by shiftwork in people, is associated

with metabolic disease.^{4,5} The master circadian clock is located in the suprachiasmatic nuclei of the anterior hypothalamus.⁶ In recent years, the existence of peripheral oscillators has been discovered and found to be critically important for organizing the metabolism of the whole body.⁷ Tissue-based clocks control the local circadian transcriptome, and are in turn synchronized to each other and to solar time by behavioral and neuroendocrine cues emanating from the suprachiasmatic nuclei.² The molecular circadian clockwork consists of interwoven positive and negative feedback loops, in which two transcription factors, CLOCK and BMAL1, form heterodimers and directly activate the transcription of *Per1*, *Per2*, *Cry1*, and *Cry2*, and so forth. These genes products are thought to enter the nucleus, inhibit the transcription activity of CLOCK-BMAL1 heterodimers, and generate a negative feedback loop.⁸ The circadian clock controls downstream events by regulating the expression of clock-controlled genes that function in the rate-limiting steps of various biological pathways.⁹

Several lines of evidence indicate that circadian rhythms are involved in both incidence and severity of drug-induced hepatic injury,^{10,11} implying that the clock genes might play a role in modulating the action of hepatotoxic effects such as fatty degeneration and hepatocellular death. Carbon tetrachloride (CCl₄) is the classic hepatotoxicant that causes acute, reversible liver injury in a range of laboratory animals.¹² It is now accepted that liver injury induced by CCl₄ involves initially the metabolism of CCl₄ to trichloromethyl(CCl₃*)-free radical by cytochrome P450 isozymes such as CYP2E1. Once the free radical is formed, it reacts with various biologically important substances such as amino acids, nucleic acids, and lipids, and then causes lipid peroxidation, membrane damage, and loss of hepatocellular calcium homeostasis.¹³ CCl₄ treatment causes morphological changes of liver mitochondria at early stages and decreases liver ATP con-

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tent because of uncoupling of oxidative phosphorylation of liver mitochondria.¹⁴

Hepatotoxicity induced by CCl₄ increases with age.¹⁵ Circadian functions are also impaired in old animals,¹⁶ in parallel to increase in drug-induced hepatic injury. That provoked us to investigate whether there is molecular relationship between circadian gene and CCl₄-induced hepatotoxicity. In this study, we found that mice deficient in the *Per2* gene, a key component of core clock oscillators, displayed increased sensitivity to CCl₄ by enhanced *Ucp2* gene expression and lowered ATP level in livers. Our findings suggest that the clock gene *Per2* plays a protective role in hepatic injury induced by hepatotoxicants.

Materials and Methods

Animals and CCl₄ Treatment

Male, 6- to 8-week-old *Per2*-null and wild-type C57BL/6 mice were used in this study. The *Per2*-null mice used in this study have been characterized previously Fu and colleagues.¹⁷ The wild-type (C57BL/6) and *Per2*-null mice (on a co-isogenic C57BL/6 background) were maintained in 12-hour light/dark cycles with lights on at 7:00 AM and off at 7:00 PM, and with free access to regular chow food and water. Mice were administrated a single intraperitoneal injection of CCl₄ in a dose of 0.3 ml/kg in olive oil. Control animals were injected with the same volume of olive oil. The time for injection and mice sacrifice is shown in Table 1. All animals had free access to food and water, only fasting 12 hours before they were sacrificed. All animal care and use procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Nanjing University of Science and Technology.

Serum Aminotransferase Activity

Serum was collected at 0, 12, and 24 hours after CCl₄ treatment and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were measured by an Olympus (Tokyo, Japan) AU2700 automatic biochemical analyzer.

Histological Analysis

At 0, 12, and 24 hours after CCl₄ treatment, a small piece of liver tissue was collected and fixed in 10% formalin.

Table 1. Time for Injection and Mouse Sacrifice

	Time	Time
Light on	7:00 am	7:00 am
Light off	7:00 pm	7:00 pm
Injection time	9:00 am	9:00 pm
Sacrifice time		
0 hours	9:00 am	9:00 pm
12 hours	9:00 pm	9:00 am (2nd day)
24 hours	9:00 am (2nd day)	9:00 pm (2nd day)

The sample was then embedded in paraffin, sliced into 5- μ m-thick sections, and stained with hematoxylin and eosin (H&E) for histological analysis under a light microscope. For liver fat analysis, samples were frozen in Tissue-Tek Cry03 DM230 (Sakura, Tokyo, Japan); 5- μ m-thick sections were loaded on poly-L-lysine-coated slides and stained with Sudan IV and counterstained with hematoxylin to show lipids in red. Necrosis areas were quantitated and percentage of necrosis was calculated using Scion Image β 4.0.3 (Scion Corp., Frederick, MD).

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA from liver samples was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Reverse transcript reaction using KeyGene (Wageningen, The Netherlands) reverse transcript enzyme according to the manufacturer's protocol. The real-time PCR reaction was performed on an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) with a cDNA sample and amplified in a 20- μ l reaction volume containing 1 $\times$ SYBR Green PCR master mix (Applied Biosystems). Primers of each gene are as follows: *Cyp2e1*: 5'-CAAGTCTTTAACCAAGTTGGCAAA-3' (forward) and 5'-CCACGATGCGCCTCTGA-3' (reverse) and *Ucp-2*: 5'-GCTGGTGGTGGTCGGAGATA-3' (forward) and 5'-ACAGTTGACAATGGCATTACGG-3' (reverse) and *Clock*: 5'-CGGCGAGAACTTGGCATT-3' (forward) and 5'-AGGAGTTGGGCTGTGATCA-3' (reverse) and *PPAR- α* : 5'-GGGTAC CACTACGGAGTTCACG-3' (forward) and 5'-GACAGGCACTTGTGAAAACG-3' (reverse) and *Gapdh*: 5'-CATCCACTGGTGCTGCCAAGGCTGT-3' (forward) and 5'-ACAACCTGGTCCTCAGTGTAGCCCA-3' (reverse). Relative expression in comparison with *Gapdh* was calculated by the comparative CT method.

Measurement of Liver Nucleotides

Liver tissue (100 mg) was homogenized and extracted from liquid nitrogen frozen samples using 0.4 N of perchloric acid. Extracts were separated and quantified by using reversed-phase high performance liquid chromatography (Waters 1525 system; Millipore Corp., Bedford, MA) analysis on a Partisphere bonded phase C18 (reverse phase) cartridge column at a flow rate of 1.0 ml/minute. As previously described by Smolenski and colleagues,¹⁸ the mobile phase was 150 mmol/L KH₂PO₄ and 150 mmol/L KCl, pH 6.0, with a superimposed 15% acetonitrile gradient: 0% for 0 to 20 seconds, 0 to 9% for 20 seconds to 7 minutes, 9 to 100% for 7 to 10 minutes, 100% for 10 to 14 minutes, 100 to 0% for 14 to 15 minutes and 0% for 15 to 20 minutes. ATP, ADP, and 5'-AMP standards were purchased from Sigma. (St. Louis, MO).

Cell Culture and Plasmid Transfections

Murine H22 cells (murine carcinoma of hepatoma 22) were maintained in Dulbecco's modified Eagle's medium

supplemented with antibiotics and 15% fetal calf serum in an atmosphere of humidified 95% air and 5% CO₂ at 37°C. The cells were transfected with the *mPer2* cDNA plasmids, indicated using transfection reagent. Cells were prepared 24 hours later for total RNA extraction and *Ucp2*, *PPAR-α*, and *Clock* expressions were examined by quantitative real-time RT-PCR as described above.

Statistics

Results are expressed as mean ± SEM. Statistical evaluation was performed using a parametric Student's *t*-test. A *P* value less than 0.05 was considered a statistically significant difference.

Results

Increased CCl₄-Induced Liver Injury in *Per2*-Null Mice

Per2-null mice display impaired clock resetting and differ from wild-type mice in their behavioral and physiological response to exterior signaling or factors.^{17,19} Therefore, we searched for a difference in liver injury induced by CCl₄ in *Per2*-null and wild-type mice. At 9:00 AM (at 2 hours after lights on), mice were injected with a dose of CCl₄ or oil, and serum aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured as markers of liver injury (Figure 1, A and B). Compared with control animals injected with oil, serum ALT and AST activities were elevated in mice injected with CCl₄. The increased levels of ALT and AST activities were similar between wild-type and *Per2*-null mice at 12 hours after CCl₄ injection, indicating no significant difference in liver injury between the two mouse genotypes at this stage. Interestingly, *Per2*-null mice had approximately two and three times higher levels of serum ALT and AST activities than wild-type mice at 24 hours after treatment with CCl₄. Moreover, in mice injected with CCl₄ at 9:00 PM (2 hours after lights off), ALT and AST activities were more strongly increased in *Per2*-null mice compared with wild-type mice at 24 hours after injection (Figure 1, C and D). Meanwhile, control animals showed no difference in ALT and AST activities after oil injection (insets in Figure 1, C and D). These data suggest that mice deficient in *Per2* gene are more sensitive to CCl₄ hepatotoxicity.

To further assess the CCl₄-induced hepatocyte injury in *Per2*-null and wild-type mice, liver samples at 0, 12, and 24 hours after CCl₄ treatment were obtained and histological analysis was taken at each point (Figure 2). Livers from both groups of animals had similar weights (data not shown). We compared lipid degeneration and extent of necrosis in H&E-stained sections from *Per2*-null and wild-type mice, respectively. As shown in Figure 1, no obvious difference was observed between *Per2*-null (Figure 2, A, E, and I) and wild-type animals (Figure 2, B, F, and J) at various time points after injection with oil as a control or at the time of injection with CCl₄ (Figure 2, C and D). Unexpectedly, *Per2*-null mice displayed many more vacuolations at 12 hours after CCl₄ treatment (Fig-

Figure 1. Serum ALT (A) and AST (B) activities were monitored after acute CCl₄ treatment. Injection times: 9:00 AM (A and B); 9:00 PM (C and D). Significant difference between *Per2*-null and WT mice was observed at 24 hours (inset, oil control). All values were the mean ± SEM (*n* = 5 to 7). ***P* < 0.01.

ure 2G), whereas fatty degeneration mainly occurred in wide-type mice (Figure 2H). At 24 hours after injection with CCl₄, centrilobular necrosis was observed in both groups of mice and the hepatocyte necrosis of *Per2*-null mice was much more predominant compared with that of wild-type mice (Figure 2, K and L). Adequate quantifications for areas of necrosis from multiple sections showed the areas of necrosis were ~2.5-fold larger in *Per2*-null mice than in wild-type mice (Figure 3A). Moreover, fat-

Figure 2. Histological analysis of WT and *Per2*-null livers after CCl_4 treatment. Paraffin-embedded sections from livers taken 0, 12, and 24 hours after CCl_4 treatments or olive oil control were stained with H&E. No differences were observed in both liver sections from WT and *Per2*-null mice treated with olive oil (A, B, E, F, I, J). No difference was observed at the time of treatment, 0 hours (C, D). At the 12-hour point, much more vacuolation was observed in *Per2*-null mice (G, inset, higher magnification) whereas fatty degeneration mainly occurred in wide-type mice (H, inset, higher magnification). At 24 hours, centrilobular necrosis was observed in both strains of mice and the necrosis of *Per2*-null mice (K) was much more severe than WT mice (L). V, centrilobular vein. Original magnifications: $\times 100$; $\times 400$ (insets).

specific Sudan staining analysis confirmed that fatty degeneration was obviously present in wild-type mice at 12 hours after CCl_4 treatment, whereas *Per2*-null mice displayed a negative reaction to Sudan staining (Figure 3B).

Cyp2e1 Down-Regulation Was Similar in Wild-Type and *Per2*-Null Mice

The enzyme *Cyp2e1* is the major enzyme responsible for CCl_4 metabolism, and it plays an important role in the modulation of CCl_4 -induced liver injury in the early stage.²⁰ Down-regulation of *Cyp2e1* after CCl_4 injection is considered as an adaptive mechanism for decreasing toxicity.¹³ Increased or stabilized *Cyp2e1* expression could result in more severe liver injury induced by CCl_4 ,²¹ and mice deficient in *Cyp2e1* are resistant to CCl_4 -induced hepatotoxicity.^{20,22} So we first examined whether *Per2* influences *Cyp2e1* expression in CCl_4 -induced liver injury. However, at various stages (0, 12, and 24 hours) of CCl_4 treatment, *Cyp2e1* expression had no significant difference between wild-type and *Per2* mice (Figure 4). The *Cyp2e1* mRNA down-regulation after CCl_4 treatment also occurred in *Per2*-null mice and had a similar tendency to that in wild-type mice. These observations suggest that *Per2* is not involved in regulating *Cyp2e1* expression in the early stage of liver injury.

Increased Hepatic Ucp2 Gene Expression and Lowered Liver ATP Content in Per2-Null Mice

Ucp2 is an inner mitochondrial membrane protein that uncouples respiration from oxidative phosphorylation.²³ Overexpression of *Ucp2* can reduce the efficiency of cellular ATP synthesis, resulting in a precipitous drop of cellular ATP level.^{24,25} The resulting ATP depletion has been proposed as causative factor in CCl_4 -induced cell death.^{13,25,26} CCl_4 induces the expression of *Ucp2* mRNA and *Ucp2* protein in hepatocytes.²⁷ Analysis of expression of *Ucp2* gene at the mRNA level indicated that *Per2*-null mice displayed a marked elevation both at 0 and 24 hours after CCl_4 injection compared with wild-type mice (Figure 5A), suggesting *Per2* functions as suppressor of *Ucp2* gene expression. Therefore overexpression of *Per2* should lower *Ucp2* gene expression. Transfecting *Per2* cDNA into H22 culture cells, a mouse hepatic tumor cell line, reduced *Ucp2* gene expression at 24-hour time points (Figure 5B). Then, we next measured liver ATP in both *Per2*-null and wild-type mice at 24 hours after CCl_4 injection. High performance liquid chromatography analysis demonstrated the ATP peak was lower in *Per2*-null mice than in wild-type mice (Figure 6, A and B). Quantification showed that levels of liver ATP were $\sim 50\%$ decreased in *Per2*-null mice compared with those in wild-type mice (Figure 6C). Furthermore, the ratio of hepatic ADP and ATP was significantly de-

Figure 3. Triglyceride distribution and necrosis quantitation associated with *Per2*-null and wild-type mice after CCl₄ treatment. **A:** Liver sections from mice at 24 hours after CCl₄ treatment were analyzed by morphometric image analysis, digitized, and quantified to calculate the percentage of necrosis. Data were presented as mean $\pm$ SEM for 8 to 10 fields. Necrosis was significantly more severe in *Per2*-null livers (* P < 0.05). **B:** Sudan IV staining of liver sections in wild-type mice and *Per2*-null mice at 12 hours after CCl₄ injection. Original magnifications, $\times 100$.

creased in CCl₄-treated *Per2*-null mice compared with wild-type mice (Figure 6D), indicating the precipitous drop of cellular ATP was associated with enhanced *Ucp2* gene expression because of the absence of *Per2*. These results provided evidence of a protective role for *Per2* by suppressing *Ucp2* gene expression in CCl₄-induced hepatotoxicity.

Figure 4. *Cyp2e1* gene expression. Liver *Cyp2e1* expression level was detected at 0, 12, and 24 hours after acute CCl₄ treatment. No statistical difference was observed between WT and *Per2*-null mice. (n = 4 to 5). Data are presented as mean $\pm$ SEM.

Figure 5. *Ucp2* gene expression *in vivo* and *in vitro*. Liver *Ucp2* gene expression was detected at the 0- and 24-hour point after CCl₄ treatment. **A:** High levels of *Ucp2* expression were observed at both time points (n = 4 to 5) in *Per2*-null mice. **B:** *Ucp2* expression was significantly lower in the *Per2* overexpression cell culture than the control group. All values were the mean $\pm$ SEM, n = 6. * P < 0.05, ** P < 0.01.

Per2 Regulated Ucp2 Gene Expression Through a Mechanism that Involves a Clock-Controlled PPAR- α Signal Transduction Pathway

In *in vitro* and *in vivo* experiments, *Ucp2* gene expression is induced by stimulating peroxisome proliferator-activated receptor- α (PPAR- α) signal transduction pathways.^{28,29} The circadian gene *Clock* is involved in the transactivation of PPAR- α mRNA in peripheral tissues.³⁰ We postulated that clock genes functioned in *Ucp2* gene regulation with PPAR- α , and that they would be up-regulated in *Per2*-null mice. To test this hypothesis, total RNA was extracted from the livers of wild-type and *Per2*-null mice. The levels of mRNAs of PPAR- α and other clock genes were examined by quantitative real-time PCR analysis. The results of three independent experiments were summarized and are presented in Figure 7. In *Per2*-null livers, the PPAR- α mRNA showed a higher expression compared with that in wild-type mice (Figure 7A). Thus, PPAR- α was a potential modulator and its expression was up-regulated in *mPer2*-null mice. We next examined the expression of the circadian gene *Clock* by mRNA level at day and night times. In *Per2*-null livers, *Clock* mRNA expression still had day and night variation but reached a 1.5-fold higher level in wild-type livers (Figure 7B). To confirm that the role of *mPer2* in regulating *Ucp2* expression was really through a mechanism that involves a

Figure 6. Liver ATP content at 24 hours after CCl₄ injection. High performance liquid chromatography profile of ATP (peak 1), ADP (peak 2), and AMP (peak 3) in livers at 24 hours after CCl₄ injection in wild-type (A) and *Per2*-null mice (B). Quantitation of nucleotides (C) and ratio of ATP, ADP, or AMP (D) in livers at 24 hours after CCl₄ injection in *Per2*-null and wild-type mice with six animals for each time point. All values were the mean $\pm$ SEM. * P < 0.05, ** P < 0.01 compared to wild-type.

Clock-controlled *PPAR- α* transduction pathway, we analyzed gene expression in hepatic tumor H22-cultured cell lines that overexpress *Per2* cDNA. As expected, the cells showed decreased expression of *PPAR- α* after 24 hours of transfection of *Per2* cDNA (Figure 7C). Also, as shown in Figure 7D, transfection of *Per2* cDNA impaired *Clock*

Figure 7. Analysis of the *Clock*-controlled *PPAR- α* signal transduction. **A:** Increased *PPAR- α* mRNA expression in livers of *Per2*-null mice (n = 5). *Clock* gene expression was detected at 11:00 AM and 11:00 PM, respectively. **B:** *Per2*-null mice showed significantly higher expression than WT mice at both time points (n = 4 to 5). Transfection assays monitoring the effects of *Per2* on *PPAR- α* (C) and *Clock* (D) gene expression. *Per2* cDNA plasmids were transfected into H22 cell lines with a 20-ng dose. Cells were harvested at 24 hours after transfection and total RNA was extracted. Relative expression in comparison with *Gapdh* was calculated with the comparative CT method by quantitative real-time RT-PCR. The results of three independent experiments are summarized and presented, all values were the mean $\pm$ SEM. * P < 0.05, ** P < 0.01.

mRNA expression. These results indicated that loss of *Per2* function resulted in abnormal transcription by *Clock*-controlled *PPAR- α* pathway, which modulates *Ucp2* gene expression.

Discussion

Recent studies have demonstrated that multiple genes are controlled by the circadian clock in a tissue-specific manner and that many of these clock-controlled genes function in the rate-limiting steps of major physiological process.^{9,31} It is commonly accepted that circadian disorder could influence the stress response. In our studies, we focused on investigating the circadian clock-controlled *PPAR-α* and *Ucp2* gene expression, and highlighted the fundamental role that the circadian clock played in protecting liver injury from oxidization stress. A nonfunctional *Per2* gene led to a feedback increase of Clock expression and as a consequence, live ATP content was decreased by activated Clock-controlled *PPAR-α* and *Ucp2* gene expression. In many cases, decreases in ATP level would inactivate Na⁺-K⁺ ATPase activity, resulting in an unbalance of extra- and intracellular sodium-potassium homeostasis.^{32,33} At the physiological level, this led to increased vacuolations in liver tissue in the early stage of CCl₄ treatment, as we observed. In addition, *PPAR-α* activation stimulated β-oxidation of fatty acids and decreased fat accumulations by amplifying the expression of lipoprotein lipase.³⁴ Thus, after CCl₄ treatment, the *Per2*-null mice had no obvious fatty degeneration because of a higher level of *PPAR-α* expression compared with wild-type mice. This implied, in *Per2*-null mice, that fatty degeneration was not a dominating factor in hepatotoxicity induced by CCl₄.

Studies in *Cyp2e1*-knockout mice, in which no liver necrosis and no increase in serum ALT and AST activities after CCl₄ treatment were seen, strongly support the contention that *Cyp2e1* was required for the CCl₄-induced hepatotoxicity.^{20,22} CCl₄ metabolism by *Cyp2e1* in hepatocytes produces high levels of oxidative stress.¹³ In this stage, loss of *Per2* function had no obvious effects in elevation of ALT and AST activities compared with wild-type mice. The oxidation stress induced by CCl₄ metabolites was correlated to induction of *Ucp2* via the stimulation of *PPAR-α* expression.^{27,29} *Ucp2* is a member of a family of proton transporters located in the mitochondrial inner membrane and involved in the regulation of superoxide and ATP synthesis.²³ The up-regulation of *Ucp2* enhanced the cyanide-induced mitochondrial dysfunction, leading to execution of necrosis.³⁵ Inhibiting *Ucp2* gene expression attenuated some drug-induced neurotoxicity.³⁶ In our observation, loss of *Per2* function resulted in resetting circadian clock functions, especially, feedback stimulated Clock gene expression. *PPAR-α*, one of the clock-controlled genes, was up-regulated in hepatocytes, leading to activation of *Ucp2* expression. Increase in *Ucp2* mRNA in the liver of CCl₄-treated animals was associated with a rise in *Ucp2* protein.²⁷ Thus, as we observed, the elevation *Ucp2* expression caused a more severe drop in ATP content of *Per2*-null livers, which was obligated for death of hepatocytes after CCl₄ treatment.

The circadian rhythm has been implicated in susceptibility of rats to chemical-induced liver damage.¹⁰ An increase in CCl₄ hepatotoxicity occurs in old animals,¹⁵ indicating genetic compounds are potential causes of

age-dependent changes in chemically induced liver damage. Age-dependent changes in the circadian gene expression in laboratory rodents and humans have been described extensively.^{37,38} An age-dependent difference was found in the case of *Per2* mRNA expression³⁹; also *Ucp2* expression increased with age.⁴⁰ Thus, the phenotype of increased CCl₄ hepatotoxicity regulated by increased *Ucp2* levels because of *Per2* gene deficiency could reflect the natural phenomena of increasing the sensitivity to toxicant with age in old animals.

Collectively, our data established *Ucp2* as a link between dysfunction of circadian clock gene *Per2* and increased CCl₄-induced liver injury.

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