Scientific paper

Interaction of PER2 with the Constitutive Androstane Receptor Possibly Links Circadian Rhythms to Metabolism

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Abstract

Period 2 (PER2) is an important factor in daily oscillations called circadian rhythms, which are emerging as one of the most important regulatory networks, responsible for homeostasis and transcriptional regulation of a number of genes. Our work shows that PER2 could act as a co-activator of the constitutive androstane receptor (CAR), a key nuclear receptor (NR) that regulates the metabolism of endobiotics and xenobiotics. Bioinformatic analysis shows that PER2 and CAR possess structural elements that could enable them to interact which was confirmed experimentally by CoIP experiment. Co-transfection of mouse hepatocarcinoma cells with plasmids overexpressing *Per2* and *Car* increases expression of *Bmal1*, a potential CAR target gene, more than transfections with *Car* only. This is the first report indicating the interaction of PER2 and CAR.

Keywords: circadian rhythms, metabolism, period 2, constitutive androstane receptor, co-activator

1. Introduction

Circadian rhythms are daily oscillations in most cells and organisms. They are governed by autonomous molecular circadian oscillators that are synchronized by external cues, such as light and food. The circadian machinery is composed of positive and negative transcriptional and translational feedback loops. The major positive loop comprises of transcriptional activators BMAL1 and CLOCK that can heterodimerize and drive their own transcription as well as that of elements of the negative loop, such as *Period* (PER) homologues, *Cryptochromes* and *Rev-erbα*. The transcriptional repression of *Bmal1* due to REV-ERBα can be counter-balanced by RORα and PPARα.[1–3]

PER2 is involved in the negative feedback loop where it directly interacts with the transactivation complex of BMAL1-CLOCK and represses its transcriptional activation capability. It also acts in the positive loop where it drives *Bmal1* expression by acting as a co-activator of NRs, eg PPARα.[1] PER2 in metabolic and mental disorders, cancer and other pathologies is currently being intensively studied.[4–8] Due to its two LXXLL structural motifs, which can interact with a hydrophobic pocket of NRs, it has been studied as a nuclear receptor co-activator.[9] Perhaps one of the most important transcriptional regulator of metabolism is CAR, a NR regulator of primary and secondary metabolism. CAR can be directly or indirectly activated by various endogenous ligands, eg bilirubin, and xenobiotics, eg barbiturates.[10–18] After activation and nuclear localization it interacts with co-activators and heterodimerization factors, most often the retinoid X receptor (RXR). The final protein complex can transactivate enzymes of the cytochrome P450 (CYP), glucuronosyltransferase (UGT) and multi drug resistance protein (MRP) families.[3,13,15,19–21]

The interaction between nuclear receptors and their co-activators is a well-documented interaction that is

highly conserved among species.[9,22] Nuclear receptors have high levels of similarity in their ligand binding domains, especially in the region of helices 3, 4 and 5. These helices form a hydrophobic cleft which interacts with leucines of the co-activators' LXXLL motifs. The two residues between the three leucines have little or no importance to binding of LXXLL motifs as they are in direct contact with the surrounding aqueous solution. Charged residues of the helices 3–5 form interactions with amino acids surrounding the LXXLL motifs which are crucial for the specificity of interactions and recognition of appropriate co-activators. The most important factor for specificity of co-activators are the two residues just before the first leucine of the LXXLL motif, usually referred as –2 and –1. Of most interest to this study was the class 3 of LXXLL motifs, according to Savkur and Burris, which encompasses motifs SXLXXLL.[9,23]

Here it was shown that CAR and PER2 interact with, and activate *Bmal1* transcription. The initial prediction was made on the basis of homology of LXXLL motifs of PER2 and several known CAR co-activators and was confirmed experimentally. Our data presents the first report of communication between drug metabolism and the circadian rhythm at the level of direct interaction between PER2 and CAR.

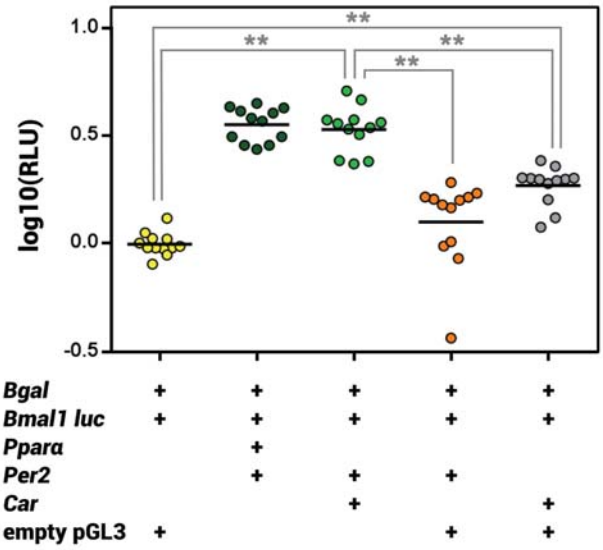

Figure 1. Co-transfection of Hepa 1–6 cells with CAR and PER2 overexpression plasmids induces promoter activity of *Bmal1 luc* reporter. This induction is similar to the one observed when performing co-transfections with *Pparα* and *Per2* ($P < 0.0001$) and different to the one observed when performing transfections with *Car* only ($P < 0.0001$). The log10 relative luciferase units of individual wells for the specific transfection mix are shown. ** - $P < 0.01$

2. Results

Bioinformatic analysis of the transcription factor binding sites using MatInspector, Matrix Library 9.4 and User-defined IUPAC strings revealed potential binding sites for CAR on the *Bmal1* promoter, eg for CAR/RXR at 414-438 (positive strand) of GXP_5050588 (*Bmal1 Mus musculus*).[24–26] This provided a sufficient basis for the transfection experiments that showed induced expression of a *Bmal1 luc* reporter when Hepa 1-6 cells were transfected with *Car* ($P < 0.01$). The induction was further enhanced when co-transfection with *Car* and *Per2* was performed ($P < 0.01$) which suggests an either direct or indirect influence of PER2 on CAR transactivation of *Bmal1* (Figure 1). Surprisingly, the transactivation of *Bmal1* with vectors overexpressing CAR and PER2 was not significantly different than transactivation with positive control overexpressing PPARα and PER2 (Figure 1) that co-immunoprecipitate at *Bmal1* regulatory sites.[1] The lack of a statistically significant difference might suggest a similar mechanism of transactivation which may lead to the exploration of structural properties of both PER2 and CAR to evaluate if a direct interaction between the proteins is plausible.

Bioinformatic analysis showed that PGC1α (PPARγC1A), a known CAR and PPARγ co-activator, may interact with CAR via its motif SLLKKLL (*Mus musculus*), which is homologous to both SXLXXLL motifs of PER2 (Figure 2), namely SGLLNLL (*Mus musculus*) and SDLLNLL (*Mus musculus*).[27–29] A further co-activator of CAR and PPARγ, PGC1β, which has three LXXLL motifs, all with a serine at a 1 or -2 position, also exists. A similar arrangement with a serine residue in front of a LXXLL motif can also be observed in NCoA6, another CAR co-activator.[28,30]

As both PER2 and PGC1α are co-activators of PPARγ, a NR involved in lipid and carbohydrate metabolism, attention was focused on the homology of the nuclear receptor. Most receptor residues that are in contact with LXXLL motifs have no charge pointing towards the co-activator motifs, besides two very distinct lysins at both the CAR and PPARγ at homologous positions. The receptors also seem to have high 3D similarity of the hydrophobic cleft as helices 3, 4, 5 are positioned in a similar manner. Both receptors seem to bind LXXLL motifs at the »end of helix 5« to the »end of helix 3«, which coincides with the lysine positioning.[31–35] Even though this could be projected onto many nuclear receptors, the position of the lysine at helix 5 could additionally explain the favouring of co-activators with a serine before LXXLL.[9,29,36]

The hypothesis that PER2 and CAR interact directly was confirmed with co-immunoprecipitation (Figure 3). For this, *Car-Flag* and *Per2-V5* co-transfection was performed. Initial release of proteins from Sepharose beads was performed at 70 °C and revealed the V5 reactive protein at an approximate size of 40 kDa. After additional heating of Sepharose at 95 °C for 2 min, a 135

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hPER2/1st    O15055    KSHENEIRYHPFRMTPYLVKVRDQQGAESQLCCLLLAERVHSGYEAPRIPPEKRI
hPER2/2nd    O15055    TSRDQQPKAPLTRDEPSDTQNSDALSTSSGLLNLLLNEDLCSASGSAASESLGSG
hPGC1α/2nd   Q9UBK2    DGDVTTDNEASPSSMPDGTPPPQEAEEPSLLKKLLLAPANTQLSYNECSGLSTQN
hPGC1α/3rd   Q9UBK2    PAIVKTENSWSNKAKSICQQQKPQRRPCSELLKYLTTNDDPPHTKPTENRNSSRD
hPGC1β/1st   Q86YN6    SASPAPSSAPPSPAPEKPSAPAPEVDELSLLQKLLLATSYPTSSSDTQKEGTAWR
hPGC1β/2nd   Q86YN6    PKACSNPSQQVRSRPWSRHHSKASWAEFSILRELLAQDVLCDVSKPYRLATPVYA
hNCoA6/1st   Q14686    PFSGAPNGNQMSCGQNPGFPVNKDVTLTSPLLVNLLQSDISAGHFGVNNKQNNTN
hNCoA6/2nd   Q14686    DGQPSDPNKLPSVEENKNLVSPAMREAPTSLSQLLDNSGAPNVTIKPPGLTDLEV
mPER2/1st    O54943    KHHENEIRYQPFRMTPYLVKVQEQQGAESQLCCLLLAERVHSGYEAPRIPPEKRI
mPER2/2nd    O54943    TSRDRQPKAPPTCNEPSDTQNSDAISTSSDLLNLLLGEDLCSATGSALSRSGASA
mPGC1α/2nd   O70343    DGAVTTDNEASPSSMPDGTPPPQEAEEPSLLKKLLLAPANTQLSYNECSGLSTQN
mPGC1α/3rd   O70343    PAIVKTENSWSNKAKSICQQQKPQRRPCSELLKYLTTNDDPPHTKPTENRNSSRD
mPGC1β/1st   Q8VHJ7    GLAAFPELDEGDTPSCTPASPAPLSAPPSPTLERLLSPASDVDELSLLQKLLLAT
mPGC1β/2nd   Q8VHJ7    PASPAPLSAPPSPTLERLLSPASDVDELSLLQKLLLATSSPTASSDALKDGATWS
mPGC1β/3rd   Q8VHJ7    PIPQACSSLSRQVQPRSRHPPKAFWTEFSILRELLAQDILCDVSKPYRLAIPVYA
mNCoA6/1st   Q9JL19    PFGGAPNGSQMSCGQNPGFPVNKDVTLTSPLLVNLLQSDISAGHFGVNNKQNNTN
mNCoA6/2nd   Q9JL19    DGQPLDPNKLPSVEENKNLMSPAMREAPTSLSQLLDNSGAPNVTIKPPGLTDLEV
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Figure 2. Alignment showing similarity between PER2 and known co-activator LXXLL motifs of CAR, namely PGC1alpha, NCoA6 and PGC1beta.[28,30] The letters h and m before the protein name designate species *Homo sapiens* and *Mus musculus*, with the sequential number of the noted LXXLL and UniProt/Swiss-Prot entry number following the protein name. The predicted LXXLL motifs with residues marked as 1-5 are shown in red, as are serines located just before LXXLL. Of note is a negatively charged side-chain, or a hydroxyl group containing residues (E, S, T) just in front of the -2 serine.[29]

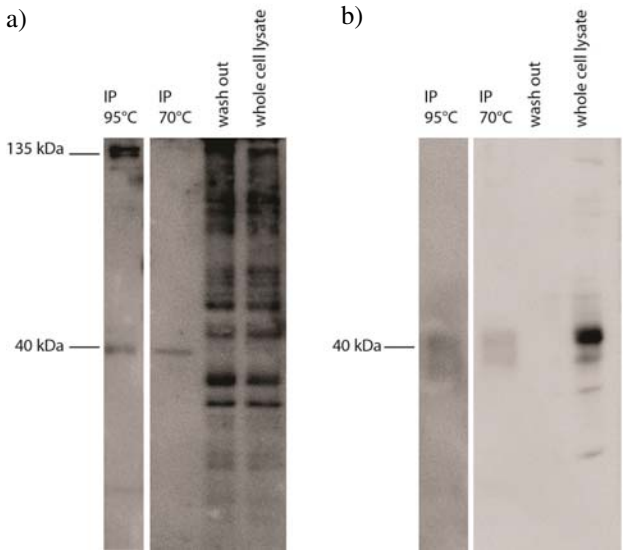

Figure 3. Co-immunoprecipitation with FLAG Ab conjugated Sepharose of *Car-Flag* and *Per2-V5*. A: Western blot with V5 Ab – PER2 shows two bands, at 40 and 135 kDa. After the final incubation of Sepharose beads at 70 °C, only the 40 kDa form was visible on the blot. The 135 kDa isoform was visible after additional incubation at 95 °C. **B**: Western blot with FLAG Ab – the signal of CAR is in the range of expected protein size (40 kDa).

kDa protein was released (Figure 3A), corresponding to the expected size of PER2.[29] The FLAG reactive protein was detected very faintly, irrespective of temperature, at 40 kDa (Figure 3B). This could correspond to CAR.[29] This corroborates the prediction that the two proteins could interact in a co-activator and nuclear receptor manner.

3. Materials and Methods

3. 1. Plasmids

Bmal1 luc, having a *Bmal1* promoter cloned into a pGL3 luciferase reporter vector, *Per2*, coding for a V5-tagged PER2, and *Pparα* constructs were kindly provided by J. Ripperger and U. Albrecht (Department of Biology, Faculty of Science, University of Fribourg, Switzerland) and have been previously described in more detail.[1]

The *Car* expression construct was kindly provided by JeanMarc Pascussi (Institut de genomique fonctionnelle, Montpellier, France) and it was constructed by Negishi Masahiko (NIH, North Carolina, USA) and has previously been described in more detail.[37]

The *Car-Flag* plasmid was kindly provided by Negishi Masahiko (NIH, North Carolina, USA) and has previously been described in more detail.[38]

An empty pGL3 basic vector (Promega) was used to perform transfections with equal ammounts of DNA. All wells were transfected with 50 ng *Bgal* – pSV-β-Galactosidase Control Vector (Promega), for normalization. All constructs besides the commercial pGl3 basic and *Bgal* represent *Mus musculus*.

3. 2. Transfections

Transfections were performed on Hepa 1-6 cells available from the European Collection of Authenticated Cell Cultures. The cells were held at 5% CO2 and 37 °C and transfered to 96-well microplates 24 hours prior to transfections with 5×10^6 cells in DMEM and 10% FBS. For transfections X-tremeGENE HP DNA (Roche) was

used according to manufacturer's instructions. Total plasmid mass was equal to 200 ng per well, with each construct of interest at 50 ng and total mass added to 200 ng with the pGL3 basic vector. The negative control represents transfections with *Bgal*, *Bmal1 luc* and pGL3 basic vector, a positive control of *Bgal*, *Bmal1 luc*, *Pparα* and *Per2* was used.

3. 3. Luciferase assay and statistical analysis of results

Cells were lysed using Passive Lysis Buffer (Promega). Luciferase assay was performed using the ONE-Glo Luciferase (Promega) according to manufacturer's instructions and measurements were performed on Synergy H4 (BioTek). Results were normalized using analysis of β-galactosidase activity and additional normalization to positive control using luciferase activity was performed for microplate comparisson. Analysis of variance was performed and results were logarithmized to achieve variance homogeneity. The t-test analysis was performed with GraphPad Prism 6.0 software. In cases where significance is very high, the software returns p values in the form p>0.0001.

3. 4. Co-immunoprecipitation (Co-IP)

HeLa cells were seeded on 6-well plates and co-transfected with *Car-Flag* and V5-tagged *Per2* using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were harvested 2 days after transfection using 200 microliters of the lysis buffer: 50 mM Tris · HCl at pH 7.5, 150 mM NaCl, 0,5% (w/v) NP-40 and complemented with protease inhibitors (Roche). The cell lysate was agitated for 10 min at 4 °C and centrifuged at 14000 g 20 min at 4 °C. Supernatant was transferred to a tube containing FLAG-coupled Sepharose beads (Sigma-Aldrich) and rotated for 2 h at 4 °C. After the centrifugation at 10000 g for 5 min at 4 °C the supernatant was then collected and stored as a whole cell lysate sample. Sepharose was washed 3 times with additional 200 microliters of lysis buffer and the first washout was collected as wash out sample. 50 microliters of Laemmli buffer was added to the Sepharose, followed by heating for 10 min at 70 °C and again for 2 min at 95 °C. Western blot was perfomed with 3 different samples: whole cell lysate, wash out and immunoprecipitate (IP) in two parallel conditions, one with anti-FLAG Ab A8592 (Sigma-Aldrich) and the other with anti-V5 Ab V8137 (Sigma-Aldrich).

3. 5. Discussion

The comparable transactivation of *Bmal1* following co-transfections with *Car*/*Per2,* or *Pparα*/*Per2* may suggest a similar mechanism of transcriptional activation of *Bmal1*. If this is the case then, in conjucntion with ChIP experiments revealing binding of PER2 and PPARα at regulatory regions of *Bmal1*,[1] this could further support the proposal that PER2 could potentially act as CAR's co-activator and that the pair can form transactivation complexes either alone or with other partners. This is further supported by finding CAR binding sites at the *Bmal1* promotor. The discovery is interesting as both proteins are important for cell homeostasis. However, PER2 is not the only co-activator of these nuclear receptors and PER2 is expressed at certain times of the day. To confidently state how important is the effect of different *Bmal1* transcriptional activity, this should be tested on reporter cell lines. We can speculate that additive effects of xenobiotic ingestion, social jet lag and nutrition overload can affect circadian clock driven endogenous liver metabolism. This can result in liver abnormalities, such as non-alcoholic fatty liver disease that could terminate in HCC.

Since some of the predictions of this study were based on homology, it is worth noting that according to Savkur and Burris the amino acid at –1 of their class 3 LXXLL motifs should be an non-polar amino acid.[9] However, since human and mouse 2nd PER2 LXXLL have G or D at -1, with high conservation of the rest of the sequence, motifs that do not follow this strict consensus of an unpolar –1 residue are shown in our alignment.[29] As well as this, the residue at –1 most probably points outwards from the hydrophobic pocket and therefore does not directly interact with helices 3-5 of NRs.[39] It would be worth exploring if it could interact with the charge clamp of NRs.[9,31,40–42]

Another interesting observation is the presence of a band at approximately 40 kDa at the western blot with anti-V5 Ab. Similar sized bands have been observed in our previous work, where a >40 kDa band was detected with western blot from mouse liver samples, with the use of a different Ab (Abcam ab467). Unfortuantely this could not be verfified using our CoIP samples with these Ab, as Abcam does not provide this specific Ab any more. Work from other researchers has shown the existence of a shorter PER2S isoform in *Homo sapiens* with the size of 45 kDa and co-IP at 55 kDa.[43] If a similar truncated form of PER2 was observed, it could be speculated that PER2 can interact with CAR with its first LXXLL. However, this does not exclude an interaction of PER2 with CAR with its second LXXLL.[1] It may be possible that a co-activator interacts with an NR with multiple LXXLL motifs, but with a different affinity. Although a cocktail of protease inhibitors were used with the lysis buffer, it is still possible that the >40kDa band is a product of protein degradation.

It is interesting to note that *Car* shows both diurnal expression patterns in liver, with *Car* mRNA levels oscillating in phase with *Bmal1*, and the possibility to be activated by ligands. The induction could very well be dependent on the phase or time of induction.[44,45] If this is the case, such findings should be considered in the pharmacoki-

netics of drug active ingredients, especially as CAR regulates the expression of several CYPs.[18,21,46] This work may therefore provide an important new link in understanding the connection between internal clock machinery, metabolism and pharmacokinetics.

As CAR can be activated by xenobiotics, such as flavonoids, cathehins and similar poliphenols, and also active ingredients of drugs, eg. barbiturates, paracetamol and some compounds with a steroid-like structure, it would also be interesting to see if such activation has any physiological effect on transcription of genes involved in circadian rhythms.[13,16,19,46] It would be possible that high levels of CAR could affect the molecular clock in the periphery, but CAR also binds other co-activators. We cannot exclude that the robust molecular clock could be affected, which would mean that different CAR activators (eg. xenobiotics) could have an effect on liver circadian regulation of various metabolic pathways.

This work also suggests that period homologues should be considered as possible NR co-activators, not only of NRs that have an established role in circadian rhythms. Perhaps such mechanisms of multiple co-activators being able to activate a single NR could provide a compensatory mechanism in case of co-activator deregulation. On the other hand, different co-activators are expressed differentially in tissues with different phases of expression, which could in fact define a NR's tissue and time specific function.

In conclusion, we show that CAR and PER2 can form an interaction which has implicaitons for circadian aspects of drug metabolism.

3. 6. Authors' contributions

T. M. performed transfections, the luciferase assay and bioinformatical and structural analyses. The bioinformatical and structural analyses were performed under expert supervission and guidance of J.S.. U.P.Z. first noticed the possibility of an interaction between CAR and PER2 during her preliminary screenings for interactions of PER2 with nuclear receptors. She also performed co-IP. Experiment planing and protocol preparation was performed by U.P.Z. and D. R. The manuscript draft was prepared by T. M. and finalised by all authors. All authors read and approved the final manuscript.

3. 7. Acknowledgements

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Povzetek

Period 2 (PER2) je pomemben faktor pri dnevnih oscilacijah, imenovanih cirkadiani ritmi. Ti so eni najpomembnejših regulatornih zank, ki so pomembne za uravnavanje homeostaze in uravnavanja prepisa velikega števila genov. Dokazali smo, da lahko PER2 deluje kot ko-aktivator konstitutivnega androstanskega receptorja (CAR), ključnega jedrnega receptorja pri uravnavanju metabolizma endobiotikov in ksenobiotikov. Bioinformatska analiza je pokazala, da PER2 in CAR vsebujeta strukturne elemente, ki omogočajo njuno interakcijo. To je bilo eksperimentalno potrjeno s CoIP poskusom. KO-transfekcija mišjih hepatokarcinomskih celic s plazmidi, ki omogočajo povečano izražanje *Per2* in *Car*, poveča izražanje *Bmal1*, potencialni tarčni gen CAR. Povečano izražanje *Bmal1* v celicah je višje, kot če so tranficirane le s *Car* plazmidom. To je prvo poročilo o interakciji PER2 in CAR.