Biochemical and Biophysical Research Communications 376 (2008) 206–210

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CREM modulates the circadian expression of CYP51, HMGCR and cholesterogenesis in the liver

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ARTICLE INFO

Article history:
Received 21 August 2008
Available online 4 September 2008

Keywords:
Cyp51
Hmgcr
Cholesterol synthesis
Circadian regulation
cAMP
Icer
Crem

ABSTRACT

We show for the first time that isoforms of the cAMP response element modulator *Crem*, regulate the circadian expression of *Cyp51* and other cholesterogenic genes in the mouse liver. In the wild type mice the expression of *Cyp51*, *Hmgs*, *Fpps*, and *Sqs* is minimal between CT12 and CT16 and peaks between CT20 and CT24. *Cyp51*, *Fpps*, and *Sqs* lost the circadian behavior in *Crem*−/− livers while *Hmgcr* is phase advanced from CT20 to CT12. This coincides with a phase advance of lathosterol/cholesterol ratio, as detected by GC–MS. Overexpression of CREMτ and ICER has little effect on the *Hmgcr* proximal promoter while they influence expression from the *CYP51* promoter. Our data indicate that *Crem*-dependent regulation of *Cyp51* in the liver results in circadian expression of this gene. We propose that cAMP signaling might generally be involved in the circadian regulation of cholesterol synthesis on the periphery.

Cholesterol synthesis is a multistep pathway represented by over 20 distinct biochemical steps. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), an early enzyme of cholesterol synthesis, is the major regulatory enzyme of this pathway. Lanosterol 14α-demethylase (CYP51) represents an enzyme of the late portion of cholesterol synthesis. It is regulated by the negative feedback through sterol regulatory element binding proteins SREBP and also by the cAMP-dependent transcription factors [1,2]. We have demonstrated previously that forskolin, a mediator of the cAMP signaling, activates *CYP51* in an immediate early fashion also in the absence of mature SREBPs. A single CRE in the *CYP51* promoter was sufficient to mediate this transactivation and the immediate early repressor ICER that arises from the *Crem* gene, was involved in attenuation of transcription [3]. While the cAMP regulation of cholesterogenic CYP51 was understood initially as important majorly for testis and ovary to produce meiosis activating sterols [4,5] it has been described later also in somatic cells [3]. However, the physiological role of the cAMP-dependent regulation of CYP51 in hepatic cells remained poorly understood.

Molecular mechanisms controlling circadian rhythms in mammals include cAMP regulation and the immediate cAMP early repressor ICER, a cAMP-dependent repressor transcribed form the *CREM* gene. ICER is rhythmically expressed in the pineal gland, with peak levels occurring at night when the transcription is induced by adrenergic input to the pineal gland from the SCN. This induction is transient because ICER represses its own transcription, which completes the CREM feedback loop [6]. After the discovery of the circadian regulation as a combination of transcriptional and translational feedback loops, operated by activators CLOCK(NPAS2)/BMAL1 and repressors of the *Period* and *Cryptochrome* families [7], ICER was majorly forgotten as a potential circadial regulator.

It is now widely accepted that peripheral tissues, including the liver, have their own biological clocks that operate independently of SCN [8]. Many aspects of the endogenous metabolism and most aspects of xenobiotic detoxification are subjected to circadian regulation [9]. According to measurements of the serum cholesterol that peaks in humans during the end of the night [10], cholesterol synthesis is among the circadially regulated processes.

Abbreviations: CREM, CRE modulator; CYP51, lanosterol 14-demethylase; Fpps, Farnesyl pyrophosphate synthase; Hmgcr, 3-hydroxy-3-methylglutaryl coenzyme A reductase; Hmgs, 3-hydroxy-3-methylglutaryl coenzyme A synthase; ICER, inducible cAMP early repressor; SCN, supraschiasmatic nucleus; Sqs, squalene synthase; SREBP, sterol regulatory element binding protein; WT, wild type

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doi:10.1016/j.bbrc.2008.08.126

To investigate the role of the CREM isoforms in the circadian regulation of *Cyp51* and some other cholesterogenic genes, we used the model of *Crem*−/− mice. The males of these mutant mice are sterile due to the arrest in spermatogenesis [11,12], while females are fertile. *Crem*-null mice of both genders showed an increase in locomotion activity that did not change during the 24-h cycle. They revealed a lower anxiety in two different behavioral models, but preserved the conditioned reactiveness to stress [13]. Our data show that the lack of the *Crem* isoforms results in modulated circadian expression of cholesterogenic genes that is reflected in a phase advance of cholesterol synthesis.

Materials and methods

Animals. The *Crem*−/− animals originate from the laboratory of Dr. P. Sassone-Corsi, IGBMC, Strasbourg, France. The protocols for animal care followed the EU regulations. Male mice (13–24 weeks old) were for 7 days under 12 h light and 12 h dark condition, followed by a 24 h dark cycle before being sacrificed. Sacrification took place in the dark, under red light. Blood was collected into EDTA-coated tubes, centrifuged (plasma), livers cut into pieces and immediately frozen in liquid N_2. Samples were stored at −80 °C for subsequent analyses. Samples were collected every 4 h starting at time 7 AM (CT0) from at least two animals at each time point.

Quantitative RT-PCR. Livers were weighted, homogenized in TRI reagent (Sigma) and total RNA isolated according to the manufacturer's instructions. RNA quality and cDNA preparation were published previously [3]. Q-RT-PCR analysis was preformed by SybrGreen technology on Applied Biosystems Prism 7900 HT sequence detection system. Sequences of primers are provided in Table 1. The PCR products were analyzed by melting curve analysis and were amplified as published before [3]. The relative amounts of mRNAs were calculated by the comparative Ct (cycle number at threshold) method (User Bulletin No. 2, Applied Biosystems, PE) using 18S rRNA as the internal control and compared to CT0 in wt animals. Data were processed by the $2^{-\Delta\Delta CT}$ method as described previously [14].

Reporter assays. Preparation of the human *CYP51* luc (−334/+316) reporter has been described previously [3]. Hamster *Hmgcr* luciferase reporter (−277/+20) was obtained from T.F. Osborne. JEG-3 cells were cultured and transfection experiments performed as described [3]. pSV-CREMτ and pSV-ICERII originate from Dr. P. Sassone-Corsi .

Sterol extraction and GC–MS analysis. Lipids were extracted from liver samples (100–400 mg) by chloroform/methanol (2:1, v/v) as described [15]. Total cholesterol and free lathosterol were assayed by isotope dilution–mass spectrometry and use of deuterated internal standards [16].

Statistical analyses. Statistics was performed with SPSS 14.0. One-way ANOVA was used for circadian rhythm determination of w.t. and *Crem* k.o. animals while two-way ANOVA was used for determining the statistically significant correlation between mouse strain (w.t. and *Crem* k.o.) and the zeitgeber time. Transfections: One-way ANOVA was performed with significance level 0.05 and Bonferroni post-hoc tests to show significant differences between experimental conditions. $p = 0.05$ was used for all statistical tests.

Table 1
Sequences of primers used for Q-RT-PCR

Gene name	Primer sequence	Reference
SREBP-2	GCGTTCTGGAGACCATGGA ACAAAGTTGCTCTGAAAACAAATCA	[27]
CYP51	ACGCTGCCTGGCTATTGC TTGATCTCTCGATGGGCTCTATC	[3]
HMG CoA synthase	GCCGTGAACTGGGTCGAA GCATATATAGCAATGTCTCCTGCAA	[27]
HMG CoA reductase	CTTGTGGAATGCCTTGTGATTG AGCCGAAGCAGCACATGAT	[27]
18S rRNA	CGCCGCTAGAGGTGAAATTC TTGGCAAATGCTTTCGCTC	[28]
FPPs	ATGGAGATGGGCGAGTTCTTC CCGACCTTTCCCGTCACA	[27]
SqS	CCAACTCAATGGGTCTGTTCCT TGGCTTAGCAAAGTCTTCCAACT	[27]

Results

CREM/ICER influence the expression of cholesterogenic genes

RT-PCR analyses of cholerestogenic *Hmgs*, *Fpps*, *Sqs*, *Cyp51*, *Srebp-2*, and *Hmgcr* from w.t. and *Crem*−/− mouse liver tissue are shown in Fig 1. In wild type animals (grey lines), the one-way ANOVA ($p < 0.05$) of *Hmgcr* expression (Fig. 1B) showed maximal expression in the dark phase at CT20 which is in accordance by previous works [17] and is consistent with the HMGCR activity [18]. The expression of other genes of cholesterol synthesis (*Hmgs*, *Fpps*, *Sqs*, *Cyp51*) is minimal between CT12 and CT16 (start of subjective night) and peaks between CT20 and CT24. A similar pattern has been described before for *CYP51* in the rat liver [19]. The circadian expression of *Srebp-2* follows the pattern of cholesterogenic genes (Fig. 1F).

In livers of *Crem* knockout animals (Fig. 1, black lines), the expression of *Srebp-2*, *Fpps*, *Sqs*, and *Cyp51* lost the circadian behavior. *Hmgs* remains circadian, but at lower amplitude with the maximum shifted from CT20 to CT24 in wild types to CT4 in *Crem* knockouts. The major regulatory enzyme of the pathway *Hmgcr* remains circadian but the peak is shifted from CT20 in wild types to CT12–CT16 in *Crem* knockouts.

Since *CYP51* and *Hmgcr* contain functional CRE elements in their proximal promoters, transactivation potentials of CREM and ICER was evaluated in promoter–reporter assays in JEG-3 cells (Fig. 2). One-way ANOVA with Bonferroni post-hoc tests showed significantly lower expression of *CYP51* reporter, containing two CRE elements, when ICER was overexpressed, and significantly higher expression of *CYP51* reporter with overexpression of CREM. It revealed a small, but significantly lower expression of *Hmgcr* reporter with co-transfection of ICER compared to non-cotransfected cells while overexpression of CREM had no effect.

CREM/ICER alter the circadian sterol profile in the mouse liver

The ratio between free lathosterol and total cholesterol is a good marker for *de novo* cholesterol synthesis [16,20]. Fig. 3 shows these ratios in the mouse plasma and liver. The two-way ANOVA of plasma showed no statistical differences between w.t. and *Crem*−/− mice with correspondence to time. Plasma cholesterol levels are, due to *de novo* synthesis, highest at CT16–CT20 both in wild type and *Crem* knockouts. Previous data confirm a maximal cholesterol synthesis in wild type mice in the dark phase [21]. However, the cholesterol synthesis takes place in the liver, followed by the transport of metabolites to the plasma. In the liver, the two-way ANOVA showed a significance ($p < 0.10$) with the maximum shifted from CT20 in w.t. to CT16 in *Crem*−/− mice.

Discussion

Liver is a central organ of the bodies homeostasis and has a unique ability to regenerate. Liver is also a circadian organ where many metabolic processes exhibit a rhythmic regulation. Increased

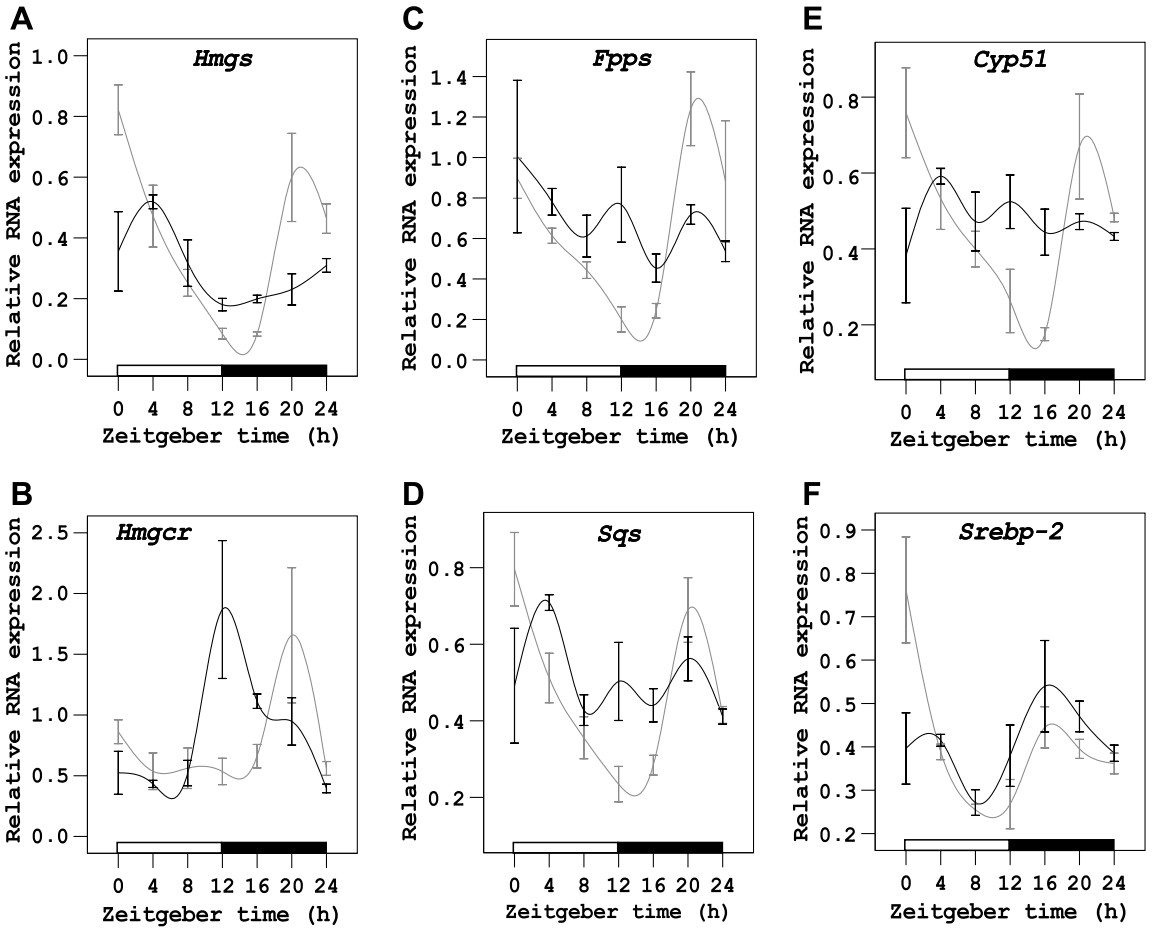

Fig. 1. Daily profiles of *Hmgs* (A), *Hmgcr* (B), *Fpps* (C), *Sqs* (D), *Cyp51* (E) and *Srebp-2* (F) mRNA expression in liver of wild type (grey line) and *Crem*−/− knockouts (black line) by RT-PCR analysis. Data are expressed as means ± SEM from two animals analyzed in triplicates. One-way ANOVA for all genes in wild type shows a significant difference ($p < 0.05$). In *Crem*−/− animals, one-way ANOVA for *Hmgs* and *Hmgcr* shows significant difference, while no significant difference is observed for *Sqs*, *Fpps*, *Cyp51*, and *Srebp-2*. The two-way ANOVA shows significant differences ($p < 0.05$) between mice strains and zeitgeber time for expression of all genes.

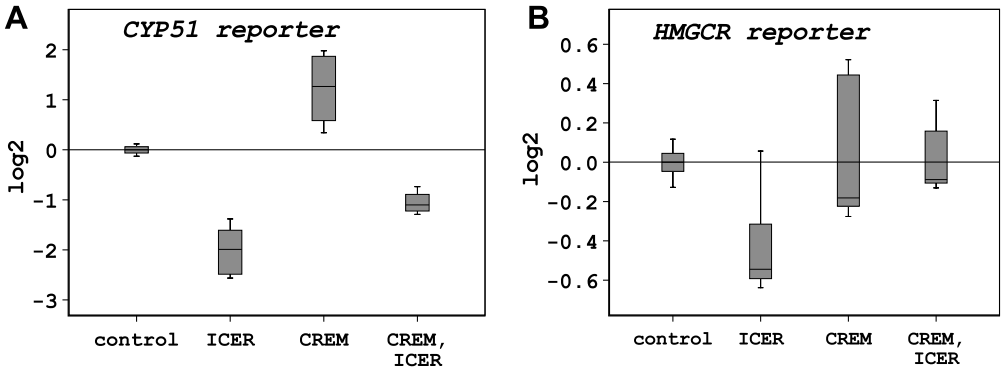

Fig. 2. Transfections of JEG-3 with the *CYP51* (−334/−316) (A) and *Hmgcr* (−277/+20) (B) promoter–reporter constructs. All transfection experiments were performed at least three times with two petri dishes for each experimental condition. Data are expressed as means ± SEM from six individual experiments. Bonferroni post-hoc test showed (A) a significant difference ($p < 0.05$) for all pairs of experimental conditions, (B) a significant difference ($p < 0.05$) only for overexpression of ICER compared to other experimental conditions. Control; ICER, overexpression of pSV-ICERII; CREM, overexpression of pSV-CREMτ.

cAMP levels and the cAMP regulatory element modulator isoforms CREM and ICER were so far shown to be involved in the hepatic regeneration and proliferation [22], ICER was overexpressed in the hepatocellular carcinoma, [23] and was induced after the forskolin treatment in immortal cell lines, including immortal hepatocytes [3,24]. Herein we show for the first time that CREM/ICER contributes also to the circadian regulation of *Cyp51* and some other genes of cholesterol synthesis in the liver. The absence of CREM isoforms in *Crem*−/− mice results in erasing the circadian behavior of three genes involved in cholesterol synthesis (*Fpps*, *Sqs*, and *Cyp51*) while *Hmgs* and *Hmgcr* remain circadian. The *Hmgs* amplitude is diminished and the circadian peak is shifted. *Hmgcr* is the major regulatory gene of cholesterol synthesis that has been previously shown to be expressed in a circadian manner [17]. It is important to note that our data from *Crem*−/− mice show the phase advance of *Hmgcr* expression which coincides with the

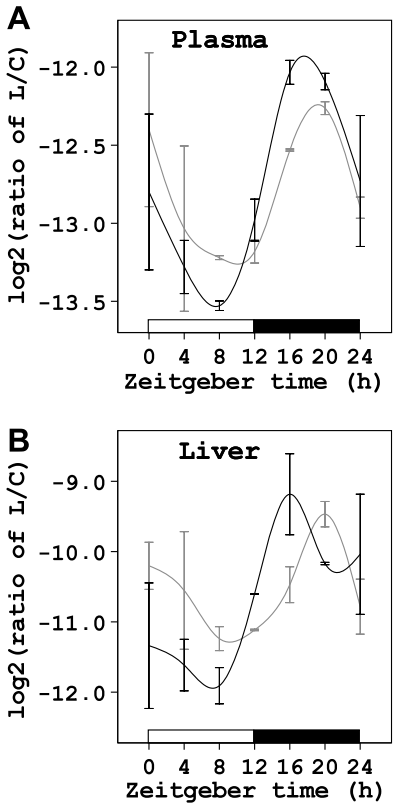

Fig. 3. Daily profiles of $\log_2$ ratios between free lathosterol (L) and total cholesterol in plasma (A) and liver (B) of wild type (grey line) and *Crem*−/− mice (black line). Data are expressed as means ± SEM from two animals. *Crem*−/− at CT24 has been excluded from statistical analyses due to problems with internal standard. (A) Plasma. One-way ANOVA for wild type shows no significant difference ($p = 0.197$) while for *Crem* knockouts there is a significant difference ($p = 0.000$). Two-way ANOVA shows no significant differences ($p = 0.375$) between mice strains and the zeitgeber time. (B) Liver. One-way ANOVA for wild type shows no significant difference ($p > 0.151$) while for *Crem* knockouts there is a significant difference ($p = 0.012$). Two-way ANOVA showed relaxed significant differences ($p < 0.087$) between mice strains and the zeitgeber time.

phase advance of *de novo* cholesterol synthesis in livers of these mice.

Interestingly, the circadian expression of *Hmgcr* was abolished in *clock* mutant mice [25]. Altogether this indicates that in addition to the normal *Clock* function, the normal *Crem* function is also important for the circadian regulation of cholesterol synthesis in the liver.

We have observed only a phase shift trend but no statistically significant difference in lathosterol/cholesterol ratio of the blood serum from wild type and *Crem*−/− mice. The reason might be in high inter-individual variation, which will be evaluated in the future when a larger colony of the infertile *Crem*−/− mice is available.

To investigate whether the *Crem* isoforms regulate the CRE-containing cholesterogenic *CYP51* and *Hmgcr* directly, the promoter–reporter analyses have been performed. The proximal *Hmgcr* promoter containing one functional CRE element shows no activation after overexpression of CREMτ and weak inhibition after overexpression of ICER, suggesting the possibility that CREM/ICER might influence *Hmgcr* expression by an indirect mechanism. Distal *Hmgcr* promoter needs to be investigated to reach the final conclusion. On the other hand, the overexpressed CREMτ transactivates the proximal *CYP51* promoter with three CRE elements, while ICER works as a cAMP-dependent *CYP51* repressor. We have shown previously by promoter–reporter assays that CREMτ binds to the *CYP51-CRE2* and that CREMτ can be displaced by ICER [3]. Lack of specific antibodies that would distinguish between bound CREM activators and the ICER repressor, represent a major obstacle for chromatin immunoprecipitation studies. It is worth mentioning again that *Icer* is transcribed from the *Crem* gene from an alternative intronic P2 promoter [26]. The amino acid sequence of ICER is, except for the first few aminoacids, identical to the sequence of CREM activators.

In conclusion, our data show for the first time that cAMP-dependent transcription factors of the CREM family are involved in the circadian regulation of *Cyp51* and cholesterol synthesis in the mouse liver.

Acknowledgments

The work was supported by the Slovenian Research Agency, Grants J1-6713, J1-9428 and P1-0104. Thanks also to FEBS (Federation of European Biochemical Societies) for fellowship grant to Jure Acimovic and to EMBO (European Molecular Biology Organization) for the fellowship to D. Rozman. Part of the work has been generated in the context of the STEROLTALK project, funded by the European Community as contract No. LSHG-CT-2005-512096 under 6th Framework Programme for Research and Technological Development in the thematic area of Life sciences, genomics and biotechnology for health.

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