Photoperiodic Regulation of PER1 and PER2 Protein Expression in Rat Peripheral Tissues

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Summary

Circadian oscillations in biological variables in mammals are controlled by a central pacemaker in the suprachiasmatic nuclei (SCN) of the hypothalamus which coordinates circadian oscillators in peripheral tissues. The molecular clockwork responsible for this rhythmicity consists of several clock genes and their corresponding proteins that compose interactive feedback loops. In the SCN, two of the genes, Per1 and Per2, show circadian rhythmicity in their expression and protein production. This SCN rhythmicity is modified by the length of daylight, *i.e.* the photoperiod. The aim of the present study was to find out whether profiles of PER1 and PER2 proteins in peripheral organs are also affected by the photoperiod. Rats were maintained under a long photoperiod with 16 h of light and 8 h of darkness per day (LD 16:8) and under a short, LD 8:16, photoperiod. The PER1 and PER2 daily profiles were measured in peripheral organs by Western blotting. The photoperiod affected significantly the PER1 profile in livers and the PER2 profile in lungs and hearts. In lungs, PER2 in the cytoplasmic, but not in the nuclear fraction, was affected significantly. The effect of the photoperiod on PER1 profiles in peripheral organs appears to differ from that in the SCN.

Introduction

The mammalian circadian system consists of a central pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus and suboscillators in peripheral tissues such as the liver, kidney, heart, lung and others (for review see Hastings *et al.* 2003, Gachon *et al.* 2004).

Circadian, i.e. circa 24 h, oscillations persist in a non-periodic environment and are generated by transcriptional-translational positive and negative feedback loops between the expression of clock genes and their proteins (for review see Reppert and Weaver 2001). The key components of the negative feedback loop are the period genes *Per1* and *Per2* and cryptochrome genes *Cry1* and *Cry2*. Their expression is activated through E-box enhancers by a heterodimer composed of two basic helix-loop-helix-PAS transcription factors *CLOCK* and *BMAL1*. Products of *Per* genes, PER proteins are temporally phosphorylated in the cytoplasm by casein kinase Iε and Iδ (Akashi *et al.* 2002) and dimerize with CRY proteins. These heterodimers are transported to the nucleus, where they inhibit their own transcription by negative interaction with CLOCK/BMAL1 dimer. The subcellular distribution of PER/CRY dimers, one of the crucial steps for transcriptional regulation, is regulated by an active nuclear import through the importin α/β system (Sakakida

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et al. 2005) and by a nuclear export (Loop *et al.* 2005).

The oscillatory mechanisms appear to be common to the central as well as peripheral oscillators. Similarly to the SCN, cells of peripheral tissues contain a set of clock genes that are expressed in a rhythmic manner, although phases of their expression vary between different organs and are delayed by 3-9 h relative to the SCN oscillation (Zylka *et al.* 1998, Yamazaki *et al.* 2000, Stokkan *et al.* 2001, Takata *et al.* 2002, Lamont *et al.* 2005). The central pacemaker in the SCN seems to coordinate phases of the individual peripheral oscillators within the body, since its destruction leads to desynchronization among various tissues (Yoo *et al.* 2004). The mechanism of such synchronization seems to be tissue-specific and involves neural and humoral factors (Balsalobre *et al.* 2000, McNamara *et al.* 2001, Terazono *et al.* 2003). Secondary factors such as the SCN-driven rhythms in locomotor activity, body temperature, feeding behavior or melatonin production, may apparently also contribute to the phasing of the peripheral oscillators (Brown *et al.* 2002).

The endogenous circadian period deviates slightly from 24 h and must be therefore synchronized with the ambient 24-h cycles. The major environmental Zeitgeber is duration of daylight, i.e. the photoperiod. Photic information is transmitted from the retina, mostly *via* the retinohypothalamic tract, to the SCN where it entrains molecular oscillations of the 24-h solar day. In temperate zones, the photoperiod changes with the season of the year and alters physiology and behavior of many mammals. Seasonally bred animals express a wide range of annual physiological rhythms as are cyclic changes of gonadal size and function, pelage, feeding behavior, body weight, hibernation etc. However, the physiology of non-seasonally bred mammals such as humans or rats may be responsive to annual changes in illumination. The photoperiod modulates the rhythmic production of melatonin in rats and humans (Illnerová 1991, Wehr *et al.* 1993), and prolactin production (Johnston 2004), levels of circulating steroid hormones (for review see Nelson and Demas 1996) and locomotor activity (Elliot and Tamarkin 1994) in rats. The length of daily illumination also modifies the response of the circadian system to environmental Zeitgebers (Illnerová and Sumová 1997). Within the SCN, the photoperiod modulates rhythmic production of neurohormones (Jáč *et al.* 2000), or rhythmic gene expression as it is the spontaneous (Sumová *et al.* 2000) and the light-induced *c-fos* expression and c-Fos protein production (Sumová *et al.*

1995). The photoperiodic modulation of the SCN rhythmicity is likely due to the direct effect of the photoperiod on the core clockwork mechanism in the SCN. Indeed, the photoperiod affects the rhythm of *Per1* gene expression and PER1 protein production in the SCN of rats (Messager *et al.* 1999, Sumová *et al.* 2002), hamsters (Carr *et al.* 2003, Tournier *et al.* 2003) and mice (Steinlechner *et al.* 2002). Moreover, the waveform, amplitude and a phase-relationship between the profiles of other clock genes in the SCN may also be affected by the photoperiod (Sumová *et al.* 2003, Tournier *et al.* 2003).

Per genes are the only clock genes inducible by acute light stimuli (Albrecht *et al.* 1997, Shearman *et al.* 1997). Therefore, they may be considered as entry for photic input into the SCN molecular clockwork mechanism. The rhythmic expression of *Per* genes in peripheral tissues is already well-established. It is, however, not clear whether this expression is also modulated by the photoperiod as in the case of SCN. The aim of the present study was thus to elucidate whether the photoperiod affects daily profiles of PER1 and PER2 proteins in rat peripheral organs, namely in the lung, heart and liver and moreover, whether and how the photoperiod affects the levels of PER proteins in the nucleus and in the cytoplasm.

Methods

Animals

Sixty-day-old male Wistar rats (Bio-Test, Konárovice, Czech Republic) were housed in a temperature of 23±2 °C with free access to food and water. For at least 4 weeks prior to the experiments, animals were maintained under a long photoperiod with 16 h of light and 8 h of darkness per day (LD 16:8) or a short photoperiod (LD 8:16). Light was provided by overhead 40-W fluorescent tubes and illumination intensity was between 50 and 200 lx, depending on the cage position.

Experimental paradigm

In order to avoid the direct effect of light on proteins rhythmicity, rats were released into darkness on the day of experiment, i.e. the morning light was not turned on and 3 animals per one time point were killed every 2 h for the next 24 h. Pieces of livers, lungs and hearts were immediately dissected and frozen on dry ice.

Preparation of protein tissue extracts

       The set of tissues from one experiment was always processed in the same assay. For the whole-cell extract preparation, the pieces of tissues were weighed and homogenized in 10 volumes of cold CelLytic MT Mammalian Tissue Lysis/Extraction Reagent (Sigma) with protease inhibitor cocktail (Sigma; 1:1000), 1 mM dithiothreitol, 0.5 mM PMSF, 1 mM Na_3VO_4 and 20 mM NaF. The samples were incubated 30 min on ice and spun at 14 000 rpm for 15 min at 4 °C. Supernatants were mixed with 80 % glycerol to give the final 10 % concentration and were used as total protein extracts.

       For nuclear and cytoplasmatic fractions, the tissues were homogenized for 5 min in 10 volumes of buffer H (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose and protease inhibitor cocktail 1:1000). The homogenates were spun for 20 min at $100 \times g$ at 4 °C to collect cellular debris. The supernatants were spun for 15 min at $1000 \times g$ at 4 °C. The resulting nuclear pellets were resuspended in one volume of buffer H. The resulting supernatants were spun at $8000 \times g$ for 10 min to collect mitochondria and finally at $70\ 000 \times g$ for 1 h to clean the fractions from membranes. The final extracts were used as cytosolic fractions.

Western blotting

       The relative amounts of total protein within one set of samples were measured by Bradford assay (Bio-Rad). Equal amounts of proteins were mixed with 2x electrophoresis Laemmli sample buffer (Bio-Rad) and incubated at 37 °C for 60 min. The 20-40 μg (depending on the tissue type and fraction) of total protein were separated on a 7.5 % polyacrylamide gel, electroblotted onto nitrocellulose membrane and then incubated overnight with primary N-terminus targeted antibody anti-PER1 and anti-PER2 (Santa Cruz) at 1:500 dilution. Anti-TFIID (TBP) antibody (Santa Cruz) was used for confirmation of the fraction´s purity. Membranes were washed and processed with horseradish peroxidase-conjugated anti-goat secondary antibody (Santa Cruz) and immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) according to manufacturer´s instructions. The samples from one type of tissue and one experiment were always loaded onto one gel. The specificity of bands was tested by preabsorbing the antiserum with the respective peptide at 20 μg/ml.

       Protein signal was visualized using an image analysis system (Fuji LAS-1000 camera) and evaluated with AIDA 2D densitometry as an integrated intensity of each specific band. Protein amounts were determined semiquantitatively by relative comparison of integrals between bands within one blot. To normalize the means of values from different experiments, each value was given as percentage of the maximal value. The final value for each animal was calculated as a mean of three immunoblots performed with the same set of tissue extracts. Data for total extracts were expressed as means of percentage ± S.E.M. from 3 animals. Data for nuclear and cytoplasmatic extracts were expressed as means ± S.D. from 2 animals.

Statistical analysis

       Data were analyzed by two-way ANOVA for photoperiod and time differences and by three-way ANOVA for the photoperiod, time and fraction differences. Also, one-way ANOVA was used for time differences with subsequent pairwise comparisons by Student-Newman-Keuls multiple-range test, with $P<0.05$ required for significance.

Results

Daily changes of PER protein levels in the total extracts of lungs, hearts and livers under a short and long photoperiod

       Bands of approximately 55-60 kDa were recognized by N-terminus-targeted antibodies anti-PER1 and anti-PER2 in lungs, hearts and livers (Figs 1 and 2). Single rows of bands were selectively suppressed by preincubation of antibodies with the appropriate peptides (data not shown). Figure 1 shows daily changes of PER1 and representative immunoblots. In lungs (Fig. 1A), the 2-way ANOVA did not reveal any difference between the long and short photoperiod and the interaction effect was also not significant. However, the effect of time was highly significant ($F=2.8$, $p<0.01$). Under both photoperiods, a daytime peak at 12:00 h was suggested. In hearts (Fig. 1B), 2-way ANOVA also did not reveal any significant difference between the PER1 profile under the long and the short photoperiod. The effect of time as well as an interaction effect were, however, significant ($F=2.6$, $p<0.05$ and $F=2.9$, $p<0.05$, respectively). The evening PER1 increase under LD 8:16 seemed to precede the evening rise below LD 16:8. Under LD 16:8, a daytime peak at 12:00 h was also indicated. In livers (Fig. 1C), the 2-way ANOVA revealed a significant difference between the PER1
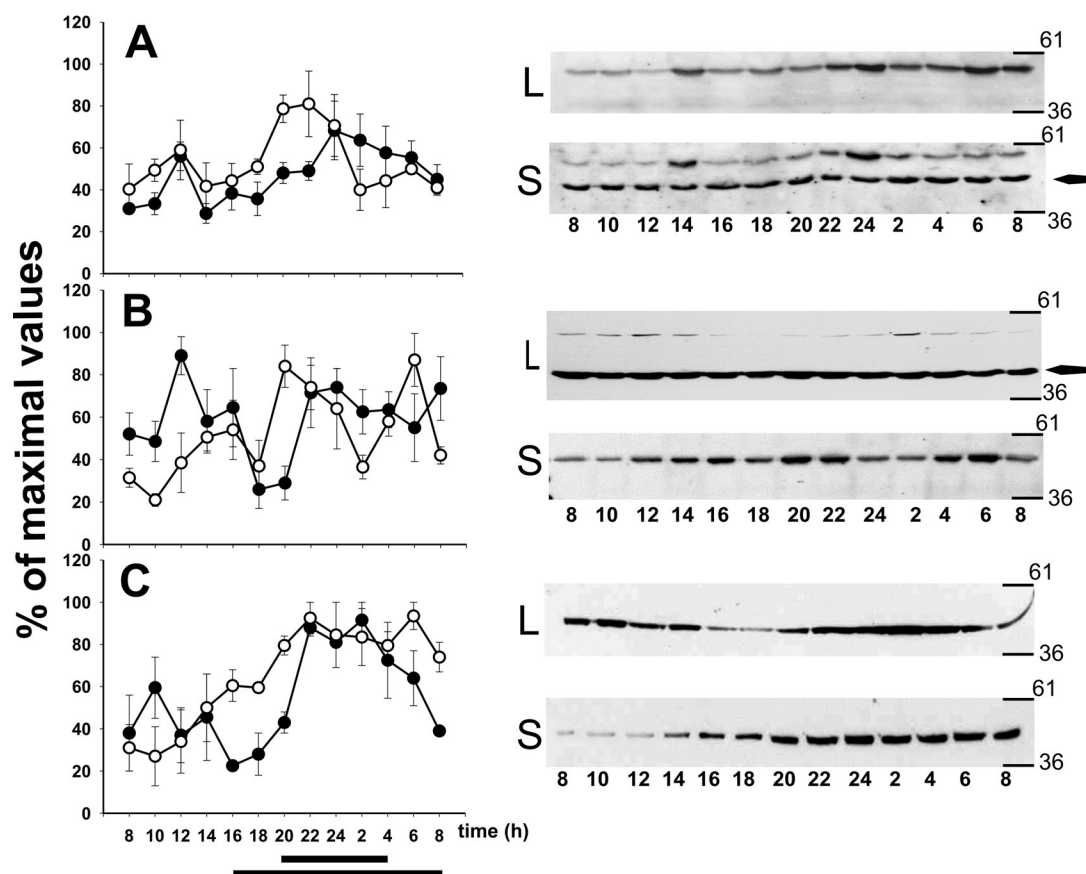
Fig. 1. Daily profiles of rPER1 levels in the lungs (A), hearts (B) and livers (C) of rats maintained under the long (full dots) and short (open dots) photoperiod. Protein levels were analyzed in total extracts and quantified by 2D densitometry from Western blots. Values are shown as percentage of maximal signal intensity. Next to each graph are the representative immunoblots for long (L) and short (S) photoperiods. Numbers beside bands indicate kDa. In all experiments, we used actin to verify the amount of loaded protein; in some images actin is indicated by arrowheads. Data are expressed as mean ± SEM from three animals. Black bars indicate the dark period.

profile under the long photoperiod and that under the short one (F=5.8, p<0.05), as well as a significant effect of time (F=6.6, p<0.01) but no significant interaction effect. As in lungs and hearts, an earlier evening PER1 rise under LD 8:16 as compared with that under LD 16:8 was only indicated. Under LD 16:8, a daytime peak around 10:00 h was suggested.

Figure 2 shows daily changes of PER2 and representative immunoblots. In the lungs (Fig. 2A), 2-way ANOVA revealed a significant difference between the short and long photoperiod (F=14.24, p<0.01), as well as a significant effect of time (F=4.45, p<0.01) and interaction effect (F=2.42, p<0.05). Under LD 16:8, a significant increase from a basal level at 18:00 h was observed at 24:00 h (18:00 vs. 24:00 h, p<0.05). Under LD 8:16, only the PER2 decrease was significant (22:00 vs. 04:00, 06:00 h, p<0.05 and 22:00 vs. 08:00 h, p<0.01) and occurred at about the same time as under LD 16:8. In hearts (Fig. 2B), the 2-way ANOVA revealed a significant difference between PER2 profile under the

short and under the long photoperiod (F=11.50, p<0.01), as well as a significant effect of time (F=5.20, p<0.01) but not a significant interaction effect. Under LD 8:16, an increase in PER2 levels occurred between 18:00 and 20:00 h (18:00 vs. 20:00 h p<0.05) followed by a sharp decrease between 20:00 and 24:00 h (for p<0.01). The waveform of the PER2 profile under LD 16:8 was shallow and the suggested maximum at 20:00 h was only by about 40 % higher than the minimum at 10:00 h. In livers (Fig. 2C), 2-way ANOVA revealed neither a difference between the PER2 profile under the short and that under the long photoperiod, nor any effect of the duration or an interaction effect.

Daily changes in PER protein levels in the nuclear (N) and cytoplasmic (C) fractions from lungs under a short and long photoperiod

Quality of fractionation was confirmed by anti-TBP (Tata-box binding protein) antibody that recognized bands of approx. 40 kDa, predominantly in the nuclear
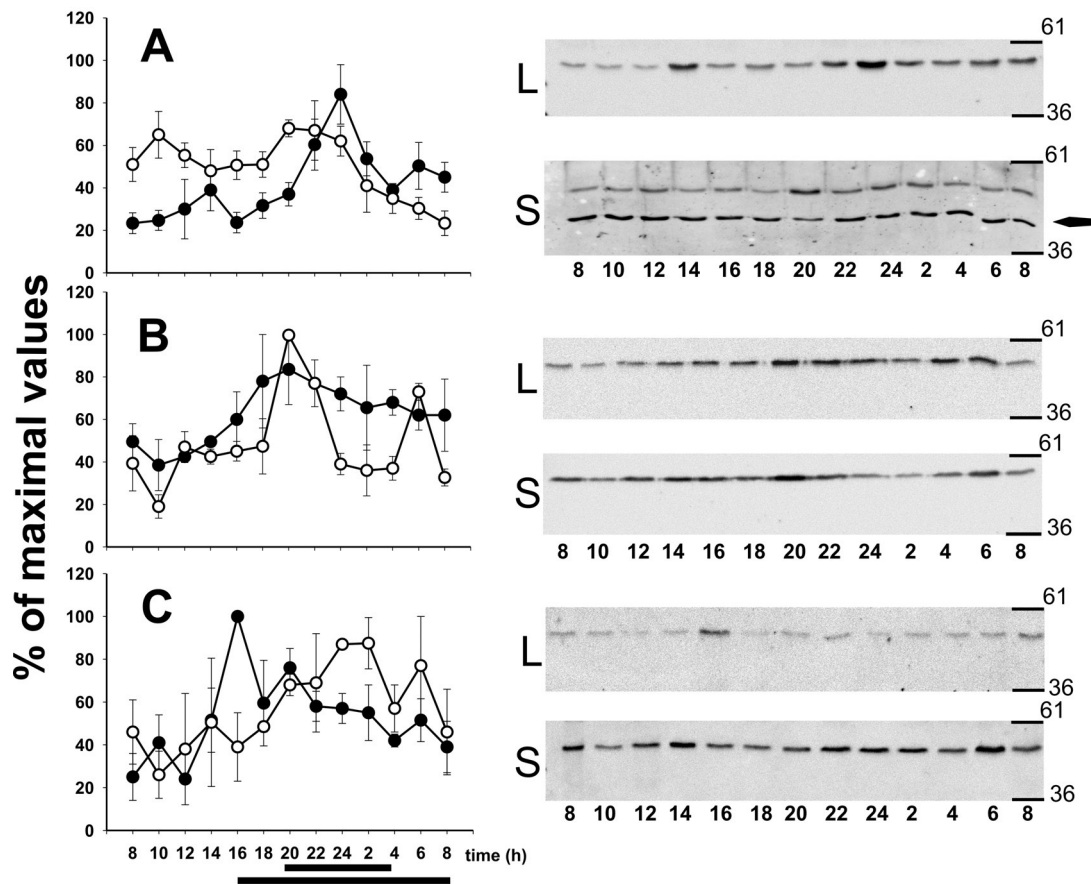
Fig. 2. Daily profiles of rPER2 levels in the lungs (A), hearts (B) and livers (C) of rats maintained under the long (full dots) and short (open dots) photoperiod. For further details see legend to Figure 1.

fractions (data not shown). Figure 3 shows daily changes of PER1 in N and C fractions and representative immunoblots in the lungs. Anti-PER1 antibody recognized a double specific band between 60 and 70 kDa in N fractions and a single band at approx. 60 kDa in C fractions. The 3-way ANOVA did not reveal any significant difference between the PER1 profile under the short and the long photoperiod, or a difference between N and C fractions. However, the effect of time was significant (F=13.6, p<0.01). The 2-way ANOVA confirmed a significant effect of time for both fractions under LD 16:8 (F=14.2, p<0.01) as well as under LD 8:16 (F=5.3, p<0.01), and also for both N fractions (F=7.6, p<0.01) and both C fractions (F=8.5, p<0.01). The interaction effect was significant for N fractions (F=2.5, p<0.05) as well as for C fractions (F=4.9, p<0.01) under both photoperiods. The interaction effect was also significant for comparison of N and C fractions under LD 16:8 (F=2.6, p<0.05), namely due to the significant PER1 decrease in C fraction level in the morning (24:00 vs. 02:00 h, p<0.05). In N and in C fractions under LD 16:8, a significant daytime peak at 14:00 h was observed

(10:00 vs. 14:00 h, p<0.01, and 14:00 vs. 20:00 and 18:00 h, p<0.05, respectively).

Figure 4 shows daily changes of rPER2 in N and C fractions and representative immunoblots in the lungs. Anti-PER2 antibody recognized a double specific band between 60 and 70 kDa in N fractions and a single band at approx. 60 kDa in C fractions. The 3-way ANOVA did not show any significant difference between the PER2 profile under the short and that under the long photoperiod or a difference between fractions but it revealed a significant effect of time (F=12.0, p<0.01). The 2-way ANOVA revealed a significant difference between the short and the long photoperiod and a significant interaction effect for cytoplasmic, but not nuclear PER2 profiles (F=6.6, p<0.05, F=2.9, p<0.05, respectively). The difference between PER2 profiles in N and C fractions was significant under LD 8:16 (F=6.9, p<0.05) but not under LD 16:8. The 2-way ANOVA also confirmed a significant effect of time for both fractions under LD 16:8 (F=15.0, p<0.01) as well as under LD 8:16 (F=4.1, p<0.01), and also for both N fractions (F=6.3, p<0.01) and both C fractions (F=6.9, p<0.01).
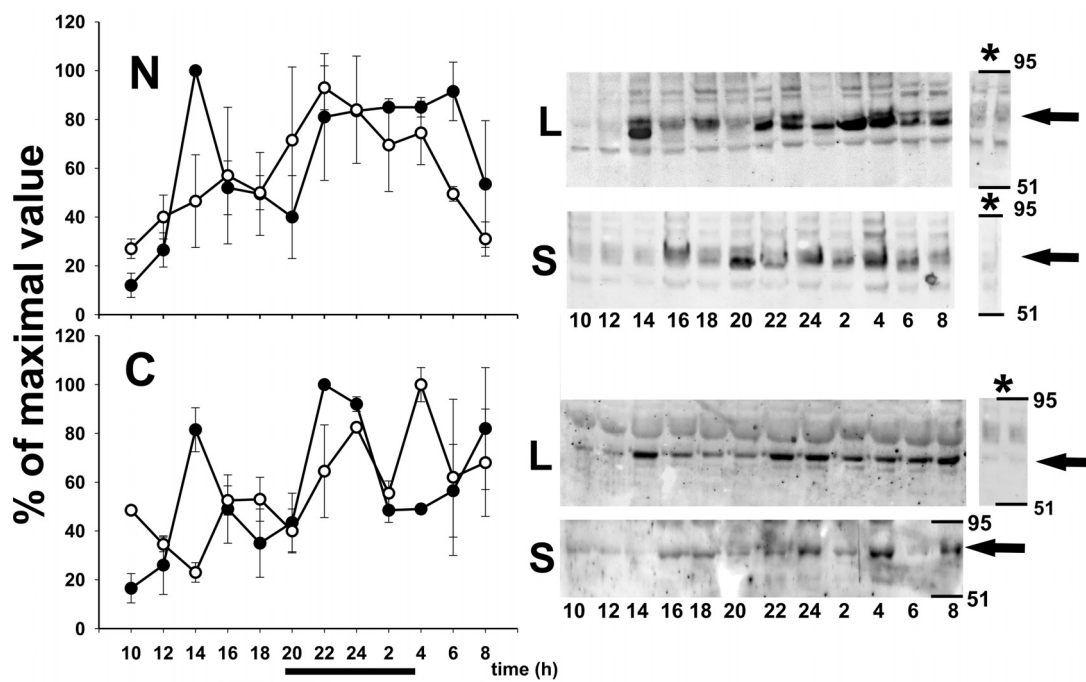
Fig.3. Daily profiles of rPER1 levels in the nuclear (N) and cytoplasmatic (C) fractions from lungs of rats maintained under the long (full dots, L) and short (open dots, S) photoperiod. Protein levels were quantified by 2D densitometry from Western blots. Values are shown as percentage of maximal signal intensity. Next to each graphics are the representative immunoblots for nuclear and cytoplasmatic fractions. Asterisks designate pre-absorption of antibody with blocking peptide. Specific bands are indicated by arrowheads. Numbers beside bands indicate kDa. Data are expressed as mean ± S.D. from two animals. Black bars indicate the dark period.

Under both photoperiods and in both fractions a PER2 daytime peak at 14:00 h, besides the night-time one, was indicated.

Discussion

The present study examined daily profiles of PER1 and PER2 proteins in total protein extracts of the lungs, hearts and livers and in nuclear and cytoplasmic fractions of lungs in rats entrained either to the long LD 16:8 or the short LD 8:16 photoperiod. In all samples, we identified bands of roughly 55-70 kDa for PER1 as well as for PER2 protein. While no comparative data for PER2 in mammalian tissues have been reported, the PER1 protein of an analogous molecular size was identified in the kidney and smaller PER1 forms in the liver, brain and insulinoma cells in the pancreas (Chilov *et al.* 2001, Muhlbauer *et al.* 2004). As in the present study, all the above mentioned data were obtained by using N-terminus targeted antibodies. Many other studies, however, detected larger PER proteins, with molecular size between 130-200 kDa, recognized mostly by C-terminus targeted antibodies. The sizes differed among organs and also according to preparation of the extracts (Hastings *et al.* 1999, Lee *et al.* 2001, Chilov *et al.* 2001, Akashi *et al.*

2002, Bendová *et al.* unpublished results). Interestingly, in contrast to peripheral tissues, we identified bands representing PER1 of approx. 110 kDa using N-terminal antibody in the SCN. Altogether, our data are in favor of the hypothesis that several isoforms of PER proteins identified by antibodies against distinct epitopes may exist and may be expressed in a tissue-specific manner. These protein isoforms could correspond to the reported splicing variants of *Per* genes (Taruscio *et al.* 2000, Hida *et al.* 2000). Lee *et al.* (2001) showed that in total extracts of the mouse liver, PER1 protein changed its electrophoretic mobility apparently due to its temporal phosphorylation. In nuclear extracts of lungs, but not in total protein extracts, we observed temporal double bands that might also represent changes in a phosphorylation state. The discrepancy with various molecular sizes could be caused by distinct protocols of protein isolation as well as by the distinct antibody used.

Densitometric measurement of bands intensity and size was used to define the relative amount of PER proteins in various samples. The measurement was, however, highly dependent on exposition time, since overexposure of immunoblots could mask differences among individual bands. Although we always endeavored to evaluate the exposures when the weakest bands were
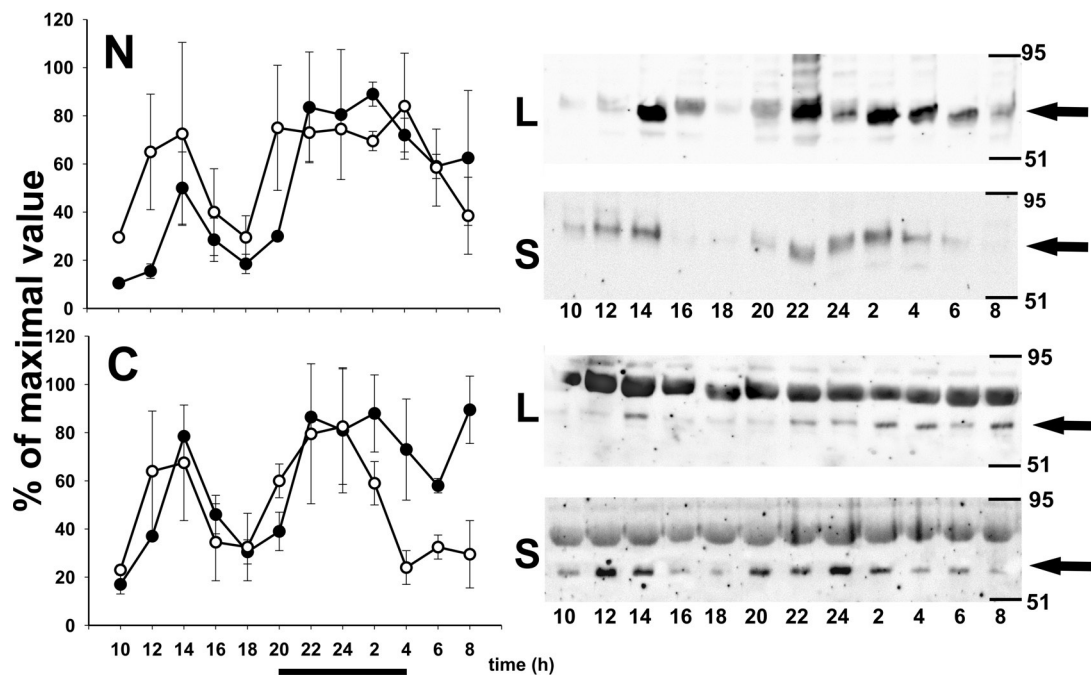
Fig.4. Daily profiles of rPER2 levels in the nuclear (N) and cytoplasmatic (C) fractions from lungs of rats maintained under the long (full dots, L) and short (open dots, S) photoperiod. For further details see legend to Figure 4.

first visible, the resulting magnitudes of amplitudes could only be expressed in one experiment and therefore no actual differences or similarities among tissues, fractions and photoperiods could be declared.

The study showed daily changes in PER protein levels, with higher values during the evening and night. The photoperiod affected significantly the PER1 profile in livers and the PER2 profile in lungs and hearts. Moreover, the photoperiod significantly modulated the PER2 profile in the cytosolic fraction from the lungs. In hamsters, the photoperiod has also been shown to modulate the amplitude and waveform of *Per1* mRNA expression in lungs and hearts (Carr *et al.* 2003). Peripheral oscillators are believed to be coordinated by the main pacemaker in the SCN (Yoo *et al.* 2004). In the rat SCN, the daytime increase of PER1 production occurs 2-4 h earlier under a long than under a short photoperiod (Sumová *et al.* 2002). Similar observations have also been made in hamsters (Messager *et al.* 1999, de la Iglesia *et al.* 2004, Johnston *et al.* 2005) and mice (Steinlechner *et al.* 2002). In contrast to the SCN, in total extracts of livers, the rise in PER1 levels under the short photoperiod preceded that under the long photoperiod. In lungs and hearts, an earlier evening PER1 rise under LD 16:8 was also indicated than in LD 8:16. Such timing of PER1 increase might indicate that in peripheral tissues, the PER1 rise is not only due to the SCN program but

also due to other factors affecting protein concentration.

Waveforms of the PER2 rhythm in lungs were similar to those of PER1: the earlier night-time maximum under the short than under the long photoperiod was also indicated. In the hearts, the difference between the PER2 profile under the long photoperiod and that under the short photoperiod was rather due to a flatter PER2 rhythmicity under the long photoperiod than to a phase shift. In the ovine liver, the peak of *Per2* mRNA expression also occurred earlier under the short day than under the long day (Andersson *et al.* 2005). To our knowledge, no photoperiodic modulation of the PER2 protein rhythmicity in the SCN has been published so far, but the profile of *Per2* mRNA was modulated similarly to that of *Per1* mRNA (Steinlechner *et al.* 2002, De la Iglesia *et al.* 2004).

It has been shown that mPER1 and mPER2 translocate from the cytoplasm into the nucleus in a time-dependent manner (Lee *et al.* 2001, for review see Tamanini *et al.* 2005). Using cell fractions from the lungs, we tested whether PER profiles in the nuclear and the cytoplasmic fractions depend on the photoperiod. In the nuclear fraction, an effect of the photoperiod on the PER1 rhythm was only indicated: the PER1 protein profile under the short photoperiod appeared to be phase advanced as compared with that under the long photoperiod. The effect of the photoperiod on the PER2

profile in the nuclear fraction was also only indicated: the evening PER2 rise under the short photoperiod also appeared to be phase advanced as compared with that under the long photoperiod. In the cytosolic fraction, however, the effect of the photoperiod on the PER2 profile was significant: while only a slight advance of the evening PER2 rise under the short photoperiod as compared with the long period was suggested, the morning PER2 decline occurred markedly earlier under the short than under the long photoperiod. Importantly, this cytosolic PER2 decline under the short photoperiod preceded the decline in the nuclear fraction by about 4 h. Since the PER2 signal in cytosolic samples is much weaker than in the nucleus, it could be masked by the high intensity signal of nuclear proteins in total extracts. These data may suggest, that, at least in lungs, the effect of the photoperiod on timing of the PERs rise may be mediated by accumulation of proteins within the nucleus. Thus, the long photoperiod might delay the evening increase of PER1 and PER2 proteins due to their later accumulation in the nucleus. Moreover, the photoperiod may also modulate timing of the PER2 morning decrease in the cytoplasm though affecting the time of its degradation. Further studies with other protein detection methods are, however, necessary to get a deeper insight into the mechanism.

Under the long photoperiod, a daytime PER1 peak in total extracts of lungs, hearts and livers was indicated. Such a PER1 peak was also indicated in the nuclear fraction of the lungs under LD 16:8. Moreover, a daytime PER2 peak was present in the nuclear as well as in the cytoplasmic fraction of the lungs under both photoperiods. Similar daytime peaks of *Per1* mRNA in hamster lungs and hearts also appeared in the data of Carr *et al.* (2003). Whether they reflect acute *Per* gene induction or an induced PER protein degradation remains to be elucidated.

In conclusion, the current study indicates that the photoperiod may affect daily profiles of PER proteins in the lungs, hearts and livers. In lungs, the effect of the photoperiod on PER2 in the cytoplasmic fraction may differ from that in the nuclear fraction. The photoperiodic control of the PER1 profile in peripheral tissues appears to be different from that described for the SCN (Sumová *et al.* 2002). The SCN rhythms may be controlled exclusively by the photoperiod, whereas the peripheral rhythms may be controlled, besides the photoperiod, by other factors, such as are e.g. food intake or locomotor activity.

Acknowledgements

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