

The metabolism of phospholipids oscillates rhythmically in cultures of fibroblasts and is regulated by the clock protein PERIOD 1¹

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SPECIFIC AIMS

We investigated temporal regulation of the biosynthesis of phospholipids in cultures of NIH 3T3 cells and of other fibroblasts (C57BL/6J and *CLOCK/CLOCK*) grown to confluence and synchronized by a 2 h 50% horse serum shock. We also looked at the expression of the clock gene *Per1* at the level of mRNA and protein under these conditions and examined its potential participation in regulation of oscillatory behavior of metabolism of phospholipids.

PRINCIPAL FINDINGS

1. Daily variation in the labeling of NIH 3T3 phospholipids

We examined the incorporation of ³²P-orthophosphate and ³H-glycerol into total phospholipids in cultures of quiescent NIH 3T3 cells after a 2 h serum shock and at different times from 0 to 62 h. The results show that there was a daily fluctuation in the biosynthesis of phospholipids of these cells treated with a high concentration of serum as indexed by the incorporation of ³²P-orthophosphate ($P < 0.000001$ by ANOVA) (Fig. 1A) or of ³H-glycerol ($P < 0.0000001$ by ANOVA) into total phospholipids. Levels of ³²P-phospholipids rapidly increased during the first half an hour of serum treatment and substantially decreased 1 h later. Then, levels peaked again 5 h after the beginning of the serum shock and remained elevated for several hours, decreasing to basal levels at 29 h (Fig. 1A). This variation was observed during the following day with the lowest levels of labeling at 58 h, exhibiting a period close to 29 h. A significant daily variation in the phospholipid labeling was also seen in the control cultures of NIH 3T3 cells treated only with a medium (DMEM) exchange at least during the first cycle. However, an overall activation of the phospholipid biosynthesis by serum stimulation was observed as compared with levels of lipid labeling in controls.

The oscillation described in the labeling of total phospholipids was accompanied by similar changes in all individual lipid species labeled. Phosphatidylinositol (PI) accounted for the species with the highest ratio of ³²P incorporation at all times examined, followed by considerable levels of phosphatidylglycerol (PG)/phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylcholine (PC), lyso-derivatives and phosphoinositides. Moreover, similar labeling patterns were observed at phases examined between both the control and shock-treated cultures. PC, PG/PE, PI and PA were lipid species with the highest ratio of precursor incorporation after ³H-glycerol administration, with slight differences in the labeling patterns of glycerophospholipids among phases examined and conditions.

2. Circadian expression of *Per1* mRNA and protein of NIH 3T3 cells

We examined the temporal expression of *Per1* mRNA and protein in NIH 3T3 cells after serum shock (Fig. 1B). We found a rapid and transient induction of *Per1* mRNA expression during the first minutes after serum stimulation and a significant daily variation that persisted at least for two cycles with the highest levels within the first hour, 27 and 55 h after treatment. These findings demonstrated that *Per1* expression oscillates rhythmically in cultures of NIH 3T3 cells as described previously, with a period close to 28 h.

The immunocytochemistry shows that PER1 also exhibited a significant daily variation with values of

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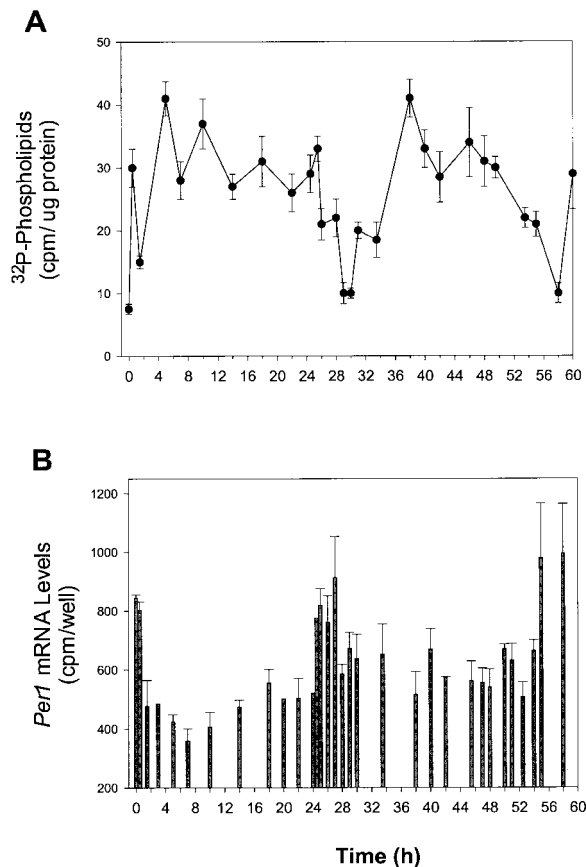


Figure 1. A) Daily variation of the ³²P-phospholipid labeling of NIH 3T3 fibroblasts grown to confluence with 5% fetal calf serum and subjected to a 2 h 50% horse serum shock (time 0). Cells were given a 30 min labeling pulse of ³²P at different times along 60 h after treatment. A daily variation was observed in the incorporation of ³²P into total phospholipids with a period close to 29 h and the lowest levels of labeling observed at 29 and 58 h after treatment ($F=6,84$; $P<0.000001$ by ANOVA). B) Circadian oscillation of *Per1* mRNA expression in NIH 3T3 cells after the serum shock as shown in A and assessed by in situ hybridization. A daily variation was observed in levels of *Per1* mRNA ($P<0.0000001$ by ANOVA) with the highest levels within the first minutes (30 min) and at 27 and 54 h after serum shock.

immunoreactivity peaking within the first h after the serum shock, declining by 3 h after treatment, and remaining low for several h until they peaked again around 26 h later (Fig. 2A). Remarkably, the immunoreactivity associated with PER1 was mainly confined to the cell nucleus while the α -tubulin positive immunostaining highlighted the rest of the cell (Fig. 2A).

3. Effect of *Per1* expression knockdown on the phospholipid labeling of NIH 3T3 cells

Taking into consideration that the metabolic oscillation described in the synthesis of phospholipids (Fig. 1A) is driven by endogenous clock mechanisms and that this oscillation is in antiphase with the circadian expression of *Per1* in NIH 3T3 cells (Figs. 1B and 2A), we investigated a potential role of this clock protein in

the rhythmic generation of lipid synthesis. When *Per1* expression was suppressed by using the specific antisense oligonucleotides given 30 min before the serum shock and maintained in the cell culture at all examined times, we found that PER1 protein was substantially reduced (Fig. 2C). Under antisense treatment, daily fluctuation of ³²P-phospholipid labeling was also significantly inhibited (Fig. 2B, gray bars). However, cell cultures treated with sense oligonucleotide before serum shock exhibited typical oscillation in phospholipid labeling with greatest levels of ³²P incorporation during the first hours post treatment and lowest levels 24-29 h later (Fig. 2B, black bars). ANOVA revealed that there was a significant time-related effect on phospholipid labeling for sense treated cultures ($P<0.003$), whereas for cultures treated with antisense oligonucleotide, no significant differences in lipid labeling were observed across time.

4. Phospholipid labeling and *Per1* expression in *CLOCK/CLOCK* fibroblasts

To further investigate temporal regulation of the biosynthesis of phospholipids in cell cultures and potential

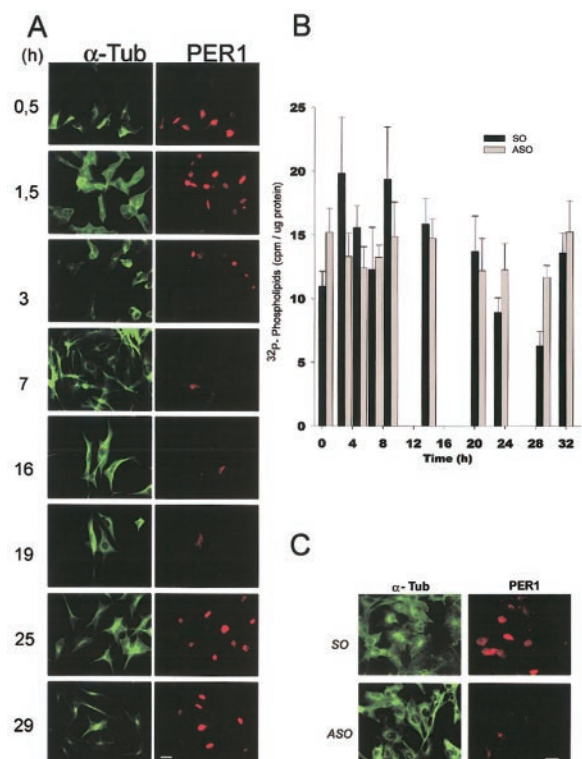


Figure 2. A) Temporal expression of PER1 protein in NIH 3T3 cells after a 2 h serum shock. PER1 immunoreactivity was localized to the cellular nucleus while the immunofluorescence associated to α -Tubulin highlighted the rest of the cell. B) Effect of the blockade of *Per1* expression on ³²P-phospholipid labeling in NIH 3T3 fibroblasts. *Per1* expression was knocked down by continuous treatment with antisense oligonucleotide (ASO) (gray bars). Controls were assessed by treatment with *Per1* sense oligonucleotide (SO) (black bars). C) Effect of administration of *Per1* SO and ASO on PER1 expression in NIH 3T3 cells 1.5 h after initiation of serum shock.

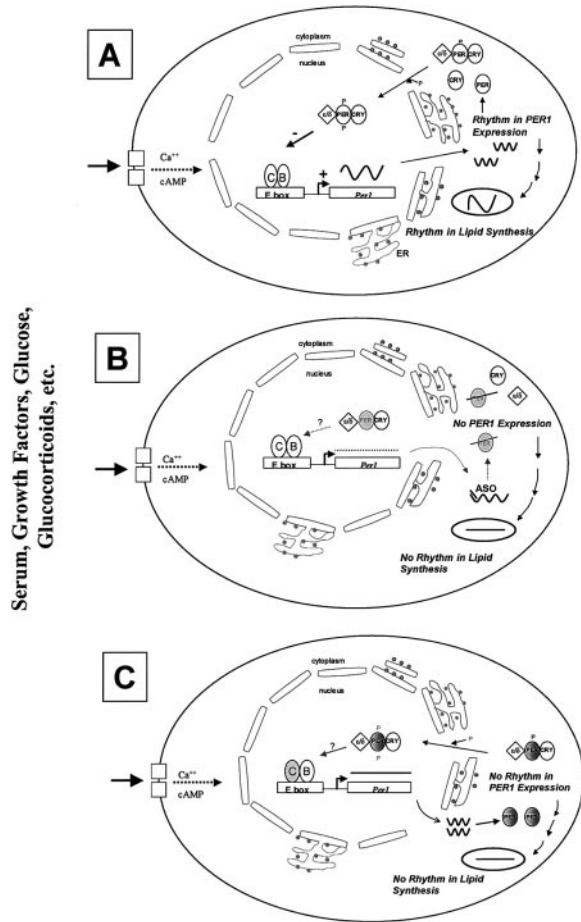


Figure 3. Circadian regulation of the biosynthesis of phospholipids in cell cultures. Different extracellular signals (serum, growth factors, glucose, glucocorticoids) synchronize immortalized cell lines grown to confluence. These cells contain peripheral oscillators that display circadian rhythms in the expression of various genes: among others, clock genes such as *Bmal1*, *period1-3* (*Per1-3*) and *cryptochromes* (*Cry1-2*). The clock mechanism comprises interactive positive (+) and negative (-) feedback loops. The transcription regulatory factors CLOCK (C) and BMAL1 (B) form heterodimers and activate transcription of *Per*, *Cry* and other clock controlled genes through E-box enhancers. Ca⁺⁺ or cAMP convey extracellular signals triggering expression of specific genes. As levels of PER proteins increase, they complex with CRY proteins and Casein Kinases I ϵ and δ (CKI ϵ and CKI δ) and are phosphorylated (P). In the nucleus, these complexes associate to CLOCK:BMAL1 heterodimers to shut down transcription (negative loop). For the positive feedback loop, other factors are involved that regulate *Bmal1* expression and activity of the protein complexes. A) The clock mechanism drives the circadian expression of *Per1*, which in turn, regulates metabolic oscillation in synthesis of phospholipids in the endoplasmic reticulum (ER). In contrast, when the clock mechanism does not operate properly as illustrated in (B) and (C), synthesis of phospholipids becomes arrhythmic. B) Knockdown of *Per1* expression by treating cells with the specific antisense oligonucleotide (ASO). C) CLOCK mutant cells displaying constant levels of PER1 expression (the *Clock* allele carries a deletion of 51 amino acids in its transcriptional activation domain). Under normal conditions, the metabolism of phospholipids oscillates daily in cell cultures by an endogenous clock mechanism involving PER1 expression. Lines and arrows in black represent processes that normally

involve of clock genes, we assessed ³²P-phosphate incorporation into phospholipids and PER1 immunoreactivity in cultures of *CLOCK/CLOCK* and of corresponding wild-type fibroblasts derived from respective male mouse pituitary glands. In wild-type cells, both phospholipid labeling and PER1 expression displayed a daily variation with profiles resembling those described for NIH 3T3 cells. In *CLOCK/CLOCK* cells, the expression of PER1 was constant and robustly observed at all times assessed and metabolic labeling of phospholipids did not significantly vary across time. Levels of labeled phospholipids in mutants were continuously high as compared with those in the wild-type at most times. A 2-way ANOVA on phospholipid labeling revealed a significant effect of time ($P < 0.04$), of cell genotype (wild-type vs. *CLOCK* mutants) ($P < 0.0000001$), and of interaction ($P < 0.002$).

CONCLUSIONS AND SIGNIFICANCE

Our findings support the idea that fibroblasts in culture are capable of oscillating in their metabolic functions as indexed by changes observed in the biosynthesis of their phospholipids (Fig. 1A and Fig. 3A). These metabolic oscillations may constitute an output of circadian oscillators located in cultured cells and may reflect a differential requirement of lipids recently synthesized for membrane biogenesis or for generation of distinct waves of lipid derived-2nd messengers varying across time. These metabolic oscillations persist at least during two cycles in cultures of NIH 3T3 cells and exhibit a period longer than 24 h. In addition, *Per1* expression is rapidly induced after the serum shock and displays circadian rhythmicity in these cells (Fig. 1B, 3A). The daily variation described in phospholipid biosynthesis occurs in an antiphase manner to the expression of *Per1*: when phospholipid labeling is high, *Per1* expression is very low and vice versa. Moreover, when *Per1* expression is knocked down by using a specific antisense oligonucleotide, metabolic oscillation observed in the incorporation of labeled precursors into phospholipids damped out (Figs. 2B, 3B), increasing levels of radioactive phospholipids at those phases at which biosynthetic levels were normally low (26–29 h after serum shock). In addition, in *CLOCK* mutant fibroblasts (Fig. 3C), our observations showed that oscillatory mechanisms are severely impaired at least for *Per1* expression and for biosynthesis of phospholipids.

Our findings demonstrate that daily fluctuations observed in the biosynthesis of phospholipids are driven by a circadian clock located in cell cultures, and that the molecular clock mechanism operating in the generation of these oscillations involved the expression of the clock gene, *Per1* (Fig. 3). [F]

occur in the cell upon stimulation or circadian regulation while dotted lines and arrows in gray represent pathways affected by the ASO treatment or the *CLOCK* mutation.