The role of the circadian clock in the regulation of human neutrophils

Doctoral theses

Krisztina Ella

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor: Dr. Krisztina Káldi, Ph.D., associate professor

Official referees:
Dr. András Dávid Nagy, Ph.D., senior lecturer
Dr. László Cervenak, Ph.D., senior research fellow

Chair of the comprehensive examination committee:
Dr. László Buday, D.Sc., professor

Members of the comprehensive examination committee:
Dr. Gabriella Sármay, D.Sc, professor
Dr. Sára Tóth, Ph.D., associate professor

Budapest
2016

INTRODUCTION

The circadian time-keeping system enables the organism to anticipate environmental changes and therefore is a crucial factor of adaptation. The circadian system has a central pacemaker in the suprachiasmatic nucleus, which may coordinate and synchronize the peripheral oscillators present in other tissues. The endogenous rhythm is generated at the cellular level by a mechanism based on the action of interconnected transcription/translation feedback loops. The negative factors of the central feedback loop are the CRYPTOCHROMEs (CRYs) and PERIODs (PERs). The CLOCK-BMAL1 positive factor complex supports transcription of PERs and CRYs that accumulate and form a complex in the cytosol. This complex enters the nucleus where it inhibits the positive factor complex, and thereby its own expression. When level of the PER-CRY complex declines in the nucleus, repression of the CLOCK-BMAL1 complex is relieved and a new 24 hour cycle is initiated. Via activation of the expression of the retinoic-acid-receptor-related orphan nuclear receptors, REV-ERBs and RORs, the CLOCK-BMAL1 complex induces an additional stabilizing loop that affects the operation of the clock: REV-ERBs repress, RORs induce

Bmal1 expression.

The CLOCK/BMAL1 complex can also induce the transcription of different clock-controlled genes, which are involved in the rhythmic operation of various physiological and biochemical processes. Based on microarray data 10% of the genome is under control of the circadian clock.

Both kinases (e.g. casein kinases, glycogen synthase kinase 3β) and phosphatases (e.g. protein phosphatase 1 and 5) are involved in the regulation of stability and/or subcellular localization of the different clock components. Therefore they are essential parts of the molecular clock, influencing the amplitude and period of the rhythm.

Both experimental and clinical data show the close relationship between circadian regulation and the immune system. The circadian clock influences the trafficking of leukocytes, inflammatory processes and it has a role in the differentiation of hemopoetic cells (e.g. B-lymphocytes, T_h17 cells). Furthermore the presence of a molecular clock has been shown in different cells with hematopoietic origin (e.g. macrophages, eosinophil granulocytes, T- and B-lymphocytes, dendritic cells, NK cells and monocytes), which could influence various cell functions, however these mechanisms have not been characterized in detail yet.

Neutrophilic granulocytes, the most abundant leukocytes in human blood constitute an essential part of the primary defense system against bacterial and fungal infections. The cell surface receptors of neutrophils recognize pathogens and trigger the activation of intracellular events leading to engulfment of microorganisms. Phagocytosed particles are then destroyed by the action of different reactive oxygen species and lytic enzymes. While reduced neutrophil activity often results in severe infectious diseases, inappropriate activation of neutrophil functions contributes to the development of various autoimmune and inflammatory diseases.

Although several immune responses linked to neutrophil functions have been described to be rhythmic, the mechanism of the circadian regulation of these cells is still not understood. Characterization of the time-of-day-specific control of neutrophil responsiveness could help to understand the pathomechanism of these inflammatory responses better and design effective chronotherapy.

OBJECTIVES

The goal of our study was to analyze time-keeping mechanisms controlling the responsiveness of human peripheral neutrophils. It was essential that our donors had the same daily rhythmicity, therefore prior to the experiments we had to determine the chronotype (middle sleep time on free days) of the donors. The main objectives of the experimental work were as follows:

1. Hungarian translation and validation of the Munich Chronotype Questionnaire (MCTQ), which can be used to determine chronotype.
2. Characterization of the clock gene expression profile of neutrophils and comparison of the results with those obtained from mononuclear cells.
3. Investigation of clock protein expression in neutrophils.
4. Investigation of the effect of systemic cues in regulation of neutrophil functions.
5. Comparison of neutrophil responses (superoxide production and phagocytosis) in different time points during the day.

METHODS

Validation of the hungarian version of the Munich Chronotype Questionnaire

We used the MCTQ to determine the chronotype of our donors. We translated the original english version to hungarian, which was back-translated for validation. We tested the questionnaire involving 780 university students studying physiology in the Semmelweis University Faculty of Medicine, Dentistry and Pharmacology. The study was approved by the Scientific and Research Committee of the Medical Research Council (Hungary) (Ethical approvals: 87/2010 és 154/2011).

Subjects and study design for investigation of leukocyte clocks

Healthy volunteers (both men and women, aged 20–35 years) were involved in this study. All participants reported both regular work schedule and sleep-wake pattern. Exclusion criteria were extreme chronotype, chronic diseases or an acute disease, shift work or jet lag in the preceding month and regular medication. Blood samples were collected at 10 am, 1 pm, 4 pm, 7 pm, 10 pm, 1 am, 4 am and 7 am. Blood

collections at night (after 10 pm) were carried out at low light intensities and with minimal disturbance of the subjects. The study was approved by the Scientific and Research Committee of the Medical Research Council (Hungary) (Ethical approvals: #10895-0/2011-EKU and 1563/2015).

Isolation of leukocytes from venous blood samples

Venous blood samples were obtained from each subject using Vacutainer® CPT^{TM} tubes (Beckton-Dickinson) containing sodium-citrate. Mononuclear cells and neutrophils were separated by ficoll gradient centrifugation. The remaining red blood cells in the neutrophil samples were eliminated by osmotic lysis. The purity of the prepared cell samples was confirmed by nuclear staining (methylen-blue) to be higher than 97%.

Measurement of plasma cortisol levels

Cortisol levels in blood plasma samples were measured in the Isotope Laboratory of the Semmelweis University using an electrochemiluminescence immunoassay (Elecsys, Roche) according to the manufacturer's instructions.

Gene expression analysis using real-time PCR

Following isolation, cells were immediately lysed using Tripure® reagent (Roche) and total RNA was extracted. Following DNase treatment, cDNA was synthesized using either the QuantiTect® reverse transcription kit (Quiagen) or the RevertAid® First Strand cDNA Synthesis Kit (Thermo Scientific). Relative expression levels of *Bmal1*, *Per1*, *Per2*, *Per3*, *Dbp*, *Rev-erbα*, *Itgb2*, $Gp91^{phox}$ and *Cxcr4* were measured in a Light Cycler® 480 system (Roche) by using TaqMan hydrolysis probes. *Gapdh* was detected as a reference gene. For data analysis the „second derivative maximum" method was performed using LightCycler® Relative Quantification Software (Roche).

Differentiation of PLB-985 cells into neutrophil-like cells

PLB-985 human myeloid cells at a starting density of $2x10^5$ cells/ml were treated with 1.25% (v/v) DMSO in RPMI 1640 medium, supplemented with L-glutamine, 10% (w/v) heat inactivated fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were then cultured for 4 days at 37°C in a humidified incubator with 5% CO_2.

Western blot analysis

Neutrophils were lysed after cell isolation in preheated (95°C) 2x SDS-PAGE Laemmli sample buffer supplemented with β-mercaptoethanol and boiled at 95°C for 10 min. Lysis of PLB-985 cells was performed by incubating the samples in lysis buffer. Then the samples were boiled in 2x Laemmli sample buffer at 95°C for 5 min. For dephosphorylation of protein samples, total cell lysates of mononuclear cells were treated with CIP Alkaline Phosphatase (New England Biolabs) according to the manufacturer's instructions. Proteins were separated on 7.5% (w/v) SDS-PAGE and then analyzed by Western blotting. For immunodetection anti-BMAL1 polyclonal antibody (1:500 dilution in 5% milk containing TBS buffer), anti-PER2 polyclonal antibody (1:200 dilution in 5% milk containing TBS buffer) and anti–β-ACTIN antibody (Sigma-Aldrich) (1:10000 dilution in 5% milk containing TBS buffer) were used.

Immunofluorescence staining of leukocytes

Cells were sedimented onto individual microscope cover slips and fixed in 4% paraformaldehyde (Sigma-Aldrich). Following permeabilization, cells were incubated in blocking solution for 30 min at room temperature. Staining

with affinity-purified polyclonal antibody against BMAL1 (dilution: 1:100) or with isotype control (rabbit IgG) (dilution: 1:100) antibodies was performed overnight at 4^{o}C. Samples were also incubated with ToPro®-3 Iodide (Life Technologies) nuclear counterstain (dilution: 1:1000). Samples were analyzed with a Zeiss LSM710 laser-scanning microscope using a 63×/1.40 oil DIC M27 immersion objective (Plan-Apochromat, Zeiss). Images were processed with ZEN 2011 SP2 black edition (Zeiss) software. Fluorescence intensity of BMAL1 staining was measured with ImageJ 1.48v software (NIH, USA).

Cortisol treatment of neutrophils

Isolated neutrophils were incubated in serum free RPMI 1640 medium (supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin) at 3x10^{5} cells/ml density for 60 minutes at 37°C, with 5% CO_2 followed by a treatment with either 300 ng/ml cortisol (Sigma-Aldrich) or vehicle for 60 minutes.

Measurement of side scattering (SSC) properties of neutrophils

Following isolation, neutrophils were fixed in 4% paraformaldehyde and stored at 4°C. SSC of all samples of the

subject was measured at the same time with a Beckman Coulter Cell Lab Quanta SC flow cytometer. Data were analyzed with WinMDI 2.9 software (Purdue University Cytometry Laboratories, West Lafayette).

Histological staining of human peripheral blood samples

Blood smears were prepared from peripheral blood collected from fingertips. Smears were stained using the standard May-Grünwald Giemsa staining protocol (Sigma-Aldrich). Following a Meander-line, 100 cells were counted in each sample and the maturation grades of peripheral neutrophils were analysed according to nuclear segmentation.

Measurement of plasma CXCL12 levels

CXCL12 levels in blood plasma samples were measured using a Human SDF-1 alpha ELISA Immunoassay (R&D Systems) according to the manufacturer's instructions.

Measurement of superoxide production of neutrophils

Superoxide production was quantified by using a chemiluminescent method. Superoxide production of the cells was stimulated with human pooled serum opsonized zymozan (Sigma-Alrich). Luminescence was measured at 37^{o}C for 30

minutes with a Mithras LB 940 Multilabel Reader (Berthold Technologies).

Quantification of phagocytosis of neutrophils

GFP-expressing serum opsonized *Staphylococcus aureus* was used to quantify the phagocytic capacity of the cells. Cells and bacteria were mixed (1:100) and sedimented onto coverslips and incubated at 37°C for 10 minutes with 5% CO_2. Samples were analyzed with a Zeiss LSM710 laser-scanning microscope using a 63×/1.40 oil DIC M27 immersion objective (Plan-Apochromat, Zeiss). Images were captured with ZEN 2011 SP2 black edition (Zeiss) software.

Statistical analysis

For statistical analysis, the SigmaPlot 11.0 software was used. Values were compared by Student's t-test and analysis of variance (ANOVA). The Tukey honest significant difference test and Bonferroni method were used for post hoc comparisons. Rhythm analysis of time series data was performed by using fixed 24-hour period cosine curve fitting with Time Series Analysis – Single Cosinor v 6.2 Software (Expert Soft Technologie). P-values<0.05 were considered statistically significant.

RESULTS

Translation of the Munich Chronotype Questionnaire and validation of the new version

Light is one of the most important signals that influence the circadian rhythm, therefore it is not surprising that chronotype is strongly correlated with the time of sunrise and latitude. We investigated if our middle sleep time data, acquired using the new hungarian version of the MCTQ, fit to the chronotype-latitude correlation published by Roenneberg and colleagues in 2007. Our data point fit well onto the regression line and the published data were reproducible, therefore the new MCTQ is validated.

Comparison of clock gene expression in neutrophils and mononuclear cells

We found different clock gene expression pattern in neutrophils compared to mononuclear cells. In mononuclear cells expression of *Per1, Per2, Per3* and *Dbp* showed clear daily variations, with phases similar to that reported in the literature. However, in neutrophils only *Per1*, *Dbp* and *Rev-erbα* levels displayed a low-amplitude oscillation.

Although relative *Per1* levels were similar, expression

of *Per2*, *Per3*, *Dbp* and *Rev-erbα* in neutrophils were significantly lower than in mononuclear cells, when *Bmal1* was used as a clock endogenous control.

Comparison of PER2 and BMAL1 protein expression in neutrophils and mononuclear cells

We compared PER2 and BMAL1 protein expression in neutrophils and in the mononuclear fraction. PER2 levels were low but detectable in mononuclear cells at all of the time points, whereas no PER2 signals could be detected in the neutrophil samples. Although in mononuclear cells BMAL1 protein expression showed moderate, but gradual increase during the day, in neutrophils only very low levels and no time-dependent changes were observed. Furthermore, two BMAL1 forms with different electrophoretic mobility were detected in mononuclear cells, whereas only one protein band with high electrophoretic mobility was present in the neutrophil sample. Treatment of the total lysates of mononuclear cells with alkaline phosphatase resulted in the appearance of a single lower band on the Western blot, suggesting that the protein forms with different electrophoretic mobility reflected the presence of disparate phosphorylation states and that phosphorylation of the protein is impaired in neutrophils.

Previous studies show that phosphorylation of BMAL1 correlates with the nuclear accumulation of the protein. In immunostained samples of neutrophils decreased nuclear level of BMAL1 was detected compared to both monocytes and lymphocytes. In addition, BMAL1 was equally distributed between the nucleus and the cytoplasm in monocytes and accumulated in the nucleus of lymphocytes, whereas in neutrophils higher BMAL1 levels were obtained in the cytoplasm than in the nucleus.

Measurement of the expression of clock components during differentiation of PLB-985 cells into neutrophil-like cells

As differentiation of the mononuclear cells and neutrophils are separated in the bone marrow, we hypothesized that clock function may undergo a modification during the neutrophil differentiation process, which cause the difference between the expression of the clock components in neutrophils and in mononuclear cells. To investigate this possibility we used PLB-985 acute myeloid leukemia cell line, which can be differentiated into neutrophil-like cells with DMSO treatment. During the differentiation process, expression of *Per1, Per2* and also the PER2 protein declined. These suggest that the molecular oscillator undergoes a modification.

Investigation the effect of cortisol on the expression of clock genes in neutrophils

As clock protein expression seems to be downregulated in mature neutrophils, we posed the question whether this peripheral clock may control time-dependent responses of the cells. Alternatively, oscillation of neutrophil responsiveness and also that of some clock genes may be a direct consequence of the rhythmic action of systemic cues. In our experiments *Per1* levels followed diurnal fluctuations in neutrophils with maxima in the early day, when high plasma cortisol levels are present. Therefore, we examined how clock gene expression of neutrophils responds to cortisol. After cortisol treatment, *Per1* expression was five fold elevated. These results suggest that the *Per1* gene in neutrophils may be under the control of the glucocorticoid receptor and therefore the observed oscillation in *Per1* expression may be a consequence of time-dependent changes in plasma cortisol levels.

Investigation of the time-dependent changes in the age composition of the peripheral neutrophil pool

As neutrophil reactivity may also change with maturation, time-dependent variations in the proportion of aged and younger cell forms in the periphery may contribute to daily

fluctuations in the responsiveness of the cells. We examined whether the maturation marker *Cxcr4* chemokine receptor expression levels display daily variations in the peripheral neutrophil pool. *Cxcr4* RNA levels showed significant oscillation peaking in the evening (8:01 pm). Granulation of neutrophils changes with maturation. Since granulation is represented by the flow cytometric parameter SSC, we analyzed the SSC properties of the human neutrophil samples. A daily oscillation of SSC could be detected with a maximum in the morning (7:50 am), suggesting again that the proportion of young and aged cells within the cell pool oscillate throughout the day with more aged neutrophil in the circulation in the evening, than in the morning. We also compared the morphological diversity of neutrophils according to nuclear segmentation in samples prepared in the morning and in the evening. While significantly more cells with aged phenotype were present in the evening samples, no difference in the proportion of aged/young cells was observed in the morning samples. In correlation with previous studies, the peripheral neutrophil number also oscillated during the day peaking in the evening (11:36 pm).

Plasma levels of CXCL12 (ligand of CXCR4) – which display high expression in the bone marrow - also oscillated

and in parallel with the fluctuation of *Cxcr4* expression. These results raise the possibility that time-dependent clearance of neutrophils is supported by daily changes of human bone marrow functions.

Investigation of superoxide production and phagocytic ability of neutrophils

In parallel the with aging of the neutrophils, *Gp91*^{phox} expression levels also display daily variations, with maxima at night (11:36 pm), when the proportion of aged cells in the peripheral pool is increased. This fluctuation of the oxidase subunit correlates with the superoxide producing capacity of the cells. We measured superoxide production in response to opsonized zymozan, and the cells isolated at 1 am produced significantly more superoxide than those isolated at 1 pm, suggesting that responsiveness of neutrophils is better at night.

In line with this, neutrophils isolated at 1 am phagocytosed significantly more opsonized *Staphylococcus aureus* bacteria than cells collected at 1 pm, suggesting again that responsiveness of more aged neutrophils is better.

CONCLUSIONS

1. Validation of the hungarian version of the MCTQ was successful and the questionnaire can be used in other experiments.
2. The expression pattern of core clock genes in human neutrophils characteristically differs from that in mononuclear cells.
3. Expression of PER2 and BMAL1 are lower in neutrophils compared to mononuclear cells. In neutrophils BMAL1 is hypophoshorilated and the nuclear accumulation of the protein is lower compared to lymphocytes and monocytes.
4. During differentiation of the PLB-985 cells into neutrophil-like cells the molecular oscillator undergoes a modification.
5. *Per1* expression in neutrophils is affected by cortisol *in vitro*, and the phase of *Per1* oscillation is in synchrony with *in vivo* changes of the plasma cortisol levels.
6. Distribution of young and aged cells within the peripheral neutrophil pool displays a daily rhythm: there are more aged cells circulating at night.
7. Superoxide production and phagocytic capacity of neutrophils are higher at night, when more aged cells are available in the circulation.

LIST OF PUBLICATIONS

My doctoral theses are based on the following publications:

Haraszti RA, **Ella K**, Gyongyosi N, Roenneberg T, Kaldi K. (2014) Social jetlag negatively correlates with academic performance in undergraduates. Chronobiol Int, 31: 603-612.
*joint first author

Ella K, Csepanyi-Komi R, Kaldi K. (2016) Circadian regulation of human peripheral neutrophils. Brain Behav Immun, 57: 209-221.

My other publications:

Gyongyosi N, Nagy D, Makara K, **Ella K**, Kaldi K. (2013) Reactive oxygen species can modulate circadian phase and period in Neurospora crassa. Free Radic Biol Med, 58: 134-143.