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Circadian rhythms and the effect of glucocorticoids on expression of the clock gene *period1* in canine peripheral blood mononuclear cells

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ABSTRACT

Circadian rhythms have a periodicity of approximately 24 h and, in mammals, are regulated by clock genes. In this study, expression profiles of clock genes (*per1*, *per2*, *clock*, *bmal1* and *cry1*) were investigated over a single 24 h period by real-time PCR in peripheral blood mononuclear cells (PBMCs) of healthy dogs and canine PBMCs treated in vitro and in vivo with glucocorticoids. Only *per1* mRNA exhibited daily rhythms in canine PBMCs. Canine PBMCs cultured with dexamethasone in vitro had dose- and time-dependent increases in *per1* mRNA expression. Intravenous injection of dexamethasone increased expression of *per1* in canine PBMCs in vivo. Rhythmic expression of *per1* in PBMCs could be used as a molecular marker for monitoring circadian rhythms and the effects of drugs on clock genes in dogs.

Introduction

Circadian rhythms have a periodicity of approximately 24 h and, in mammals, are regulated by clock genes (Huang et al., 2011). The mammalian circadian clock system is organised in a hierarchical manner. The central circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus; peripheral clocks are present in the skin, liver and kidney, as well as in individual cells (Ko and Takahashi, 2006). The central circadian oscillation in the SCN is entrained by light signals from the retina and coordinates peripheral clocks through neural and humoral signals. The axis of central and peripheral clocks regulates time-of-day dependent variations in a variety of physiological processes.

Glucocorticoids secreted from the adrenal gland exhibit robust circadian rhythms, which in mice are regulated by the central clock in the SCN and the adrenal peripheral clock (Son et al., 2008; Chung et al., 2011). Glucocorticoids influence various aspects of biological rhythms, including locomotor activity, carbohydrate and lipid metabolism, and higher brain functions in rodents (Chung et al., 2011). In addition, glucocorticoids can reset peripheral clocks, but not the central clock, in mice (Balsalobre et al., 2000). Thus, glucocorticoids represent a potent humoral signal that links the central and peripheral clocks.

The 24 h circadian clock in mammals is regulated by positive and negative molecular loops coordinated by clock genes (Lowrey and Takahashi, 2004; Ko and Takahashi, 2006). The positive components of the circadian clock comprise Clock and Bmal1, which form a heterodimer and activate transcription of target genes, including the negative feedback loops of clock genes, such as *period1*, *period2* and *period3* (*per1–3*) and *cryptochrome1* and *cryptochrome2* (*cry1–2*). Heterodimers of Per1–3 and Cry1–2 translocate into the nucleus and inhibit Clock/Bmal1 activity, thereby repressing their own transcription. The clock genes contributing to the positive and negative feedback loops are expressed in the SCN, as well as in peripheral organs and cells, although they also exhibit tissue-specific expression profiles (Ko and Takahashi, 2006).

Circadian rhythms recognised in dogs include fluctuations in body core temperature (Refinetti and Piccione, 2003), blood pressure and heart rate (Piccione et al., 2005), intraocular pressure and tear production (Giannetto et al., 2009) and serum cortisol concentrations (Palazzolo and Quadri, 1987). In the present study, we determined expression profiles of clock genes in peripheral blood mononuclear cells (PBMCs) in healthy dogs and investigated whether glucocorticoids regulated clock gene expression in canine PBMCs.

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Table 1
Nucleotide sequences of oligonucleotide primers.

Gene	Primer	Sequence (5′–3′)	Size (base pairs)	GenBank accession number
Per1	Forward Reverse	GCCTTGTGCCATGGATATGTC TGGTTGCGAAGCGTGTACTC	77	XM_546606
Per2	Forward Reverse	GGTTCAAGGAGATGCGGAAAC CACTCAACTGCCCCTTGGA	83	XM_543306
Clock	Forward Reverse	CGCCGCTCTATAACACTATGGTGATC TACTGCGGCGCTCTGGGTACTG	100	XM_532376
Bmal1	Forward Reverse	AGAAAACGACCAAGGCTCAAGTAG TGCTTCCAAGAGACTCATAATGACA	75	XM_534063
Cry1	Forward Reverse	GCATCAACAGGTGGCGATTTTTG TGCTGGCTGTCCACGAATCAC	100	XM_857660
GAPDH[a]	Forward Reverse	TCAACGGATTTGGCCGTATTGG TGAAGGGGTCATTGATGGCG	89	NM_001003142

[a] Glyceraldehyde 3-phosphate dehydrogenase.

Materials and methods

Dogs

Four healthy dogs were used in this study, comprising one beagle and three mixed breed dogs (beagle, Greyhound and Labrador retriever), two intact males and two intact females, with a mean age of 1.9 ± 0.1 years and a mean bodyweight of 17.9 ± 2.4 kg. The dogs were housed in individual cages and fed a commercial diet (Science Diet Adult; Hill's-Colgate) once daily at 10.00 h, with water provided ad libitum. The temperature of the room was maintained at 25 ± 1 °C. Lights were turned on automatically at 07.00 h and off at 19.00 h. The dogs were acclimatised under these conditions and did not receive any treatment for 2 weeks before the commencement of the experiment. Rectal temperatures were measured during the experiment with an electronic thermometer every 3 h when blood samples were collected. Experiments were conducted during a single 24 h period and data are expressed as the average of four dogs. All procedures were approved by the Institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology (approval number 22-12; date of approval 16 April 2010).

Collection of peripheral blood mononuclear cells and serum

Blood samples were collected from the jugular veins using syringes and needles every 3 h over a single 24 h period, beginning at 09.00 h; a weak light was used only for a short time when collecting blood during the hours of darkness. Heparinised blood (3 mL) was used for separation of PBMCs using Ficoll-Paque Plus (GE Healthcare Biosciences) immediately after blood sampling. Blood was diluted 1:1 with phosphate buffered saline, layered onto Ficoll-Paque Plus and centrifuged at 400 *g* for 30 min at room temperature. PBMCs at the interface were collected with a Pasteur pipette. Serum was harvested from 1 mL blood for measuring concentrations of cortisol.

Estimation of cortisol concentrations and stimulation of PBMCs with dexamethasone

Cortisol concentrations in serum were measured by ELISA (canine cortisol ELISA kit; catalogue number ERK C2003, Endocrine Technologies). Sera diluted 1:1 with the sample diluent and cortisol standards (0–552 nM) were added into appropriate wells in the plate. Cortisol Enzyme Conjugate Solution (Endocrine Technologies) was added to each well and the plate was incubated for 1 h at room temperature. After washing, 3,3′,5,5′-tetramethylbenzidine colour reagent was added to each well. After incubation for 20 min at room temperature, the reaction was terminated with Stopping Solution (Endocrine Technologies). Absorbance was measured at 450 nm with a microplate reader (Immuno Mini NJ2300; Microtec). The minimum detectable concentration of cortisol using this kit was estimated by the manufacturer to be 2.8 nM. For in vitro experiments, PBMCs were held in serum-free Roswell Park Memorial Institute medium (RPMI Medium 1640; catalogue number 31800-022, Gibco) at 4 °C overnight.

PBMCs in serum-free RPMI medium (5×10^5 cells/mL) were stimulated with dimethyl sulphoxide (DMSO) (molecular grade, catalogue number 043-07216, Wako), 1 nM dexamethasone (molecular grade, catalogue number BML-EI126–0001, Biomol International) at 37 °C for 4 h, or 50% horse serum at 37 °C for 2 h. Previous studies have shown that 50% horse serum synchronises clock gene expression in mammalian cells, including human PBMCs (Balsalobre et al., 1998; Ebisawa et al., 2010). To ascertain the effects of dose of dexamethasone, PBMCs were stimulated with DMSO or 0.001–1 nM dexamethasone at 37 °C for 4 h. To ascertain the effects of time of exposure to dexamethasone, PBMCs were incubated with 1 nM dexamethasone at 37 °C for 0–12 h.

To determine the effects of dexamethasone on clock gene expression in vivo, dogs were injected with saline intravenously at 15.00 h, then blood samples were collected for isolation of PBMCs 4 h later at 19.00 h. Two days later, the same dogs were injected IV with 1 mg/kg dexamethasone (Decadron, medical grade, catalogue number 872454, MSD) at 15.00 h, then blood samples were collected 4 h later at 19.00 h.

Reverse transcriptase PCR and real-time PCR

Total RNA was extracted from PBMCs using the FastPure RNA kit (Takara Bio), treated with DNase, then reverse-transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara Bio). cDNA samples were subjected to reverse transcriptase (RT)-PCR analysis with GoTaq Hot Start Polymerase (Promega) and real-time PCR analysis with SYBR Premix EX Taq™ II (Takara Bio) using primer pairs listed in Table 1. For RT-PCR, the PCR thermal cycle was 95 °C for 2 min, 35 cycles of

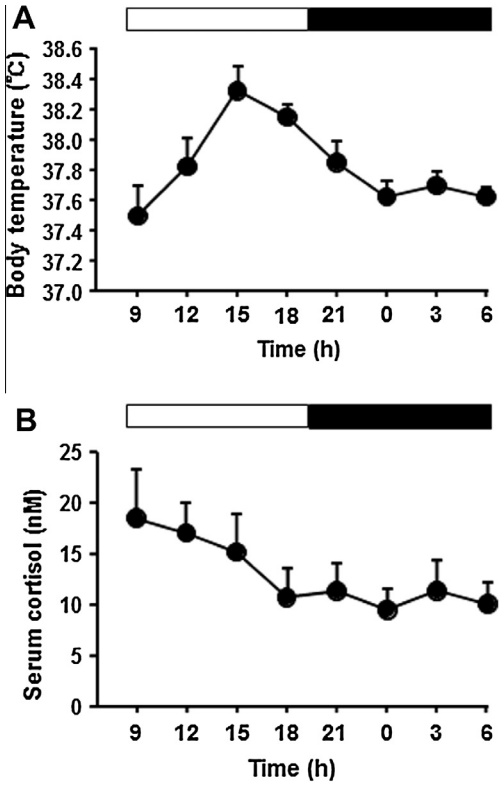

Fig. 1. Daily rhythms of rectal temperature and serum cortisol levels in healthy dogs. Rectal temperature was measured with an electronic thermometer (A) and serum cortisol levels were determined by ELISA (B) in healthy dogs every 3 h during a day. The white and black bars above the graphs indicate the light and dark periods, respectively. Data represent the means of four healthy dogs ± standard errors. Single cosinor analysis revealed significant circadian rhythms ($P < 0.05$) in both rectal temperature and serum cortisol levels.