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SHORT COMMUNICATION

Effect of lipopolysaccharide on circadian clock genes *Per2* and *Bmal1* in mouse ovary

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Abstract In mammals, circadian rhythms are associated with multiple physiological events. The aim of the present study was to examine the effect of lipopolysaccharide (LPS) on circadian systems in the ovary. Immature female mice were received an intra-peritoneal injection of equine chorionic gonadotropin (eCG) and LPS. Total RNA was collected from the ovary at 6-h intervals throughout a 48 h of experimental period. The expression of the circadian genes *period 2* (*Per2*) and *brain and muscle ARNT-like 1* (*Bmal1*) such as circadian genes was measured by quantitative PCR. Although expression of *Per2* and *Bmal1* in the ovary did not display clear diurnal oscillation, LPS suppressed the amplitude of *Per2* expression. Additionally, LPS inhibited the expression of *cytochrome P450 aromatase* (*CYP19*) and *luteinizing hormone receptor* (*LHr*) genes in the ovary of eCG-treated mice. Our data suggest that *Per2* may be associated with the inhibition of *CYP19* and *LHr* expression by LPS in the ovaries of immature mice.

Keywords Lipopolysaccharide · Circadian rhythm · Ovary · Liver

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Introduction

In mammals, circadian rhythms are associated with multiple physiological events and are regulated by "central clock" located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus [1]. Circadian rhythms have also been observed in peripheral tissues includeing liver, kidney [2, 3], uterus and ovary [4], which are referred to as "peripheral clocks". The peripheral clocks synchronize with central clocks through neuronal and hormonal systems [5]. Circadian oscillations are generated by a set of clock genes forming a transcriptional autoregulatory feedback loop. In mammals, *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2* are associated with this transcriptional feedback loop.

The mammalian ovary is an organ in which follicular development, ovulation and the formation of corpus luteum can occur periodically. These physiological events in the ovary are referred to as the estrous cycle (animal) or menstrual cycle (human). The functions of granulosa and theca cells that are the major components of ovarian follicles is associated with the estrous cycle (or menstrual cycle) in mammals. In mammals, follicle-stimulating hormone (FSH) from the pituitary enhances follicular development by promoting granulosa cell function, including estradiol production and cell proliferation. Estradiol is synthesized by the enzyme *CYP19* (*P450aromatase*) in the granulosa cells of growing follicles and subsequently induces the gene expression of *luteinizing hormone receptor* (*LHr*) in granulosa cells. Follicle-derived estradiol exerts positive feedback on both the hypothalamus and the pituitary to trigger a luteinizing hormone (LH) surge that precedes ovulation.

Recent studies support a role of circadian clock genes on granulosa and theca cell function in the ovary. The rhythmic expression of clock genes was observed in both

granulosa and theca cells in rodent ovary [6, 7]. Recent evidence indicates that FSH can induce the expression of circadian clock genes in the granulosa cells [8]. It has also been reported that clock genes expressed in granulosa cells are involved in the steroidogenesis. *Bmal1* is associated with the production of progesterone (P4) and prostaglandin (PGE2) in rat granulosa cells [9]. Moreover, *Clock* is associated with estradiol (E2) production by enhancing mRNA expression of *LHr* and *CYP19* in granulosa cells [10]. Thus, circadian clock genes play an important role for ovarian cellular functions in mammals.

Ascending infection of the upper female genital tract with gram-negative bacteria can lead to the development of pelvic inflammatory disease (PID) in women [11] or endometritis in dairy cattle [12]. Both are pathophysiological conditions that have been associated with infertility. Lipopolysaccharide (LPS), the major component of the outer membrane of gram-negative bacteria, can disturb normal ovarian function. Injection of intravenous LPS can inhibit in peripheral plasma estradiol concentrations despite normal plasma luteinizing hormone (LH) levels in the rhesus monkey [13]. Moreover, LPS can delay ovulation by attenuating the increased preovulatory estradiol in heifer [14]. LPS can also been shown to disturb estradiol production in granulosa cells [15] and progesterone production in theca cells [16] in vitro. Thus, LPS can induce ovarian dysfunction by affecting the functions of follicular cells such as granulosa and theca cells.

Although circadian clock genes are associated with ovarian function, it is still unknown whether LPS affects the circadian rhythm of clock genes during follicular development in the ovary. We hypothesized that LPS affects follicular development by disturbing the circadian rhythm of clock genes. To test this hypothesis, we examined the effect of exogenous LPS treatment on the circadian rhythm of *Per2* and *Bmal1* during ovarian follicular development that is induced by exogenous hormone treatment (equine chorionic gonadotropin).

Materials and methods

Animals and sample collection

ICR female mice (4-week-old; 22–25 g) were purchased from SANKYO LABO SERVICE Co. Inc. The animals were housed with free access to food and water at all times and were maintained on a 12-h light (AM 8:00, zeitgeber time 0, ZT 0): 12-h dark (PM 8:00, ZT 12) cycle at a controlled temperature (22–24 °C). The animals were divided into two randomly assigned groups. In the control group, the mice received an intra-peritoneal (i.p.) injection of equine chorionic gonadotropin (eCG, 5 IU, ASKA Animal Health Co., Ltd. Tokyo, Japan) and saline at ZT 0 on day 1 and saline alone at ZT0 on day 2. In the LPS group, the mice received an i.p. injection of eCG (5 IU) and LPS (1.0 μg/g body weight) at ZT 0 on day 1, and LPS alone at ZT 0 on day 2. In both groups, the mice were sacrificed by decapitation every 6 h: ZT 0, 6, 12, 18, and 24 on day 1 and day 2, and the livers and ovaries were removed rapidly. The removed tissues were placed in Trizol (Life Technologies, Inc., DriveRockville, MD, USA) and were stored −80 °C until RNA extraction.

RNA extraction, reverse transcription (RT), and quantitative polymerase chain reaction (PCR)

Collected liver and ovary samples were homogenized in Trizol reagent and total RNA was extracted from each ovary and liver according to the manufacturer's instructions and then frozen at −80 °C. Before the RT reaction, samples were treated with DNase and single-strand cDNA was then reverse transcribed from total RNA using a commercial kit (PrimeScript^{TM} RT Reagent Kit with gDNA Eraser; TAKARA BIO INC., Shiga, Japan). The RT reaction conditions were as follows: 15 min of cDNA synthesis at 37 °C and 5 s of inactivation at 85 °C. The mRNA levels of *Per2* and *Bmal1*, steroid-synthesis related genes such as *LHr* and *CYP19* were quantified by real-time PCR using an iQcycler (Bio-Rad Laboratories, Inc., Tokyo, Japan) and a commercial kit (QuantiTectTM SYBR® Green PCR; QIAGEN GmbH, Hilden, Germany).Each primer used is showed in Table 1. The amplification program included 10 min of activation at 95 °C followed by 50 cycles of PCR (95 °C for 10 s, specific temperature of each primer's annealing for 30 s and 72 °C for 20 s). Values were normalized using *β-actin* as the internal standard.

Statistical analysis

All data are presented as mean ± SEM. A one-way ANOVA was used to test for any diurnal variations in *Per2* and *Bmal1* expression in each tissue type. Student's t test was used to investigate any gene expression differences between control and LPS-treated mice for expression of multiple genes. Analyses were considered to be statistically significant at $P < 0.05$.

Results

Effect of LPS treatment on circadian expression of Per2 and Bmal1 in the liver

The expressions of *Per2* and *Bmal1* mRNA in the liver of the control group displayed diurnal rhythms and anti-phase

Table 1 Primer pairs used for detection of mRNAs

Genes	Primer sequence	Size (bp)	GeneBank accession no.
Per2	Forward: 5′-GGCACATCTCGGGATCG-3′	112	NM_011066
	Reverse: 5′-GAGCAGAGGTCCTCGCC-3′		
Bmal 1	Forward: 5′-GGAGAAGGTGGCCCAAA-3′	135	NM_001243048
	Reverse: 5′-AGGCGATGACCCTCTTA-3′		
CYP19	Forward: 5′-CATGGTCCCGGAAACTGTGA-3′	186	NM_007810.3
	Reverse: 5′-CTAGTAGTTGCAGGCACTTC-3′		
LHr	Forward: 5-TGAGTCCATCACGCTGAAAC-3′	80	NM_013582.2
	Reverse: 5′-AGATTAGCGTCGTCCCATTG-3′		
β-Actin	Forward: 5′-CACACCTTCTACAATGAGCTGC-3′	108	NM_007393.5
	Reverse: 5′-CATGATCTGGGTCATCTTTTCA-3′		

(Figs. 1a, 2a). However, in the LPS treatment group, whilst the expression of *Per2* mRNA showed a diurnal rhythm, levels during the experimental period were suppressed (Fig. 1b). Additionally, LPS inhibited the mean level of *Per2* mRNA at day 1 and day 2 (Fig. 1c, d). In contrast with the expression pattern of *Per2*, LPS treatment did not affect the diurnal rhythms of *Bmal1* (Fig. 2b), or the mean level of *Bmal1* at day 1 or day 2 (Fig. 2c, d).

Effect of LPS treatment on circadian expression of Per2 and Bmal1 and the expression of CYP19 and LHr genes in the ovary

In control ovary tissues, the diurnal rhythm of *Per2* expression was observed at day 2 but not at day 1 (Fig. 3a). LPS treatment disturbed the diurnal rhythm of *Per2* expression at day 2 (Fig. 3b) and the average level of *Per2* expression decreased at day 2 (Fig. 3d). The diurnal rhythms of *Bmal1* expression in the ovary with or without LPS treatment was not observed at day 1 and day 2 (Fig. 4a, b). Additionally, the average levels of *Bmal1* expression was the same between both groups (Fig. 4c, d). The expression of *CYP19* and *LHr* in LPS treated ovary was suppressed at day 2 (Fig. 5).

Discussion

This study examined the effect of LPS treatment on liver and ovarian circadian rhythm in mice. In the present study, we used exogenous hormone-treated immature mice to induce ovarian follicular development. Although administration of LPS did not affect the circadian rhythm of *Per2* and *Bmal1* in the ovary, change to the circadian rhythm of

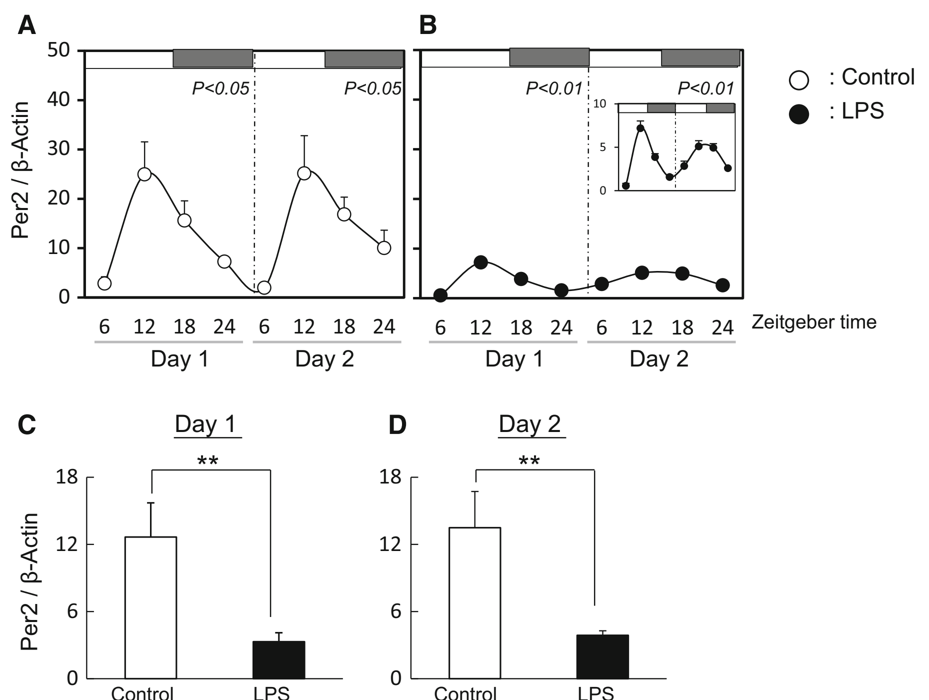

Fig. 1 Effect of LPS on circadian rhythm of *Per2* in mouse liver The quantified data of *Per2* at day 1 and day 2 in liver from mice with (**b**) or without (**a**) LPS treatment. White and *black bars* at the top of the figure indicate the time of lights-on and lights-off, respectively. Data are presented as mean ± SEM, $n = 3$–4, at each time point and analyzed by one-way ANOVA to determine diurnal variations in clock gene expression. Mean expression level of *Per2* is shown during day 1 (**c**) and day 2 (**d**) in liver from mice with or without LPS treatment. Data are presented as mean ± SEM and analyzed by *t* test to investigate any differences between control and LPS-treated mice (*$P < 0.05$, **$P < 0.01$)

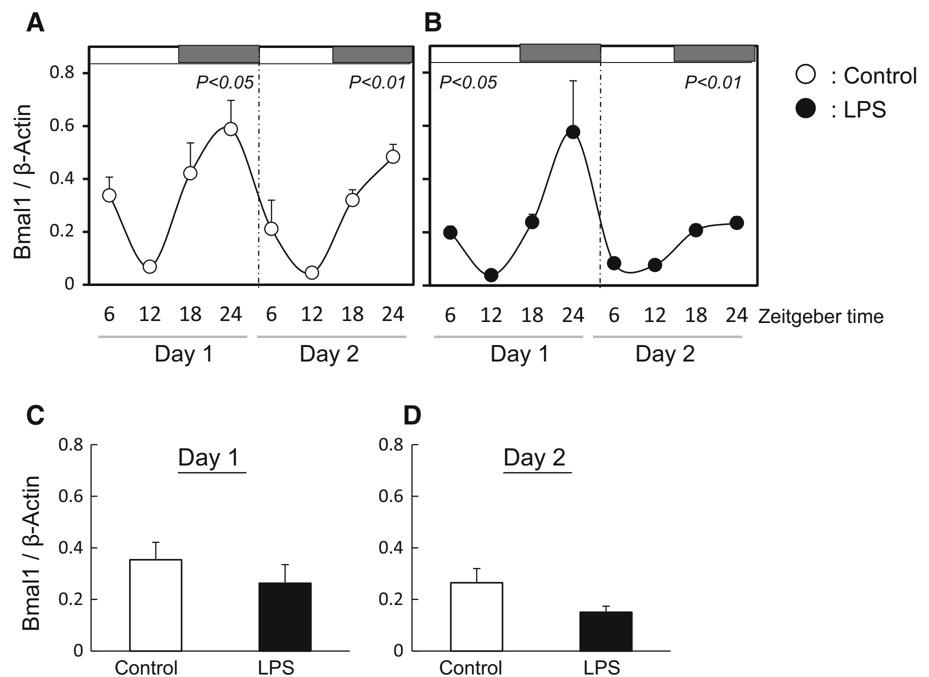

Fig. 2 Effect of LPS on circadian rhythm of *Bmal1* in mouse liver The quantified data of *Bmal1* at day 1 and day 2 in liver from mice with (**b**) or without (**a**) LPS treatment. *White and black bars* at the top of the figure indicate the time of lights-on and lights-off, respectively. Data are presented as mean ± SEM, $n = 3$–4, at each time point and analyzed by one-way ANOVA to investigate diurnal variations in clock gene expression. Mean expression level of *Bmal1* is shown during day 1 (**c**) and day 2 (**d**) in liver from mice with or without LPS treatment. Expression data are presented as mean ± SEM

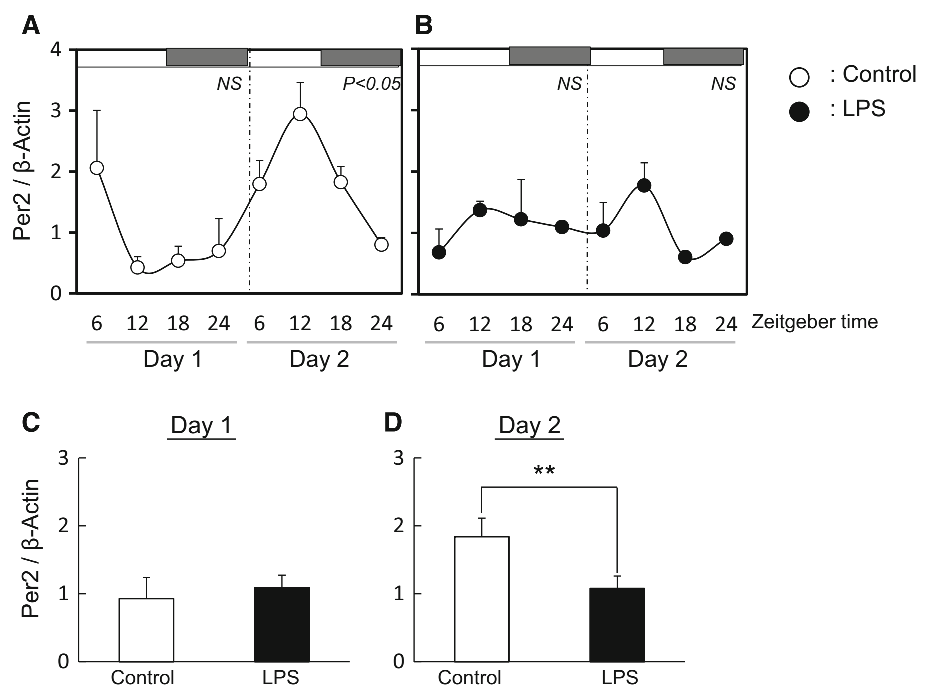

Fig. 3 Effect of LPS on circadian rhythm of *Per2* in mouse ovary The quantified data of *Per2* at day 1 and day 2 in ovary from mice with (**b**) or without (**a**) LPS treatment. *White and black bars* at the top of the figure indicate the time of lights-on and lights-off, respectively. Data are presented as mean ± SEM, $n = 3$–4, at each time point and analyzed by one-way ANOVA to examine diurnal variations in clock gene expression. Mean expression level of *Per2* is shown during day 1 (**c**) and day 2 (**d**) in ovary from mice with or without LPS treatment. Data are presented as mean ± SEM analyzed by *t* test to determine any difference between control and LPS-treated mice (**$P < 0.01$)

these genes were observed in the liver. The liver is a well-established peripheral oscillator that displays robust circadian rhythms of clock gene expression. To verify the correct analysis of circadian rhythm in ovary, mRNA expression of *Per2* and *Bmal1* in the liver was measured. Periodic rhythm and antiphase form of *Per2* and *Bmal1* was observed in the liver. However, injection of LPS suppressed *Per2* expression in the liver at day 1 and day 2. This observation is consistent with the published literature that indicates that a single dose of LPS (1 mg/kg) significantly suppressed the expression levels of *Per2* in the liver at day 1 [17]. Several clock-controlled elements are present in the *Per2* promoter region [18]. A noncanonical E-box enhancer (CATGTG, −497 in human and −163 in mouse, and CACGTT, −356 in human and −23 in mouse) drives circadian expression of Per2 through Clock/Bmal1-mediated transcriptional activation [19, 20]. Another important regulatory element is the DBP/E4BP4-binding element (D-

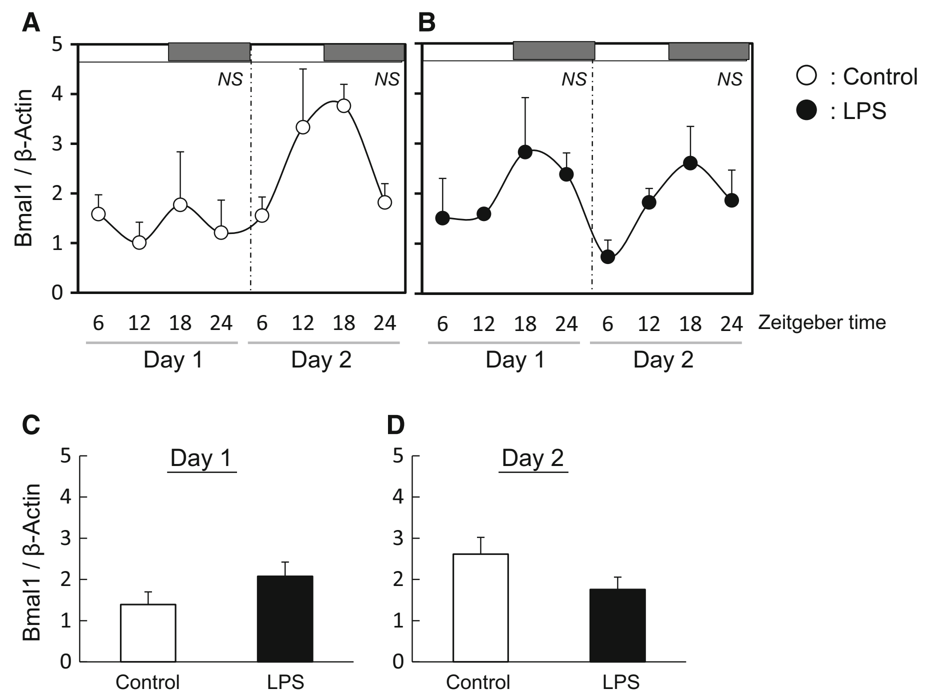

Fig. 4 Effect of LPS on circadian rhythm of *Bmal1* in mouse ovary The quantified data of *Bmal1* at day 1 and day 2 in ovary from mice with (**b**) or without (**a**) LPS treatment. *White and black bars* at the top of the figure indicate the time of lights-on and lights-off, respectively. Data are presented as mean ± SEM, $n = 3$–4, at each time point and analyzed by one-way ANOVA to determine diurnal variations in clock gene expression. Mean expression level of *Bmal1* is shown during day 1 (**c**) and day 2 (**d**) in ovary from mice with or without LPS treatment. Data are presented as mean ± SEM

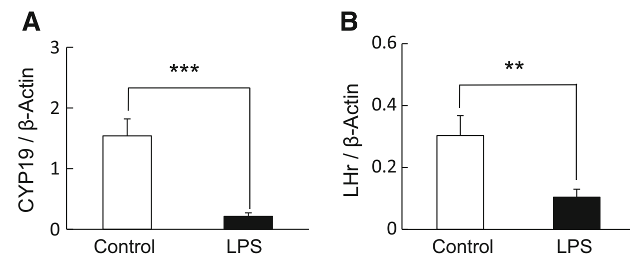

Fig. 5 Effect of LPS on CYP 19 and LHr in the mouse ovary The quantified data of *CYP19* (**a**) and *LHr* (**b**) expression in ovary (ZT 24 on day 2) from mice with ($n = 4$) or without ($n = 4$) LPS treatment 48 h after eCG injection (**$P < 0.01$, ***$P < 0.001$). Data are presented as mean ± SEM

box). This element influences *Per2* expression by a repressor-antiphasic-to-activator mechanism, which generates high-amplitude transcriptional activity [18]. Thus, LPS may inhibit transcription of *Per2* by inhibiting transcription factor binding to E-box or D-box in mouse liver.

Rhythmic expression of clock genes in the ovary has been observed in mature rat [6, 7, 21] and mouse [22]. Rhythmic expression of *Per2* was induced within 24 h after FSH treatment in immature rat granulosa cells within in vitro culture [7, 23]. We observed that the circadian rhythms of *Per2* and *Bmal1* developed in mouse ovary at day 2 after eCG treatment without LPS. These results suggest that the generation of clock gene rhythmicity by gonadotropin in immature granulosa cells may occur prior to rhythmicity in the ovarian tissue. Although LPS did not affect the rhythmic expression of *Per2* and *Bmal1*, LPS suppressed the mean level of *Per2* at day 2 in the ovary. As in the liver, LPS may inhibit the transcription of *Per2* by inhibiting transcription factor binding to E-box or D-box in the ovary.

In the present study, we demonstrated that expression of *CYP19* and *LHr* genes was inhibited in the mouse ovary treated with LPS 48 h after eCG injection. CYP19 is a critical enzyme in the production of estradiol in granulosa cells of the growing follicles in the mammalian ovary. Intraperitoneal injection of LPS in mice can lead to increased levels of inflammatory cytokines such as interleukin (IL)-10, IL-6 and tumor necrosis factor alpha (TNF-α) in blood [24, 25]. TNF-α and IL-6 inhibit the expression of *CYP19* (*P450aromatase*) and *LHr* in cultured granulosa cells [26–28]. Therefore, IL-6 and TNF-α activation by LPS may be associated with the observed decrease in the expression of *CYP19* and *LHr* in the mouse ovary. Interestingly, we observed that the mean level of *Per2* expression at day 2 decreased in the ovaries treated with LPS compared to those in the control group. Our previous study reported that siRNA knock-down of *Per2* decreased the expression of *LHr* gene in bovine granulosa cells [10]. Together, these results suggest that LPS or LPS-induced cytokines may suppress *LHr* expression by inhibiting *Per2* in the mouse ovary.

In conclusion, our data indicate that the amplitude of *Per2*, but not of *Bmal1*, in the liver is influenced by LPS and that *Per2* may be associated with the inhibition of *CYP19* and *LHr* expression by LPS in the ovary. This study suggests that *Per2* is a target factor of LPS in peripheral tissues. Our results contribute to the understanding of ovarian pathophysiological functions in mammals.

Compliance with ethical standards

Conflict of interest None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this manuscript.

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