Effects of a selective casein kinase 1δ and ε inhibitor on FcεRI expression and IgE-mediated immediate-type cutaneous reactions in dogs

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ABSTRACT. The molecular clock network in mast cells has been shown to be a factor responsible for circadian regulation of allergic inflammation. PF670462 is a selective inhibitor of casein kinase 1δ and ε (CK1δ/ε) that control the posttranslational modification of clock proteins. The aims of this study were to evaluate the effects of PF670462 on gene and protein expression of FcεRI, the high-affinity IgE receptor, in canine mast cells and on IgE-mediated immediate-type cutaneous reactions in dogs. PF670462 decreased mRNA expression of *FcεRIα* and *β*, but not *γ*, and protein expression of FcεRI in a canine mast cell line. Furthermore, PF670462 suppressed IgE-mediated immediate-type cutaneous erythema in dogs. These findings indicate that CK1δ/ε function as regulators for FcεRI expression and IgE-mediated cutaneous reactions in dogs.

KEY WORDS: circadian rhythm, dog, IgE, mast cell

Circadian rhythms generate a periodicity of approximately 24 hr in various physiological events in the body. The central clock in the suprachiasmatic nucleus of the hypothalamus coordinates peripheral clocks independently present in each organ and in individual cells [5, 6]. The 24 hr circadian rhythms in mammals are regulated by positive and negative molecular loops created by clock genes. The positive molecular loop consists of a heterodimer of circadian locomotor output cycles kaput (CLOCK) and brain and muscle arnt-like 1 (BMAL1), which activate transcription of target genes, including the negative feedback loops of clock genes, such as *period* (*per*) *1*, *per2*, and *per3* and *cryptochrome* (*cry*) *1* and *cry2*. PER and CRY proteins form a heterodimer and translocate into the nucleus, where they suppress their own transcription by inhibiting CLOCK/BMAL1 activity [6]. Circadian rhythms are also controlled by the posttranslational modification and degradation of clock proteins. Casein kinase 1δ and ε (CK1δ/ε) can complex with PER and CRY [7] and phosphorylate PER proteins [2], leading to their proteasome-mediated degradation and nuclear translocation [1, 3], thereby contributing to determine the circadian period length [8].

Accumulating evidence indicates that the clinical severity and pathogenesis of allergic diseases are under the control of circadian rhythms [14]. Although detailed mechanisms underlying time-of-day-dependent variations in allergic reactions remain elusive, recent studies have clarified that the molecular clock network in mast cells is one of the factors responsible for circadian regulation of allergic inflammation [11, 13]. In mast cells, CLOCK was shown to regulate circadian expression of the β subunit of FcεRI, a high-affinity IgE receptor, and facilitate the cellular response to IgE [13].

We previously demonstrated time-of-day-dependent oscillations in mRNA expression of the clock gene in canine peripheral blood mononuclear cells (PBMCs) [16] and in intradermal reactivity to histamine in healthy dogs [4]. Dogs develop various allergic diseases, such as atopic dermatitis [9] and food allergy [10]. These findings indicate that dogs are a good model to investigate the role of the molecular clock network in the development of allergic reactions.

A recent study has reported that PF670462, a selective CK1δ/ε inhibitor, suppressed IgE-mediated reactions in mice in

association with increased expression of PER2 which acts as a negative regulator of CLOCK in mast cells or basophils [12]. However, the effects of PF670462 have never been investigated in dogs. The aims of this study were to evaluate the effects of PF670462 on gene and protein expression of FcεRI in canine mast cells and to determine whether PF670462 attenuated IgE-mediated immediate-type cutaneous reactions in dogs.

Five healthy intact male beagles were used in this study. The mean age ± standard deviation (SD) of the dogs was 8.8 ± 2.7 years (range, 4–10 years), and the mean body weight ± SD was 12.7 ± 1.0 kg (range, 11.0–13.7 kg). The dogs were housed in individual cages and fed a commercial diet (Science Diet Adult; Hill's-Colgate Ltd., Tokyo, Japan) once daily. Water was provided *ad libitum*. The ambient temperature was maintained at 24°C (range, 23–25°C). Lights were automatically turned on at 07:00 hr and off at 19:00 hr. All procedures were approved by the Institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology.

Blood samples were collected from the cephalic veins of healthy dogs using syringes and needles. PBMCs were separated and collected from heparinized blood (2 m*l*) using Ficoll-Paque Plus (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, U.K.) according to a previous report [16].

For analysis of cell viability, PBMCs (2×10^5 cells/m*l*) were incubated in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% FBS (Biowest, Nuaille, France) and 1% penicillin and streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in the presence of saline or 1–10 μM PF670462 (Sigma-Aldrich) for 0–24 hr. As canine mast cells, HRMC cells (2×10^5 cells/m*l*), a canine mast cell tumor cell line, were cultured in serum-free AIM-V medium (Thermo Fisher Scientific, Waltham, MA, U.S.A.) [15] in the presence of saline or 1–10 μM PF670462 for 0–24 hr. Cell viability was determined by the trypan blue exclusion test (Wako Pure Chemical Industries).

To examine the effects of dose of PF670462 on mRNA expression of *FcεRIα*, *β*, and *γ*, canine mast cells (5×10^5 cells/m*l*) were stimulated with 0–10 μM PF670462 for 24 hr in serum-free AIM-V medium. To explore the effects of time of exposure to PF670462, canine mast cells (5×10^5 cells/m*l*) were incubated with 10 μM PF670462 at 37°C for 0–24 hr. After incubation, canine mast cells were immediately preserved in RNA*later* solution (Thermo Fisher Scientific) for RNA extraction.

Total RNA was extracted from canine mast cells using NucleoSpin RNA (Takara Bio, Kusatsu, Japan) and reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara Bio). The cDNA samples were subjected to real-time PCR analysis as described previously [16]. Primers for real-time PCR (Supplementary Table 1) were designed by a Perfect Real Time support system (Takara Bio). *Glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) was used as an internal control and relative mRNA expression of the target genes was determined by the $2^{-\Delta\Delta Ct}$ method [16], where $\Delta CT=CT^{sample}–CT^{gapdh}$. Each value was presented as an *n*-fold change of mRNA expression relative to that in the control group.

Cell surface expression of FcεRI was measured by flow cytometric analysis in canine mast cells in the presence or absence of PF670462 according to the previous report [15]. In brief, HRMC cells (2×10^5 cells/m*l*) were cultured with or without 10 μM PF670462 at 37°C for 24 hr in serum-free AIM-V medium. After stimulation, cells were incubated with 10% high *Dermatophagoides pteronyssinus* (DP)-specific IgE serum from a dog with canine atopic dermatitis. Serum specific IgE levels to DP measured by a quantitative ELISA [17] was 844 *ng*/m*l* in the dog (positive IgE levels: >100 *ng*/m*l*). Cell surface IgE bound to FcεRI was detected with fluorescein isothiocyanate (FITC)-labeled goat anti-dog IgE antibody (Bethyl Laboratories, Montgomery, TX, U.S.A.). Cells were analyzed by the Guava® easyCyte system (Merck, Tokyo, Japan), and the mean fluorescence intensity (MFI) was calculated with InCyte software (Merck).

In the Prausnitz–Küstner (PK) test, the hair coat on the left lateral thorax of healthy dogs was clipped before the experiment. Dogs were sedated with intravenous injection of butorphanol (0.2 mg/kg; Vetorphale; Meiji Seika Pharma, Tokyo, Japan). Using a syringe with a 26-gauge needle, 0.05 m*l* of 5-fold diluted high DP-specific IgE serum in saline was intradermally injected. Each injection site was marked with a permanent marker. Twenty-four hours later, 0.05 m*l* of 10-fold diluted DP antigen (final concentration, 1:10,000 w/v; Stallergenes Greer, London, U.K.) in saline was intradermally injected into the same site of the serum injection site. The same volume of saline and histamine diluents (5 μg/0.05 m*l*; Sigma-Aldrich) was injected as negative and positive controls, respectively. Since IgE is heat-labile [18], heat-inactivated high DP-specific IgE serum (56°C for 4 hr) was also intradermally injected as a control. To evaluate the wheal formations, edema sizes and erythema scores were measured 30 min after the DP injection. The edema sizes were expressed as the mean of the vertical and horizontal diameters of the area of edema. The erythema scores were graded from 0–3, where 0=none, 1=mild erythema, 2=moderate erythema, and 3=severe erythema according to a previous report [19]. Edema sizes and erythema scores induced by intradermal injections of high DP-specific IgE serum and DP antigen were significantly larger and higher, respectively, than those evoked by intradermal injections of serum alone, antigen alone, saline or heat-inactivated serum ($P<0.05$; Supplementary Fig. 1A and 1B). Edema and erythema were observed 30 min after the intradermal injection of DP antigen and lasted at least 6 hr, whereas those were not detected 24 hr after the injection (Supplementary Fig. 2A and 2B). Thus, the PK test in this study was suitable for evaluation of IgE-mediated immediate-type cutaneous reactions in dogs.

Effects of PF670462 were investigated using the PK test. In this experiment, 0.05 m*l* of saline or PF670462 (1 and 2.5 μmol/site; Merck Millipore, Burlington, MO, U.S.A.) dissolved in saline was intradermally injected to heathy dogs. Twenty-four hours later, high DP-specific IgE serum was intradermally injected into the same site of saline or PF670462 injection site, followed by the DP injection at a 24 hr interval. The edema sizes and erythema scores were determined as described above.

The normality of all data was analyzed by the Shapiro–Wilk test. Data among the groups were analyzed by the Kruskal-Wallis test, followed by the Shirley-Williams test, the Steel-Dwass test, or the Steel test, the Friedman test, followed by the Scheffe test, or one-way analysis of variance (ANOVA), followed by the Williams test or the Tukey test, depending on the normality. Data between

two groups were analyzed by the unpaired *t*-test. Statistical analyses were performed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan) software. $P<0.05$ was considered statistically significant.

We first investigated possible cytotoxic effects of PF670472 on canine PBMCs as normal canine immune cells and HRMC cells as canine mast cells at the concentrations of 0–10 μM for 0–24 hr of incubation. PF670462 did not affect the cell viability of canine PBMCs and mast cells under this condition ($P>0.05$; Supplementary Fig. 3A and 3B).
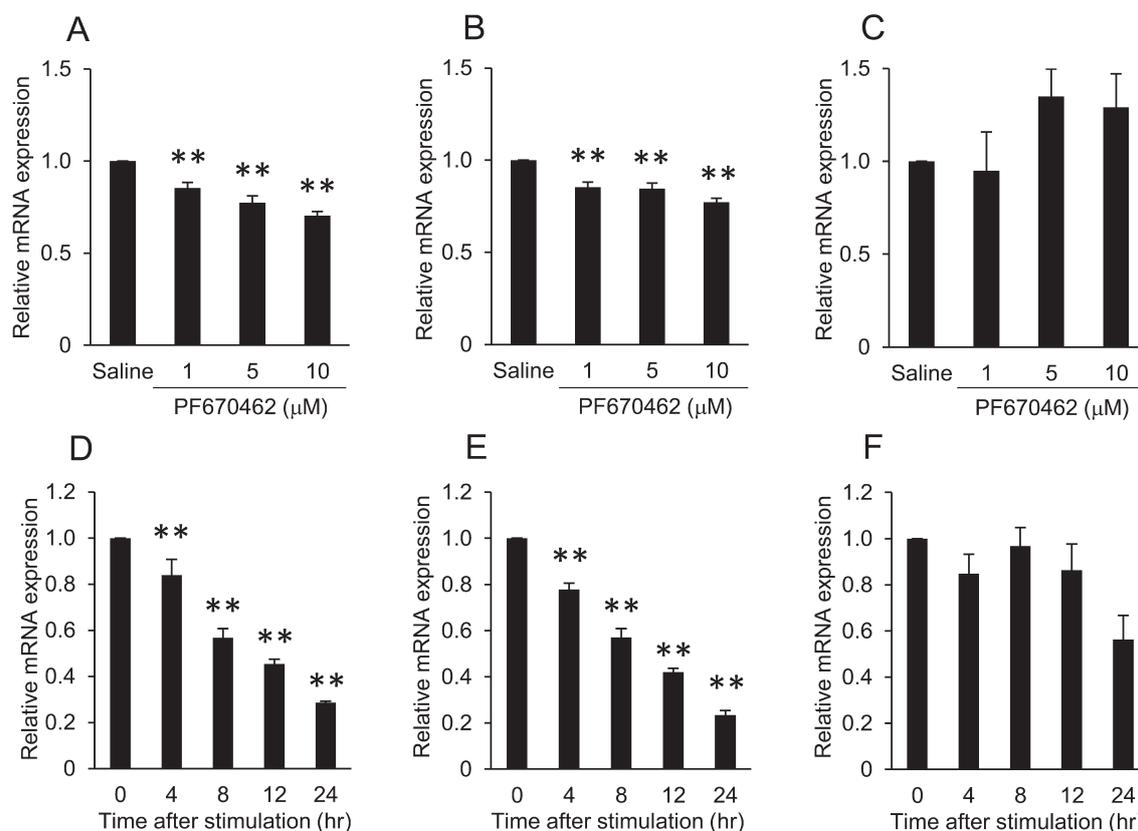
We next examined effects of PF670462 on mRNA expression of *FcεRIα*, *β*, and *γ*, components of the high-affinity IgE receptor, in canine mast cells. Increasing concentrations of PF670462 decreased mRNA expression of *FcεRIα* and *β*, but not *γ*, in canine mast cells in a dose-dependent manner (Fig. 1A–C); the expression levels of *FcεRIα* and *β* were significantly lower at 1–10 μM of PF670462 stimulation than saline stimulation ($P<0.01$; Fig. 1A and 1B), and reached a minimum at 10 μM. Transcription of *FcεRIα* and *β*, but not *γ*, mRNA also decreased depending on the incubation time of canine mast cells with PF670462 (Fig. 1D–F); the expression levels of *FcεRIα* and *β* were significantly lower at 4–24 hr incubation than at 0 hr incubation ($P<0.01$; Fig. 1D and 1E), and reached a minimum at 24 hr incubation.

Effects of PF670462 on protein expression of FcεRI were further evaluated in canine mast cells. Flow cytometric analysis revealed that PF670462 decreased cell surface expression of FcεRI in canine mast cells (Fig. 2A). MFI of FcεRI was significantly lower in canine mast cells cultured in the presence of PF670462 than those in the absence of PF670462 ($P<0.05$; Fig. 2B).

We finally determined the effects of PF670462 on IgE-mediated immediate-type cutaneous reactions in dogs. In the PK test, an intradermal injection of PF670462 (2.5 μmol/site) before sensitization with high DP-specific IgE serum significantly suppressed DP-induced erythema, but not edema, in the skin of healthy dogs ($P<0.01$; Fig. 3A, 3B, and Supplementary Fig. 4).

In this study, we clarified that PF670462 decreased mRNA expression of *FcεRIα* and *β*, but not *γ*, in canine mast cells in dose- and incubation time-dependent manners. We also demonstrated that PF670462 reduced cell surface expression of FcεRI in canine mast cells at a protein level. Furthermore, PF670462 was shown to suppress IgE and antigen-induced cutaneous erythema in the PK test. These findings suggest that PF670462 has suppressive effects on IgE-mediated immediate-type cutaneous reactions in dogs possibly due to attenuated degranulation of IgE-activated canine mast cells.

A previous study reported that PF670462 induced PER2 expression in murine mast cells [12]. PER2 inhibits CLOCK activity

Fig. 1. Dose- and incubation time-dependent effects of PF670462 on mRNA expression of *FcεRIα*, *β*, and *γ* in canine mast cells. (A–C) HRMC cells were incubated with saline or 1–10 μM PF670462 at 37°C for 24 hr. Relative mRNA levels of *FcεRIα* (A), *β* (B), and *γ* (C) were determined by real-time PCR. (D–F) HRMC cells were incubated with 10 μM PF670462 at 37°C for 0–24 hr. Relative mRNA levels of *FcεRIα* (D), *β* (E), and *γ* (F) were determined by real-time PCR. Data represent the mean of three independent experiments ± standard error. Data among the groups were analyzed by the Kruskal-Wallis test, followed by the Shirley-Williams test. **$P<0.01$, significant difference from the controls determined by saline (A–C) or 0 hr (D–F).
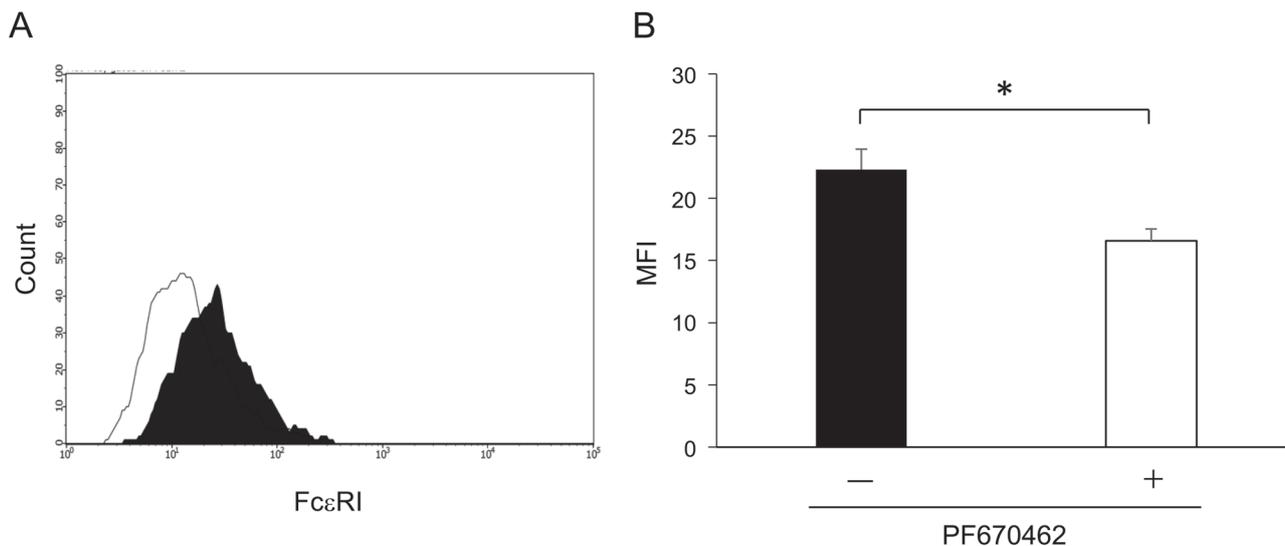
Fig. 2. Effects of PF670462 on cell surface expression of FcεRI in canine mast cells. (A, B) HRMC cells were cultured with 10 μM PF670462 (A, white histogram; B, white bar) or without PF670462 (A, black histogram; B, black bar) at 37°C for 24 hr in serum-free AIM-V medium. Cell surface expression of FcεRI was measured by flow cytometric analysis using high IgE serum and FITC-labeled goat anti-dog IgE antibody. Cell surface IgE bound to FcεRI was shown as histograms (A) and the mean fluorescence intensity (MFI) (B). Data represent one of four independent experiments (A) or the mean of four independent experiments ± standard error (B). Data between two groups were analyzed by the unpaired t-test. *$P<0.05$.
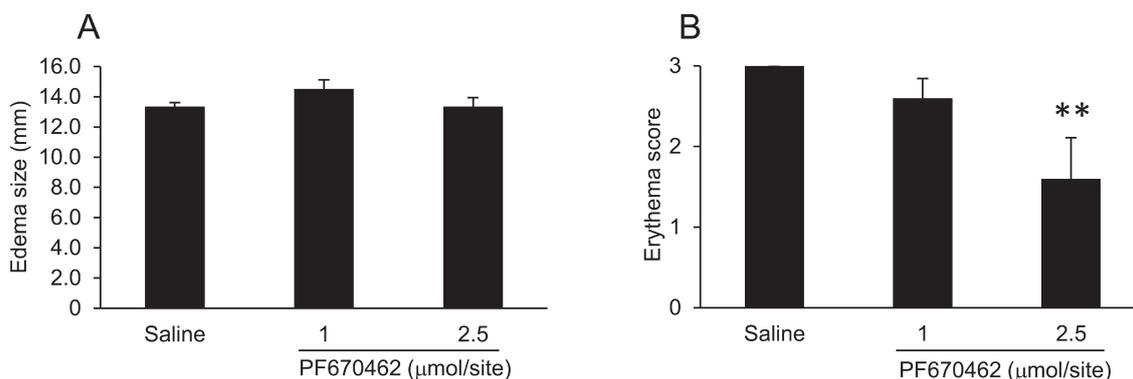
Fig. 3. Effects of PF670462 on IgE-mediated immediate-type cutaneous reactions. (A, B) Saline or PF670462 (1 and 2.5 μmol/site) were injected intradermally to heathy dogs. Twenty-four hours later, high *Dermatophagoides pteronyssinus* (DP)-specific IgE serum was intradermally injected into the same site of the saline or PF670462 injection site, followed by the intradermal injection of DP at a 24 hr interval. The edema sizes (A) and erythema scores (B) were measured at 30 min after the DP injection. Data represent the mean of five healthy dogs ± standard error. Data among the groups were analyzed by one-way ANOVA, followed by the Williams test (A), and the Kruskal-Wallis test, followed by the Shirley-Williams test (B). **$P<0.01$, significant difference from the controls determined by saline.

that was shown to regulate gene expression of *FcεRIβ* by directly binding to the promotor region in murine mast cells [13]. It is, therefore, assumed that PF670462-induced PER2 decreased gene expression of *FcεRIβ*, resulting in downregulation of cell surface expression of FcεRI in murine mast cells [12]. In this study, we clearly demonstrated that PF670462 decreased mRNA expression of *FcεRIα* and *β*, but not *γ*, in canine mast cells. PF670462-induced reduction in *FcεRIβ* gene in canine mast cells could be explained by the same mechanism in murine mast cells. However, our study suggests that PF670462 has other unknown mechanisms that diminish gene expression of *FcεRIα* in canine mast cells. To elucidate how PF670462 down-regulates gene expression of *FcεRIα* and *β* in canine mast cells, further studies are required.

In the dose- and incubation time-dependent effects of PF670462 on mRNA expression of *FcεRIα*, *β*, and *γ* in canine mast cells, transcription of *FcεRIα* and *β* appeared to be more severely affected in the incubation time-dependent experiment rather than the dose-dependent experiment. However, the controls used in the two experiments were slightly different. In the dose-dependent experiment, the control was canine mast cells cultured for 24 hr in the medium in the presence of saline instead of PF670462. In contrast, in the incubation time-dependent experiment, the control was canine mast cells that were not cultured in the medium

containing PF670462. Therefore, as transcription of *FcεRIβ* in murine mast cells was shown to be under the circadian control [13], intrinsic expression rhythms of *FcεRIα* and *β* in canine mast cells might have influenced the results in this study.

In the current study, an intradermal injection of PF670462 significantly suppressed IgE-mediated immediate-type cutaneous erythema in dogs. However, PF670462 did not affect edema sizes in the PK test. This discrepancy might be explained by the mild suppressive effects of PF670462 on IgE-mediated immediate-type cutaneous reactions in dogs. Although the concentrations of PF670472 in the PK test were determined based on those used in mice (50 mg/kg) [12], PF670462 did not completely inhibit cutaneous erythema in dogs, suggesting that mast cell degranulation still remained. In addition, an intradermal injection of saline only could induce some degrees of edema without erythema, as shown in Supplementary Fig. 1A. In this condition, PF670462 might have attenuated IgE-mediated immediate-type cutaneous erythema without affecting edema sizes in dogs.

In conclusion, we demonstrated that PF670462 suppressed IgE-mediated immediate-type cutaneous erythema in dogs. The suppressive effects of PF670462 were at least in part mediated by decreased cell surface expression of FcεRI through down-regulation of *FcεRIα* and *β* genes in canine mast cells. Since the suppressive effects of PF670462 on IgE-mediated immediate-type cutaneous reactions in dogs were mild even at high concentrations, PF670462 may not be suitable for clinical use in dogs with allergic diseases. However, the results in this study indicate that CK1δ/ε function as the regulators for FcεRI expression and IgE-mediated cutaneous reactions in dogs.

REFERENCES

1. Akashi, M., Tsuchiya, Y., Yoshino, T. and Nishida, E. 2002. Control of intracellular dynamics of mammalian period proteins by casein kinase I epsilon (CKIepsilon) and CKIdelta in cultured cells. *Mol. Cell. Biol.* **22**: 1693–1703. [Medline] [CrossRef]
2. Camacho, F., Cilio, M., Guo, Y., Virshup, D. M., Patel, K., Khorkova, O., Styren, S., Morse, B., Yao, Z. and Keesler, G. A. 2001. Human casein kinase Idelta phosphorylation of human circadian clock proteins period 1 and 2. *FEBS Lett.* **489**: 159–165. [Medline] [CrossRef]
3. Eide, E. J., Woolf, M. F., Kang, H., Woolf, P., Hurst, W., Camacho, F., Vielhaber, E. L., Giovanni, A. and Virshup, D. M. 2005. Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. *Mol. Cell. Biol.* **25**: 2795–2807. [Medline] [CrossRef]
4. Goto, S., Shimizu, S., Watanabe, M., Osada, H., Sasaki, K., Shimoda, M., Nagai, M., Shirai, J., Itoh, H. and Ohmori, K. 2016. Influence of glucocorticoids on a time-of-day-dependent variation in intradermal reactivity to histamine in dogs. *Vet. J.* **214**: 86–90. [Medline] [CrossRef]
5. Huang, W., Ramsey, K. M., Marcheva, B. and Bass, J. 2011. Circadian rhythms, sleep, and metabolism. *J. Clin. Invest.* **121**: 2133–2141. [Medline] [CrossRef]
6. Ko, C. H. and Takahashi, J. S. 2006. Molecular components of the mammalian circadian clock. *Hum. Mol. Genet.* **15**: R271–R277. [Medline] [CrossRef]
7. Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S. and Reppert, S. M. 2001. Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107**: 855–867. [Medline] [CrossRef]
8. Lee, H., Chen, R., Lee, Y., Yoo, S. and Lee, C. 2009. Essential roles of CKIdelta and CKIepsilon in the mammalian circadian clock. *Proc. Natl. Acad. Sci. U.S.A.* **106**: 21359–21364. [Medline] [CrossRef]
9. Marsella, R. and Girolomoni, G. 2009. Canine models of atopic dermatitis: a useful tool with untapped potential. *J. Invest. Dermatol.* **129**: 2351–2357. [Medline] [CrossRef]
10. Mueller, R. S. and Unterer, S. 2018. Adverse food reactions: Pathogenesis, clinical signs, diagnosis and alternatives to elimination diets. *Vet. J.* **236**: 89–95. [Medline] [CrossRef]
11. Nakamura, Y., Harama, D., Shimokawa, N., Hara, M., Suzuki, R., Tahara, Y., Ishimaru, K., Katoh, R., Okumura, K., Ogawa, H., Shibata, S. and Nakao, A. 2011. Circadian clock gene Period2 regulates a time-of-day-dependent variation in cutaneous anaphylactic reaction. *J. Allergy Clin. Immunol.* **127**: 1038–45.e1, 3. [Medline] [CrossRef]
12. Nakamura, Y., Nakano, N., Ishimaru, K., Ando, N., Katoh, R., Suzuki-Inoue, K., Koyanagki, S., Ogawa, H., Okumura, K., Shibata, S. and Nakao, A. 2016. Inhibition of IgE-mediated allergic reactions by pharmacologically targeting the circadian clock. *J. Allergy Clin. Immunol.* **137**: 1226–1235. [Medline] [CrossRef]
13. Nakamura, Y., Nakano, N., Ishimaru, K., Hara, M., Ikegami, T., Tahara, Y., Katoh, R., Ogawa, H., Okumura, K., Shibata, S., Nishiyama, C. and Nakao, A. 2014. Circadian regulation of allergic reactions by the mast cell clock in mice. *J. Allergy Clin. Immunol.* **133**: 568–575. [Medline] [CrossRef]
14. Nakao, A. 2018. Clockwork allergy: How the circadian clock underpins allergic reactions. *J. Allergy Clin. Immunol.* **142**: 1021–1031. [Medline] [CrossRef]
15. Ohmori, K., Kawarai, S., Yasuda, N., Tanaka, A., Matsuda, H., Nishimura, R., Sasaki, N., Tsujimoto, H. and Masuda, K. 2008. Identification of c-kit mutations-independent neoplastic cell proliferation of canine mast cells. *Vet. Immunol. Immunopathol.* **126**: 43–53. [Medline] [CrossRef]
16. Ohmori, K., Nishikawa, S., Oku, K., Oida, K., Amagai, Y., Kajiwara, N., Jung, K., Matsuda, A., Tanaka, A. and Matsuda, H. 2013. Circadian rhythms and the effect of glucocorticoids on expression of the clock gene period1 in canine peripheral blood mononuclear cells. *Vet. J.* **196**: 402–407. [Medline] [CrossRef]
17. Okayama, T., Matsuno, Y., Yasuda, N., Tsukui, T., Suzuta, Y., Koyanagi, M., Sakaguchi, M., Ishii, Y., Olivry, T. and Masuda, K. 2011. Establishment of a quantitative ELISA for the measurement of allergen-specific IgE in dogs using anti-IgE antibody cross-reactive to mouse and dog IgE. *Vet. Immunol. Immunopathol.* **139**: 99–106. [Medline] [CrossRef]
18. Tsukui, T., Sakaguchi, M., Kurata, K., Maeda, S., Ohmori, K., Masuda, K., Tsujimoto, H. and Iwabuchi, S. 2012. Measurement for canine IgE using canine recombinant high affinity IgE receptor α chain (FcεRIα). *J. Vet. Med. Sci.* **74**: 851–856. [Medline] [CrossRef]
19. Watanabe, M., Osada, H., Shimizu, S., Goto, S., Nagai, M., Shirai, J., Sasaki, K., Shimoda, M., Itoh, H. and Ohmori, K. 2016. Characterization of platelet-activating factor-induced cutaneous edema and erythema in dogs. *Am. J. Vet. Res.* **77**: 969–975. [Medline] [CrossRef]