Changes in the expression of gallinacins, antimicrobial peptides, in ovarian follicles during follicular growth and in response to lipopolysaccharide in laying hens (\textit{Gallus domesticus})

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Abstract

The aim of this study was to identify the types of gallinacin genes (GALs) expressed in ovarian follicles and to determine the changes in their expression during follicular growth and in response to lipopolysaccharide (LPS). Follicles at different stages of growth were collected from laying hens ($n = 5$) and LPS-injected hens ($n = 3$). The expression of GALs in the theca and granulosa layers was examined by semi-quantitative RT-PCR. The expression of GAL-1, -2, -7, -8, -10, and -12 in the theca layer and GAL-1, -8, -10, and -12 in the granulosa layer was identified in white and yellow follicles. The expression of these genes was not changed in the theca and granulosa layers during follicular growth except for a decrease in that of GAL-1 in theca. The expression of GAL-1, -7, and -12 in the theca layer of the third largest follicles was increased in response to LPS at a dose of 1 mg/kg body weight and this increase was induced within 3 h and maintained until 12 h postinjection. Granulosa layers did not respond to LPS until 12 h injection. These results show that six and four types of GALs are expressed in the theca and granulosa layers of healthy follicles respectively, and their levels do not change with follicular growth except for GAL-1 in theca. Elevated levels of GAL-1, -7, and -12 expression in theca in response to LPS suggest that the theca cells expressing these GALs function to eliminate LPS-containing bacteria.

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Introduction

The ovaries of mature hens consist of cortical follicles embedded in the ovarian stroma, and numerous white follicles (WF), several hierarchical yellow follicles, and postovulatory follicles (POF) protruding from the ovarian surface (Johnson 2000, Barua et al. 2001). The follicular wall consists of granulosa and theca layers, but ovarian follicles change in structure and function during follicular growth: namely, theca interna and externa develop with a decrease in the production of estrogen and increase in that of progesterone before ovulation (Gilbert 1979, Bahr et al. 1983). Ovarian follicles are the site in laying hens where foreign agents circulating in the blood accumulate (Yoshimura & Okamoto 1998). Pathogenic microorganisms may infect hen ovarian tissues and subsequently be transmitted to the eggs (Keller et al. 1995). \textit{Salmonella enteritidis} (SE) bacteria were isolated from ovaries following oral or i.v. inoculation (Keller et al. 1995, Withanage et al. 1998). Adaptive immunity via the major histocompatibility complex (MHC) and T cells has been examined in the ovarian follicles of healthy and infected birds, and the theca layers were suggested to be the major site where these immunocompetent cells reside (Barua & Yoshimura 2004a, 2004b, Subedi & Yoshimura 2005).

Recently, there are reports that defensin, a family of antimicrobial peptides, is one of the keys to the innate immune system which provides the initial defense against pathogens (Sugiarto & Yu 2004). The family is divided into $\alpha$, $\beta$, and $\theta$ defensins. Only $\beta$-defensin exists in chickens where it is known as gallinacin (Zhao et al. 2001, Xiao et al. 2004). Gallinacins attack a wide range of microorganisms including Gram-positive and Gram-negative bacteria, fungi, and yeast (Evans et al. 1995, Harmon 1998, Sugiarto & Yu 2004). A total of 13 different gallinacin genes (GALs), designated GAL-1 to -13, have been identified (Xiao et al. 2004). GAL-1 to -7 are predominantly expressed in bone marrow and respiratory tract, whereas GAL-8 to -13 are restricted to the liver and urogenital tract (Xiao et al. 2004). The expression of GAL-3 was observed in the ovary of immature chicken (Zhao et al. 2001). However, the expression of GALs in follicular tissue of laying hens has not been profiled. Changes in expression during
follicular growth or during infection in ovarian tissue have not been examined either. As ovarian follicles undergo dramatic changes in structure and function during follicular growth, the immunity in this tissue may also be changed. To confirm that gallinacins participate in the defense against pathogens, it is necessary to examine whether their expression is enhanced in response to microorganisms or their components. Lipopolysaccharide (LPS) is the major cell wall constituent of Gram-negative bacteria such as *Escherichia coli* and *Salmonella* (Morrison & Ryan 1987) and can be used to mimic bacterial infections and inflammation (Sunwoo et al. 1996, Leshchinsky & Klasing 2003). The goal of this study was to determine the types of gallinacins that play a role in the elimination of pathogens in the follicles of laying hens, specifically, which types of GALs are expressed in ovarian follicles and whether their expression changes with follicular growth in healthy birds. Furthermore, we studied the changes in the expression of GALs in response to an injection of LPS in vivo in order to show the possibility that specific GALs expressed by follicular cells participate to attack pathogens.

**Materials and Methods**

**Experimental birds**

White Leghorn hens approximately 400-day-old and laying five or more eggs in a sequence were used. They were kept in individual cages under a 14 h light:10 h darkness photoperiod with water and allowed to feed *ad libitum*. Hens were euthanized under anesthesia with sodium pentobarbital 18–20 h before the estimated time of ovulation (5 h after oviposition) to collect ovarian follicles as described previously (Subedi & Yoshimura 2005). Handling of chickens was done in accordance with regulation of Hiroshima University for animal experiments.

**Experimental design**

In experiment 1, the types of GALs expressed in ovarian stroma and follicles, and changes in the level of their expression with follicular growth were observed. White follicles (3–5 mm in diameter), the fifth and third largest follicles (F5 and F3 respectively), the largest follicles (F1), and postovulatory follicles were collected (n = 5). Ovarian stroma was also collected from the ovarian cortical tissue. The outer connective tissue surrounding the surface of each follicle was removed, and theca and granulosa layers were isolated separately from the follicles as described previously (Porter et al. 1989). For the POF sample, only the theca layer was isolated.

In experiment 2, the effects of LPS on the expression of GALs in F3 were observed *in vivo*. A stock solution of LPS was prepared by dissolving LPS from *E. coli* 0111:B4 (Wako Pure Chemical Industries, Osaka, Japan) in Dulbecco’s phosphate buffer (Nissui Pharmaceutical Co., Tokyo, Japan) at 0, 2, 4, and 8 mg/ml. Laying hens were injected intravenously with different doses of LPS, namely at 0, 0.5, 1, and 2 mg/kg body weight (BW) using the 0, 2, 4, and 8 mg/ml stock solutions, respectively, to know the dose-dependent effects of LPS (n = 3 each). The expression of each GAL was examined in the theca and granulosa layers at 3 h. Since the expression of GAL-1, -7, and -12 in the theca was most enhanced by LPS at a dose of 1 mg/kg BW, this concentration was used for examining the expression of GALs at different time points. The time course of changes in the expression of GALs in the theca and granulosa layers was examined at 0, 3, 6, and 12 h after the injection of LPS (1 mg/kg BW) (n = 3).

To examine the tissue dependency of GAL-1, -7, and -12 expression, their expression in the kidney and liver was also examined before and 3 h after the injection with 1 mg/kg BW LPS.

**RT-PCR and nucleotide sequencing**

RNA was extracted using sepasol RNA I super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s directions. The RNA sample was resuspended in TE buffer (10 mM Tris (pH 8.0) with 1 mM EDTA), and treated with 10 U DNase I (Roche Diagnostic GmbH, Pensburg, Germany) at 37 °C for 1 h, 80 °C for 30 min, and 4 °C for 5 min. Then the concentration of total RNA was determined using Gene Quant pro (Amersham Pharmacia Biotech, Cambridge, UK) and the RNA samples were stored at −80 °C until use.

Semi quantitative RT-PCR was performed to examine the expression of gallinacin mRNAs. The RNA samples were reverse transcribed using Rever Tra Ace (Toyobo Co. Ltd., Osaka, Japan) as described previously (Chowdhury et al. 2003). The reaction mixture (10 μl) consisted of 1 μg total RNA, 1 × RT buffer, 1 mM of each dNTP, 20 U RNase inhibitor, 0.5 μg oligo (dT)20, and 50 U Rever Tra Ace. The RT was performed at 42 °C for 30 min, followed by heat inactivation for 10 min at 99 °C using a Programmable Thermal Controller, the PTC-100 (MJ Research Inc., Waltham, MA, USA). The PCR was performed in the PTC-100 in a reaction mixture of 25 μl containing cDNA corresponding to 1 μg of the initial total RNA, 1 × PCR buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, and 0.625 U Takara Taq (Takara Bio Inc., Shiga, Japan). In order to determine the types of GALs expressed in the ovarian stroma, theca and granulosa layers of WF and F3, cDNA samples of these tissues were amplified using primers for all types of GALs (Table 1) at an annealing temperature ranging from 52 to 60 °C and with 40 PCR cycles. For the GALs expressed in the theca (GAL-1, -2, -7, -8, -10, and -12), different cycles of PCR, namely 30, 35, 40, and 45, were tested to optimize the amplification using the cDNA sample of theca tissue of F3. A linear response for the 30 through 45

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cycles was observed, and 35 cycles for GAL-12 and 40 cycles for GAL-1, -2, -7, -8, and -10 were considered optimal. Each observed GALs was amplified using an optimal number of cycles to examine changes in their expression level during follicular growth and in response to LPS in both theca and granulosa layers. The cycle parameters were denaturation at 94°C for 30 s, 30 cycles (for β-actin) or 35 cycles (for GAL-12) or 40 cycles (for GAL-1 to -11) of denaturation at 94°C for 30 s, annealing at 58°C (for β-actin, GAL-3, -4, -5, -6, -7, -9, -10, and -11) or 60°C (for GAL-1, -2, -8, and -12) for 30 s for all GALs or 1 min for β-actin, and extension at 72°C for 1 min, and a final extension at 72°C for 6 min. β-actin was used for standardization for each gallinacins. PCR products were separated by electrophoresis on 3% (w/v) agarose gels containing ethidium bromide (0.5 mg/mL) and photographed under UV illumination. Densitometry was performed using a Gel-Pro analyzer (Media Cybernetics, Silver Spring, MD, USA), and the ratio of gallinacin mRNA to β-actin mRNA was obtained.

The PCR products of obtained GALs were sequenced using a Big Dye terminator Sequence Kit (ver. 3.1, Applied Biosystems, Foster City, USA) with an ABI 3100 automated sequencer (Applied Biosystems, Foster City, USA), as described previously (Ohashi et al. 2005) to confirm correspondence to the sequences in GenBank.

**Results**

**Experiment 1**

Out of the 13 GALs reported, 6 GALs (GAL-1, -2, -7, -8, -10, and -12) were observed in the theca layer and 4 GALs (GAL-1, -8, -10, and -12) in the granulosa layer of white and yellow follicles, WF and F3 (Fig. 1A and B respectively). Twelve types of GALs (GAL-1 to -12) were observed in the ovarian stroma (Fig. 1C). The expression of GAL-13 was not observed in both ovarian stroma and follicles. The expression of GAL-3, -4, -5, -6, -9, and -11 was also not observed in ovarian follicles. The expression of GAL-1 was significantly decreased in the theca layer with follicular growth (Fig. 2A). However, the levels of expression of GAL-2, -7, -8, -10, and -12 in the theca layer were not different among WF, F3, F1, and POF (Fig. 2B–E respectively). The expression of GAL-1, -8, -10, and -12 in granulosa layer did not show a significant difference among WF, F3, and F1 (data not shown).

**Statistical analysis**

The results were expressed as the actual mean ± s.e.m. of the ratio of GAL to β-actin mRNA. Prior to analysis, data were first analyzed by Bartlett’s test to ensure homogeneity of variance. If significance was found for a particular parameter (where raw data were heterogeneous), square root transformations were performed. The significance of differences was examined using a one-way ANOVA followed by Duncan’s multiple range test. The Kruskal–Wallis one-way ANOVA was used when unequal variances were found even after transformation. Experiment levels of GALs in liver and kidney were compared between control and treated groups using Student’s t-test. A difference with a P value of < 0.05 was considered statistically significant.
granulosa layer did not respond to LPS at doses of 0.5, 1, and 2 mg/kg BW (Fig. 4B–D respectively).

Changes in the expression of GALs in the theca layer of F3 0, 3, 6, and 12 h after the administration of LPS (1 mg/kg BW) are shown in Fig. 5. The expression of GAL-1, -7, and -12 was significantly increased at 3 h when compared with 0 h and remained relatively high until 12 h postinjection (Fig. 5A, C, and F respectively). The GAL-2, -8, and -10 in the theca layer did not respond to LPS during 12 h (Fig. 5B, D, and E respectively).

The expression of GAL-8 and -10 in the granulosa layer of F3 did not show a significant difference until 12 h after the administration of LPS (Fig. 6B and C respectively). However, the expression of GAL-1 and -12 in the granulosa layer of F3 decreased at 3 h and was low at or until 12 h (Fig. 6A and D respectively).

Unlike in the theca layer, the levels of the expression of GAL-1, -7, and -12 in kidney and liver were not affected by 3-h postinjection of 1 mg/kg BW of LPS (data not shown).

Discussion

We are the first to report the expression of gallinacins, antimicrobial peptides, in ovarian follicles and changes in them during follicular growth and in response to LPS. The noteworthy findings were: (1) out of the 13 GALs reported to date, 12, 6, and 4 types were expressed in the ovarian stroma, theca, and granulosa layers of WF and hierarchical yellow follicles (F3) respectively, (2) mRNA levels of these GALs in the theca and granulosa layers did not change with follicular growth, except for a decline in the expression of GAL-1 in the theca layer, and (3) the injection of LPS in vivo enhanced the expression of GAL-1, -7, and -12 in the theca layer of F3 but not in the granulosa layer.

The current study showed that GAL-1, -2, -7, -8, -10, and -12 were expressed in the theca cells and GAL-1, -8, -10, and -12 in the granulosa cells of laying hens. Theca layer is a heterogeneous tissue consisting of fibroblast-like cells, interstitial cells, nerve cells, and endothelial cells of capillaries (Gilbert 1979, Yoshimura & Bahr 1995). GAL-2 and -7 that observed in the theca but not in the granulosa might be expressed in these theca cells. Although Zhao et al. (2001) found the expression of GAL-3 in the ovary of immature hens, this expression was not observed in both theca and granulosa layers in the current study. Our study showed that the ovarian stromal tissue expressed 12 types of GALs (GAL-1 to -12) including GAL-3. Stromal cells are likely to express more GALs than theca and granulosa cells. Thus, the expression of GAL-3 in the ovary of immature hen observed by Zhao et al. (2001) might be due to the expression in the stroma rather than follicular cells.
Out of six types of theca GALs, GAL-2, -7, -8, -10, and -12 did not change in their expression, whereas the level of GAL-1 decreased during follicular growth and postovulation. None of the four types of GALs in the granulosa cells changed with follicular growth. The most distinct change in the matured ovarian tissue is the development of follicles during follicular growth, including the differentiation of theca into theca externa and interna layers with the development of a vascular architecture and proliferation of granulosa cells (Gilbert 1979). We have observed that the theca cell proliferation was high in growing follicles, although it was decreased from F3 to F1 (Yoshimura et al. 1996). The current study suggests that although the follicular structure are changed with growth, the expression of GALs in the theca and granulosa layers, except for theca GAL-1, is unchanged during follicular growth under normal conditions. Only the theca expression of GAL-1 that decreased in association with follicular growth might be affected by the follicular cell population and/or the change in their functions during the growth of follicles.

The expression of theca GAL-1, -7, and -12 was enhanced in response to 1 mg/kg BW of LPS within 3h injection and remained at relatively high levels until 12 h, although 2 mg/kg BW of LPS did not increase the expressions. The expression of GAL-8 was not enhanced by 1 mg/kg BW of LPS until 12 h, whereas it was enhanced by 2 mg/kg BW of LPS. However, the expression of GAL-2 and -10 was not affected by LPS. Thus, we show that at least the theca cells are able to respond to LPS and the expression of GAL-1, -7, and -12

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**Figure 3** Changes in the expression of gallinacin mRNA (GALs) in the theca layer of F3 at doses of 0, 0.5, 1, and 2 mg/kg BW of LPS after 3h injection. (A) GAL-1, (B) GAL-2, (C) GAL-7, (D) GAL-8, (E) GAL-10, and (F) GAL-12. The values are the mean ± S.E.M. of the ratio of GAL to β-actin mRNA (n = 3). Means with different letters differ significantly (P<0.05).

**Figure 4** Changes in the expression of gallinacin mRNA (GALs) in the granulosa layer of F3 at doses of 0, 0.5, 1, and 2 mg/kg BW of LPS after 3h injection. (A) GAL-1, (B) GAL-8, (C) GAL-10, and (D) GAL-12. See Fig. 3 for other explanations.

**Figure 5** Changes in the expression of gallinacin mRNA (GALs) in the theca layer of F1 at different time points after the injection of 0, 3, 6, and 12 h of LPS (1 mg/kg BW). The values are the mean ± S.E.M. of the ratio of GAL to β-actin mRNA (n = 3). (A) GAL-1, (B) GAL-2, (C) GAL-7, (D) GAL-8, (E) GAL-10, and (F) GAL-12. Means with different letters differ significantly (P<0.05).
is enhanced. It remains to be studied whether theca cells expressing GAL-2 and -10 are stimulated by other pathogenic agents. The expressions of GAL-1, -7, and -12 were not enhanced in kidney and liver within 3 h LPS injection (1 mg/kg BW), which enhanced the expressions in theca. These results show that the effects of LPS on GALs expression was different among the tissues, and the theca layer is more sensitive to LPS and/or respond earlier to it than kidney and liver.

The theca gallinacins, whose gene expression was enhanced by LPS, are expected to act on pathogenic microorganisms such as Salmonella and E. coli to eliminate them from the follicular tissues. Furthermore, these gallinacins may play a role in inducing adaptive immunity to antigens by inducing the formation of immunocompetent cells. In mammals, β-defensins exert chemotactic effects on dendritic cells and resting memory T cells to link innate immunity with the adaptive immune system (Yang et al. 1999, Oppenheim et al. 2003, Wu et al. 2003). We have demonstrated that the i.p. or i.v. inoculation of laying hens with Salmonella antigens increased numbers of MHC class II expressing cells, and CD4+ and CD8+ T cells in the theca layer of white and larger yellow follicles (Yoshimura & Takata 2002, Barua & Yoshimura 2004a). The theca GALs whose level of expression was increased by LPS may mediate part of that process.

In the granulosa cells, four types of GALs, GAL-1, -8, -10, and -12 were expressed, but were not enhanced by LPS unlike in the theca layer, and rather, the expression of GAL-1 and -12 tended to decline after the injection of LPS (Figs 4 and 6). The reason why the expression of some of the GALs was decreased is unknown. We suggest that the theca layer is the primary site where gallinacins are synthesized in response to bacterial antigens in vivo.

In conclusion, we show that 12 types of GALs in the stroma, 6 in the theca layer and 4 in the granulosa layer are expressed in healthy follicles, and their expression in the follicles does not change with follicular growth except for a decrease in theca GAL-1. Among the follicular cells expressing these GALs, those expressing GAL-1, -7, and -12 could respond to LPS, suggesting that GALs are involved in defense against Gram-negative bacteria. The theca tissue where GAL expression was increased in response to LPS may be the primary site for such a system of defense.

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Figure 6 Changes in the expression of chicken gallinacin mRNA (GALs) in the granulosa layer of F3 at different time points after the injection of 0, 3, 6, and 12 h of LPS (1 mg/kg BW). (A) GAL-1, (B) GAL-8, (C) GAL-10, and (E) GAL-12. See Fig. 5 for other explanations.

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