Changes in the expression of gallinacins, antimicrobial peptides, in ovarian follicles during follicular growth and in response to lipopolysaccharide in laying hens (*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 12h postinjection. Granulosa layers did not respond to LPS until 12h 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.

Reproduction (2007) **133** 127–133

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). *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 α , β , and θ defensins. Only β -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 (F_5 and F_3 respectively), the largest follicles (F_1), 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 F_3 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, Painsburg, 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 μ l) consisted of 1 μ g total RNA, 1 $\times$ RT buffer, 1 mM of each dNTP, 20 U RNase inhibitor, 0.5 μ g 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 μ l containing cDNA corresponding to 1 μ g of the initial total RNA, 1 $\times$ PCR buffer, 0.2 mM of each dNTP, 0.4 μ M 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 F_3 , 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 F_3 . A linear response for the 30 through 45

Table 1 The PCR primer sequences with their accession numbers used in the tissue expression profiling of *GALs* and β -actin.

Target	Primers 5'-3'	Accession numbers
<i>GAL-1</i>	F-AAACCATGCGGATCGTGACCTGC	R-CAATGCTAAACTGCACACCTTTA
<i>GAL-2</i>	F-GTTCTGTAAAGGAGGGTCTGCCAC	R-ACTCTACAACACAAAACATATTGC
<i>GAL-3</i>	F-CTGCCGCTTCCACACATAG	R-GCAATGCCAACTGCACGCCTTTA
<i>GAL-4</i>	F-ATCGTGCTCTCTTTGTGGCAGTTCA	R-CTACAACCATCTACAGCAAGAATACT
<i>GAL-5</i>	F-ATGCAGATCCTGCCTCTCTCTTTGCT	R-TCAGGAATACCATCGGCTCCGGCAGCAGAA
<i>GAL-6</i>	F-GATCCTTTACCTGCTGCTGTCT	R-TCCTCACACAGCAAGATTTAGTC
<i>GAL-7</i>	F-CTGCTGTCTGCTCTCTTTGTGG	R-CATTTGGTAGATGCAGGAAGGA
<i>GAL-8</i>	F-ACAGTGTGAGCAGGCAGGAGGGA	R-CTCTTCTGTTTCAGCCTTTGGTG
<i>GAL-9</i>	F-ATGAGAATCCTTTCTTCCTGTGTC	R-TTAGGAGCTAGGTGCCCATTTGCAGC
<i>GAL-10</i>	F-CTGTTCTCTCTTCTCTTCCAG	R-AATCTTGGCAGCAGTCTTAACA
<i>GAL-11</i>	F-ACTGCATCCGTTCCAAAGTCTG	R-TCGGGCAGCTTCTCTACAAC
<i>GAL-12</i>	F-CCCAGCAGGACCAAGCAATG	R-GTGAATCCACAGCCAATGAGAG
<i>GAL-13</i>	F-CATCGTTGTCTTCTCTCTC	R-ACTTGCAGCGTGTGGGAGTTG
β -actin	F-TTCCAGCCATCTTCTTG	R-TCCTTCTGCATCTGTCA

F, forward; R, reverse.

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.

Statistical analysis

The results were expressed as the actual mean $\pm$ 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. Expression 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.

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. 1 A 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, F₅, F₃, F₁, and POF (Fig. 2B–F respectively). The expression of *GAL-1*, -8, -10, and -12 in granulosa layer did not show a significant difference among WF, F₅, F₃, and F₁ (data not shown).

Experiment 2

The expression of *GAL-1*, -7, and -12 in the theca layer of F₃ was enhanced by LPS at a dose of 1 mg/kg BW, although levels declined to those of the control at 2 mg/kg BW (Fig. 3A, C, and F respectively). Only the expression of *GAL-8* in the theca layer was enhanced by LPS at a dose of 2 mg/kg BW (Fig. 3D). The expression of *GAL-2* and -10 was not enhanced in response to LPS at the doses examined in this study (Fig. 3B and E respectively). The expression of *GAL-1* in the granulosa layer of F₃ was decreased by LPS at 0.5–2 mg/kg BW (Fig. 4A). The expression of *GAL-8*, -10, and -12 in the

Figure 1 Expression pattern of gallinacin mRNA (GALs) determined by RT-PCR analysis in the (A) theca and (B) granulosa layers of WF and F₃, and (C) ovarian stroma of laying hen. Out of 13 reported gallinacins, GAL-1, -2, -7, -8, -10, and -12 are observed in the theca layer, whereas GAL-1, -8, -10, and -12 are expressed in the granulosa layer.

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

Figure 2 Changes in the expression of gallinacin mRNA (GALs) in the theca during follicular growth in laying hens. (A) GAL-1, (B) GAL-2, (C) GAL-7, (D) GAL-8, (E) GAL-10, and (F) GAL-12. The values are the mean $\pm$ S.E.M. of the ratio of GALs to β -actin mRNA ($n=5$). WF, white follicles; F₅, the fifth largest follicles; F₃, the third largest follicles; F₁, the largest follicles; and POF, the largest postovulatory follicles. Means with different letters differ significantly ($P<0.05$).

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.

Figure 3 Changes in the expression of gallinacin mRNA (GALs) in the theca layer of F_3 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 $\pm$ S.E.M. of the ratio of GAL to β -actin mRNA ($n=3$). Means with different letters differ significantly ($P<0.05$).

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

Figure 4 Changes in the expression of gallinacin mRNA (GALs) in the granulosa layer of F_3 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 F_3 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 $\pm$ 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 ($P<0.05$).

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 F_3 to F_1 (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*

Figure 6 Changes in the expression of chicken gallinacin mRNA (GALs) in the granulosa layer of F_3 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 (D) GAL-12. See Fig. 5 for other explanations.

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 they 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.

Acknowledgements

Authors would like to thank Dr T Bungo for helpful suggestions for statistical analysis and Mr Y Yamashita for analysis of the density of PCR bands. This work was supported by a Grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 27 June 2006

First decision 23 September 2006

Accepted 2 October 2006