The aim of this study was to determine the mechanism by which expression of avian β-defensins (AvBD) in the follicular theca tissue was regulated. It was examined whether their expression was stimulated directly by LPS or indirectly through proinflammatory cytokines (IL-1β and IL-6) induced by LPS. Theca tissues of ovarian follicles were collected from White Leghorn hens. The specimens of these theca tissues were cultured in TCM-199 culture medium and stimulated by lipopolysaccharide from Salmonella minnesota (LPS), recombinant chicken IL-1β, or recombinant chicken IL-6. In the first experiment, changes in the expression of IL-1β, IL-6, AvBD10, and AvBD12 in response to LPS stimulation were examined by quantitative reverse-transcription PCR. The AvBD10 and 12 had been known to be expressed in the theca. In the second experiment, changes in the expression of AvBD10 and 12 in response to recombinant chicken IL-1β or IL-6 stimulation were examined by quantitative reverse-transcription PCR. Density of AvBD12 protein after IL-1β stimulation that showed changes in the gene expression was analyzed by Western blotting. In the first experiment, LPS was able to induce IL-1β and IL-6, but not AvBD10 or AvBD12. In the second experiment, IL-1β was able to upregulate significantly the expression of AvBD12 mRNA and protein. However, IL-6 did not exert significant effects on the expression of AvBD10 and AvBD12. It is suggested that LPS may stimulate theca cells to produce proinflammatory cytokines, whereas, in turn, IL-13 stimulates those cells to synthesize AvBD12, which may be able to attack infectious gram-negative bacteria.

Key words: hen ovarian follicle, β-defensin, lipopolysaccharide, interleukin

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types of AvBD genes and immunoreactive AvBD8, 10, and 12 have been identified (Subedi et al., 2007b; Abdelsalam et al., 2010; Michaillidis et al., 2012). Intravenous injection of chickens with LPS caused an increase in the expressions of some AvBD including AvBD12 as well as IL-1β and IL-6 in the theca layer of ovarian follicles (Subedi et al., 2007b; Abdelsalam et al., 2011), whereas the AvBD expression in the granulosa cells was not increased by LPS treatment (Subedi et al., 2007b). Increases in the expression of several AvBD in the oviduct and cecal tonsil infected by Salmonella (Yoshimura et al., 2006; Akbari et al., 2008) and in the oviduct stimulated by LPS are also reported (Abdel Mageed et al., 2008). In humans, it is reported that human β-defensin (DEFB)-4 expression was induced in different cells by IL-1β (Liu et al., 2003; McDermott et al., 2003). In chickens, intravenous injection with LPS modulated AvBD expression in the theca tissue where TLR4 recognizing LPS is expressed (Subedi et al., 2007a). However, it remains unknown whether the expression of AvBD was induced by the direct effect of LPS stimulation or mediated by cytokines synthesized in response to LPS.

The aim of this study was to determine the mechanism by which expression of AvBD in the follicular theca tissue was regulated. It was examined whether their expression was stimulated directly by LPS or indirectly through IL-1β and IL-6 induced by LPS. We choose AvBD10 and 12 among 14 types of AvBD because they responded differently to LPS in previous in vivo study; namely, expression of AvBD12 was increased by LPS, but change in the expression of AvBD10 was not significant (Subedi et al., 2007b).

MATERIALS AND METHODS

Birds and Tissue Sampling

White Leghorn hens approximately 300-d-old and laying 5 or more eggs in a sequence were used. Hens were kept in individual cages under a lighting regimen of 14L:10D and provided with feed and water ad libitum. The hens were euthanized under anesthesia with Somnopentyl (Kyoritsu Pharmaceutical Co. Ltd., Tokyo, Japan). Six hours after oviposition, the second (F2) and third (F3) largest preovulatory follicles were collected. Birds were handled in accordace with the Animal Experiment Committee regulation of Hiroshima University. Superficial connective tissue of the theca was removed. The theca was separated from the granulosa layer and washed in PBS. Theca tissue was cut into small pieces (approximately 5 × 5 mm) and placed in a sterile tube for culture (Greiner Bio-one Co. Ltd., Tokyo, Japan) containing 4 mL of culture medium.

Experimental Design

The theca tissue was cultured as described below. The effects of LPS on cytokines and AvBD expression (experiment 1) and that of cytokines on AvBD gene and protein expression (experiment 2) were examined.

**Experiment 1. Effects of LPS on the Expression of Cytokines and AvBD**

The dose dependency and time course of the effects of LPS on the cytokines (IL-1β and IL-6) and AvBD (AvBD10 and 12) were examined. The theca layer isolated from F2 was used for dose-dependency examination and F3 for time-course analysis (n = 5 birds). They were cultured as described below and stimulated by LPS (lipopolysaccharide from Salmonella minnesota; Invitrogen, San Diego, CA) at concentrations of 0, 10², 10³, or 10⁴ ng/mL for 3 h (dose dependency) or 10³ ng/mL of LPS for 0, 0.5, 1, or 3 h (time course).

**Experiment 2. Effects of IL-1β and IL-6 on AvBD Expression**

The dose dependency and time course of the effects of IL-1β and IL-6 on AvBD10 and 12 gene and protein expression in the cultured theca were examined. The theca of F2 and F3 were isolated for dose and time dependency analysis of AvBD gene expression, respectively (n = 5 and 4 birds for IL-1β and IL-6 stimulation, respectively; different birds from those used in experiment 1). The isolated theca tissues were cultured as described below and stimulated with IL-1β (recombinant chicken interleukin-1β; Abderseere Ltd., Oxford, UK) or IL-6 (recombinant chicken IL-6; Abderseere Ltd.) dissolved in sterile PBS at 0, 10², or 10³ ng/mL for 3 h (dose dependency); 10² ng/mL for 0, 1, or 3 h (time course). The theca tissue of these follicles were also incubated with 0 to 10³ ng/mL of IL-1β for 5 h to examine the effects on the AvBD12 protein expression, whose gene expression was upregulated (n = 5; different birds from those used for other experiments).

**Tissue Culture**

Incubation was performed in a CO² incubator at 37°C under a humidified atmosphere of 5% CO₂ and 95% air. The culture medium was TC-199 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Cosmo Bio, Tokyo, Japan).

**Real-Time PCR Analysis**

Total RNA was extracted from cultured tissues using Sepazol 1 super (Nacalai Tesque Inc., Kyoto, Japan) as described previously (Abdelsalam et al., 2011). It was purified by incubating at 42°C for 45 min with DNase I (TaKaRa Bio Inc., Shiga, Japan) at a concentration of 1 U/μg of RNA. Purified RNA samples were reverse transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) following the manufacturer’s instruc-
ations. Briefly, 10 μL of reaction mixture containing 1 μg of total RNA, 1 × RT buffer, 1 mM each deoxyribonucleotide triphosphate mixture, 20 U RNase inhibitor, 0.5 mM oligo (dT), and 50 U ReverTra Ace was incubated at 42°C for 30 min using Programmable Thermal Controller PTC-100 (MJ Research Inc., Waltham, MA). Quantitative PCR analysis was performed for *IL-1β*, *IL-6*, *AvBD10*, and *AvBD-12* expression using the Roche Light Cycler (Roche Applied Science, Indianapolis IN) as described previously (Abdelsalam et al., 2011). Expression of ribosomal protein S17 (*RPS17*) was examined as an internal control to normalize the values of each sample. A total of 20 μL of reaction mixture containing 1 × SYBR Premix Ex TaqII (Ta-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Accession No.</th>
</tr>
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<tbody>
<tr>
<td><em>AvβD-10</em></td>
<td>Forward: CTGTTCCTCTTCCTCTCCAG</td>
<td>NM_001001609</td>
</tr>
<tr>
<td></td>
<td>Reverse: AATCTTGGCAGAGGTTAACA</td>
<td></td>
</tr>
<tr>
<td><em>AvβD-12</em></td>
<td>Forward: GGAACCTTTGTTTCGTGTTCA</td>
<td>AY534898</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAGAATGACGGGTTCAAGGC</td>
<td></td>
</tr>
<tr>
<td><em>IL-1β</em></td>
<td>Forward: ACTGGGCATCAAGGGCTA</td>
<td>NM_204524</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGTAGAAGATGAAGCGGGTC</td>
<td></td>
</tr>
<tr>
<td><em>IL-6</em></td>
<td>Forward: AGAAATCCCTCCTCGCCAAT</td>
<td>NM_204524</td>
</tr>
<tr>
<td></td>
<td>Reverse: AATAGCGAAGCGGCCCTCA</td>
<td></td>
</tr>
<tr>
<td><em>RPS17</em></td>
<td>Forward: AAGCTGCGAGAGGAGGAGG</td>
<td>NM_204217</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGTTGGACAGGCTGCGGAAG</td>
<td></td>
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</tbody>
</table>

1 *avβD* = avian β-defensin.
2 *RPS17* = ribosomal protein S17.

Figure 1. Effects of lipopolysaccharide (LPS) stimulation on the mRNA expression of *IL-1β* and *IL-6* in the theca layer. A and C: Expression level of *IL-1β* and *IL-6* mRNA in theca tissue stimulated with 0 to 10⁴ ng/mL of LPS for 3 h. B and D: Expression level of *IL-1β* and *IL-6* mRNA in theca tissue stimulated with 10³ ng/mL of LPS for 0 to 3 h. Each value is the mean ± SE of fold changes in expression (n = 5). Bars with different letters (a–c) are significantly different (P < 0.05).
KaRa Bio Inc.), 0.2 μM of each forward and reverse primers (Table 1), 1 μL of cDNA and DNase free water was placed into 20-μL capillaries (Roche Diagnostics GmbH, Mannheim, Germany). Following denaturation at 95°C for 30 s, PCR was carried out with a thermal protocol of 95°C for 5 s and 60°C for 20 s. Specificity of the amplified products was verified by melting curve analysis and by running the products on 2% (wt/vol) agarose gel. Data analysis was performed as described previously by Abdelsalam et al. (2011). Namely, the ΔCT was calculated for each sample by subtracting the threshold cycle (CT) value of RPS17 (internal control) from the CT of the respective target gene. For relative quantification, the ΔCT value of RPS17 was then subtracted from the ΔCT of each experimental sample to generate the ΔΔCT. The ΔΔCT value was therefore fit to the formula $2^{-\Delta\Delta CT}$ to calculate the approximate fold difference.

Figure 2. Effects of lipopolysaccharide (LPS) stimulation on the mRNA expression of avian β-defensin (AvBD) 10 and AvBD12 in the hen ovarian theca layer. A and C: Expression level of AvBD10 and AvBD12 mRNA in theca tissue stimulated with 0 to $10^4$ ng/mL of LPS for 3 h. B and D: Expression level of AvBD10 and AvBD12 in theca tissue stimulated with $10^3$ ng of LPS/mL for 0 to 3 h. Each value is the mean ± SEM of fold changes in expression (n = 5).

SDS-PAGE and Western Blot

Theca tissue was homogenized separately in a 5× volume of homogenization buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 0.1% (wt/vol) SDS, and 1 mM phenylmethylsulfonylfluoride using a polytron homogenizer (Polytron PT1200c, Kinematica AG, Littau, Switzerland). The samples were centrifuged at 12,000 × g for 20 min at 4°C. The supernatant was collected and the protein concentration was measured using a protein assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumen as the standard protein.

The samples were separated by Tricine-sodium dodecyl sulfate-PAGE (Tricine SDS-PAGE; 16% separating gel and 4% stacking gel) as described by Abdelsalam et al. (2011). Samples were mixed with sample buffer composed of 30% (vol/vol) glycerol, 5% (vol/vol)
mercaptoethanol, 4% (wt/vol) SDS, 0.06% (wt/vol) bromophenol blue, and 150 mM Tris-HCl, pH 7.0, at a sample protein concentration of 1 μg/μL and boiled for 5 min. Each 10-μL sample mixture was run on gels.

After SDS-PAGE, the proteins in the gel were electrophoretically transferred onto a PVDF membrane (Bio-Rad) at 270 mA for 1 h. The membrane was soaked in methanol for 10 min and then washed briefly with Tris-buffered saline containing 0.1% Tween20 [TBS-T; 20 mM Tris HCl, pH 7.6, 0.8% (wt/vol) sodium chloride, and 0.1% (vol/vol) Tween 20]. It was incubated with 5% (wt/vol) casein milk (Roche, Mannheim, Germany) solution in TBS-T for 60 min and then incubated with rabbit anti-chicken AvBD12 polyclonal antibody (Abdel Mageed et al., 2009) diluted at a concentration of 10 μg/mL in TBS-T or mouse anti-chicken β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5,000 in TBS-T overnight at 4°C. The membrane was then washed in TBS-T for 30 min (10 min × 3 times) before incubation with peroxidase labeled anti-rabbit IgG for AvBD12 or anti-mouse IgG for β-actin (GE Healthcare, Buckinghamshire, UK) diluted at 1:5,000 in TBS-T for 1 h at room temperature. The membrane was washed with TBS-T for 30 min (10 min × 3 times) and the immunoprecipitates on the membrane were treated by Amersham ECL Western blotting detection reagents (GE Healthcare) for 1 min. Images were taken using an ATTO cooled CCD camera system E2-Capture II (Atto Co., Tokyo, Japan). Band intensity was measured using computer software CS analyzer version 3 (Atto Co.).

**Statistical Analysis**

The significance of differences in the expressions of IL-1β, IL-6, and AvBD gene and proteins among different treatment groups with LPS, IL-1β, or IL-6 was determined by one-way ANOVA, followed by Tukey’s test. Differences were considered significant at P < 0.05.

**RESULTS**

**Experiment 1. Effect of LPS on the Expression of Cytokines and AvBD**

Figure 1 shows the effect of LPS stimulation on IL-1β and IL-6 expression in the theca of the ovarian follicle.
The expressions of IL-1β and IL-6 were significantly upregulated with the increase of LPS doses from 0 to $10^4$ ng/mL with a peak at $10^3$ ng/mL. Expressions of both cytokines also increased with time after stimulation with $10^3$ ng/mL.

Figure 2 shows the effect of LPS stimulation on the expression of AvBD10 and AvBD12 in the theca. There was no significant change in the expression of both AvBD in response to different doses of LPS from 0 to $10^4$ ng/mL, and to different times of incubation (0 to 3 h) with $10^3$ ng/mL of LPS.

**Experiment 2. Effects of IL-1β and IL-6 on AvBD Expression**

Figure 3 shows the effect of recombinant IL-1β stimulation on the expression of AvBD10 and AvBD12 in theca tissue. The IL-1β stimulation showed no significant effect on the expression of AvBD10. Meanwhile, stimulation of theca tissue for 3 h resulted in the increased expression of AvBD12 at doses $10^2$ and $10^3$ ng/mL. Also, AvBD12 expression significantly increased in the theca stimulated by $10^2$ ng/mL of IL-1β with the incubation time from 0 to 3 h with a peak at 3 h.

In contrast, IL-6 did not affect the expression of AvBD10 and 12 by incubating at different doses (at 0 to $10^3$ ng/mL for 3 h) or with different time (for 0 to 3 h, at $10^2$ ng/mL; Figure 4).

The results of Western blot analysis for AvBD12 in the theca after stimulation for 5 h with different doses of IL-1β (0 to $10^3$ ng/mL) are shown in Figure 5. The density of an immunoreactive band for AvBD12 was increased significantly by $10^2$ and $10^3$ ng/mL of IL-1β compared with tissue incubated without IL-1β.

**DISCUSSION**

We are reporting that the expression of AvBD12 in the theca was upregulated by IL-1β that may be synthesized in response to LPS. Significant findings of this study were 1) LPS was able to induce the mRNA expression of proinflammatory cytokines, IL-1β and IL-6, although it did not induce AvBD10 or -12; and 2) IL-1β, but not IL-6, induced AvBD12 mRNA and protein expression, although it did not affect the expression of AvBD10.

Proinflammatory cytokines play a key role in initiating an innate immune response and assist in generating a local inflammatory response (Staeheli et al., 2001). In the current in vitro study, it was found that LPS increased the expression of proinflammatory cytokines IL-1β and IL-6 in a dose- and time-dependent manner. These results support our previous in vivo study showing that injection of chickens with LPS resulted in a marked increase of proinflammatory cytokine genes and protein expression in the theca (Abdelsalam et al.,...
In broilers, *S. enteritidis* infection resulted in the increased expression of proinflammatory cytokines (*IL-1β* and *IL-6*) in the spleen and cecum (Cheeseman et al., 2007). In newly hatched chicks, oral infection with *Salmonella* resulted in the upregulation of mRNA expression of proinflammatory cytokines of the intestinal and liver tissues in correlation with inflammatory signs (Withanage et al., 2004). The current study used LPS of *Salmonella*, and thus it is assumed that the expressions of *IL-1β* and *IL-6* may be upregulated in the theca in response to some *Salmonella*, as observed in other organs.

In a previous study, intravenous injection of chickens with LPS increased the expression of *AvBD12* but not *AvBD10* in the theca of follicles (Subedi et al., 2007b). The expression of *AvBD12* was also upregulated in hen ovarian tissues by oral *Salmonella* inoculation (Michailidis et al., 2012). In contrast, the current in vitro study showed that LPS did not significantly affect the expression of *AvBD10* and *AvBD12* in theca tissue. One of the possibilities for the difference in the thecal *AvBD12* expression in response to LPS between in vivo and in vitro studies is that the induction of *AvBD12* by LPS in this tissue might occur indirectly through the production of cytokines, which in turn might induce *AvBD12*.

In experiment 1, LPS stimulation upregulated expression of *IL-1β* and *IL-6*, but not *AvBD10* and *AvBD12*. The results of experiment 2 showed that stimulation of theca tissue with *IL-1β* increased the gene and protein expression of *AvBD12*, although *AvBD10* gene expression was not affected. Thus, it is suggested that, in theca tissue, *IL-1β* is synthesized in response to LPS, and then *IL-1β* stimulates the expression of *AvBD12*. Because the expression of *AvBD10* and *AvBD12* was not changed significantly by *IL-6*, *IL-6* may not play roles in the regulation of expression of those *AvBD*. In humans also, *DEFB4* expression in gingival keratinocyte cultures was increased by *IL-1β* up to ~16-fold, whereas it was increased by LPS only up to ~5-fold (Mathews et al., 1999). It remains unknown why the expressed *IL-1β* did not affect *AvBD12* expression by a paracrine manner within a cultured tissue. One of the possibilities is that the amount or biological activity of *IL-1β* synthesized in vitro was not enough to stimulate *AvBD12* expression.

In conclusion, the results of this study suggest that theca tissue expresses *IL-1β* and *IL-6* in response to LPS, and then *IL-1β* stimulates *AvBD12* expression. This process of *AvBD12* synthesis may occur against infection by gram-negative bacteria in ovarian follicles.

**ACKNOWLEDGMENTS**

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