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Expression of β-defensins in intestines of chickens injected with vitamin D_3 and lipopolysaccharide

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ABSTRACT. The objective of this study was to evaluate the effect of vitamin D_3 (VD_3) on the regulation of chicken intestinal β-defensin genes under normal and lipopolysaccharides (LPS) conditions. Four treatment groups were used, including a negative control group, VD_3-injection group, LPS-injection group, and both VD_3-injection and LPS-injection group. At 4, 24, and 48 h post-injection, intestines were collected and RNA was isolated to measure the chicken β-defensin genes with putative vitamin D responsive elements using quantitative polymerase chain reaction. Expressions of all 7 chicken β-defensin genes was detectable in the intestines. Significant increases in *GAL-6*, *-7* and *-9* were found following LPS injection treatment at 4, 24, and 48 h post-injection, respectively, whereas VD_3 injection did not affect the expression of any investigated genes under normal conditions.

However, the expression of *GAL-4*, *-5*, *-6*, and *-10* were synergistically upregulated by VD_3 in combination with LPS. These results suggest that VD_3 enhances the immune immunity during LPS challenge by inducing the expression of chicken β-defensin genes when birds are exposed to immune stressors.

Key words: β-defensin; Chicken; Host defense peptide; Vitamin D3; Lipopolysaccharide

INTRODUCTION

Host defense peptides (HDPs), also known as antimicrobial peptides, are ancient antibacterial weapons produced by the host itself (Zasloff, 2002). Because of their broad-spectrum antibacterial activity to bacterium, viruses, and parasites (Rozek et al., 2000; Lamberty et al., 2001; Date-Ito et al., 2002), particularly drug-resistant bacteria, HDPs have been recognized as a new generation of antimicrobial drugs. There are 2 therapeutical options: directly use or induced expression. Significant progress has been made in developing new antimicrobial peptides agents, but the expense and difficulty of preparing large amounts of peptide and the uncertainty in the systemic use of these peptides have slowed their development. Moreover, those pathogens were found to be highly resistant to HDPs (Peschel, 2002). The primary limitation to the induction approach is identifying an adequate inducer with minimum side effects.

Vitamin D_3 (VD_3) was initially recognized as a hormone that maintains the homeostasis of calcium and phosphorous (Findling et al., 1982) obtained from dietary sources or synthesized in the skin by ultraviolet irradiation (Jones et al., 1998). The active form of VD3, $1,25(OH)_2D_3$, is generated through 2 hydroxylation reactions in the liver and kidney, respectively. Apart from regulating calcium homeostasis and controlling cellular differentiation and proliferation, $1,25(OH)_2D_3$ has been found to be highly potent in regulating the expression of human HDPs through the VDR pathway (Wang et al., 2004; Liu et al., 2009). In this process, the $1,25(OH)_2D_3$ enters the target cell from the circulation via vitamin D binding protein and binds to the vitamin D receptor (VDR) in the cytoplasm. Similar to several nuclear receptors, VDR functions as a heterodimer with the retinoid X receptor. The heterodimer subsequently enters the nucleus and finally regulates the expression of targets genes by binding to vitamin D receptor-binding elements (VDREs). Two bovine defensins can be induced by $1,25(OH)_2D_3$, while 3 bovine cathelicidin genes with potential VDREs are not (Nelson et al., 2012).

There are 2 main families of HDPs in vertebrate, defensin and cathelicidin. In chicken, 14 β-defensin (*GAL1-14*) and 4 cathelicidin (*Fowlicidin1-3* and *cathelicidin-B1*) genes have been identified in the chicken (Xiao et al., 2004, 2006; Lynn et al., 2007). Although there are some discrepancies between studies analyzing the tissue expression of chicken HDPs, the crucial role of HDP in chicken innate immunity is well-documented, particularly chicken β-defensins, which show widespread antimicrobial activity against bacteria and fungi (Cuperus et al., 2013). Additionally, chicken β-defensins have been found to be important in immune regulation, such as anti-inflammation by blocking LPS-induced inflammation and promotion of wound-healing. Similar to in mammals, multiple chicken HDPs are inducible in response to fatty acids and microbial products or infection. With the growing problem of resistance to conventional antibiotics, the regulation of endogenous HDPs by these dietary compounds may provide novel therapeutic uses (Michailidis et al., 2012; Sunkara et al., 2012). However,

whether VD_3 can regulate the endogenous expression of chicken β-defensin genes remains unknown. The purpose of this study was to investigate the effects of VD_3 on innate host defenses in chicken with and without an inflammatory challenge, as characterized by intestinal β-defensin gene expression.

MATERIAL AND METHODS

Animal and experimental design

A total of 154 1-day-old and healthy male Taihe silky chicks were used and provided with feed and water *ad libitum*. In order to maintain all the tested individuals at a similar body weight (BW), 2.0-2.1 kg, birds with significant lower or higher body weight were excluded. At 84 days of age, the 72 chickens selected were randomly divided into 4 groups. The treatment groups were as follows: i) negative control (saline-injection) (NC) group, ii) VD_3-injection group, iii) LPS-injection group, and iv) VD_3- and LPS-injection group. VD_3 (Sigma, St. Louis, MO, USA) and LPS (Sigma) were dissolved in 75% ethyl alcohol and diluted with saline, and chickens were intraperitoneally injected with 5000 IU/kg and 500 μg/kg body weight, respectively. All procedures were approved by the Institutional Animal Care and Use Committee of the Sichuan Agricultural University. Six chickens from each group were randomly selected and euthanized after 4, 24, and 48 h post-injection (p.i.). The small intestines were immediately removed and frozen in liquid nitrogen. All samples were collected within 20 min for RNA isolation and stored at -80°C.

RNA isolation and reverse transcription

Total RNA was extracted by using RNAiso Plus (Takara Bio, Shiga, Japan) according to the manufacturer protocol and eluted with 50 μL RNase-free water. Purified RNA was examined by 1% agarose gel electrophoresis and an Agilent 2100 bio-analyzer (Agilent, Santa Clara, CA, USA). RNA was reverse-transcribed using the PrimeScript RT reagent Kit (Takara Bio) according to the kit instructions. cDNA samples were diluted 1:5 in sterile water and stored at -20°C.

Promoter analysis

The published 14 chicken β-defensin cDNA sequences (National Center for Biotechnology Information, http: http://www.ncbi.nlm.nih.gov/), containing 2000 bp in their positions relative to the 5' ends of the genes, were investigated for putative transcription factor binding sites of VDR using NHR-Scan (http://nhrscan.genereg.net/cgi-bin/nhr_scan.cgi) (Sandelin and Wasserman, 2005).

Real-time polymerase chain reaction (PCR)

Quantitative PCR was performed using the Bio-Rad CFX 96 system (Bio-Rad, Hercules, CA, USA) according to manufacturer instructions. Reactions contained of 12.5 μL SYBR® Premix Ex Taq™ II kit (Takara Bio), 0.5 μL of each forward and reverse primers, 2 μL cDNA, and 9.5 μL diluted water. Relative RNA expression levels were measured for

7 chicken β-defensin genes with putative VDREs. Primer specificity was determined by gel electrophoresis and melting curve analysis. Primers were designed from corresponding cDNA sequences (Table 1).

All templates were amplified by using the following protocol: 95°C for 2min; followed by 40 cycles of 95°C for 5 s and n°C (the optimal temperature of each chicken β-defensin gene, listed in Table 1) for 30 s, ending with 60°C for 30 s. Relative quantification of mRNA transcripts was accomplished using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The glyceraldehydes 3-phosphate dehydrogenase gene was used as the reference gene. The control sample was used for calibration, and the expression of each gene is reported as fold change relative to the control.

Table 1. Primer sequences for real-time PCR.

Gene	Strand	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)	Accession No.
GAL-3	F	AGGATTCTGTCGTGTTGGGAGC	143	62	NM_204650.2
	R	TTCCAGGAGCGAGAAGCCAC			
GAL-4	F	GGCTATGCCGTCCCAAGTATT	106	60	NM_001001610.2
	R	CCAAATCCAACAATGCAAGAAG			
GAL-5	F	AGCCGATGGTATTCCTGATGG	107	61	NM_001001608.2
	R	TGGTGATTGTTGCCTCTGGTG			
GAL-6	F	TGGCAGTGGACTAAAATCTTGC	197	59	NM_001001193.1
	R	TTTCACAGGTGCTGATAGGGA			
GAL-7	F	ATGGAATAGGCTCTTGCTGTG	119	58	NM_001001194.1
	R	GCCAGATAGAATGGAGTTGGAG			
GAL-9	F	AACACCGTCAGGCATCTTCACA	131	62	NM_001001611.2
	R	CGTCTTCTTGGCTGTAAGCTGGA			
GAL-10	F	AACTGCTGTGCCAAGATTCCG	112	62	NM_001001609.1
	R	AGGAGGAATCCATCACAATCAGC			
GAPDH	F	AGGACCAGGTTGTCTCCTGT	153	62	NM_204305.1
	R	CCATCAAGTCCACAACACGG			

Statistical analysis

All quantitative PCR experiments were performed in triplicate and results are reported as mean ΔΔCt value ± standard error. All data were analyzed according to a completely randomized design, consisting of 4 treatment and 6 replicates, fitting into a General Linear Models analysis using SAS (SAS Institute, Cary, NC, USA). Treatment means were compared using the Tukey multiple range test, and the level of significance was set at $P \leq 0.05$.

RESULTS

We identified VDREs from 2000 bp upstream of the promoters of chicken β-defensin genes. VDREs were detected in the promoter domain in 7 (respectively are *GAL-3*, *-4*, *-5*, *-6*, *-7*, *-9*, *-10*) of 14 chicken β-defensin genes (Table 2). We found a duplicated VDRE type (DR3, direct repeats separated by 3-bp) in the promoter region of the *GAL-3* and *GAL-4*, while *GAL-5*, *-6*, *-7*, *-9*, and *-10* had only one VDRE. The other VDRE type, ER6 (everted repeats with 6-bp spacing) was only found in *GAL-5*, *-7*, and *-9*. Except for *GAL-5*, VDREs were found in the -500 to +0 bp regions of the genes (the first nucleotide of translation start codon was designated as +1). Figure 1A shows the expression pattern of 7 chicken β-defensin genes with putative VDREs obtained from the intestines of the negative control group. Regardless of the

treatment of group and time point, expression of the antimicrobial peptide genes investigated in this study was detected in the intestines of chickens. Relative expression of the 7 chicken β-defensin genes from high to low was *GAL-7*, *GAL-4*, *GA-6*, *GAL-5*, *GAL-10*, and *GAL-3*, normalized to *GAL-3*.

Table 2. Predicted VDREs in the promoters of chicken β-defensin genes.

Gene	Site type	Location	5'-Half	Spacer	3'-Half
GAL-3	DR3	-2065	AGATCA	GCA	AGGCCA
	DR3	-426	TGAACT	GCC	TGCACT
GAL-4	DR3	-1625	TGGACT	GGA	TGACCT
	DR3	-458	TGGACT	AGA	TGACCT
GAL-5	ER6	-1916	TGAGCT	CCTCTT	TGTCCA
GAL-6	DR3	-102	AGTGCA	AGA	AGGCCA
GAL-7	ER6	-116	CAACCT	CATGTG	AGTTCA
GAL-9	ER6	-244	TGGTCC	TTGTTC	AGGTCA
GAL-10	DR3	-25	GGGGCA	CGC	AGTCCA

DR3: Direct repeats separated by 3 bp, ER6: everted repeats with 6 bp spacing.

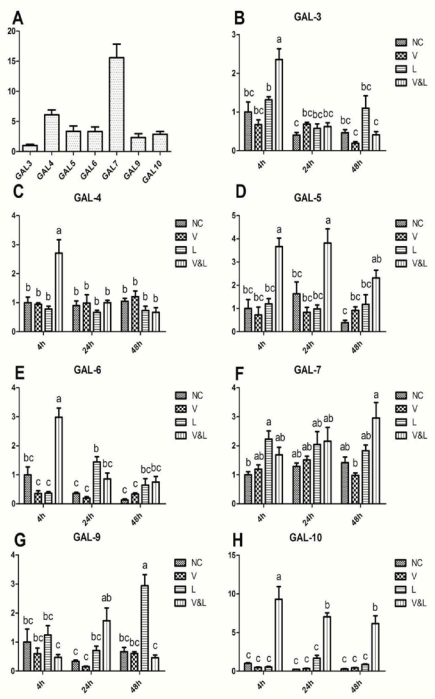

Figure. 1. A. Expression pattern of *GAL-3*, *-4*, *-5*, *-6*, *-7*, *-9*, and *-10* obtained from the intestines of the negative control group, and expression levels of chicken β-defensin genes were calculated relative to that of GAL-3 using GAPDH as a reference gene. Relative expression levels of *GAL-3* (**B**), *GAL-4* (**C**), *GAL-5* (**D**), *GAL-6* (**E**), *GAL-7* (**F**), *GAL-9* (**G**), and *GAL-10* (**H**) in the intestines of chickens. Treatment groups were as follows: negative control (NC; saline-injection), vitamin D-injected group (V), LPS-injected group (L), and vitamin D- and LPS-injected group (V&L). Target gene expression is presented relative to *GAPDH* expression and normalized to the NC group at 4 h post-injection. Error bars represent standard errors of the means. The differences in gene expression among groups at each point were tested by the Tukey range test and were considered to be significant at $P \leq 0.05$.

The effects of VD_3 and LPS injection effects on the relative expression of *GAL-3*, *-4, -5*, *-6*, *-7*, *-9*, and *-10* are illustrated in Figure 1 (B-H). As expected, no significant difference was observed in the NC group for the relative expression of all 7 chicken β-defensin genes among 3 time points.The same result was observed for the VD_3 injection group. In the LPS injection group, the relative expression of GAL-6 at 24 h p.i. was significantly higher than at 4 h p.i., while for GAL-9, the upregulation of genes following LPS injection increased over time and a significant difference was observed between 4 and 24h p.i. Interestingly, in the VD_3- and LPS-injection group, the relative expression of *GAL-3*, *GAL-4*, *GAL6*, and *GAL-10* at 4 h p.i. were all significantly higher than either at the other 2 time points. The relative expression of *GAL-6*, *-7*, and *-9* following the LPS injection were significantly higher than in controls at different time points of 4, 24, and 48 h p.i., respectively, while the others were unresponsive to LPS injection. Although the relative expression of all 7 chicken β-defensin genes was not affected by VD_3 injection compared with the NC group, the combination of LPS injection and VD_3 injection resulted in significantly higher expression of the *GAL-10* gene relative to the other groups at all 3 time-points, as well as *GAL-3* at 4 h p.i., *GAL-4* at 4 h p.i*., GAL-5* at 4 h and 48 h p.i. and *GAL-6* at 4 h p.i.

DISCUSSION

Intestine is an important frontier of the body, which not only regulates the selective entry of nutrients but also remains vigilant in the defense against pathogens. Bacterial and viral infections of the intestines are some of the most serious causes of growth retardation and death in chicken. In this study, the effect of VD_3 and LPS on the regulation of chicken HDPs was investigated by measuring the relative expressions of *GAL-3*, *-4*, *-5*, *-6*, *-7*, *-9*, and *-10* in the intestines of chickens. The expression of the 7 chicken β-defensin genes with putative VDREs were all detected in the intestines of chicken. In contrast with our results, *GAL-1*, *-2*, *-4*, *-5*, *-7*,*-10* genes were found to be moderately expressed in the intestines of chicken among *GAL1-10* (Lynn et al., 2004), while in a subsequent study only low levels of *GAL-13* were expressed in the intestines (Xiao et al., 2004). In addition to the different experimental conditions and primers used for PCR, the different breeds and ages of chickens used may have contributed to the discrepancies between the studies analyzing tissue expression in chicken β-defensin genes.

Our results indicate that although LPS injection significantly induced the expression chicken β-defensin genes in the intestines of chickens. Consistently, it was previously shown that multiple microorganisms or microbial products could trigger the immune response and induce HDP expression in chickens (Michailidis et al., 2012; Anastasiadou et al., 2014). Enhanced expression of β-defensin genes is typically associated with pathogenic infections and inflammations. Although inflammation is necessary for an immune response, it is detrimental to the host and production traits of animals in a chronic inflammatory state (Klasing, 2007). Thus LPS is not an ideal inducer for upregulating the expression of HDPs because of the unwanted effect of proinflammation. In addition to the differences in the relative expression of *GAL-6*, *GAL-7*, and *GAL-9* between the LPS injection and the NC group, all significant differences were presented between the VD_3 injection and LPS injection group and the other 3 groups (the NC group, VD injection group, and LPS injection). Furthermore, higher relative levels of *GAL-6*, *GAL-7*, and *GAL-9* gene expression in birds injected with LPS occurred at different times and only several, but not all 7 chicken β-defensin genes were significantly

upregulated by LPS. Chicken *CATH1* was shown to be induced by *Salmonella* in cecal tonsil, while no significant responses were observed in the jejunum of chicken (Akbari et al., 2008; Van Dijk et al., 2009). Therefore, chicken β-defensins are regulated differently in response to LPS.

In humans, $1,25(OH)_2D_3$ plays crucial role in enhancing the expression of human HDP (Liu et al., 2006). In this study, we did not observe a significant change in the expressions of the 7 chicken β-defensin genes with putative VDREs (*GAL-3*, *-4*, *-5*, *-6*, *-7*, *-9*, and *-10*) in the group that was injected with VD_3 compared with the NC group. In agreement with our results, 3 bovine cathelicidins with potential VDREs were unresponsive to $1,25(OH)_2D_3$ (Nelson et al., 2010). Additionally, the regulation of cathelicidin gene was retained in primates because the functional VDRE in the cathelicidin promoter is conserved in primates (Wang et al., 2004). VDREs have been suggested to be essential in the response to VD_3 in a large number of $1,25(OH)_2D_3$-dependent target genes. However, some studies reported that VD_3 enhanced the expression of *CATH-1* and *AvBD-1* (which have no VDREs in the promoter) in the bursa and thymus of chicken (Zhang et al., 2011). Moreover, the gene neutrophil gelatinase-associated lipocalin responded positively to $1,25(OH)_2D_3$ (Goetz et al., 2002). Therefore, whether VDREs exists are essential for $1,25(OH)_2D_3$-dependent target genes in response to VD_3 requires further investigation.

Although $1,25(OH)_2D_3$ has been shown to be a direct inducer of human HDPs, when and how $1,25(OH)_2D_3$ functions have not been determined (Wang et al., 2004). A later study showed that activation of Toll-like receptors triggers an antimicrobial pathway that is dependent on the endogenous production and action of $1,25(OH)_2D_3$ (Liu et al., 2006). In this study, no increases in chicken β-defensin genes were detected in the VD_3 injection group compared with the NC group, but when VD_3 was combined with LPS, there may be a direct synergistic effect on elevating the expression of all 7 chicken β-defensin genes except for *GAL-7*. This suggests that VD_3 only upregulated the expression of endogenous HDPs when the host was exposed to inflammatory challenge. Furthermore, VD_3 was shown to inhibit the expression of inflammatory cytokines (Staeva-Vieira and Freedman, 2002; Szeto et al., 2007).

In conclusion, our results indicated that VD_3 is useful for mediating the immune response only when animals are exposed to immune stressors via synergistically upregulating chicken β-defensin gene expression to resist diseases in chickens. Further studies are required to determine whether VD_3 can modulate the expression of chicken β-defensin genes *in vitro*, as well as reveal the mechanisms of how VD_3 modulates chicken β-defensin genes.

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