Profile of Toll-like receptor expressions and induction of nitric oxide synthesis by Toll-like receptor agonists in chicken monocytes

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Abstract

Toll-like receptors (TLRs) play a major role in the innate immune system for initial recognition of microbial pathogens and pathogen associated components. Nitric oxide (NO) is generated in immune cells in response to microbial stimulation and is involved in pathogenesis and control of infection. We used RT-PCR analysis to examine the TLR expression profile on chicken monocytes and demonstrated these cells express chicken TLR2, 3, 4, 6, and 7. TLR5 was not detected by the TR-PCR. We also investigated the differential induction of NO synthesis in chicken monocytes by TLR agonists, including flagellin (FGN, from Salmonella typhimurium), synthetic lipoprotein Pam3CSK4 (PAM), lipopolysaccharide (LPS, from Salmonella enteritidis), lipoteichoic acid (LTA, from Staphylococcus aureus), the synthetic double stranded RNA analog (poly I:C), the guanosine analog, loxoribine (LOX), and synthetic CpG oligodeoxynucleotide (CpG-ODN). Our results indicate that there was a vast difference among these agonists for their ability to induce NO production. CpG-ODN and LPS were the most potent stimuli and induced significant quantities of NO in cultured monocytes, whereas LTA stimulated significant NO production only at high concentrations. Other agonists such as FGN and poly I:C stimulated very little NO, while PAM, LOX, and nCpG-ODN (control ODN) did not induce NO production. RT-PCR analysis demonstrated that LPS, LTA, and CpG-ODN induced inducible nitric oxide synthase (iNOS) expression in monocytes; whereas the other agonists did not. The presence of TLRs on chicken monocytes and the differential induction of NO production in chicken monocytes by various TLR agonists suggest the differentiation of signaling pathways downstream of individual TLRs.

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1. Introduction

The innate immune system of vertebrates recognizes structurally conserved pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) and allows immediate host immune responses to limit the invading microbes (Janeway and Medzhitov, 2002). PRRs are located either at the cell surface or in the cytosol of the immune cells and respond to PAMPs that are either extracellular or intracellular that have crossed the plasma membrane (Atman and Philpott, 2004). Toll-like receptors (TLRs), which are vertebrate homologies of the Drosophila Toll protein, are type 1 transmembrane proteins and represent a newly defined family of vertebrate PRRs that recognize specific structural motifs expressed by microbes (Akira et al., 2001; Netea et al., 2004). Eleven TLRs have been identified in humans and mice, with each member recognizing and responding to different microbial components (Akira et al., 2001; Netea et al., 2004; Zhang et al., 2004). For example, TLR2 recognizes bacterial lipoproteins, peptidoglycans (PGN), and lipoteichoic acids (LTA) from gram-positive bacteria; TLR3, virus-derived double-stranded RNA and its DNA analog [poly I:C]; TLR4, lipopolysaccharide (LPS) from Gram-negative bacteria; TLR5, bacterial flagellin; TLR9, CpG motif of bacterial DNA. TLR7 and TLR8, which are close phylogenetic relatives and form an evolutionary cluster with TLR9 (Du et al., 2000), have been recently found...
to mediate recognition of single-stranded RNA (Diebold et al., 2004; Lund et al., 2004) and are receptors for small synthetic guanosine-based antiviral molecules, such as loxoribine (LOX) (Heil et al., 2003). TLRs are also known to form complexes to confer a further degree of specificity. For example, heterodimers of TLR1/2 recognize triacylated bacterial lipopeptides, whereas TLR2/6 recognizes diacylated Mycoplasma lipopeptides (Takeda et al., 2002). A new member of TLRs, TLR11, has recently been identified in mice, which responds specifically to uropathogenic bacteria (Zhang et al., 2004). The natural ligands for TLR10 have not been identified.

The expression profiles of TLRs, their functions, and signaling pathways have been elucidated in various mammalian immune cell types (Muzzio and Mantovani, 2001; Muzzio et al., 2000; Takeda and Akira, 2004), but limited information is available for immune cells in avian species. Chicken TLR2 has been cloned and its functional isoform type 2 was found to respond to both bacterial lipoproteins and LPS, suggesting that the chicken TLR2 may also play the role of TLR4 (Fukui et al., 2001). Later, however, chicken TLR4 has also been cloned and its allelic variation has been linked to resistance to infection with Salmonella enterica serovar Typhimurium in chickens (Leveque et al., 2003). Functionally, TLR2, 4, and 9 mediated immune responses have been demonstrated in chicken heterophils, monocytes, and a macrophage cell line (Farnell et al., 2003; He et al., 2003; He and Kogut, 2003). Recently, several TLR homologous sequences have been discovered using bioinformatics tools, including TLR1/6/10, TLR3, TLR5, and TLR7/8/9 in chicken EST libraries (Smith et al., 2004), and full length coding sequences of TLR3, TLR6, and TLR7 have been predicted by automated computational analysis of genomic sequence (NCBI: http://www.ncbi.nlm.nih.gov/).

Nitric oxide (NO) is a multi-functional mediator with diverse physiological and pathological roles in vasodilatation, neurotransmission, and host defense against infectious agents and tumors (Cooke and Dzau, 1997; Dawson and Dawson, 1998; MacMicking et al., 1997; Stuehr and Nathan, 1989). There are three distinct isoforms of nitric oxide synthase: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS or NOS-3) (Hiki et al., 1991). The constitutive isoforms, eNOS and nNOS, found in endothelial cells and neurons produce small quantity of NO that acts primarily as a cell signaling molecule (Hussain and Qureshi, 1998) and differential Toll-like receptor-4 expression in chicken macrophages (DiI and Qureshi, 2002). In the present study, we examined the gene expression of TLRs and TLR-mediated NO induction in peripheral blood monocytes from neonatal chickens. A panel of TLR agonists were used to stimulate the monocytes while their ability to induce iNOS mRNA in the cells and NO accumulation in cell culture media were evaluated.

2. Materials and methods

2.1. Reagents

The synthetic lipoprotein Pam3CSK4 (palmitoyl-3-cysteine-serine-lysine-4; PAM), the synthetic dsRNA analog, poly I:C, flagellin (FGN, from Salmonella typhimurium), and the guanine analog loxoribine were all purchased from InvivoGen (San Diego, CA). LPS (from Salmonella enteritidis) and LTA (from Staphylococcus aureus) were purchased from Sigma (St. Louis, MO). Synthetic ODNs were purchased from BioSource International (Camarillo, CA, USA) and further purified by ethanol precipitation. The sequences of synthetic ODNs used in the present study were: CpG-ODN, GTC GTT GTC GTT GTC GTT (He et al., 2003) and a control ODN without CpG motif (GCCCCCCGGGG). All media and additives for cell culture were purchased from Sigma.

2.2. Cell isolation

Chicken peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood collected from 2- or 3-day-old chickens as described by Kogut et al. (1995). Briefly, peripheral blood from approximately 100 chicks was pooled, mixed with 1% methylcellulose (1:1, v/v), and centrifuged at 250 × g for 60 min. The PBMC layer at the 1.077/supernatant interface was collected, washed, and resuspended in RPMI-1640.

2.3. Macrophage culture

Aliquots of 200 μl of PBMC (1 × 10⁷ cells/ml) were dispensed to a round-bottom 96-well plate and incubated at room temperature for 2 h. After incubation, non-adherent cells were removed by washing twice with the culture medium [Dulbecco’s Modified Eagles Medium (DMEM) containing 10% chicken serum, antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml), and 1.5 mM 1-glutamine]. The adherent monocytes in 200 μl culture medium per well were stimulated with TLR agonists...
for 48 hr at 41°C in a 5% CO₂ and 95% humidity incubator.

2.4. Nitrite assay

Nitrite, a stable metabolite of nitric oxide, produced by activated monocytes was measured by the Greiss assay (Green et al., 1982). Briefly, an aliquot of 100 μl culture supernatant from each well was transferred to the wells of a new flat-bottom 96-well plate and combined with 50 μl of 1% sulfanilamide and 50 μl of 0.1% naphthylenediamine (both were prepared in 2.5% phosphoric acid solution). After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density (ODs100) of each well using a SPECTRA MAX microplate reader (Molecular Devices, Sunnyvale, CA). Sodium nitrite (Sigma) was used as a standard to determine nitrite concentrations in the cell-free medium.

2.5. Expression of TLRs and nitric oxide synthase (iNOS) mRNA in chicken monocytes

Monocytes from the initial seeding of PBMC (2 × 10⁷ cells/well) were cultured in the 12-well plate and stimulated with various TLR agonists in the DMEM medium. Total RNA from unstimulated monocytes were used for RT-PCR analysis of TLR expressions. Total RNA from the monocytes stimulated with various agonists were used for RT-PCR analysis of iNOS expression. The cDNA was synthesized using ThermoScript™ Reverse Transcriptase (Invitrogen, Carlsbad, CA) and using poly(dT)₂₀ as primer. RNase free-DNase I (Promega, Madison, WI, USA) was used to remove genomic DNA contamination in RNA samples at 37°C for 30 min and heat-denatured at 75°C for 5 min prior to RT reaction. PCR amplification was conducted with gene-specific primers (Table 1). The BD Advantage™ 2 Polymerase mix (BD Biosciences Clontech, Palo Alto, CA) was used for PCR reaction. PCR started with an initial denaturation at 94°C for 1 min followed by 30 cycles (for β-actin), 40 cycles (for iNOS) of 94°C for 30 s and 68°C for 1 min (except for TLR5, 40 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 1 min were used), and ended with a final extension at 68°C for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose/ethidium bromide gel.

2.6. Data analysis

Nitric oxide production data were pooled from three independent experiments. Within each experiment, there were four or five replicate assays for each treatment. Data were analyzed by one-way ANOVA followed by multiple comparisons (Tukey test) with the non-stimulated control using SigmaStat® software (Jandel Scientific, San Rafael, CA). The value of p < 0.05 is considered to be significant.

3. Results

3.1. Expression of TLRs in chicken monocytes

Several chicken homologous TLRs have been identified in the chicken EST library and predicted based on chicken genome sequence (Smith et al., 2004; NCBI: http://www.ncbi.nlm.nih.gov). PCR primers specific to

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sequences of PCR primers</th>
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<tbody>
<tr>
<td>β-Actin</td>
<td>Forward: GATTTGACAGGAGATGAGCCACAG</td>
</tr>
<tr>
<td>Reverse: GATCCACATCTGTGAAGTTGAC</td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>Forward: TGCCTGGAAGCAACATCTCTCTCA</td>
</tr>
<tr>
<td>Reverse: TTTCCACAGTGGAGAAGTTCT</td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>Forward: TCCCTGAGATGATGTGGTCT</td>
</tr>
<tr>
<td>Reverse: AATGACGAAAACATCAGATCTCCT</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>Forward: TGGACAGGACAGAGACATCTCT</td>
</tr>
<tr>
<td>Reverse: AAGCTCAGACAGAGACATCTCT</td>
<td></td>
</tr>
<tr>
<td>TLR5</td>
<td>Forward: TGCAGACGTTTTCTCTGAGTT</td>
</tr>
<tr>
<td>Reverse: CCACATCGAATTTCTCTCT</td>
<td></td>
</tr>
<tr>
<td>TLR6</td>
<td>Forward: AAGCTGACAGGAGGCTGTTGTTGTA</td>
</tr>
<tr>
<td>Reverse: AAGCTCAGCTTTCTCAAGGGA</td>
<td></td>
</tr>
<tr>
<td>TLR7</td>
<td>Forward: TGACGACAGGTGTTTCTGAGGTT</td>
</tr>
<tr>
<td>Reverse: ATCCAGGCTTTCTCTCAGCC</td>
<td></td>
</tr>
</tbody>
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* The coding sequences were predicted by automated computational analysis of genomic sequence and supported by EST evidence (NCBI: http://www.ncbi.nlm.nih.gov).

* Primer sequences are from Iqbal et al. (unpublished data).
TLR2 type 2, TLR3, TLR4, TLR5, TLR6, and TLR7 were designed to detect the expression of those TLRs in total RNA from chicken monocytes. Our results show that monocytes from neonatal chickens express, TLR2, 3, 4, 6, and 7 (Fig. 1). However, TLR5 was not detected in the monocytes.

3.2. Induction of iNOS mRNA in chicken monocytes by TLR agonists

TLR agonists LPS, LTA, and CpG-ODN strongly up-regulated the expression of iNOS mRNA as demonstrated by RT-PCR analysis (Fig. 2), which were correlated with the amounts of nitrite in the culture media. The TLR agonists LPS, LTA, and CpG-ODN, induced significant iNOS gene expression after 18 h stimulation. Similar results were also obtained from the monocytes at 24 h after stimulation with TLR agonists. At 4 h post stimulation, however, no difference of iNOS expression was detected among the treatments (data not shown).

3.3. TLR agonist stimulated NO production in chicken monocytes

Monocytes from neonatal chickens were readily induced to produce NO when stimulated with LPS, CpG-ODN, and LTA. Other LTR agonists, including Pam, FGN, poly I:C, and LOX, stimulated little or no NO production (Fig. 3). LPS and CpG-ODN were most potent stimuli for NO production; while LTA induced significant amount NO only at high concentration (100 μg/ml). mCpG-ODN did not induce any NO production in monocytes.

4. Discussion

Monocytes/macrophages are mononuclear phagocytes, playing critical roles in innate immune responses and as antigen processing and presenting cells in initiating the acquired immune response. These cells are originated in the bone marrow and subsequently enter the circulation as blood monocytes. As a component of the first line immunological defense, monocytes/macrophages play an important role in recognition of infectious agents. This recognition can be mainly attributed to the family of TLRs (Akira et al., 2001; Athman and Philpott, 2004; Netea et al., 2004). Recognition of PAMP by TLRs induces the production of reactive oxygen and nitrogen intermediates (ROI and RNI), pro-inflammatory cytokines, and up-regulates expression of costimulatory molecules, subsequently initiating the adaptive immunity (Werling et al., 2004; Werling and Jungi, 2003). Identification of TLRs and their microbial agonists is critical to the understanding of pathogen–host interactions. In mammalian species, a repertoire of 11 TLRs has been identified and understanding of their roles in innate immune control of microbial infections has been greatly advanced (Quesniaux and Ryffel, 2004).

Bioinformatics analysis of chicken EST database and chicken genomic sequence has identified a number of chicken homologs of TLRs, including TLR3, TLR5, TLR6, and TLR7 (Smith et al., 2004), in addition to the previously cloned chicken TLR2 and TLR4 (Fukui et al., 2001; Leveque et al., 2003). Full length coding sequences of several chicken TLRs, including TLR3, TLR6, and TLR7, have been identified by automated computational analysis of genomic sequence (NCBI: http://www.ncbi.nlm.nih.gov/). In contrast to mammalian, chicken TLR1, TLR6, and TLR10 appear to be represented by just one gene as indicated by a comprehensive analysis of chicken EST database (Smith et al., 2004). Our analysis of sequence alignment (http://www2.igh.cnrs.fr/bin/align-guess.cgi) indicated that chicken TLR6 (XM_423937) shows equivalent sequence identity to all three human TLR1 (NP_003254. 48.7%), TLR6 (NP_006059. 47.1%), and TLR10 (NP_112218. 47.5%). Chicken TLR9 has not been identified in chicken EST database. Previous research (Smith et al., 2004) suggested that one chicken gene may carry out function of TLR7/8/9 based on a phylogenetic analysis of chicken EST database; however, the full length chicken TLR7 (XM_416836) predicted by automated computational analysis of genomic sequence (NCBI: http://www.ncbi.nlm.nih.gov/) shows that the chicken TLR7 shears much greater identity to human TLR7 (AAH33651, 63.6%) than TLR8 (AAQ80663, 41.6%) and TLR9 (AAQ89443, 36.4%). Therefore, the possible existence of a chicken TLR9 cannot be ruled out. We have previously demonstrated that the TLR9 agonist CpG-ODN is recognized by chicken immune cells and that it induces functional activation of heterophils and monocytes (He and Kogut, 2003; He et al., 2003, 2004).
Information on the TLR expression and role of TLRs in the immune response to microbial molecule stimulation in avian immune cells is limited. In the present study, the expression profile of chicken TLRs in blood monocytes from neonatal chickens was examined by RT-PCR using primers specific for chicken TLR2, 3, 4, 5, 6, and 7. The monocytes from the neonatal chickens express all these TLRs except for TLR5. PCR amplification resulted in intensive DNA bands of TLR2, 4, 6, and 7 followed by less intense TLR3, indicating expression of these receptors in the monocytes. These results are significant, because they suggest monocytes from neonatal chickens are capable of sensing infectious agents and inciting immunological responses. However, no TLR5 mRNA was detected in the monocytes under the condition of this
study. The absence of TLR5 expression in monocytes indicates this receptor is restricted to certain cell types, since chicken heterophils are found to express TLR5 (Kogut et al., unpublished data). TLRs are known to express differentially in tissues and cellular subset of leukocytes (Hornung et al., 2002; Werling et al., 2004; Zarembek and Godowski, 2002). This differential expression profile ensures that different types of infections induce distinct types of immune responses by specific immune cell population.

TLRs have been demonstrated to share largely common signaling pathways and stimulation of distinct TLRs can give overlapping responses while in some cases, a specific response can arise to a particular TLR (Netea et al., 2004; Quesniaux and Ryffel, 2004). Several recently discovered adaptor molecules that interact with TLRs appear to modulate the responses downstream of the individual TLR activation (Athman and Philpott, 2004). NO is an important proinflammatory mediator, involved in macrophage antimicrobial and tumoricidal activities (Eisenstein, 2001). NO has been shown to mediate host defense against intracellular pathogenic microorganisms such as Salmonella (Eisenstein, 2001; Nathan and Shilooh, 2000) and virus proliferation (Djebbari et al., 2002; Nathan and Shilooh, 2000). Production of NO is considered an important innate immune response by activated monocytes/macrophages. In this study, TLRs mediated immune response was evaluated by using a panel of TLR agonists to stimulate the chicken monocytes. The induction of NO synthesis by TLR agonists were measured by both accumulated nitrite in the culture media and iNOS mRNA in the cells. Our results showed a differential induction of NO by TLR agonists: TLR2, 4, and 9 agonists LTA, LPS, and CpG-ODN strongly induced both iNOS mRNA and NO production in chicken monocytes while TLR3 agonist poly IC stimulated little NO production and no induction of iNOS mRNA. Neither TLR5 nor TLR7 agonists, FGN and LOX, respectively, induced NO production or iNOS mRNA. These results demonstrated that TLR2, 4, and 9 and their signaling pathways are involved in regulating iNOS gene expression in chicken monocytes in response to microbial molecule stimulations. Interestingly, PAM, also a TLR2 ligand, induced neither iNOS mRNA nor NO production in the monocytes. The discrepancy between LTA and PAM needs to be further investigated. One possible explanation is that PAM is a synthetic compound which has been shown to be TLR2-specific, whereas LTA is a product purified from gram-positive bacteria, which has been shown to contain trace amount of LPS in commercial product (Gao et al., 2001; Morath et al., 2002). This minute amount LPS in LTA may contribute to the NO induction in chicken monocytes when high concentration of LTA was used for stimulation. In addition, the lack of response of chicken monocytes to PAM stimulation suggested that induction of NO by high concentration of LTA is not mediated by TLR2. In a murine macrophage RAW 264.7 cells, poly I:C induced iNOS mRNA and NO production while it alone did not induce iNOS mRNA and produced much less NO (Mizel et al., 2003). Our results were in line with their observations that poly I:C alone appeared to have no effect on iNOS mRNA and have only minimal effect on NO production. Since TLR5 mRNA was not detected in the chicken monocytes, it remains to be seen if TLR5 agonist FGN is able to stimulate NO production in other cell types. However, studies have shown that FIC, a FGN from Gram-negative Salmonella enteritidis, induced NO production of human monocytes-macrophages (Moors et al., 2001) and a heteromeric complex of TLR5 and TLR4 may be involved in this activity (Heitemeier et al., 1998). LOX, an antiviral compound and a ligand of human and murine TLR7, showed no effect on chicken monocyte iNOS expression and NO production, although TLR7 mRNA was expressed in the cells. This result suggested that TLR7 signaling pathway is less likely involved in the induction of iNOS. In contrast, LOX stimulation seemed to induce certain degrees of negative response of NO production in the monocytes. It will be interesting to see the effects of combination of LOX with other TLR agonists on NO production. Since the sequence of chicken TLR9 was not available, we were not able to examine TLR9 expression in the monocytes. However, our results strongly indicate existence of a functional homolog of TLR9 in chickens, because CpG-ODN, the TLR9 specific ligand, was one of the most potent stimuli to induce chicken monocyte iNOS mRNA and NO production, whereas control ODN with no CpG motif had no effect on NO production. Although TLR7, 8, and 9 are phylogenetically more closely related and are believed to form an evolutionarily conserved cluster within the TLR family, our results, based on functional assay, do not support the notion that one chicken gene carries the function of TLR7, 8, and 9 (Smith et al., 2004).

Acknowledgements

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References


