Pharmacological analysis of signal transduction pathways required for oxidative burst in chicken heterophils stimulated by a Toll-like receptor 2 agonist

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Abstract

Toll-like receptors (TLRs) play an important role in the innate immune response of avian heterophils. We previously used the pharmacological inhibitors genistein, verapamil, chelerythrine, and pertussis toxin to investigate the upstream signaling events involved in TLR2-mediated oxidative burst in chicken heterophils. Only chelerythrine, a protein kinase C inhibitor, was found to significantly inhibit oxidative burst stimulated by the TLR2 agonist lipoteichoic acid (LTA). In the present study, we used selective pharmacological inhibitors to investigate the roles of phosphatidylinositol-3\(^\text{V}\)-kinase (PI3-K), phospholipase C (PLC), calcium-dependent protein kinase C (PKC), extra-cellular signal regulated kinase (ERK), and nuclear translocation factor kappa B (NF-\(\kappa\)B) on TLR2-mediated oxidative burst. U-73122 (a PLC inhibitor), wortmannin (a PI3-K inhibitor), PD 98059 (an ERK inhibitor), Go¨ 6976 (a PKC inhibitor) and Bay 11-7082 (a NF-\(\kappa\)B inhibitor) significantly decreased LTA-stimulated oxidative burst in heterophils by 77%, 30%, 36%, 78%, and 61%, respectively. Activated TLR2 utilizes PI3-K, PLC, PKC, ERK, and NF-\(\kappa\)B as signaling factors that mediate the oxidative burst of chicken heterophils.

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Keywords: Heterophils; Oxidative burst; Chicken; Toll-like receptor; Signal transduction

1. Introduction

Heterophils, the avian equivalent to the mammalian neutrophil, are essential cellular components of the avian innate immune system [1,2]. These granulocytic phagocytes kill pathogens by the release of toxic oxygen metabolites (oxidative burst) and the release of lytic enzymes and antimicrobial peptides (degranulation) [3]. Heterophils can respond to invading bacteria within 30 min of infection and are more efficient at phagocytizing and killing than macrophages [4,5].

Abbreviations: ERK, Extra-cellular signaling kinase; LDCL, Luminol-dependent chemiluminescence; LTA, Lipoteichoic acid; NF-\(\kappa\)B, Nuclear transcription factor kappa B; PAMP, Pathogen-associated molecular pattern; PBS, Phosphate-buffered saline; PI3-K, phosphatidylinositol-3\(^\text{V}\)-kinase; PLC, Phospholipase C; PMN, Polymorphonuclear cell; PRR, Pattern recognition receptor; SA, Staphylococcus aureus; TLR, Toll-like receptor.

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Toll-like receptors (TLRs) enable chicken heterophils to identify pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) or lipoteichoic acid (LTA) [6–8]. Recognition of PAMPs by TLRs initiate signal transduction pathways that trigger an oxidative burst in chicken heterophils [6–8]. While TLR signal transduction pathways have been studied extensively in mammalian leukocytes, knowledge of signal transduction pathways involved in TLR-mediated oxidative burst in avian heterophils is limited [8].

We have previously investigated the mechanisms by which TLRs lead to functional activation in heterophils from neonatal chickens [8]. These studies revealed that oxidative burst stimulated by Salmonella enteritidis lipopolysaccharide (LPS) is regulated by a pertussis toxin sensitive, protein kinase C-dependent, Ca\(^{2+}\)-dependent, G-proteins. Alternatively, Staphylococcus aureus lipoteichoic acid (LTA)-stimulated heterophils were not regulated by tyrosine kinases, Ca\(^{2+}\) ions, or G proteins, but instead were regulated by protein kinase C. The study demonstrated the ability of TLRs to recognize and differentiate between molecules derived from Gram-positive and Gram-negative bacteria.

The objective of the present study was to observe the effects of more specific intermediary enzymes and secondary messengers involved in LTA-stimulated oxidative burst. Five prominent signal transduction factors or secondary messengers involved in neutrophil activation include phospholipase C (PLC), phosphatidylinositol-3-kinase (PI3-K), extracellular signal regulated kinase (ERK), protein kinase C (PKC), and nuclear translocation factor kappa B (NF-\(\kappa\)B) [9]. In these experiments, we compared the effects of U-73122 (a PLC inhibitor), wortmannin (a PI3-K inhibitor), PD 98059 (an ERK inhibitor), Gö 6976 (a PKC inhibitor) and Bay 11-7082 (a NF-\(\kappa\)B inhibitor) on the ability of LTA to stimulate an oxidative burst in vitro.

2. Materials and methods

2.1. Experimental animals

Single-comb white Leghorn male chickens (Hy-Line W-36) were obtained on the day-of-hatch from a commercial hatchery (Hy-Line International, Bryan, TX) and placed in floor pens with supplemental heat. Chicks were provided water and a balanced, unmedicated, corn–soybean ration ad libitum that met or exceeded the National Research Council guidelines for chicken nutrition [10].

2.2. Isolation of heterophils

Blood was collected by decapitation and EDTA was used as an anticoagulant. Blood was pooled from 50–100 neonates and peripheral heterophils isolated as previously described [2]. Briefly, blood was mixed with 1% methylcellulose (Sigma, St. Louis, MO) in a 1.5:1 ratio and centrifuged at 250 \(\times\) g for 15 min. The supernatant was removed and resuspended in Hanks balanced salt solution without calcium and magnesium at a 1:1 ratio. The suspension was then layered over a 1.077/1.119 Ficoll-Hypaque gradient (Sigma) and centrifuged at 250 \(\times\) g for 60 min. Following a RPMI wash, the cells were quantitated using a Neubauer hemacytometer and the concentration was adjusted to 4 \(\times\) 10\(^6\) heterophils/ml. Cell viability was routinely >95% as determined by trypan blue exclusion. The purity of the heterophil suspensions was assessed by microscopic examination of Diff-Quick-stained cytospin (Shandon Scientific, Pittsburgh, PA) smears. Heterophil preparations obtained by this method were typically >95% pure.

2.3. Agonists

LTA (Sigma) was dissolved in DMSO and diluted in RPMI to stock concentrations of 1 mg/ml and stored at −20 °C until used. The final concentration of DMSO in the experiments was less than 0.5%. The effective concentration of 8 \(\mu\)g of LTA per ml of cells (4 \(\times\) 10\(^6\) heterophils/ml) was previously determined by a dose response [6].

2.4. Inhibitors

The inhibitors used in these studies and their activities are listed in Table 1. Wortmannin, U-73122, Gö 6976, PD 98059, and Bay 11-7082 were dissolved in DMSO and stock solutions were stored at 4 °C until used. Inhibitors were obtained from Calbiochem (La
Jolla, CA) with the exception of Bay 11-7082, which was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Working concentrations of the inhibitors were prepared in RPMI 1640 tissue culture medium (Sigma) from the stock concentrations. The final concentration of DMSO in the experiments was less than 0.5%. Wortmannin, U-73122, Go¨ 6976, and PD 98059 were used at concentrations of 1, 10 and 100 μM. Bay 11-7082 was used at concentrations of 25 and 50 μM.

Heterophils were pre-incubated with inhibitors prior to stimulation with LTA; at 39 °C for 30 min in 15 ml screw capped polypropylene conical tubes on a rocker plate.

2.5. Luminol-dependent chemiluminescence (LDCL) assay

Oxidative burst of heterophils was measured by use of a luminol-dependent chemiluminescence assay adapted from a previously described procedure [11]. Heterophils (4 × 10⁵) and luminol (0.1 M; Sigma) in 0.5 ml of RPMI 1640 were placed in Beckman polypropylene scintillation vials. The luminol-dependent chemiluminescence of chicken heterophils was measured in a LKB 1219 series liquid scintillation counter using the tritium channel and the coincidence mode following stimulation with opsonized SE. LDCL was quantified as counts per minute (cpm) for 1 h. All samples were assayed in replicates of five vials. Results are expressed as peak cpm/10⁶ heterophils.

2.6. Experimental design

To determine which signal transduction pathways are involved in TLR-mediated oxidative burst, cells were incubated for 30 min at 39 °C in a 15-ml conical tube with the appropriate amount of inhibitor or left untreated. After this incubation with inhibitors, LTA was immediately added to the cells and incubated in the dark for 30 min at room temperature prior to LDCL measurement. Each trial was repeated in three identical experiments.

2.7. Statistical analysis

Data were pooled for each group and compared to a stimulated control using a paired t-test for the LTA treatment from Sigma Stat 2.0 statistical software (Jandel, Chicago, IL).

3. Results

3.1. Effect of PI-3K inhibition on TLR2-mediated oxidative burst

PI-3K has been shown to bind directly to the interleukin-1 receptor (IL-1R), which has a cytoplasmic region that is homologous to the TLR [12]. The IL-1R/PI-3K binding domain is conserved between human, mouse, and chicken cells [13]. Once stimulated, PI-3K activates PLC and is involved in mediating oxidative burst in neutrophils [14,15]. To determine if PI-3K is involved in TLR2-mediated oxidative burst, we used wortmannin, a selective inhibitor of PI-3K. Chicken heterophils pre-incubated with 100 μM wortmannin demonstrated a 30% reduction in oxidative burst (Fig. 1).

3.2. Effect of PLC inhibition on TLR2-mediated oxidative burst

PLC has been shown to play a central role in signal transduction pathways leading to oxidative burst and degranulation in mammalian neutrophils [16,17]. A specific inhibitor of PLC, U-73122, was used to investigate the role of PLC in the TLR2-mediated stimulation of oxidative burst in chicken heterophils. Pretreatment of heterophils with U-73122 attenuated the oxidative burst response in a concentration-dependent manner (Fig. 2). The highest concentration of U-73122, 100 μM, inhibited TLR2-mediated oxidative burst by as much as 77% (Fig. 2).
Fig. 1. The effect of wortmannin, a phosphatidylinositol-3'-kinase (PI3-K) inhibitor, on lipoteichoic acid (LTA) stimulated luminol-dependent chemiluminescence of neonatal chicken heterophils. Heterophils were pre-incubated with the inhibitor for 30 min at 39 °C. Heterophils were then incubated with LTA for 30 min in luminol and LCDL was measured as an indicator of oxidative burst. Data was pooled from three repetitions and presented as the percent of stimulated control. Significant differences were determined using a paired t-test (* represents p < 0.05 compared to control heterophils).

Fig. 2. The effect of U 73122, a phospholipase C (PLC) inhibitor, on lipoteichoic acid (LTA) stimulated luminol-dependent chemiluminescence of neonatal chicken heterophils. Heterophils were pre-incubated with the inhibitor for 30 min at 39 °C. Heterophils were then incubated with LTA for 30 min in luminol and LCDL was measured as an indicator of oxidative burst. Data was pooled from three repetitions and presented as the percent of stimulated control. Significant differences were determined using a paired t-test (* represents p < 0.05 compared to control heterophils).

Fig. 3. The effect of Go 6976, a protein kinase C (PKC) inhibitor, on lipoteichoic acid (LTA) stimulated luminol-dependent chemiluminescence of neonatal chicken heterophils. Heterophils were pre-incubated with the inhibitor for 30 min at 39 °C. Heterophils were then incubated with LTA for 30 min in luminol and LCDL was measured as an indicator of oxidative burst. Data was pooled from three repetitions and presented as the percent of stimulated control. Significant differences were determined using a paired t-test (* represents p < 0.05 compared to control heterophils).

Fig. 4. The effect of PD 98059, an extracellular signal-regulated kinase (ERK) inhibitor, on lipoteichoic acid (LTA) stimulated luminol-dependent chemiluminescence of neonatal chicken heterophils. Heterophils were pre-incubated with the inhibitor for 30 min at 39 °C. Heterophils were then incubated with LTA for 30 min in luminol and LCDL was measured as an indicator of oxidative burst. Data was pooled from three repetitions and presented as the percent of stimulated control. Significant differences were determined using a paired t-test (* represents p < 0.05 compared to control heterophils).
3.3. Effect of PKC on TLR2-mediated oxidative burst

The PKC family of isozymes catalyze numerous protein phosphorylations that are essential in various signal transduction cascades and cellular activation [18]. PKC is particularly important to neutrophil oxidative burst. To determine the role of PKC in TLR-stimulated oxidative burst in chicken heterophils we used a selective inhibitor of PKC, Go6976. Go6976 inhibited the oxidative burst response in a concentration-dependent manner (Fig. 3). The PKC inhibitor reduced oxidative burst by 78% at a concentration of 100 μM (Fig. 3).

3.4. Effect of ERK on TLR2-mediated oxidative burst

Activation of ERK is coupled to the stimulation of cell-surface proteins via several different upstream signaling pathways, and contributes to the regulation of oxidative burst by participating in the phosphorylation of p47 phox [19,20]. PD 98059, a selective inhibitor of ERK was used to determine the role of ERK in the TLR-mediated stimulation of oxidative burst in avian heterophils. Pretreatment of heterophils with PD 98059 reduced oxidative burst by 26–41% at concentrations of 1, 10, and 100 μM (Fig. 4).

3.5. Effect of NF-κB on TLR2-mediated oxidative burst

NF-κB is a family of nuclear transcription factors that initiate the production of immune proteins. NF-κB has been shown to be activated by TLRs and is an important part of the oxidative burst signaling pathway in chicken macrophages [12,21–24]. We used an inhibitor of NF-κB, Bay 11-7082, to determine if NF-κB is involved in the TLR mediated oxidative burst pathway. Bay 11-7082 decreased LTA-stimulated oxidative burst by 56% and 61% at concentrations of 25 and 50 μM, respectively (Fig. 5).

4. Discussion

Intracellular signal transduction is a series of elaborate biochemical pathways regulated by multiple enzyme systems and secondary messengers [3]. Several of these signal transduction enzymes and secondary messengers have been shown to be involved in the oxidative burst of mammalian and avian phagocytes including PI3-K, PLC, PKC, ERK, and NF-κB [9,25–29]. The majority of reports on oxidative burst examines interactions with Fc or complement receptors [3,15,28]. However, preliminary reports demonstrate the involvement of TLRs in oxidative burst [8,18,23,25,29]. The present study utilized a TLR2 agonist, *S. aureus* lipoteichoic acid, to evaluate the signal transduction pathways involved in TLR-mediated oxidative burst in chicken heterophils.

The IL-1R has a binding domain that is homologous to the TLR cytoplasmic domain [30]. The activated interleukin-1 receptor (IL-1R) can elicit proliferation, differentiation, or changes in metabolism, and the majority of these biological effects are mediated through NF-κB as has been similarly demonstrated in TLRs [13,21]. The IL-1R has a binding site for PI3-K that is an important upstream transduction factor for NADPH oxidase activation and phagocytosis in neutrophils [15]. Although IL-1R and TLR may have different extra-cellular signals it is likely that they have
common signaling pathways [31]. We hypothesized that PI3-K is also involved in TLR-mediated oxidative burst, and demonstrated the importance of this enzyme as part of the TLR activated cascade leading to heterophil oxidative burst through the use of the specific PI3-K inhibitor, wortmannin (Fig. 1). Wortmannin significantly inhibited oxidative burst in chicken heterophils, and we must conclude that PI3-K participates physiologically in the oxidative burst of heterophils. These results are consistent with the role of PI3-K in signaling pathways leading to the activation of oxidative burst in mammalian neutrophils [15,27].

Activated PLC plays a central role in signal transduction pathways leading to oxidative burst in mammalian neutrophils [16]. PLC functions in the cell by converting phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) (an activator of PKC) and inositol 1,4,5-triphosphate (release of intracellular stores of Ca\(^{2+}\)) [9,27]. Employing the specific PLC inhibitor U-73122, we demonstrated that PLC is a required second messenger in the signaling cascade of TLR-mediated oxidative burst in avian heterophils (Fig. 2).

PKC is a well-known activator of oxidative burst as it promotes the phosphorylation and translocation of p47 phox as the initial step for NADPH oxidase activation [3,9,15]. Similarly, we have previously reported that chelerythrine (a PKC inhibitor) significantly reduced LTA-stimulated oxidative burst in chicken heterophils [8]. Members of the PKC family have been grouped into three categories of cPKC, aPKC, and nPKC. The cPKCs and aPKCs are calcium-dependent and are activated by DAG or phorbol ester, while the nPKCs are neither calcium-dependent nor responsive to DAG or phorbol ester [32]. Since PKC is involved in the TLR signaling pathway, we used a more selective inhibitor Gö 6976 that inhibits the Ca\(^{2+}\)-dependent PKC α-isozyme [32]. Gö 6976 significantly inhibited LTA-stimulated oxidative burst indicating that Ca\(^{2+}\)-dependent PKC is involved in TLR-mediated oxidative burst (Fig. 3). Further research utilizing calcium chelators such as BAPTA and EDTA will be required to determine the role of Ca\(^{2+}\) in TLR2-mediated oxidative burst.

In mammalian neutrophils, the mitogen-activated protein kinase (MAPK) super family is a signal transduction pathway common to many stimuli [17]. ERK, a member of the MAPK family, has been reported to be activated by PKC and is involved in the phosphorylation of p47 (phox) when stimulated with bacterial agonists [19,25]. Similarly, in the present experiments, the ERK inhibitor (PD 98105) significantly decreased oxidative burst in chicken heterophils. These results provide indirect evidence implying that the PKC → ERK → phosphorylation of p47 phox pathway is also activated in chicken heterophils following TLR ligation (Fig. 4). Further experiments are planned to fully prove this hypothesis.

TLRs and NF-κB have been reported to be important components of the oxidative burst signaling pathway [6,7,23,29]. NF-κB is inactive when associated with the inhibitor IκB-α [33]. The TLR signaling pathway is reported to phosphorylate IκB-α allowing NF-κB to translocate into the nucleus and up regulate defense-related and pro-inflammatory gene expression [22,23,29,33,34]. To define the role of NF-κB in TLR-mediated oxidative burst we applied an inhibitor of IκB-α phosphorylation (Bay 11-7082), which prevents the activation of NF-κB [33]. Bay 11-7082 significantly decreased LTA stimulated oxidative burst demonstrating the involvement of NF-κB in TLR mediated oxidative burst (Fig. 5). Similar results have been reported in chicken macrophages [23].

In summary, by employing selective pharmacological inhibitors, we have been able to identify those signaling pathways activated by cross-linking of TLR2 on the surface membrane of chicken heterophils that results in oxidative burst. These results are similar to what has been reported on the signaling pathways involved in mammalian neutrophils [9,15,19,25,26,29]. Due to the conserved nature of mammalian and avian TLRs we can speculate that engaging TLR2 on chicken heterophils activates a PI3K → PLC → PKC → ERK → NF-κB signal transduction pathway that induces oxidative burst.

References


