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ScienceDirect
Molecular Immunology 45 (2008) 1001–1008
Molecular Immunology
www.elsevier.com/locate/molimm

Thrombocytes respond to lipopolysaccharide through Toll-like receptor-4, and MAP kinase and NF-κB pathways leading to expression of interleukin-6 and cyclooxygenase-2 with production of prostaglandin E2

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Received 9 June 2007; accepted 27 July 2007
Available online 6 September 2007

Abstract

Chicken thrombocytes are equivalent in hemostatic function to mammalian platelets. Platelets are enucleated components of mammalian blood, while thrombocytes are nucleated blood leukocytes of chickens. Platelets and thrombocytes share characteristics that contribute to innate immunity. Experiments were conducted to determine if thrombocytes could respond in vitro to lipopolysaccharide (LPS) of *Salmonella minnesota* through Toll-like receptor-4 (TLR4). The aim was to activate the signal pathways leading to expression of interleukin-6 (IL-6) and inducible cyclooxygenase (COX-2) and to production of prostaglandin E2 (PGE2). Chicken thrombocytes were found to express TLR4, and LPS-induced an increase in thrombocyte mRNA expression of IL-6 and COX-2 with release of PGE2 into culture media. An increase of COX-2 and PGE2 due to LPS stimulation was inhibited by MEK1 inhibitor PD98059, but IL-6 expression was unaffected by PD98059. The IKK-2 inhibitor BMS345541 inhibited IL-6 and COX-2 with reduction of PGE2 concentrations. Therefore, the MAP kinase (MAPK) pathway activates expression of COX-2 and ultimately PGE2 production, but this pathway has little or no influence on IL-6 expression in thrombocytes. The NF-κB pathway also influences COX-2 expression and PGE2 production, and it is a primary activation signaling cascade for IL-6 gene expression in chicken thrombocytes. Thrombocytes represent a major component of the innate immune system of chickens in response to LPS and possibly other microbial products.

Keywords: Thrombocytes; Toll-like receptor-4; Interleukin-6; Cyclooxygenase-2; Prostaglandin E2; MEK1; NF-κB

1. Introduction

Chicken thrombocytes are nucleated blood leukocytes that produce and elaborate several bioactive proteins and compounds, which align these cells with the same or similar functions of mammalian platelets. β-Thromboglobulin (Wachowicz and Krajewski, 1979), thrombocyte-derived growth factors (Horiuchi et al., 1990) and lysosomal compounds (Daimon and Uchida, 1982) are produced by these cells. More recently thrombocytes were found to contain MIP-1β and other chemotactic factors (Lam, 2002), platelet-derived growth factor-A and -B chains (Horiuchi et al., 2001; Horiuchi et al., 2002) and leukotriene B_4 (Jha et al., 2005). Just like mammalian platelets, expression of CD40L (CD154) occurs on chicken thrombocytes (Tregaskes et al., 2005), and this observation gives weight to the importance of thrombocytes in integration and modulation of immunity.

The list of biologically active compounds synthesized or released by mammalian platelets upon activation is long and varied (Elzey et al., 2005), and many of the compounds have chemotactic, adhesion or vasoactive properties. In many cases these platelet compounds are pre-formed and stored in vacuoles while others are synthesized by enzymatic pathways. Due to the fact that platelets are enucleated cells that bleb-off megakaryocytes in the bone marrow, there has been interest in whether platelets do or do not possess cytoplasmic mechanisms for expression initiated through cell surface receptors. With regard to surface receptors, platelets express CD40L (CD154) (Chakrabarti et al., 2005; Martinson et al., 2004), P-selectin (CD62) (Nagata et al., 1993), FasL (Ahmad et al., 2001), chemokine receptors (Boehlen and Clemetson, 2001), Toll-like receptors (TLR) (Cognasse et al., 2005) and FcγRIIA

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doi:10.1016/j.molimm.2007.07.035

and αIIbβ3 (Pampolina and McNicol, 2005). The presence of these receptors indeed reveals a ligand reactive and stimulatory nature for platelets. This has been substantiated by the presence of cytokine (IL-7, SCF and TGF-β) mRNA (Soslau et al., 1997); release of cytokines, CC and CXC chemokines (Boehlen and Clemetson, 2001; Klinger and Jelkmann, 2002), leukotriene B_4 (Palmantier and Borgeat, 1991) and thromboxane A_2 (Pampolina and McNicol, 2005); and generation of reactive oxygen intermediates (Chakrabarti et al., 2005).

Recognizing the parallel functions of mammalian platelets and chicken thrombocytes, it is also intriguing to question the function of nucleated thrombocytes as blood leukocytes possessing the potential to mediate immune responses. Of interest is the probable role of these cells in the mediation of inflammation in support of innate immunity. With this in mind and knowledge of the apparent expression of receptors and some mediators of innate immunity, a series of experiments was undertaken to determine if chicken thrombocytes could respond to lipopolysaccharide (LPS). Also, could a response occur through TLR4-linked signaling pathways resulting in increased expression of interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) mRNA with the release of the inflammatory mediator prostaglandin E2 (PGE2)? Furthermore, the effects of both MAPK kinase (MEK1) and IκB kinase-2 (IKK-2) inhibitors on the expression of IL-6 and COX-2 mRNA and the production of PGE2 were examined to determine if one or both of the MAPK and/or NF-κB pathways are functional in chicken thrombocytes leading to pro-inflammatory responses.

2. Materials and methods

2.1. Chickens

Ten- to 12-week-old Single Comb White Leghorn female chickens were used for experiments. Different groups of chickens were used for each of the three experiments examining the effects of lipopolysaccharide (LPS), a MEK1 inhibitor and an IKK-2 inhibitor, respectively. Chickens were reared and maintained at the Morgan Poultry Center, Clemson University, Clemson, SC.

2.2. Thrombocyte recovery

Whole blood (3 mL) was drawn by syringe from the wing vein of each chicken into a 0.1 mL volume of 10% EDTA solution. The blood samples were immediately placed on ice for transport to the laboratory. Under a sterile bench, each blood sample was diluted at 1:1 with Ca^{2+} and Mg^{2+} free Hank's balance salt solution (HBSS). Three milliliters of diluted blood were layered on top of 3 mL of lymphocyte separation medium (density = 1.077 g/mL, Mediatech Inc.) in 15 mL conical tubes. The conical tubes were centrifuged at 1700 × *g* for 30 min at RT according to a previously established procedure (Horiuchi et al., 1990). The thrombocyte cell layer at the plasma/medium interface was removed with a sterile Pasteur pipette and transferred to a sterile, round-bottom polystyrene tube. The cells were washed twice with 2 mL HBSS by centrifugation at 450 × *g* for 5 min at RT. The final cell pellets were resuspended in 2 mL HBSS and placed on ice. Cell counts for each sample were done via trypan blue exclusion method. Centrifugation was repeated and each cell sample was resuspended in RPMI-1640 culture medium so that 1×10^7 cells could be transferred in appropriate volumes to 1.5 mL sterile, RNase- and DNase-free microcentrifuge tubes for short-term culture.

2.3. Thrombocyte culture

As stated above, 1×10^7 thrombocytes were transferred to 1.5 mL microcentrifuge tubes in appropriate RPMI-1640 culture medium volumes with addition of LPS and/or inhibitors (or DMSO control) to give a final volume of 1 mL. All incubations were performed at 41 °C in a humidified, water-jacketed CO_2 (5%) incubator on a rocking platform, with tubes gently and constantly agitating end-to-end.

2.3.1. First experiment

Thrombocytes were cultured in the presence of 0, 0.1, 1 and 10 μg/mL LPS (*Salmonella minnesota* Ultra pure LPS, InvivoGen) for 1 h.

2.3.2. Second experiment

Thrombocytes underwent initial 30 min incubation with 10 μM of the MEK1 inhibitor PD98059 (Biomol International) or DMSO control. Following the 30 min incubation, 10 μg/mL LPS was added to the appropriate sample tubes for 1 h culture.

2.3.3. Third experiment

Thrombocytes were cultured as above with the following changes. Cells were initially incubated with 5 μM of the IKK-2 inhibitor BMS345541 (Calbiochem) or DMSO control for 30 min after which 10 μg/mL LPS was added to the appropriate sample tubes for 1 h treatment.

At the conclusion of each culture period, the tubes were immediately spun in a microcentrifuge for 2 min at 5000 × *g*. The resulting supernatants were recovered and transferred to sterile 1.5 mL microcentrifuge tubes and stored at −20 °C, and the pellets were resuspended in 100 μL RNA*later*™ and frozen (−20 °C).

2.4. RNA isolation and real-time reverse transcriptase polymerase chain reaction

Isolated cells (1×10^7) frozen in RNA*later*™ were processed for mRNA isolation according to the Qiagen RNeasy® mini-column procedure with on-column DNase digestion. The RNA content and purity was then determined with a spectrophotometer (Eppendorf BioPhotometer).

Real-time reverse transcriptase PCR was performed with the QuantiTech SYBR Green RT-PCR kit (Qiagen). Individual RT-PCR reactions were established with 10 ng of target gene mRNA, 0.5 μM primers for each target (Table 1), 2.5 mM $MgCl_2$ and all other components for the reactions according to the kit manufacturer's recommendations. Amplification and detection of target genes was performed using an Eppendorf Mastercycler®