Involvement of $G_i$ proteins and Src tyrosine kinase in TNF$\alpha$ production induced by lipopolysaccharide, group B Streptococci and Staphylococcus aureus

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Abstract

Previous studies have suggested that heterotrimeric $G_i$ proteins, Src tyrosine kinase and phosphatidylinositol-3 kinase (PI3 Kinase) are involved in signaling events induced by lipopolysaccharide (LPS) leading to pro-inflammatory cytokines gene expression. To investigate the involvement of these mediators in Gram-positive bacteria induced pro-inflammatory cytokine expression, LPS (10 ng/ml), heat killed group B Streptococci (GBS 1 l$g$/ml) and Staphylococcus aureus (SA 10 l$g$/ml) were used to induce TNF$\alpha$ production in the murine J774A.1 macrophage (MØ) cell line and human promonocytic THP-1 cell line. Pertussis toxin (PTx, 1 l$g$/ml), an inhibitor of $G_i$ protein; pyrazolopyrimidine-2 (PP2, 1 or 25 l$M$), a Src tyrosine kinase inhibitor; and LY294002 (100 nM), an inhibitor of PI3 Kinase were used to examine the involvement of $G_i$, Src tyrosine kinase and PI3 Kinase, respectively, in TNF$\alpha$ production. In J774A.1 cells, pretreatment with PTx and PP2 attenuated TNF$\alpha$ production induced by LPS (60 $\pm$ 9% and 81 $\pm$ 11% inhibition, $n = 3$, $p < 0.05$, respectively), GBS (95 $\pm$ 1% and 80 $\pm$ 6% inhibition, $n = 3$, $p < 0.05$, respectively) and SA (51 $\pm$ 18% and 68 $\pm$ 16% inhibition, $n = 4$, $p < 0.05$, respectively). However, pretreatment with LY 294002 inhibited LPS induced TNF$\alpha$ production (60 $\pm$ 9% and 81 $\pm$ 11% inhibition, $n = 3$, $p < 0.05$), but did not inhibit GBS or SA induced TNF$\alpha$ production. In THP-1 cells, pretreatment with PTx, PP2 and LY 294002 inhibited TNF$\alpha$ production induced by LPS (84 $\pm$ 3%, 59 $\pm$ 12% and 84 $\pm$ 4% inhibition, $n = 3$, $p < 0.05$, respectively) and SA (56 $\pm$ 7%, 87 $\pm$ 1% and 35 $\pm$ 6% inhibition, $n = 3$, $p < 0.05$, respectively). These data support our hypothesis that $G_i$-coupled and Src tyrosine kinase-coupled signaling pathways are involved in both Gram-negative and Gram-positive bacteria induced pro-inflammatory cytokine expression. However, unlike LPS, involvement of PI3 Kinase in Gram-positive bacteria induced signaling pathways are species dependent.

1. Introduction

Both Gram-negative and Gram-positive bacteria are potent inducers of pro-inflammatory responses that can cause fatal sepsis syndrome [1,2]. Whereas the membrane glycolipid lipopolysaccharide (LPS) appears to be the main mediator of immune responses to Gram-negative bacteria, several components including peptidoglycans, lipoteichoic acid and lipoproteins on the cell surface of Gram-positive bacteria mediate cell activation [3–5]. Many of the manifestations of septic shock have been related to the exaggerated release of pro-inflammatory cytokines upon interaction of host cells with microbial products [6]. Understanding of the cell activation mechanism involved in sepsis may be critical.
to develop therapies aimed at reducing cytokine production.

The primary cause of sepsis syndrome is activation of MO pro-inflammatory cytokine gene expression by LPS, group B Streptococci (GBS) and Staphylococcus aureus (SA) through different cell receptors. The first identified LPS receptor is CD14, a 55-kDa glycosylphosphatidylinositol (GPI)-anchored protein expressed on the surface of monocytes and neutrophils, which binds LPS with high affinity [7–9]. Recently, human Toll-like receptors were discovered, and it was demonstrated that Toll-like receptor 4 (TLR4) plays a critical role in LPS mediated immune response [10,11]. Many other proteins including MD-2 [12], complement receptor type III (CR3) [13,14], as well as other Toll-like receptors, heat shock proteins, chemokine receptor CXCR4, growth differentiation factor 5 (GDF5) [15] and caspase activator Nod-1 [16] have all been implicated in the initial steps of LPS recognition and signaling. The Gram-positive bacteria SA induced signaling pathways are mainly mediated through Toll-like receptor 2 (TLR2) coupled with CD14 [3–5,17,18]. The receptors for the Gram-positive bacteria GBS remain unknown. GBS induced TNFα production is mediated by MyD88 suggesting that TLRs coupled with CD14 are also involved in GBS recognition, but it was found that TLR1, 2, 4 or 6 did not recognize GBS [19]. Blockade of CD14 or CR3 decreased GBS induced TNFα or nitric oxide (NO) production, which suggests that CR3 coupled with CD14 are the main receptors for GBS [20–22].

The extent to which Gram-negative or Gram-positive bacteria show common post-receptor signaling protein remain uncertain. Heterotrimeric guanine nucleotide binding regulatory (G) proteins are involved in LPS signaling. It has been shown that Gαi proteins are physically associated with the CD14 receptor [23]. Studies utilizing pertussis toxin (PTx), which specifically inhibits receptor-Gi coupling by catalyzing the ADP-ribosylation of Gαi proteins, have demonstrated inhibition of LPS-induced transcription of TNFα, IL-1 mRNA, activation of mitogen-activated protein (MAP) kinase, p38, JNK, ERK1/2 and production of prostaglandin E2 (PGE2), thromboxane B2 (TxB2) and NO in different cell types [24–27]. It was reported that SA induced cytokine expression was inhibited by mastoparan, which serve as a G protein antagonist [28].

Src family tyrosine kinase proteins are frequently associated with the Gi proteins [29–31]. Activation of Gi protein coupled receptors results in the tyrosine phosphorylation of adapter proteins such as SH2 containing protein (Shc) [32]. Subsequent association of tyrosine-phosphorylated Shc with Grb2 serves to direct the guanine nucleotide exchanger SOS to Ras. Src family tyrosine kinases are also involved in the LPS signaling pathway. Hck, Fgr and Lyn are three members of Src tyrosine kinase, Hck and Fgr knockout mice are remarkably resistant to endotoxin shock [33]. The Src family kinase inhibitor pyrazolopyrimidine-1 (PP1) blocked LPS induced TNFα production and NO synthesis [34].

The phosphatidylinositol-3 kinase (PI3 Kinase) signaling pathway is also involved in LPS signaling [35,36]. The PI3 Kinase-Akt pathway has been shown to regulate negatively NFκB and expression of pro-inflammatory cytokine genes induced by LPS [37]. On the other hand, it was also shown that PI3 Kinase-Akt pathway positively regulated LPS induced signaling [38,39]. PI3 Kinase signaling pathway were also necessary for heat killed SA induced NFκB activation [40]. As with Src tyrosine kinase, activation of PI3 Kinase leading to MAP kinase activation appears to be Gi coupled. The Gαi subunit can bind and regulate subunits of PI3 Kinase directly [41].

In this study, we hypothesize that Gi proteins, Src tyrosine kinase and PI3 Kinase are involved in both Gram-negative and Gram-positive bacteria induced pro-inflammatory cytokine expression. LPS, GBS and SA induced TNFα production in murine MO J774A.1 cells and human promonocytic THP-1 cells were investigated. Specific inhibitors PTx, pyrazolopyrimidine-2 (PP2) and LY294002 were used to inhibit LPS, GBS and SA stimulation.

2. Results

2.1. Effects of PTx on LPS, GBS or SA induced TNFα production

To test the hypothesis that Gi proteins are involved in both Gram-negative and Gram-positive bacteria induced pro-inflammatory cytokine release, J774A.1 cells were pretreated with PTx (1 μg/ml) followed by stimulation with LPS, GBS or SA. PTx inhibited LPS induced TNFα production by 60±9% (n = 3, p < 0.05, Fig. 1A), GBS induced TNFα production by 95±1% (n = 3, p < 0.05, Fig. 1B) and SA induced TNFα production by 51±18% (n = 4, p < 0.05, Fig. 1C). These data were extended in human promonocytic THP-1 cells. Our data demonstrated that unlike J774A.1 cells, THP-1 cells were unresponsive to GBS (1–100 μg/ml) stimulation (data not shown). However, pretreatment of PTx inhibited TNFα production induced by LPS (84±3% inhibition, n = 3, p < 0.05, Fig. 2A) and SA (56±7% inhibition, n = 3, p < 0.05, Fig. 2B).

2.2. Effects of PP2 on LPS, GBS or SA induced TNFα production

Src tyrosine kinase mediated signaling pathways are regulated by Gi proteins. To investigate the involvement of Src tyrosine kinase in LPS, GBS and SA
induced TNFα production, the J774A.1 cells were pretreated with PP2 (1 μM) and stimulated with LPS, GBS or SA. PP2 pretreatment significantly decreased TNFα production induced by LPS (81 ± 11% inhibition, n = 3, p < 0.05, Fig. 3A), GBS (80 ± 6% inhibition, n = 3, p < 0.05, Fig. 3B) and SA (68 ± 16% inhibition, n = 4, p < 0.05, Fig. 3C). A higher concentration of PP2 (25 μM) was necessary for blockade of TNFα production induced by LPS and SA in THP-1 cells. Pretreatment of THP-1 cells with PP2 inhibited TNFα production induced by LPS (59 ± 12% inhibition, n = 3, p < 0.05, Fig. 4A) and SA (87 ± 1% inhibition, n = 3, p < 0.05, Fig. 4B).

2.3. Effects of LY294002 on LPS, GBS or SA induced TNFα production

To test the involvement of PI3 Kinase in LPS, GBS and SA induced TNFα production, the J774A.1 cells were pretreated with LY294002 (100 nM) and stimulated with LPS, GBS or SA. LY294002 pretreatment significantly inhibited LPS induced TNFα production (82 ± 13% inhibition, n = 3, p < 0.05, Fig. 5A) but did not inhibit GBS or SA induced TNFα production (Fig. 5B, C).
However, in THP-1 cells LY294002 pretreatment significantly inhibited TNFα production induced by LPS (84±4% inhibition, n = 3, p < 0.05, Fig. 6A) and SA (35±6% inhibition, n = 3, p < 0.05, Fig. 6B).

3. Discussion

These studies show that in J774A.1 cells pretreatment with PTx attenuated TNFα production in THP-1 cells. THP-1 cells were pretreated with PP2 (25 μM) for 1 h followed by stimulation with LPS (10 μg/ml) (A) or heat killed SA (10 μg/ml) (B) for 24 h. LPS and SA induced TNFα production in the presence of vehicle was arbitrarily set at 100%. Data represent the mean±SE from three independent experiments. *, p < 0.05 PP2 pretreated group stimulated with LPS, GBS and SA versus LPS, GBS and SA stimulated control group. #, p < 0.05 basal group versus LPS and SA stimulated control group.
part MyD88 dependent signaling suggesting that GBS may couple to TLRs, which remain to be identified. Apparently the receptors responsible for GBS activation are missing in THP-1 cells as GBS failed to induce TNF-$\alpha$ in these cells (data not shown). Despite the uncertainties of GBS receptors, our results support the hypothesis that Gi proteins play a common role in both Gram-positive and Gram-negative bacteria induced signaling pathways leading to pro-inflammatory cytokine gene expression. Whether Gi proteins directly bind with TLR2, TLR4, CR3 or other receptors or post-receptor protein MyD88 will need further investigation.

It was reported that treatment with TNF-$\alpha$ significantly increased the expression of $\zeta_i$ proteins in different cells including human airway smooth muscle cells, rat cardiomyocytes and polymorphonuclear leukocytes [43–45]. Our studies suggest that Gi proteins play a common role in bacteria induced TNF-$\alpha$, which
suggests the possibility that the cells may use this positive feedback mechanism to enhance signaling.

In J774A.1 cells, pretreatment with PP2 decreased LPS, GBS and SA induced TNFα production suggesting that Src family tyrosine kinase proteins are involved in LPS, GBS and SA induced TNFα gene expression. Our previous studies demonstrated that pretreatment of THP-1 cells with PP2 blocked LPS induced TxB2 and a NFκB reporter gene construct [46]. However, the effect of PP2 on GBS and SA was not investigated in the previous studies. Our present data suggested that down stream of Gi proteins, Src tyrosine kinase also plays a common role in both Gram-negative and Gram-positive bacteria induced pro-inflammatory cytokine gene expression.

Pretreatment of murine J774A.1 MØ with the PI3 Kinase inhibitor LY 294002 blocked LPS induced TNFα production but did not inhibit GBS and SA induced signaling pathway. In THP-1 cells, pretreatment with LY 294002 inhibited both LPS and SA induced TNFα production. However, LY 294002 inhibited LPS induced TNFα production by 84 ± 4%, whereas the inhibition of SA induced TNFα production was only 35 ± 6%. Thus even in THP-1 cells the role of the PI3 Kinase appears to be more predominant in LPS signaling. These data also suggest that involvement of PI3 Kinase in Gram-positive bacteria induced signaling pathways may be species dependent. Our data suggest that the PI3 Kinase positively regulates LPS induced TNFα production, which is in agreement with other studies [38,39]. However, Guha and Mackman [37] demonstrated that PI3 Kinase-Akt pathway negatively regulates LPS induced TNFα production. Such differences in PI3 Kinase function may reflect variations in species or cell types in addition to differences in Gram-positive and Gram-negative bacteria stimuli. Arbibe et al. [40] reported that PI3 Kinase positively regulates SA induced NFκB activation, which agree with our data.

These data support the hypothesis that Gi-coupled and Src tyrosine kinase-coupled signaling pathways are involved in both Gram-negative and Gram-positive bacteria induced pro-inflammatory cytokine gene expression. The incidence of Gram-positive sepsis is increasing [47], with increased risk of polymicrobial sepsis. Solomon et al. [23] reported that mastoparan, a ligand agonist/antagonist of Gz, proteins protects rats from LPS shock. Therefore targeting Gi proteins with pharmacological agents may be an effective method of treating septic shock resulting from polymicrobial sepsis.

4. Materials and methods

4.1. Cell culture

J774A.1 cells and THP-1 cells (American Type Culture Collection, Manassa, VA) were grown in RPMI 1640 medium (Cellgro Mediatech Inc., Herndon, VA), supplemented with heat inactivated 10% fetal calf serum (FCS) (Sigma, St. Louis, MO), 50 U/ml penicillin and 50 μg/ml streptomycin (Cellgro Mediatech) in 150 cm² tissue culture flasks and maintained at 37°C in 5% CO2, 95% incubator air. The confluent J774A.1 cells within 40 passages were used for experiments. THP-1 cells within 10 passages were used for experiments.

4.2. Cell stimulation

For J774A.1 cells stimulation, 2 ml of J774A.1 cells at a density of 1×10⁶ cells/ml were added in six-well plates. The cells were pretreated with PTx (List Biological Laboratories Inc., Campbell, CA) at a concentration of 1 μg/ml for 6 h, PP2 (1 μM; Calbiochem, San Diego, CA) for 1 h or LY294002 (100 nM; Sigma) for 1 h. After pretreatment, the cells were washed and stimulated with LPS (10 ng/ml) from Salmonella enteritidis (Sigma), heat killed GBS (1 μg/ml) or SA (10 μg/ml) (heat killed GBS and SA were kind gifts from Dr Giuseppe Teti, Medical University of Messina, Messina, Italy) for 20 h. For stimulation of THP-1 cells, 10 ml of THP-1 cells at a density of 1×10⁶ cells/ml were pretreated with PTx at a concentration of 1 μg/ml for 6 h, PP2 (25 μM) for 1 h or LY294002 (100 nM) for 1 h. After pretreatment, the cells were washed and stimulated with LPS (10 μg/ml) or heat killed SA (10 μg/ml) for 24 h.

Viability was quantitated with Trypan Blue exclusion LPS, GBS, SA, PTx, PP2 and LY294002 at the concentration of drugs used in this paper do not affect cell viability (>95% viability).

4.3. TNFα assay

TNFα was measured using an enzyme-linked immunosorbant assay (ELISA) with mouse TNFα ELISA kits (eBioscience, San Diego, CA).

4.4. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined by unpaired Student’s t-test or by analysis of variance (ANOVA) with Fisher’s probable least-squares difference test using Statview software (SAS Institute Inc., Cary, NC). p < 0.05 was considered significant.

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References


