

Interleukin-1 β -induced interleukin-6 production in A549 cells is mediated by both phosphatidylinositol 3-kinase and interleukin-1 receptor-associated kinase-4

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Abstract

The aim of this study is to investigate whether PI3K (phosphatidylinositol-3-kinase) is involved in IL-1 β (interleukin-1 β)-induced IL-6 production in A549 (human lung adenocarcinoma epithelial cell) and human RASF (rheumatoid arthritis synovial fibroblast). PI3K inhibitor, LY294002 significantly reduced IL-1 β -induced IL-6 production in A549 cells but not in RASF, indicating that IL-1 β -induced IL-6 production was partially mediated by PI3K in A549 cells but not in RASF. siRNA (small interfering RNA) of IRAK4 (IL-1 receptor-associated kinase 4) treatment decreased IRAK4 mRNA level by up to 90% in A549 cells. In this condition, IL-1 β -induced increase of IL-6 mRNA and protein level was decreased by up to 93% and 70%, respectively. Furthermore, the combination of IRAK4 siRNA and LY294002 treatment decreased protein induction level of IL-6 in A549 cells compared with that of IRAK4 siRNA or LY294002 alone. These results indicate that IL-1 β -induced IL-6 production in A549 cells is mediated by both PI3K and IRAK4 and suggest that involvement of PI3K in the IL-1-induced IL-6 production is cell type specific.

Keywords: interleukin-6 (IL-6); interleukin-1 receptor associated kinase-4 (IRAK4); phosphatidylinositol 3-kinase (PI3K); rheumatoid arthritis synovial fibroblast (RASF); signal transduction; small interfering RNA (siRNA)

1. Introduction

IL-6 (interleukin-6) is a pleiotropic cytokine produced by a diverse set of cell populations and plays a crucial role in immune and inflammatory responses. Levels of IL-6 are increased in blood as well as the sites of diseases, such as in patients with COPD (chronic obstructive pulmonary disease) and RA (rheumatoid arthritis) (Madhok et al., 1993; Bucchioni et al., 2003), and therefore, IL-6 has been implicated in the pathogenesis of a variety of diseases. Inflammatory cytokines, such as IL-1 and TNF- α (tumour necrosis factor- α) induce IL-6. IL-1 is a key modulator of immune and inflammatory processes by mediating inflammation by activating macrophages, recruiting neutrophils and stimulating growth and differentiation of B cells and T cells. IL-1 is also responsible for mediating several diseases such as local and systemic infection, degenerative arthritis and autoimmune diseases (Dinarello, 1998). IL-1 binds to the IL-1 receptor that belongs to the TIR (Toll/IL-1R) family (O'Neill and Dinarello, 2000) characterized by an intracytoplasmic TIR domain, which mediates recruitment of the IRAK (IL-1 receptor-associated kinase) complex with adaptor proteins, such as MyD88 (myeloid differentiation protein 88) (Wesche et al., 1997). Activation of IRAK4 leads to phosphorylation of IRAK1 resulting in the

subsequent phosphorylation/activation of downstream substrates, such as NF- κ B (nuclear factor- κ B) (Beutler, 2000). On the other hand, recent evidence has shown that several additional molecules are involved in IL-1 signalling, such as PI3K (phosphatidylinositol 3-kinase) (Reddy et al., 1997; Marmiroli et al., 1998), indicating that a complex integrated circuitry may be at work. However, it is unclear whether PI3K pathway is involved in IL-1 β -mediated IL-6 production in lung epithelial cells and RASF (rheumatoid arthritis synovial fibroblasts). In the current study, we confirm that IL-1 β -mediated IL-6 production in A549, lung epithelial cells is mediated by both IRAK and PI3K signalling, while only the IRAK pathway is involved in IL-1 β -mediated IL-6 production in RASF.

2. Materials and methods

2.1. Cell culture and treatment

A549 cells were purchased from the ATCC (American Type Culture Collection) and cultured according to the protocol provided by the ATCC. F-12 medium (Invitrogen) containing 10% FBS (fetal bovine serum, Sigma–Aldrich) was used as a

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Abbreviations: ATCC, American Type Culture Collection; COPD, chronic obstructive pulmonary disease; FBS, fetal bovine serum; IL-1 β , interleukin-1 β ; IL-1RI, IL-1 receptor type I; IL-6, interleukin-6; IL-1RAcP, IL-1R accessory protein; IRAK4, IL-1 receptor-associated kinase 4; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation protein 88; NF- κ B, nuclear factor- κ B; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; qRT, quantitative real-time; RASF, rheumatoid arthritis synovial fibroblast; siRNA, small interfering RNA; TAB1, TAK1-binding protein; TAK1, transforming growth factor- β activated kinase 1; TIR, Toll/IL-1R; TNF- α , tumour necrosis factor- α .

culture medium. Human rheumatoid arthritis synovial fibroblast was established in house and cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 15% FBS. PI3K inhibitor, LY294002 was purchased from Tocris Bioscience. A549 cells ($3\text{--}5 \times 10^4$ cells/well) or RASF ($4\text{--}5 \times 10^4$ cells/well) were plated on to 96-well culture plate and then cultured overnight. On the next day, cells were pretreated with LY294002 for 45 min, and IL-1 β (1 ng/ml; R&D Systems) was added in the culture medium and cells were further incubated for 4 h (A549) or 20 h (RASF).

2.2. siRNA treatment

IRAK4 siRNA (ON-TARGET plus SMART pool) and control siRNA (siCONTROL Non-Targeting siRNA pool) was purchased from Thermo Scientific. A549 cells (2.5×10^4 cells/96-well) were plated on to 96-well culture plate and then cultured overnight. On the next day, siRNA (control siRNA and IRAK4 siRNA, final concentration: 100 nM) was added into cells and further incubated for 72 h. Then, cells were pretreated with LY294002 for 45 min and IL-1 β (1 ng/ml; R&D Systems) was added in the culture media, and cells were further incubated for 4 h.

2.3. qRT (quantitative real-time)-PCR assay and measurement of IL-6

IL-6 level in the culture media was determined using MSD MULTI-ARRAY and MULTI-SPOT kits (Meso Scale Discovery) according to the manufacturer's directions. In qRT-PCR experiments, cells were lysed with Nucleic Acid Purification Lysis Solution (Applied Biosystems), and total RNA was isolated using 6100 Nucleic Acid PrepStation according to the manufacturer's directions (Applied Biosystems). pRT-PCR was conducted using qScript™ One-Step qRT-PCR kit (Quanta BioSciences). Amplified reactions were quantified on an ABI PRISM 7900 Sequence detection system (Applied Biosystems). Relative gene quantities were obtained using the comparative C_t method after normalization to appropriate

control gene (cyclophilin A: NM_021130.3). TaqMan probes were also purchased from Applied Biosystems.

3. Results

3.1. Involvement of PI3K in IL-1 β -induced IL-6 production

To determine if PI3K is involved in IL-1 β -induced IL-6 production in A549 cells and RASF, A549 cells and RASF were treated with IL-1 β in the absence or presence of PI3K inhibitor LY294002. IL-1 β -induced IL-6 production from A549 cells was significantly decreased by LY294002 in a concentration-dependent manner, while LY294002 at a highest concentration (20 μ M) did not completely inhibit the IL-1 β -induced IL-6 production from A549. Also, similar results were obtained using other PI3K inhibitors specific to PI3K p110 α or PI3K p110 β (data not shown). These results indicate that IL-1 β -induced IL-6 production in A549 cells was partly mediated by PI3K (Figure 1a). In contrast, no decrease in the IL-1 β -induced IL-6 production in RASF was observed by LY294002, indicating that PI3K was not involved in the production of IL-1 β -induced IL-6 production in RASF (Figure 1b).

3.2. Effect of IRAK4 siRNA on IL-1 β -induced IL-6 production

Next, we investigated whether IL-1 β -induced IL-6 production was mediated by IRAK4 using IRAK4 siRNA in A549 cells. IRAK4 mRNA level was decreased by up to 90% by IRAK4 siRNA (Figure 2). In this condition, IL-1 β -induced increase of IL-6 mRNA level was decreased by 93%, and the level of IL-6 production was also decreased by 70% (Figures 3 and 4). Furthermore, combination of IRAK4 siRNA and LY294002 further decreased the protein induction level of IL-6 in A549 cells, indicating that IL-1 β -induced IL-6 production in A549 cells is mediated by both PI3K and IRAK4 (Figures 3 and 4).

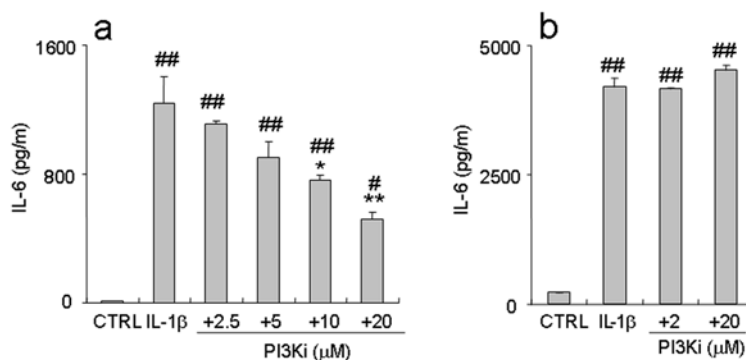


Figure 1 IL-1 β -induced IL-6 production in A549 (a) or RASF (b)

PI3Ki: PI3K inhibitor (LY294002); IL-1 β (1 ng/ml). Data were indicated by means \pm S.E.M. ($n=3$). # $P<0.001$ compared with CTRL; ## $P<0.0001$ compared with CTRL; * $P<0.001$ compared with IL-1 β alone; ** $P<0.0001$ compared with IL-1 β alone (one-way ANOVA with Bonferroni, GraphPad Prism ver. 4.03, GraphPad).

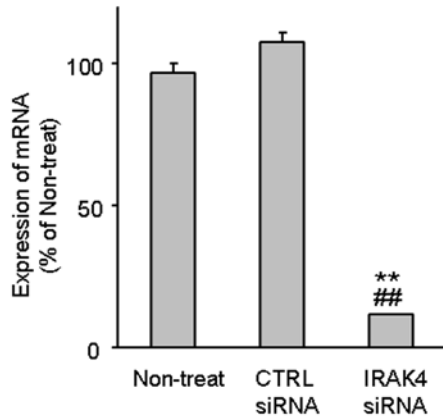


Figure 2 Reduction of IRAK4 mRNA expression by siRNA in A549

Relative gene quantities were obtained using the comparative C_t method after normalization to human cyclophilin A (NM_021130.3). Data [means \pm S.E.M ($n=3$)] were indicated by fold change of gene expression calculated by dividing the value of treatment group by the non-treatment. ** $P<0.001$ compared with non-treat; ## $P<0.001$ compared with CTRL siRNA (one-way ANOVA with Bonferroni, GraphPad Prism ver. 4.03).

4. Discussion

In this study, we have demonstrated that IL-1 β -induced IL-6 production is mediated by both PI3K and IRAK4 in A549 cells, while only IRAK4 mediates IL-1 β -induced IL-6 production in RASF. The involvement of PI3K in IL-1 β -mediated signalling pathway has been reported (Reddy et al., 1997; Marmioli et al., 1998; Sizemore et al., 1999; Neumann et al., 2002; Cenni et al., 2003; Cahill and Rogers, 2008). PI3K inhibitor did not decrease the IL-1 β -induced IL-6 production in Swiss 3T3 and SaoS2 cells, indicating that PI3K is not involved in the IL-1 β -induced IL-6 production in these cell lines (Neumann et al., 2002; Cenni et al., 2003). In contrast, IL-1 β -induced IL-6 mRNA and IL-6 production in Caco-2 cells were completely inhibited by PI3K inhibitor, LY294002, indicating that PI3K-dependent pathway is dominating in the IL-1 β induction of IL-6 in Caco-2 cells (Cahill and Rogers, 2008). These results suggest

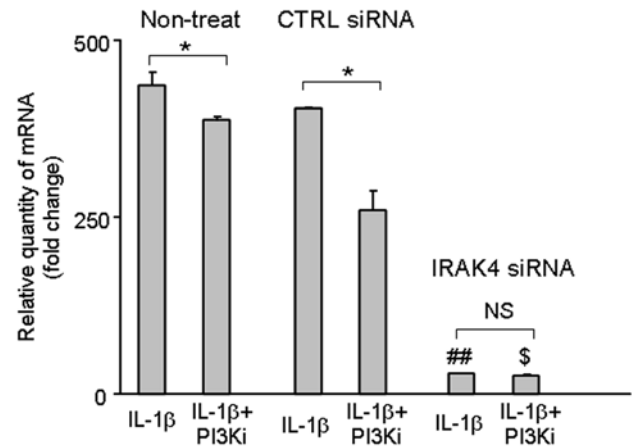


Figure 3 Effect of IRAK4 siRNA and PI3K inhibitor on the IL-1 β -induced IL-6 mRNA expression in A549

PI3Ki: PI3K inhibitor (LY294002: 20 μ M), IL-1 β (1 ng/ml). Relative gene quantities were obtained using the comparative C_t method after normalization to human cyclophilin A (NM_021130.3). Data were indicated by means \pm S.E.M ($n=3$). * $P<0.05$ compared with IL-1 β . ## $P<0.01$ compared with CTRL siRNA treated with IL-1 β ; \$ $P<0.05$ compared with IRAK4 siRNA treated with IL-1 β and PI3Ki (Welch test, GraphPad Prism ver. 4.03).

that the involvement of PI3K in the IL-1-induced IL-6 production is cell type specific. Our results are in accordance with these reports as discussed above. The involvement of PI3K in IL-1-induced IL-6 production depends on the cell types; PI3K is partially involved in the IL-1 β -induced IL-6 production in A549 cells but not in RASF.

IRAK4 is the most proximal kinase mediating IL-1 signalling and plays an essential role in IL-1/TLR-mediated signalling (Suzuki et al., 2002). In the present study, ablation of IRAK4 expression in A549 with siRNA suppressed IL-1 β -induced IL-6 production. This result clearly demonstrates that IRAK4 has a critical role in the IL-1 signalling pathway in A549 cells. On the other hand, it has been reported that p85, regulatory subunit of PI3K directly interacts with IL-1RI (IL-1 receptor type I) or with IL-1RAcP (IL-1R accessory protein) (Reddy et al., 1997; Marmioli et al., 1998). The catalytic subunit of PI3K, p110 subsequently associates with p85, which results in the full activation

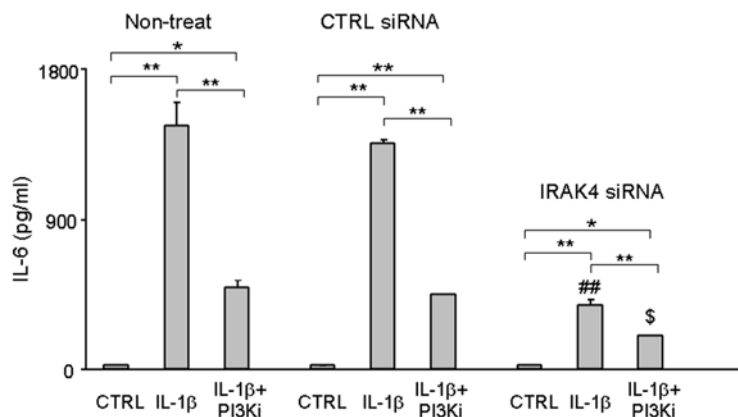


Figure 4 Effect of IRAK4 siRNA and PI3K inhibitor on the IL-1 β -induced IL-6 production in A549

PI3Ki: PI3K inhibitor (LY294002: 20 μ M); IL-1 β (1 ng/ml). * $P<0.01$; ** $P<0.001$ (one-way ANOVA with Bonferroni, GraphPad Prism ver. 4.03). ## $P<0.001$ compared with CTRL siRNA treated with IL-1 β ; \$ $P<0.01$ compared with IRAK4 siRNA treated with IL-1 β and PI3Ki (Welch test, GraphPad Prism ver. 4.03).

of PI3K and consequently phosphorylates Akt/PKB (protein kinase B). In this signalling pathway, IRAKs are not required. PI3K inhibitor reduced IL-1 β -induced IL-6 production in A549 cells, while PI3K inhibitor did not inhibit IL-1 β -induced IL-6 production in RASF in this study. These results suggest that PI3K (p85 and/or p110) interacts with IL-1RI or with IL-1RAcP in A549 cells but not in RASF. It has been reported that there is an interaction of IRAK1 and IRAK2 with PI3K (Cenni et al., 2003; Neumann et al., 2002). Interaction of IRAK1 with Akt/PKB, target for PI3K has been reported (Neumann et al., 2002). On the other hand, kinase-defective mutant of Akt impairs IRAK2-dependent but not IRAK1-dependent NF- κ B activity (Cenni et al., 2003). Our preliminary study showed that the reduction of IRAK1 expression using IRAK1 siRNA did not decrease the IL-6 expression. Furthermore, IRAK2 expression level was significantly increased when A549 cells were treated with PI3K inhibitor (data not shown). At the present time, however, it is still unclear what mechanisms regulate the interaction of PI3K with IRAK4. Further investigation on the interaction between PI3K and IRAK4 is required.

Differences in the downstream signalling of PI3K may define what extent PI3K is involved in the IL-1 β -induced IL-6 production. Both PI3K and IRAK signalling pathway culminate in the activation of NF- κ B. PI3K is reportedly involved in the IL-1-mediated activation of NF- κ B (Reddy et al., 1997; Marmioli et al., 1998; Sizemore et al., 1999). Recently, Cahill reported that a novel PI3K-dependent Akt/I κ B kinase α pathway is involved in the IL-6 production in response to IL-1 (Cahill and Rogers, 2008). On the other hand, there is much evidence demonstrating the central role of MyD88-IRAK-TRAF6 (TNF-receptor-associated factor 6) module in the signalling of IL-1, and the signal is relayed to MAPK (mitogen-activated protein kinase) pathway and TAK1 (transforming growth factor- β activated kinase 1)-TAB1 (TAK1-binding protein) (Schmidt et al., 2001; Jiang et al., 2002; Frączek et al., 2008). In fact, we confirmed the IL-1 β -induced phosphorylation of p38 MAPK as well as TAB1 in A549 cells (data not shown). These signals lead to the activation of NF- κ B. Whether these multiple signalling pathways are involved in the IL-1 β -induced IL-6 production in A549 or RASF are to be investigated; namely, correlation between phosphorylation of Akt/PKB and phosphorylation of IKK α , activation of p65 NF- κ B subunit and nuclear translocation of NF- κ B.

In conclusion, we have shown that the involvement of PI3K in IL-1-induced IL-6 production depends on the cell types. Cell type-dependent involvement of PI3K in the IL-1-induced IL-6 production should be taken into account when PI3K is considered to be a potential target molecule for disease treatment, i.e. COPD and RA.

Author contribution

Hiroyuki Eda initiated the project idea, designed, performed experiments and wrote the manuscript. Barry Burnette provided the experimental idea, and designed and performed the experiments. Hideaki Shimada provided the experimental idea and designed the experiments. Heidi Hope interpreted the data and supervised the project. Joseph Monahan supervised the project.

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