Regulation of Epidermal Growth Factor Receptor Expression and Morphology of Lung Epithelial Cells by Interleukin-1 β

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Regulation of epidermal growth factor receptor expression and morphology of lung epithelial cells by interleukin-1β

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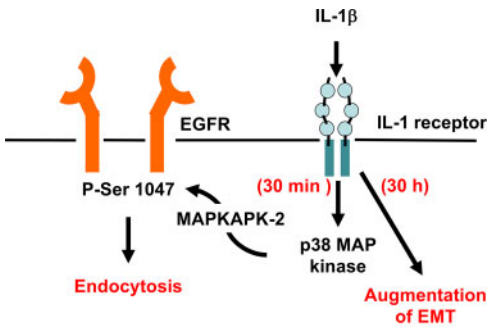
Graphical Abstract



[A549 cells: Lung alveolar epithelial cells]

Accumulating evidences suggested that the overactivation of epidermal growth factor receptor (EGFR) was involved in the development of adult respiratory distress syndrome and pulmonary fibrosis. Elucidation of the mechanisms that regulate EGFR residence on the plasma membrane during inflammatory lung conditions is important for identifying potential therapies. We have demonstrated that flagellin phosphorylated EGFR at Ser1047 and induced transient EGFR internalization. In this study, we examined the molecular pathway and effect of interleukin 1 beta (IL-1β) on EGFR in alveolar epithelial cells. Treatment of A549 cells with IL-1β induced the activation of p38 mitogen-activated protein kinase (MAP kinase) and MAP kinase-activated protein kinase-2 (MAPKAPK-2), as well as EGFR phosphorylation at serine 1047. Both MAPKAPK-2 activation and EGFR phosphorylation were inhibited by SB203580, a p38 MAP kinase inhibitor. In addition, MK2a inhibitor (a MAPKAPK-2 inhibitor) suppressed EGFR phosphorylation. Assessment of the biotinylation of cell surface proteins indicated that IL-1β induced EGFR internalization. Furthermore, long-term treatment of A549 cells with IL-1β caused morphological changes and loss of cell–cell contact. Moreover, IL-1β augmented the effect of transforming growth factor beta 1 on the epithelial–mesenchymal transition. These results suggested that IL-1β regulates EGFR functions and induces morphological changes of alveolar epithelial cells.

Keywords: alveolar epithelial cells; EGF receptor; IL-1 beta; MAPKAPK-2; p38 MAP kinase.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial–mesenchymal transition; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; IL-1β, interleukin-1 beta; IL-1R, interleukin-1 beta receptor; MAP kinase, mitogen-activated protein kinase; MAPKAPK-2, mitogen-activated protein kinase-activated protein kinase-2; rhIL-1Ra, recombinant human interleukin-1 receptor antagonist; PBS, phosphate-buffered saline; TLR5, Toll-like receptor 5; TGF-β1, transforming growth factor beta 1; TNFα, tumour necrosis factor alpha.

The epidermal growth factor receptor (EGFR) is essential for cell proliferation and differentiation (*1*, *2*). When epidermal growth factor (EGF) binds to the EGFR, dimerization of the receptor occurs with autophosphorylation of multiple tyrosine residues, resulting in receptor activation and stimulation of various signal transduction pathways (*1*, *3*). The EGF–EGFR complex undergoes rapid internalization by receptor-mediated endocytosis (*4*), after which the internalized EGFR is transported to lysosomes for degradation. Such desensitization of the EGFR is crucial for regulating cellular events triggered by its stimulation.

In addition to desensitization of the EGFR after activation, there is evidence that desensitization of this receptor is induced after phosphorylation of serine residues in various cells, including alveolar epithelial cells (*2*, *5*, *6*). Several studies have shown that various cellular stressors induce this type of ligand-independent EGFR endocytosis, including starvation, hypoxia, oxidative stress, ultraviolet irradiation and exposure to tumour necrosis factor α (TNFα) (*7–9*). TNFα is a cytokine with roles in various cellular functions (*5*) that has been reported to activate the p38 mitogen-activated protein kinase (MAP kinase) pathway and induce transient EGFR phosphorylation at serine 1047 (Ser1047) (*2*). After phosphorylation, the receptor is internalized via a clathrin-dependent mechanism (*6*). Interestingly, it was reported that the internalized receptor does not undergo lysosomal degradation, and that inactivation of the p38 MAP kinase pathway results in EGFR dephosphorylation and recycling back to the cell surface (*6*).

A549 cells are an immortalized alveolar epithelial cell line, which has been employed to investigate alveolar epithelial cell responses to several treatments (*10*, *11*). Flagellin is a primary structural component of bacterial flagella, and it is known to stimulate Toll-like receptor 5 (TLR5) (*12*). We previously found that flagellin activates the p38 MAP kinase pathway in A549 cells and induces EGFR phosphorylation at Ser1047 (*13, 14*). We also found that MAP kinase-activated protein kinase-2 (MAPKAPK-2) induced the phosphorylation of Ser1047 after treatment of A549 cells with TNFα and flagellin (*14*). Furthermore, we confirmed that EGFR internalization was induced by flagellin (*15*). EGFR internalization by TNFα and flagellin may achieve desensitization to EGF during inflammatory processes such as bacterial infection. Moreover, we reported that long-term treatment of A549 cells with flagellin augmented the epithelial–mesenchymal transition (EMT) in response to transforming growth factor beta 1 (TGF-β1) (*13*). So far, endogenous substances with similar effects to bacterial flagellin have not been reported.

Interleukin 1 beta (IL-1β) and its receptor are major mediators of infectious, inflammatory and degenerative diseases (*16*). The IL-1β receptor (IL-1R) and TLR5 belong to the same receptor superfamily and share common cytosolic adapter proteins (*17*). In this study, we investigated EGFR phosphorylation and internalization after short-term treatment of A549 cells with IL-1β. We also examined the effects of long-term IL-1β treatment on cell morphology.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) from Sigma Chemical Co. (St. Louis, MO, USA); foetal calf serum (FCS) from HyClone (Logan, UT, USA); DynaMarker Protein MultiColor from BioDynamics Laboratory Inc. (Tokyo, Japan); U0126 (*9903*), anti-MAPKAPK-2 antibody (Cat# 3042, RRID: AB_10694238), rabbit anti-phospho-MAPKAPK-2 (Thr222) monoclonal antibody (9A7) (anti-P-Thr222 MAPKAPK-2 antibody, Cat# 3316, RRID: AB_2141311), rabbit anti-phospho-MAPKAPK-2 (Thr334) monoclonal antibody (27B7) (anti-P-Thr334 MAPKAPK-2 antibody, Cat# 3007, RRID: AB_490936), anti-p38 MAP kinase antibody (Cat# 2307, RRID: AB_659929), anti-phospho-p38 MAP kinase antibody (Cat# 4631, RRID: AB_331765) and anti-E-cadherin antibody (Cat# 3195, RRID: AB_2291471) from Cell Signalling Technology (Beverly, MA, USA); anti-extracellular signal-regulated kinase (anti-ERK) antibody (Cat# M5670, RRID: AB_477216) from Sigma Chemical Co. (St. Louis, MO, USA); anti-active ERK antibody (Cat# V8031, RRID: AB_430866) from Promega Co. (Madison, WI, USA); mouse anti-EGFR monoclonal antibody (6F1) (IgG_{2b}) (Cat# CSA-330E, RRID: AB_2262025) from Assay Designs (Ann Arbor, MI, USA); mouse anti-phospho-EGFR (Ser1047) monoclonal antibody (1H9) (anti-P-Ser1047 EGFR antibody, Cat# AM00036PU-N, RRID: AB_980924) from Acris Antibodies Inc. (San Diego, CA, USA); mouse anti-phospho-EGFR (Tyr1173) monoclonal antibody (9H2) (anti-P-Tyr1173 EGFR antibody, Cat# 324864-1SET, RRID: AB_243240) from Calbiochem, Merck (Darmstadt, Germany); anti-fibronectin antibody (Cat# ab23750, RRID: AB_447655) from Abcam (Cambridge, UK); anti-GAPDH antibody (Cat# GTX82899, RRID: AB_625904) from GeneTex, Inc. (Los Angeles, CA, USA); recombinant human TGF-β1 and IL-1β from R&D Systems (Minneapolis, MN, USA); recombinant human interleukin-1 receptor antagonist (rhIL-1Ra) from Wako Pure Chemical Industries (Osaka, Japan); SB203580 [4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1H-imidazole] and MK2a inhibitor [4-(2'-fluorobiphenyl-4-yl)-*N*-(4-hydroxyphenyl)-butyramide] from Calbiochem (Darmstadt, Germany). Other chemicals were of analytical grade.

Culture of A549 cells and preparation of cell extracts

A549 cells (Cat# A549, RRID: CVCL_0023) were cultured in 60 mm Petri dishes (Nunc, Roskilde, Denmark) with DMEM containing 4.5 g/l of glucose and 10% (vol/vol) heat-inactivated FCS (*10*, *11*). Then the medium was exchanged for DMEM containing 0.1% FCS, the cells were incubated for 2 h, and were subsequently treated with IL-1β in the presence of 0.1% FCS. The concentrations of signal transduction inhibitors (rhIL-1Ra, U0126, SB203580 and MK2a inhibitor) were selected according to the instructions of the manufacturers. Cells in the Petri dishes were washed once in PBS and subjected to lysis with 300 μl of 1 × SDS-PAGE sample buffer containing 2% (wt/vol) SDS, 62.5 mM Tris-HCl, pH 6.8, 5% (vol/vol) 2-mercaptoethanol, 5% (vol/vol) glycerol and 0.01% (wt/vol) bromophenol blue (*14*, *18*). Then cell extracts underwent sonication for 20 s on ice and heating at 98°C for 5 min, followed by storage at −80°C until use.

SDS-PAGE and immunoblotting

SDS-PAGE was done by the method of Laemmli (*18*) and was followed by immunoblotting (*14*, *19*). Then immunoreactive proteins were detected with an enhanced chemiluminescence kit (GE Healthcare UK Ltd., Little Chalfont, UK) according to the manufacturer's directions. Phosphorylation of proteins was assessed by quantification of phosphoproteins using an ImageQuant LAS 4000 mini (GE Healthcare UK Ltd., UK) and Multi Gauge software (version 3.1, RRID: SCR_014299). To estimate molecular weights by chemiluminescence, the MagicMark XP Western Protein Standard (Invitrogen, Carlsbad, CA, USA) was also loaded onto each SDS-PAGE gel. For the EGFR, the 170 kDa position was estimated by using 220 kDa and 120 kDa standards. Before reprobing, each membrane was incubated with stripping buffer [62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% (wt/vol) SDS] for 30 min at 50°C (*14*). Then each membrane was washed at room temperature with blocking solution that contained 5% (wt/vol) skim milk, 100 mM Tris-HCl (pH 7.5), 0.9% (wt/vol) NaCl and 0.1% (vol/vol) Tween-20, and with Tris-buffered saline containing 100 mM Tris-HCl (pH 7.5), 0.9% (wt/vol) NaCl and 0.1% (vol/vol) Tween-20, before being subjected to immunoblotting.

Biotinylation of cell surface proteins

Cell surface proteins were biotinylated and isolated by using a Cell Surface Protein Isolation kit (Pierce Biotechnology, Rockford, IL, USA) (*20*). After A549 cells were grown to 90–95% confluence in 100 mm Petri dishes (Nunc) and treated with IL-1β, incubation was done for 30 min at 4°C with PBS containing sulphosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (sulpho–NHS–SS–biotin),

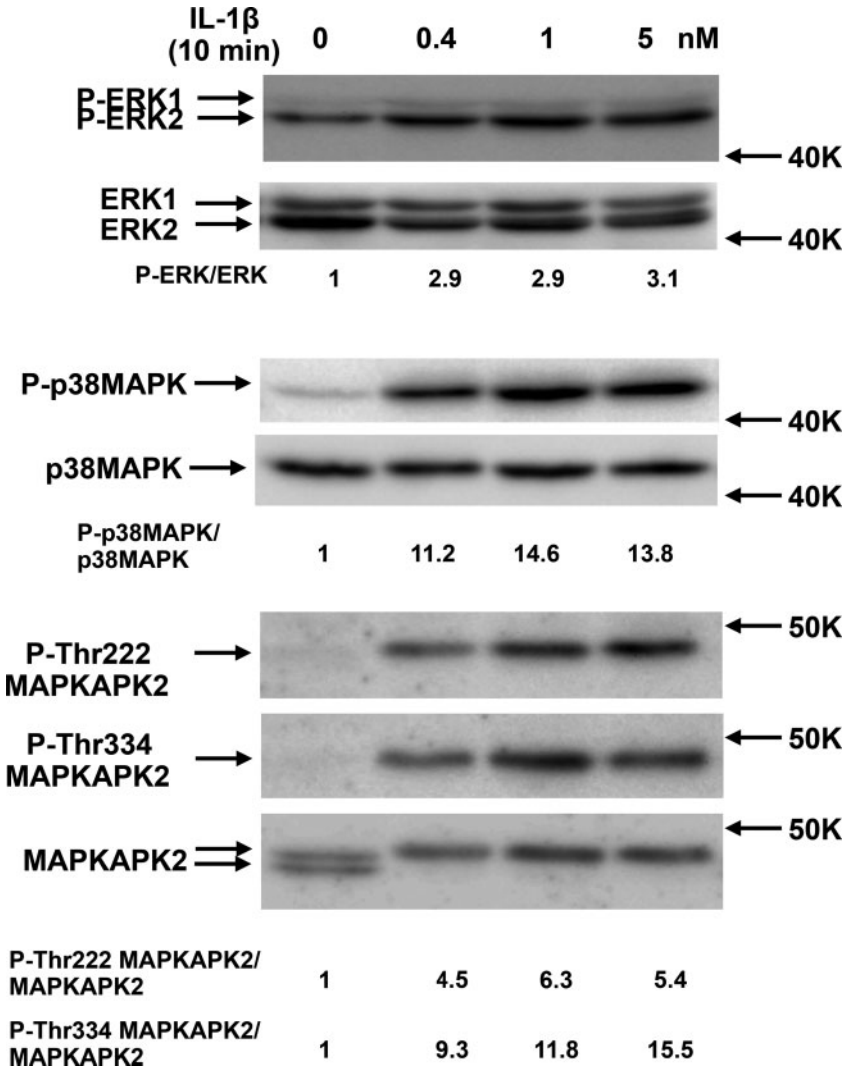


Fig. 1. Phosphorylation of ERK1/2, p38 MAP kinase and MAPKAPK-2 by IL-1β. A549 cells were treated with IL-1β at the indicated concentrations for 10 min. The cell extracts (31.7 μg) were subjected to SDS-PAGE on 12% (wt/vol) acrylamide gel for ERK1/2 or 10% (wt/vol) acrylamide gel for p38 MAP kinase and MAPKAPK-2. Then immunoblotting was performed with anti-active ERK antibody (1:750), anti-phospho-p38 MAP kinase antibody (1:500), anti-P-Thr222 MAPKAPK-2 antibody (1:750) or anti-P-Thr334 MAPKAPK-2 antibody (1:750). After the primary antibodies were stripped, immunoblotting was performed with anti-ERK antibody (1:1,000), anti-p38 MAP kinase antibody (1:500) or anti-MAPKAPK-2 antibody (1:750), respectively. For the quantification of phosphorylation level of each protein, the ratio of the signal of phosphorylation to that of the protein of each protein (P-ERK/ERK, P-p38MAPK/p38MAPK, P-Thr222 MAPKAPK2/MAPKAPK2 and P-Thr334 MAPKAPK2/MAPKAPK2) was determined. The phosphorylation level of the control was taken as 1, and from this value, each value was calculated. Bands for ERK1/2 (P-ERK1/2 and ERK1/2), p38 MAP kinase (P-p38MAPK and p38MAPK) and MAPKAPK-2 (P-Thr222 MAPKAPK-2, P-Thr334 MAPKAPK-2 and MAPKAPK-2) are indicated. We repeated the same experiments three times with reproducible results and representative results are shown.

a membrane-impermeable biotinylation reagent. Then cells were lysed in the presence of protease inhibitor cocktail, following which biotinylated proteins were isolated using NeutrAvidin agarose. Subsequently, the isolated proteins were released by incubation at room temperature for 60 min with SDS-PAGE sample buffer containing dithiothreitol, followed by immunoblotting.

Examination of cell morphology

A549 cells were cultured in 60 mm Petri dishes (Nunc) and were treated with IL-1β and TGF-β1 in the presence of 10% FCS. Phase contrast images were recorded at 30 h using an EVOS fl (Advanced Microscopy Group, Bothell, WA, USA).

Other procedures

Protein concentrations were measured by using a Qubit Protein Assay kit and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Because pilot experiments obtained linear protein concentration curves in the presence of 0.04% (wt/vol) SDS, 0.1% (vol/vol) 2-mercaptoethanol and 0.1% (vol/vol) glycerol, the cell extracts were diluted 50-fold with water for quantification. Each experiment was at least performed in triplicate and representative data are shown. Results are expressed as the mean ± standard error (SE). Statistical analysis was performed by one-way ANOVA with Duncan's multiple range test using StatView (version 5.0). In all analyses, $P < 0.05$ was considered to indicate statistical significance.

Results

ERK, p38 MAP kinase and MAPKAPK-2 activation by IL-1β

It has been reported that MAPKAPK-2 was activated by ERK and p38 MAP kinase, we first examined whether IL-1β activated ERK and p38 MAP kinase (*21*). ERK showed about 3-fold activation relative to

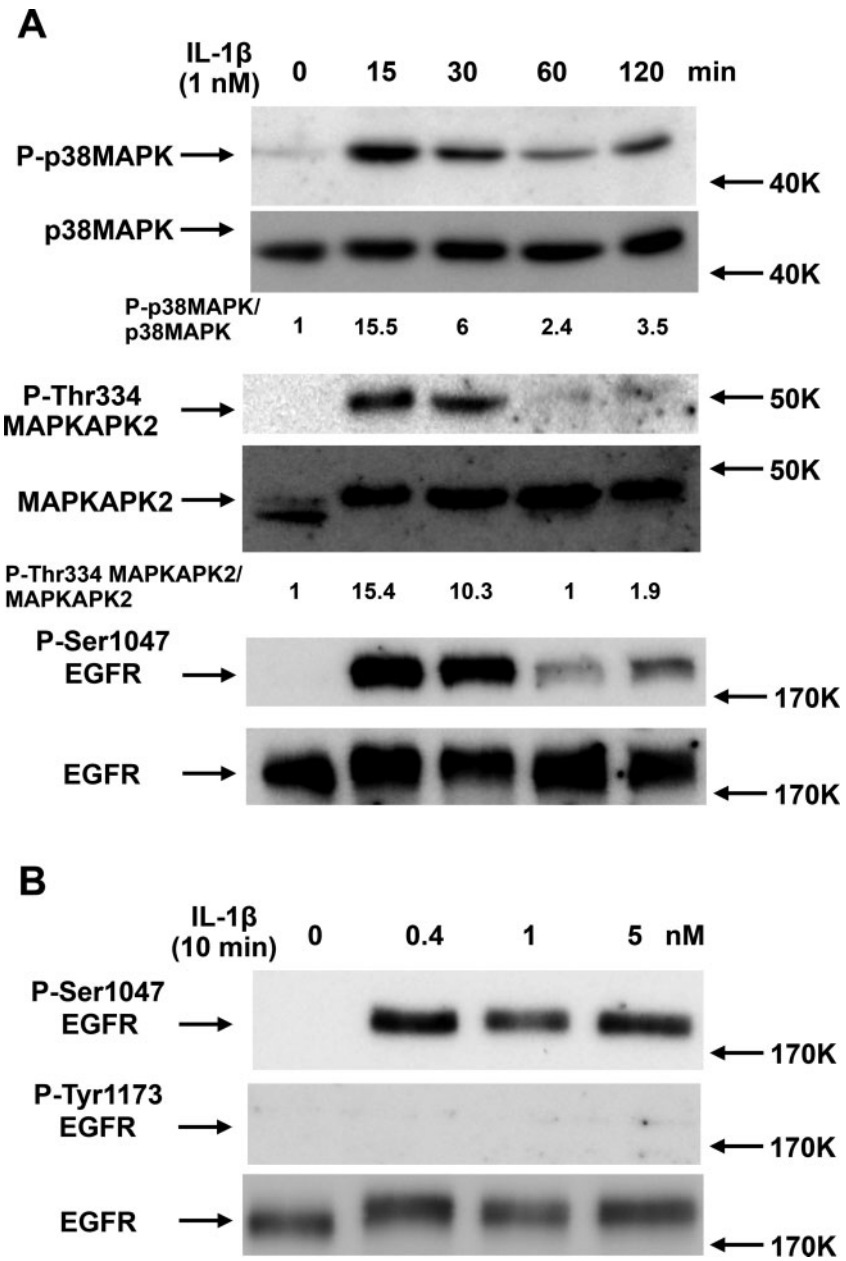


Fig. 2. Time course of p38 MAP kinase and MAPKAPK-2 activation by IL-1β and EGFR phosphorylation. (**A**) A549 cells were treated with 1 nM IL-1β for the indicated periods. Cell extracts (58 μg) were subjected to SDS-PAGE on 10% (wt/vol) acrylamide gels for p38 MAP kinase and MAPKAPK-2 or 7.5% (wt/vol) acrylamide gel for EGFR. Immunoblotting analysis of p38 MAP kinase and MAPKAPK-2 was performed as described in the legend of Fig. 1. Immunoblotting analysis of EGFR was performed with anti-P-Ser1047 EGFR antibody (1:100) and anti-EGFR antibody (1:600). (**B**) A549 cells were treated with IL-1β at the indicated concentrations for 10 min. Then cell extracts (38.4 μg) were subjected to SDS-PAGE on 7.5% (wt/vol) acrylamide gel and immunoblotting was performed with anti-P-Ser1047 EGFR antibody (1:100) and anti-P-Tyr1173 EGFR antibody (1:300) as described above. After the anti-P-Tyr1173 EGFR antibody was stripped, immunoblotting was performed with anti-EGFR antibody (1:600). Bands for p38 MAP kinase (P-p38MAPK and p38MAPK), MAPKAPK-2 (P-Thr334 MAPKAPK-2 and MAPKAPK-2) and EGFR (P-Ser1047 EGFR, P-Tyr1173 EGFR and EGFR) are indicated. We quantified the phosphorylation level of each protein as described in the legend of Fig. 1. The phosphorylation level of p38 MAP kinase and MAPKAPK-2 of the control was taken as 1, and from this value, each value was calculated. We repeated the same experiments three times with reproducible results and representative results are shown.

the control after 10 min of treatment with IL-1β at 0.4, 1 and 5 nM (Fig. 1). In contrast to this moderate level of ERK activation, p38 MAP kinase showed 11.2-, 14.6- and 13.8-fold activation by IL-1β at 0.4, 1 and 5 nM, respectively. We previously reported that p38 MAP kinase activated MAPKAPK-2 after A549 cells were stimulated with TNFα and flagellin (*14*). Because p38 MAP kinase was strongly activated by IL-1β, we subsequently investigated whether MAPKAPK-2 was activated by this cytokine. It was reported that MAPKAPK-2 activation involves phosphorylation of Thr222, Ser272 and Thr334 (*21*). In this study, we examined Thr222 and/or Thr334 phosphorylation using the antibodies that were available. There was marked phosphorylation of both Thr222 and Thr334 after incubation with IL-1β at 0.4 nM

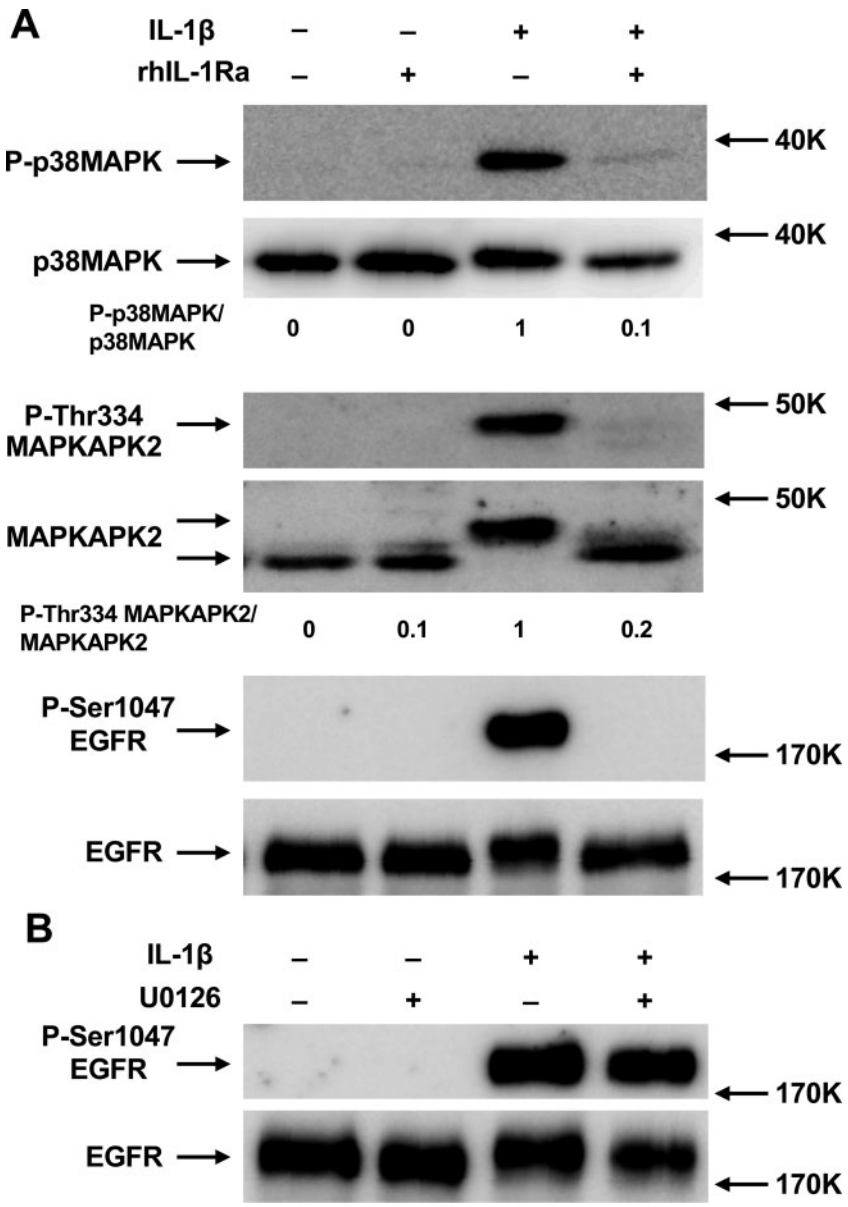


Fig. 3. Effects of rhIL-1Ra and U0126 on MAPKAPK-2 activation and EGFR phosphorylation at Ser1047 by IL-1β. (**A**) A549 cells were pre-treated with or without 200 ng/ml rhIL-1Ra for 30 min and incubated with or without 1 nM IL-1β for 10 min in the presence or absence of rhIL-1Ra. Cell extracts (26.6 μg) were subjected to SDS-PAGE on 10% (wt/vol) acrylamide gels for p38 MAP kinase and MAPKAPK-2 or 7.5% (wt/vol) acrylamide gel for EGFR, and immunoblotting was performed as described in the legends of Figs 1 and 2. (**B**) A549 cells were pre-treated with or without 10 μM U0126 for 30 min and incubated with or without 1 nM IL-1β for 10 min in the presence or absence of U0126. Then the cell extracts (114 μg) were subjected to SDS-PAGE on 7.5% (wt/vol) acrylamide gel and immunoblotting analysis of EGFR was performed as described in the legend of Fig. 2. We quantified the phosphorylation levels of p38 MAP kinase and MAPKAPK-2 as described in the legend of Fig. 1. Because the phosphorylation level of the control was 0, that in the presence of IL-1β alone was taken as 1, and each value was calculated. Bands for p38 MAP kinase (P-p38MAPK and p38MAPK), MAPKAPK-2 (P-Thr334 MAPKAPK-2 and MAPKAPK-2) and EGFR (P-Ser1047 EGFR and EGFR) are indicated. We repeated the same experiments three times with reproducible results and representative results are shown.

(Fig. 1). Phosphorylation of Thr222 was increased by 4.5-, 6.3- and 5.4-fold following incubation with IL-1β at 0.4, 1 and 5 nM, respectively, while Thr334 showed a 9.3-, 11.8- and 15.5-fold increment of phosphorylation at 0.4, 1 and 5 nM, respectively. An anti-MAPKAPK-2 antibody detected two bands in the absence of IL-1β (Fig. 1). In contrast, a single MAPKAPK-2 band was detected after IL-1β treatment of cells and this band was located at a higher molecular weight than the two bands in the control culture. These results suggested that phosphorylation of MAPKAPK-2 decreased its electrophoretic mobility. Because the other bands were not detected, it seems that MAPKAPK-2 was almost completely activated by IL-1β.

Time course of p38 MAP kinase and MAPKAPK-2 activation and EGFR phosphorylation by IL-1β

Figure 2A compares the time course of p38 MAP kinase with MAPKAPK-2 activation by 1 nM IL-1β.

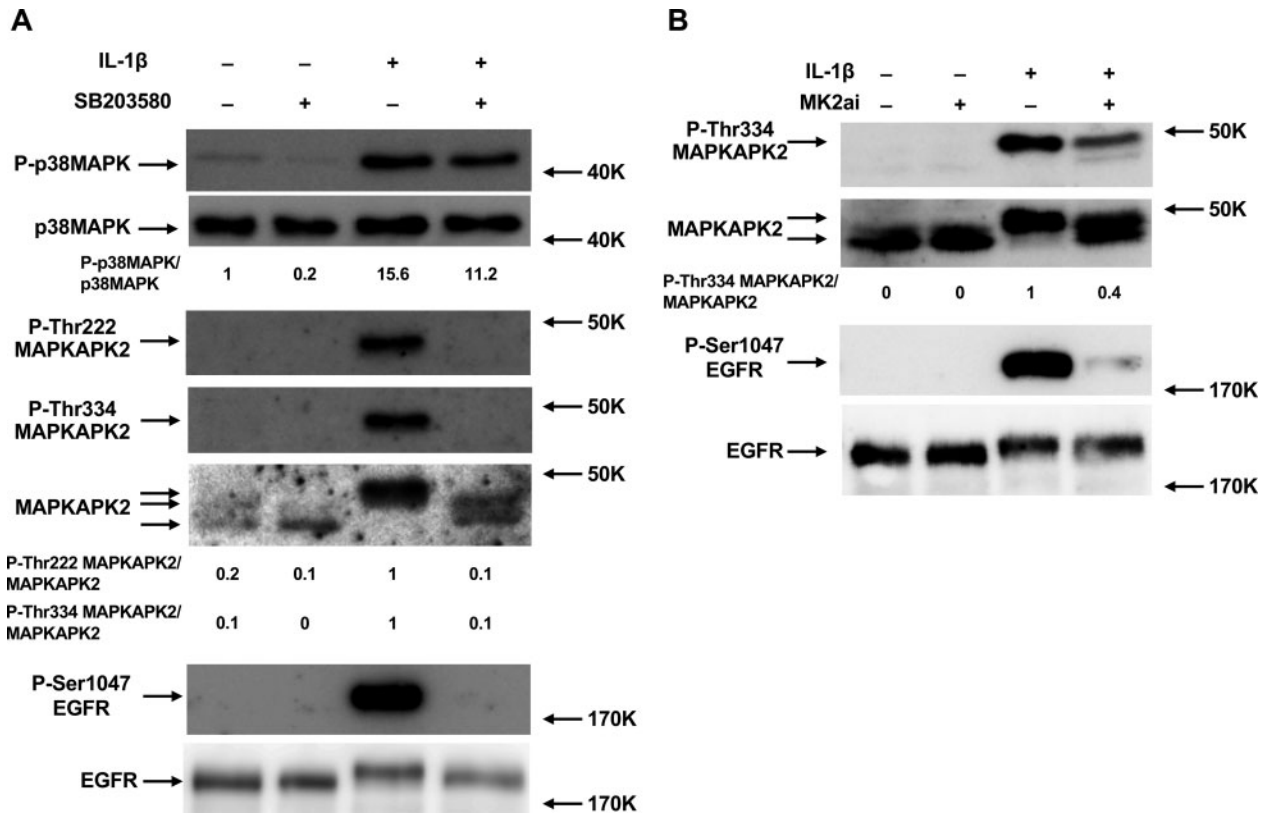


Fig. 4. Effects of SB203580 and MK2ai on p38 MAP kinase and MAPKAPK-2 activation, and EGFR phosphorylation at Ser1047 by IL-1β. (**A**) A549 cells were pre-treated with or without 5 μM SB203580 for 30 min and incubated with or without 1 nM IL-1β for 10 min in the presence or absence of SB203580. Cell extracts (26.3 μg) were subjected to SDS-PAGE on 10% (wt/vol) acrylamide gels for p38 MAP kinase and MAPKAPK-2 or 7.5% (wt/vol) acrylamide gel for EGFR, and immunoblotting was performed as described in the legends of Figs 1 and 2. (B) A549 cells were pre-treated with or without 20 μM MK2ai for 30 min and incubated with or without 1 nM IL-1β for 10 min in the presence or absence of MK2ai. Then the cell extracts (39.5 μg) were subjected to SDS-PAGE on 10% (wt/vol) acrylamide gel for MAPKAPK-2 or 7.5% (wt/vol) acrylamide gel for EGFR, and immunoblotting was performed as described above. We quantified the phosphorylation levels of p38 MAP kinase and MAPKAPK-2 as described in the legend of Fig. 1. Because the phosphorylation level of MAPKAPK-2 of the control was 0, that in the presence of IL-1β alone was taken as 1, and each value was calculated. Bands for p38 MAP kinase (P-p38MAPK and p38MAPK), MAPKAPK-2 (P-Thr222 MAPKAPK-2, P-Thr334 MAPKAPK-2 and MAPKAPK-2) and EGFR (P-Ser1047 EGFR and EGFR) are indicated. We repeated the same experiments three times with reproducible results and representative results are shown.

Activation of both p38 MAP kinase and MAPKAPK-2 was maximal at 15 min, when both showed an approximately 15-fold increase of activation. Then activation of both protein kinases declined from 30 min, but p38 MAP kinase activation was still observed at 120 min. Phosphorylation of Thr334 of MAPKAPK-2 was slight at 60 min and the MAPKAPK-2 protein remained at the high molecular weight position at 120 min.

We previously reported that stimulation of TLR5 induced EGFR phosphorylation at Ser1047 by MAPKAPK-2 in A549 cells (*14*). After detecting marked activation of MAPKAPK-2 by IL-1β, we next examined the influence on EGFR phosphorylation (Fig. 2A). At 0 min, EGFR phosphorylation at Ser1047 was undetectable under our experimental conditions. In contrast, Ser1047 phosphorylation was prominent after 15 min of IL-1β treatment. While Ser1047 phosphorylation decreased from 60 min, it was still observed at 120 min. When EGFR protein was examined, there were no significant changes in response to IL-1β. We previously reported that the electrophoretic mobility of EGFR decreased in response to treatment with flagellin and TNFα (*14*). In this study, the electrophoretic mobility of EGFR was also decreased by treatment with IL-1β (Fig. 2A). When cells were incubated with various IL-1β concentrations for 10 min, both Ser1047 phosphorylation and gel shift of EGFR were observed from 0.4 nM (Fig. 2B). However, Tyr1173 phosphorylation was not observed, indicating that IL-1β did not cause EGFR activation. When EGFR protein levels were examined, there were no significant changes in response to IL-1β.

Investigation of signalling pathways leading to EGFR phosphorylation at Ser1047

Involvement of the IL-1β receptor in EGFR phosphorylation by IL-1β

Next, we examined the signalling pathways that caused EGFR phosphorylation after IL-1β treatment. We first investigated whether the IL-1R had a role in the effects of IL-1β (Fig. 3A). Recombinant human interleukin-1 receptor antagonist (rhIL-1Ra) is a natural IL-1R antagonist. We found that rhIL-1Ra

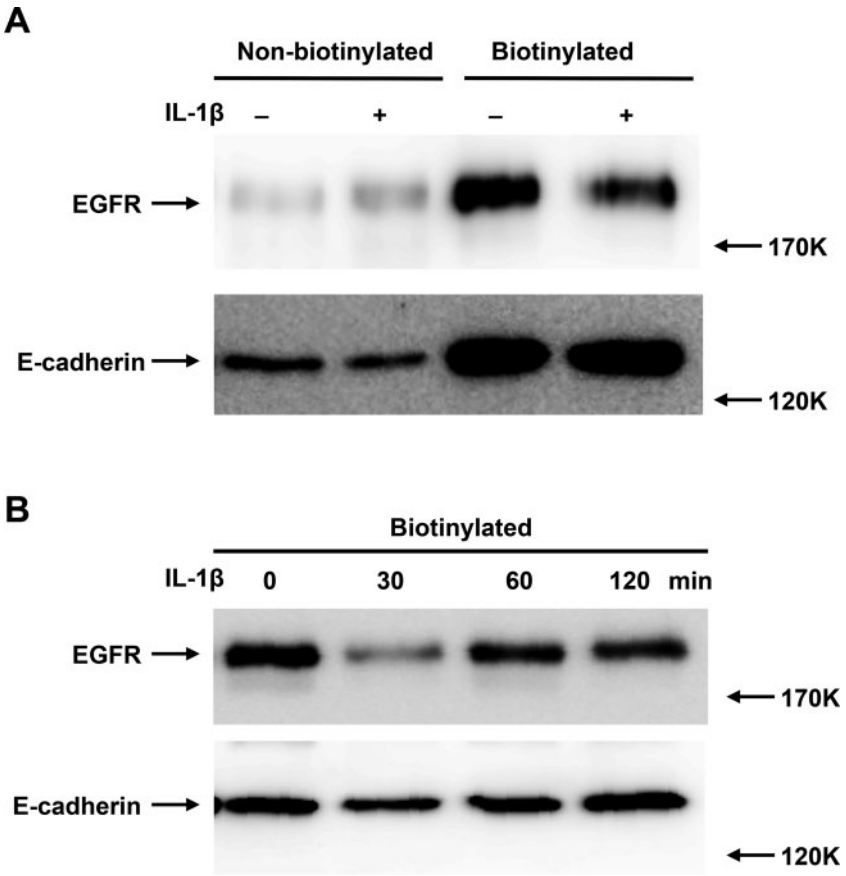


Fig. 5. Reduction of biotinylated cell surface EGFR by incubation with IL-1β. (**A**) A549 cells were incubated with or without 1 nM IL-1β for 30 min and then were cultured at 4°C for 30 min with PBS containing sulpho–NHS–SS–biotin as described in Materials and Methods. After the cells were lysed, biotinylated proteins were isolated with NeutrAvidin agarose. Isolated proteins were released by incubation for 60 min at room temperature with 1 × SDS-PAGE sample buffer containing dithiothreitol. Non-biotinylated (19.6 μg) and biotinylated (12 μg) proteins were subjected to SDS-PAGE on 7.5% (wt/vol) acrylamide gel, and immunoblotting was performed with anti-EGFR antibody (1:600) or anti-E-cadherin antibody (1:750). We repeated the same experiments five times (EGFR) and four times (E-cadherin) with reproducible results, and representative results are shown. (**B**) A549 cells were treated with 1 nM IL-1β for the indicated periods. Biotinylated proteins (12 μg) were subjected to immunoblotting analysis for EGFR and E-cadherin. Bands for EGFR and E-cadherin are indicated. We repeated the same experiments three times with reproducible results and representative results are shown.

almost completely inhibited the activation of p38 MAP kinase and MAPKAPK-2 induced by IL-1β, as well as EGFR phosphorylation at Ser1047. Gel shift of MAPKAPK-2 and EGFR was inhibited by IL-1R, although MAPKAPK-2 and EGFR protein levels were unchanged. These findings suggested that p38 MAP kinase and MAPKAPK-2 activation, as well as EGFR phosphorylation, by IL-1β were receptor-mediated processes.

Role of ERK in EGFR phosphorylation by IL-1β

Because ERK was activated by IL-1β, we examined whether ERK had a role in EGFR phosphorylation at Ser1047 (Fig. 3B). We found that a MEK inhibitor (U0126) reduced IL-1β-induced phosphorylation of Ser1047 by about 30%. This result suggested that ERK was not important for EGFR phosphorylation at Ser1047.

Role of p38 MAP kinase in EGFR phosphorylation by IL-1β

We also investigated the possible role of p38 MAP kinase in MAPKAPK-2 activation and EGFR phosphorylation (Fig. 4A). SB203580 is an ATP-competitive inhibitor of p38 MAP kinase; we found that it inhibited IL-1β-induced activation of p38 MAP kinase by about 30% via an unknown mechanism. In addition, SB203580 almost completely inhibited MAPKAPK-2 activation and EGFR phosphorylation. Gel shift of both MAPKAPK-2 and EGFR was strongly inhibited by SB203580, although MAPKAPK-2 and EGFR protein levels were unchanged. These findings strongly suggested that p38 MAP kinase was mainly involved in MAPKAPK-2 activation and EGFR phosphorylation after stimulation with IL-1β.

Role of MAPKAPK-2 in EGFR phosphorylation by IL-1β

We previously reported that MAPKAPK-2 phosphorylates EGFR at Ser1047 in A549 cells treated with flagellin and TNFα (*14*). Therefore, we examined whether MAPKAPK-2 was involved in IL-1β-induced EGFR phosphorylation. MK2a inhibitor binds MAPKAPK-2 and prevents activation by p38 MAP kinase. We found that MK2a inhibitor reduced MAPKAPK-2 phosphorylation at Thr334 by about 60%, indicating that it partially inhibited the activation of MAPKAPK-2 (Fig. 4B). In contrast, MK2a inhibitor strongly inhibited EGFR phosphorylation at Ser1047. MAPKAPK-2 and EGFR protein levels were not altered by any of the treatments tested. Taken together, IL-1β induced EGFR

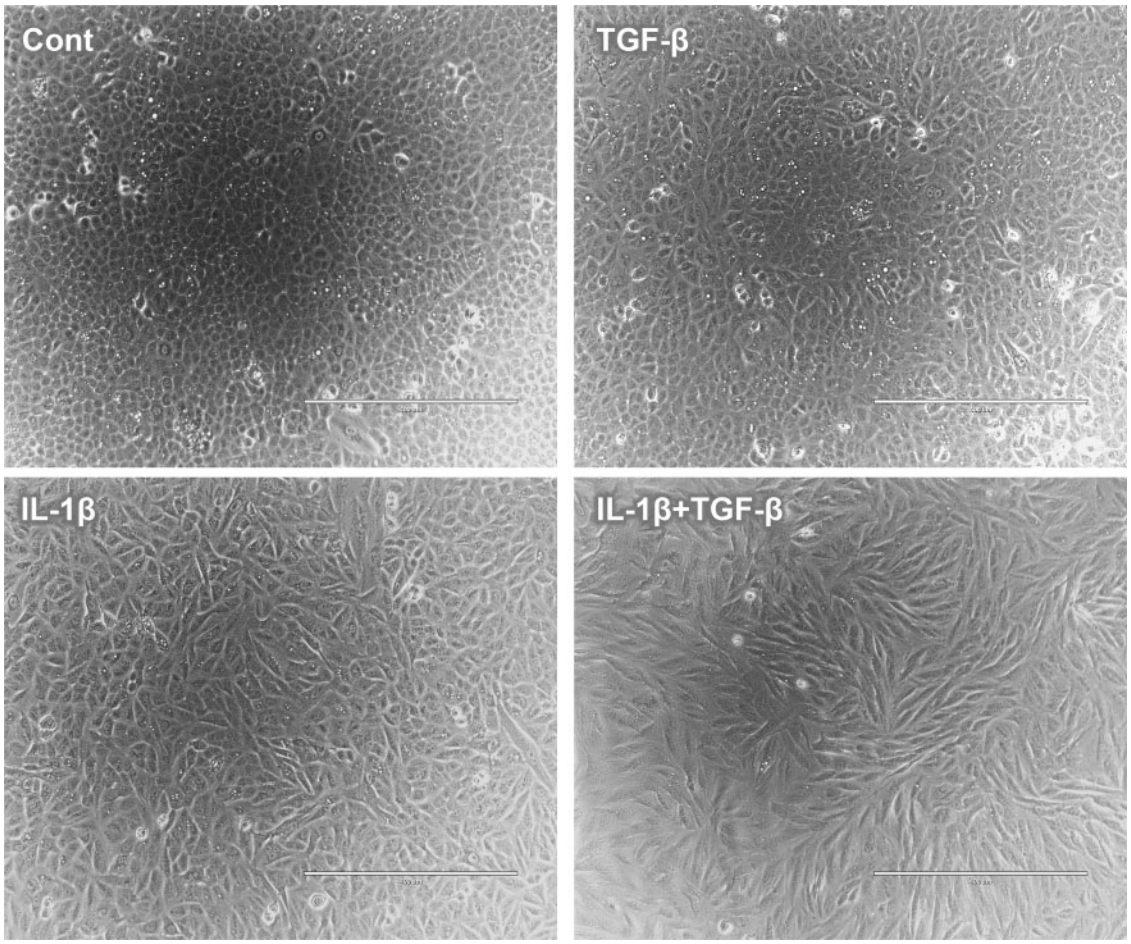


Fig. 6. Morphological changes of A549 cells after treatment with IL-1β and/or TGF-β1 for 30 h. A549 cells were treated with or without 1 nM IL-1β and/or 10 ng/ml TGF-β1 for 30 h in the presence of 10% FCS. Scale bars indicate 400 μm. We repeated the same experiments four times with reproducible results and representative results are shown.

phosphorylation at Ser1047 by activating the p38 MAP kinase/MAPKAPK-2 pathway.

EGFR internalization in response to IL-1β

EGFR phosphorylation at Ser1047 by TNFα was reported to induce internalization of this receptor (*2*, *6*). We previously confirmed that EGFR phosphorylation by flagellin induced receptor internalization (*15*). Therefore, we assessed whether IL-1β induced internalization of the EGFR. After incubation of A549 cells with IL-1β for 30 min, the cells were cooled to 4°C and then incubated for 30 min with sulpho–NHS–SS–biotin. We found that treatment with IL-1β reduced biotinylated EGFR protein, but non-biotinylated EGFR protein increased slightly (Fig. 5A). In contrast, biotinylated E-cadherin protein was unchanged by treatment with IL-1β. When the EGFR protein level was divided by the E-cadherin protein level, non-biotinylated EGFR protein was increased to 331 ± 30% ($n = 3$, $P < 0.05$) of the control level, whereas biotinylated EGFR protein was decreased to 51 ± 12% ($n = 3$, $P < 0.05$) of the control level. These data showed that EGFR was internalized after treatment with IL-1β. When the time course of EGFR internalization was examined (Fig. 5B), we confirmed that the biotinylated EGFR protein level was reduced at 30 min, but was elevated from 60 min. When the EGFR protein level was divided by the E-cadherin protein level, the ratio was 32, 72 and 51% of the control at 30, 60 and 120 min, respectively. These findings suggest that recycling of internalized EGFR was occurring at 60 min.

Morphological changes of A549 cells during long-term treatment with IL-1β

We previously reported that 24 h of treatment with flagellin augmented the EMT induced by TGF-β1 (*13*). Accordingly, we examined whether long-term treatment of A549 cells with IL-1β induced morphological changes by incubating A549 cells with TGF-β1 and IL-1β for 30 h (Fig. 6). Control A549 cells had a cobblestone appearance, whereas cells treated with TGF-β1 or IL-1β showed enlargement with loss of cell–cell contact. Combined TGF-β1 and IL-1β treatment induced more pronounced morphological changes and the cells became spindle-shaped.

Changes of fibronectin and E-cadherin associated with IL-1β

These morphological changes suggested that IL-1β might induce the EMT in A549 cells, so we examined whether early biochemical signs of the EMT could be

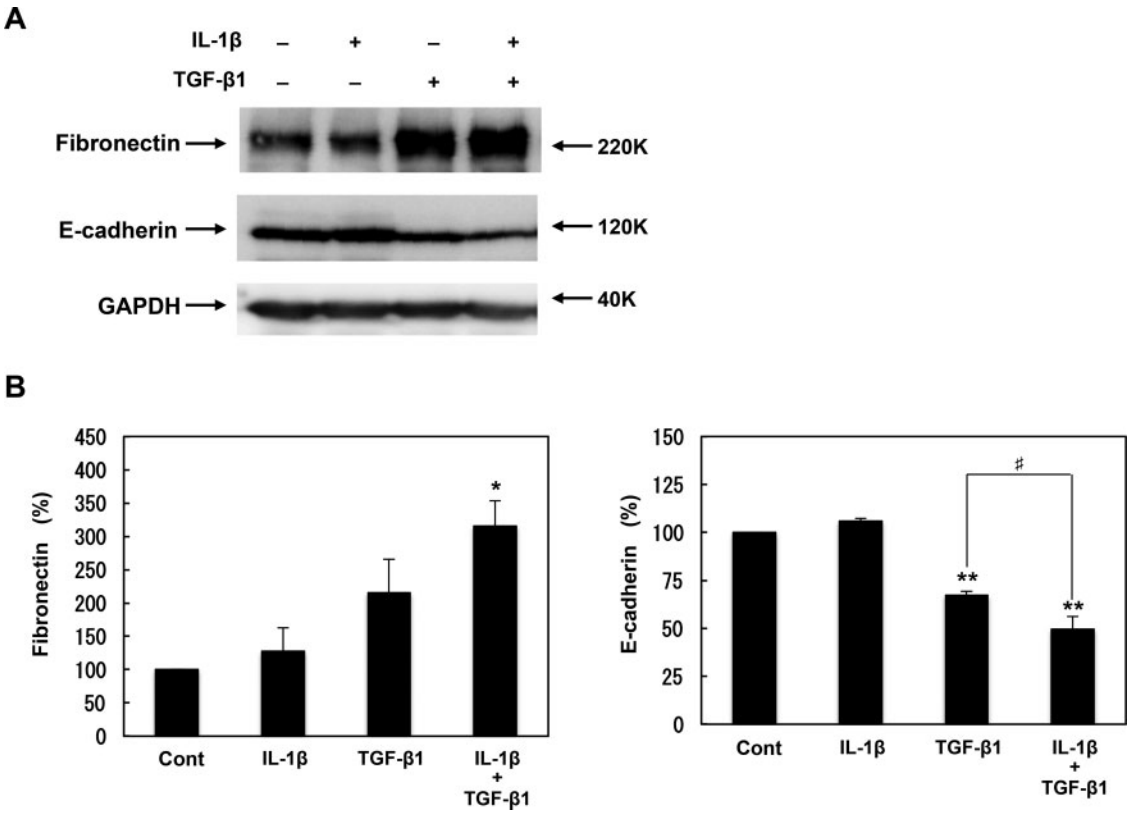


Fig. 7. Changes of fibronectin and E-cadherin expression after treatment with IL-1β and/or TGF-β1. (**A**) A549 cells were treated with or without 1 nM IL-1β and/or 10 ng/ml TGF-β1 for 30 h in the presence of 10% FCS. Cell extracts (57.1 μg) were subjected to SDS-PAGE on 7.5% (wt/vol) acrylamide gels for fibronectin and E-cadherin or 10% (wt/vol) acrylamide gel for GAPDH, and immunoblotting was performed with anti-fibronectin antibody (1:500), anti-E-cadherin antibody (1:750) or anti-GAPDH antibody (1:3,000). We repeated the same experiments four times (fibronectin and E-cadherin) and three times (GAPDH) with reproducible results and representative results are shown. (**B**) Results from three independent experiments are summarized. The bars present fibronectin and E-cadherin protein levels as the mean ± SE ($n = 3$), expressed as a percentage of the corresponding control. $^{*}P < 0.05$; $^{**}P < 0.01$ (versus control). Significant difference of the E-cadherin protein level between cells treated with TGF-β1 alone and cells treated with IL-1β plus TGF-β1 ($^{\#}P < 0.05$).

detected. It has been reported that increase of fibronectin and reduction of E-cadherin are early biochemical signs of EMT (*22*). Therefore, we examined the changes in the protein levels of fibronectin and E-cadherin after IL-1β and TGF-β1 treatments (Fig. 7). We found that treatment with TGF-β1 for 30 h increased fibronectin protein levels in A549 cells, but decreased E-cadherin protein levels (Fig. 7A). On the other hand, IL-1β did not increase fibronectin protein levels or reduce E-cadherin protein levels (Fig. 7A). However, IL-1β augmented the effect of TGF-β1 on fibronectin and E-cadherin. GAPDH protein was not changed by any of the treatments (Fig. 7A). Results from three independent experiments are summarized in Fig. 7B. Fibronectin increased significantly to 315.6 ± 37.4% ($P < 0.05$) of the control value in the presence of both TGF-β1 and IL-1β. In contrast, E-cadherin was reduced to 67.3 ± 1.9% ($P < 0.01$) and 49.7 ± 6.6% ($P < 0.01$) of the control level by treatment with TGF-β1 or TGF-β1 plus IL-1β, respectively. IL-1β significantly augmented the suppression of E-cadherin by TGF-β1 (Fig. 7B). These findings indicated that IL-1β augmented the effects of TGF-β1 on biochemical indicators of the EMT in A549 cells.

Discussion

Gefinitib, an EGFR tyrosine kinase inhibitor, is useful for the treatment of lung cancer, indicating a crucial role of the EGFR in lung cancer progression. It has been reported that resistance to these inhibitors develops during cancer treatment (*23*) and there have been reports indicate that ligand-independent EGFR internalization is involved in this resistance (*24*). In addition to lung cancer, there is accumulating evidence that overexpression and/or hyperactivation of EGFR is involved in adult respiratory distress syndrome and pulmonary fibrosis (*25*, *26*). Therefore, understanding the mechanisms that regulate EGFR residence on the plasma membrane is important for identifying the molecular pathways involved in these pathological changes and therapies. It has also been reported that the inactive EGFR after ligand-independent internalization is involved in various cellular functions, such as induction of autophagy, expression of proteins, DNA replication and regulating the intracellular glucose concentration, although the molecular mechanisms of these effects have not been studied precisely (*8*, *27*, *28*).

We have examined EGFR phosphorylation in response to various endogenous substances that are

reported to be involved in inflammation (*14*, *15*, *29*). In this study, IL-1β caused a marked increase in phosphorylation of the EGFR at Ser1047, whereas Tyr1173 was not phosphorylated, indicating that IL-1β did not activate the EGFR. TNFα and hydrogen peroxide phosphorylated both Ser1047 and Tyr1173 (*14*, *15*). Bradykinin did not induce Tyr1173 phosphorylation and Ser1047 phosphorylation was much lower than with IL-1β (*29*). We found that IL-1β had the greatest effect on phosphorylation of Ser1047 among TNFα, hydrogen peroxide, bradykinin and flagellin, which suggests that IL-1β is crucial for EGFR functions related to inflammation.

In this study, the EGFR underwent internalization after IL-1β treatment and the time course of internalization was similar to that of Ser1047 phosphorylation (Figs 2A and 5B). These findings suggested that Ser1047 phosphorylation induced internalization of the EGFR. To our knowledge, this is the first report about EGFR phosphorylation and internalization by IL-1β. Our data indicated that IL-1β induced the internalization of EGFR without the activation of EGFR. Therefore, the present data suggested that the EGFR activation was suppressed by IL-1β. It has been reported that phosphorylation of Ser1047 induced the internalization of EGFR, while EGFR did not undergo lysosomal degradation (*6*). Therefore, IL-1β may inhibit the EGF-induced degradation of EGFR. In the present study, we found that the amount of EGFR was not increased after IL-1β treatment. These results may suggest that culture medium did not contain EGF under our assay conditions. The effects of IL-1β on the amount of EGFR in the presence of EGF should be examined for the future study.

The physiological phenomena after the internalization of EGFR are not clear at present. Because the long-term treatment of A549 cells with IL-1β and TGF-β1 induced EMT-like morphological changes, the internalization of EGFR may be involved in the induction of EMT. The molecular mechanisms of receptor internalization and its pathophysiological significance warrant further investigation.

IL-1β is one of the first pro-inflammatory cytokines to be activated at sites of injury. In addition to its early effects, IL-1β is involved in tissue repair in the late phase, such as remodelling and scarring (*30*–*32*). There have been many reports that IL-1β is implicated in pulmonary fibrosis (*33*, *34*). Long-term IL-1β treatment of human umbilical vein endothelial cells was reported to induce the EMT (*35*). The present study demonstrated that long-term IL-1β treatment of A549 cells caused morphological changes. However, we could not confirm that the morphological changes were due to the EMT because expression of E-cadherin and fibronectin did not change after treatment with IL-1β alone. Accordingly, the molecular mechanisms of the morphological changes caused by IL-1β should be examined precisely in the future.

In conclusion, short-term IL-1β treatment of A549 cells induced EGFR phosphorylation at Ser1047 via the IL-1R/p38 MAP kinase/MAPKAPK-2 pathway, resulting in transient EGFR internalization. In addition, long-term treatment with IL-1β induced morphological changes of the cells. Our results suggested that IL-1β is deeply involved in ligand-independent EGFR desensitization, and may play an important role in pulmonary fibrosis and inflammatory conditions.

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Conflict of Interest

None declared.

Data availability statement

Research data are not shared.

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