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培養肺胞上皮細胞における過酸化水素とフラジェリンによる上皮成長因子受容体の制御機構

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Differential regulation of epidermal growth factor receptor by hydrogen peroxide and flagellin in cultured lung alveolar epithelial cells

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ABSTRACT

In previous studies, we found that stimulation of Toll-like receptor 5 (TLR5) by flagellin induced the activation of mitogen-activated protein kinase (MAPK)-activated protein kinase-2 (MAPKAPK-2) through activation of the p38 MAPK pathway in cultured alveolar epithelial A549 cells. Our studies strongly suggested that MAPKAPK-2 phosphorylated epidermal growth factor receptor (EGFR) at Ser1047. It has been reported that phosphorylation of Ser1047 after treatment with tumor necrosis factor α (TNFα) induced the internalization of EGFR. In the present study, we first found that treatment of A549 cells with hydrogen peroxide induced the activation of MAPKAPK-2 and phosphorylation of EGFR at Ser1047 within 30 min. This was different from flagellin treatment because hydrogen peroxide treatment induced the phosphorylation of EGFR at Tyr1173 as well as Ser1047, indicating the activation of EGFR. We also found that KN93, an inhibitor of CaM kinase II, inhibited the hydrogen peroxide-induced phosphorylation of EGFR at Ser1047 through inhibition of the activation of the p38 MAPK pathway. Furthermore, we examined the internalization of EGFR by three different methods. Flow cytometry with an antibody against the extracellular domain of EGFR and biotinylation of cell surface proteins revealed that flagellin, but not hydrogen peroxide, decreased the amount of cell-surface EGFR. In addition, activation of extracellular signal-regulated kinase by EGF treatment was reduced by flagellin pre-treatment. These results strongly suggested that hydrogen peroxide activated the p38 MAPK pathway via activation of CaM kinase II and that flagellin and hydrogen peroxide regulate the functions of EGFR by different mechanisms.

Keywords: A549 cells, CaM kinase II, Epidermal growth factor receptor, Flagellin, Hydrogen peroxide, TNFα

1. Introduction

The alveolar epithelium is involved in the production of surfactant and serves as a physical barrier. Injury to alveolar epithelial cells is a pathological hallmark of acute respiratory distress syndrome (ARDS) (Sugahara et al., 2006, Finigan et al., 2012). ARDS is marked by the recruitment and activation of leukocytes, which release cytotoxic reactive oxygen species (ROS) as well as proteases and cytokines. It has been increasingly reported that ROS are key substances modulating the pulmonary vascular endothelial damage that characterizes ARDS (Sarma et al., 2011). Although ROS reportedly activate the p38 mitogen-activated protein kinase (MAPK) pathway in other cell systems (Saitoh et al., 1998), the consequences of activation of the p38 MAPK pathway on inflammation have not been elucidated. In addition, activation of the p38 MAPK pathway by ROS in alveolar epithelial cells has not been fully examined. Recent studies suggested that members of the epidermal growth factor receptor (EGFR) family are activated in alveolar epithelial cells during ARDS and regulate alveolar epithelial barrier functions (Finigan et al., 2012). However, the effects of ROS on the functions of EGFR are not clear at present.

Tumor necrosis factor α (TNFα), a cytokine especially important in inducing inflammatory responses, activates the nuclear factor-κB signaling pathway (for review, see Mizutani et al., 2010). In addition to activation of the nuclear factor-κB signaling pathway, TNFα reportedly activated the p38 MAPK pathway and induced the transient phosphorylation of EGFR at serine 1047 (Ser1047) (Nishimura et al., 2009). Interestingly, phosphorylated EGFR was internalized through a clathrin-mediated mechanism (Singhirunnusorn et al., 2007). We have been examining the intracellular signaling pathways that are activated after TNFα treatment of cultured alveolar epithelial A549 cells (Mizutani

et al., 2010). Recently, we found that MAPK-activated protein kinase-2 (MAPKAPK-2) was activated by TNFα treatment through activation of the p38 MAPK pathway (Noguchi et al., 2013). Our studies strongly suggested that MAPKAPK-2 was responsible for the phosphorylation of EGFR at Ser1047 (Noguchi et al., 2013).

Flagellin, a primary structural component of bacterial flagella, binds to and activates Toll-like receptor 5 (TLR5) in vertebrates (Hayashi et al., 2001). We found that stimulation of TLR5 by purified flagellin induced activation of the p38 MAPK pathway in cultured alveolar epithelial cells, including A549 cells (Kondo et al., 2012). Furthermore, we found the activation of MAPKAPK-2 and phosphorylation of EGFR at Ser1047 through activation of the p38 MAPK pathway by treatment of A549 cells with flagellin (Noguchi et al., 2013). These results may suggest that flagellin induces the internalization of EGFR in the case of pulmonary infection by flagellated bacteria, such as *Legionella pneumophila* (for review, see Yamamoto et al., 2014). So far, the internalization of EGFR after flagellin treatment has not been examined.

In the present study, we first examined whether or not hydrogen peroxide treatment of A549 cells activated MAPKAPK-2 through the activation of p38 MAPK. We found that hydrogen peroxide activated MAPKAPK-2, followed by the phosphorylation of EGFR at Ser1047. Therefore, internalization of EGFR was compared after treatment of A549 cells with hydrogen peroxide, flagellin, and TNFα.

2. Materials and methods

2.1. Materials

The following chemicals and reagents were obtained as indicated: Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS), Sigma Chemical Co. (St Louis, MO, USA); fetal calf serum (FCS), HyClone (Logan, UT, USA); protease inhibitor cocktail and protein phosphatase inhibitor cocktail (EDTA free), Nacalai Tesque (Kyoto, Japan); U0126, anti-MAPKAPK-2 antibody, anti-phospho-MAPKAPK-2 (Thr222) rabbit monoclonal antibody (9A7), anti-phospho-MAPKAPK-2 (Thr334) rabbit monoclonal antibody (27B7), anti-p38 MAPK antibody, anti-phospho-p38 MAPK antibody, anti-MAPK kinase 3 (MKK3) antibody, anti-MKK6 antibody, anti-phospho- MKK3/MKK6 antibody, anti-E-cadherin antibody, anti-phospho-HSP27 (Ser82) rabbit monoclonal antibody (D1H2), anti-rodent HSP27 rabbit polyclonal antibody, anti-HSP27 mouse monoclonal antibody (G31), Cell Signaling Technology (Beverly, MA, USA); KN93, anti-extracellular signal-regulated kinase (ERK) antibody, Sigma Chemical Co.; anti-active ERK antibody, Promega Co. (Madison, WI, USA); anti-EGFR mouse monoclonal antibody (6F1)(IgG_{2b}), Assay Designs (Ann Arbor, MI, USA); anti-phospho-EGFR (Thr669) mouse monoclonal antibody (5F10)(anti-P-Thr669 EGFR antibody) and anti-phospho-EGFR (Ser1047) mouse monoclonal antibody (1H9) (anti-P-Ser1047 EGFR antibody), Acris Antibodies Inc. (San Diego, CA, USA); anti-phospho-EGFR (Tyr1173) mouse monoclonal antibody (9H2)(anti-P-Tyr1173 EGFR antibody), SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole], and MK2a inhibitor [4-(2'-fluorobiphenyl-4-yl)-N-(4-hydroxyphenyl)-butyramide], Calbiochem

(Darmstadt, Germany); purified flagellin from *B. subtilis*, InvivoGen (San Diego, CA, USA); and TNFα, Wako (Osaka, Japan). Other chemicals were of analytical grade.

2.2. Culture of A549 and MLE12 cells, and preparation of cell extracts

A549 and MLE12 cells were kindly provided by Dr. Y. Isohama of Tokyo University of Science. The cells were grown on Petri dishes (Nunc, Roskilde, Denmark) in a culture medium consisting of 500 mL DMEM containing 4.5 g/L glucose and 10% (vol/vol) heat-inactivated FCS, as described previously (Kuwahara et al., 2006; Mizutani et al., 2010). The cells were treated without or with hydrogen peroxide, TNFα, or flagellin as indicated. We treated the cells with hydrogen peroxide over a wide range of concentrations from low (0.005 mM) to high (5 mM). In a previous study, we found that MAPKAPK-2 was strongly activated by 100 ng/mL TNFα and 2 μg/mL flagellin (Noguchi et al., 2013). Therefore, we treated the cells with TNFα and flagellin at these concentrations. We chose the concentrations of inhibitors of signal transduction (U0126, KN93, SB203580, and MK2a inhibitor) as directed by the manufacturer's instructions. The cells were washed once in PBS and lysed in 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 (Laemmli, 1970). The cell extract was sonicated for 20 sec at room temperature, heated to 98°C for 5 min, and kept at -80°C until use (Higa-Nakamine et al., 2011).

2.3. Determination of cell viability

Cell viability was examined using a trypan blue-exclusion test with TC10 trypan blue dye that contained 0.4% (wt/vol) trypan blue dye in 0.81% (wt/vol) NaCl and 0.06% (wt/vol) potassium phosphate dibasic solution. The numbers of live and dead cells in the cell suspension were estimated using a TC10 cell counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.4. SDS-PAGE and immunoblotting analysis

SDS-PAGE was performed by the method of Laemmli (1970) followed by immunoblotting analysis (Mizutani et al., 2011; Towbin et al., 1979). Immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (GE Healthcare UK Ltd., Little Chalfont, UK) in accordance with the manufacturer's instructions and quantified using an ImageQuant LAS 4000 mini (GE Healthcare UK Ltd.). For reprobing, the membrane was incubated with stripping buffer [62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% (wt/vol) SDS] at 50°C for 30 min (Yamanaka et al., 2007). The membrane was then washed with a blocking solution containing 5% (wt/vol) skim milk, 100 mM Tris-HCl, pH 7.5, 0.9% (wt/vol) NaCl, and 0.1% (vol/vol) Tween-20 followed by Tris-buffered saline with Tween-20 (TTBS) containing 100 mM Tris-HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween-20 at room temperature and subjected to immunoblotting analysis.

2.5. Flow cytometry analysis

Cell surface expression of EGFR was investigated by flow cytometry analysis using an antibody against the extracellular domain of EGFR (LA22) (Wu et al., 1989). A549 cells were grown to more than 8×10^6 cells in 100-mm Petri dishes (Nunc) and treated for 30 min

with hydrogen peroxide, TNFα, or flagellin as indicated. Thereafter, the cells were fixed with fixation buffer for 20 min on ice without permeabilization of the cell membrane. After being washed with Wash Buffer, the cells were incubated with a fluorescently labeled antibody against EGFR (anti-EGFR PerCP) (Merck Millipore, Darmstadt, Germany) on ice for 60 min and analyzed using a Guava^{TM} Flow cytometer (Merck Millipore). The internalization of EGFR was estimated using the average of the fluorescence intensities (mean fluorescence) of EGFR on the cell surface.

2.6. Cell surface biotinylation

The biotinylation of cell surface proteins and isolation of biotinylated proteins were performed using a Cell Surface Protein Isolation kit (Pierce Biotechnology, Rockford, IL, USA) (Higa-Nakamine, 2012). A549 cells were grown to 90–95% confluency in 100-mm Petri dishes (Nunc) and treated with hydrogen peroxide, TNFα, or flagellin as indicated. Furthermore, the cells were incubated at 4°C for 30 min with PBS containing sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-Biotin), a thiol-cleavable amine-reactive biotinylation reagent. Cells were lysed in the presence of protease inhibitor cocktail, and the biotinylated proteins were isolated with NeutrAvidin agarose. Isolated proteins were released by incubating with SDS-PAGE sample buffer containing dithiothreitol for 60 min at room temperature. Debiotinylated and released proteins were subjected to immunoblotting analysis.

2.7. Other procedures

Protein concentrations were determined using a Qubit Protein Assay Kit with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). In pilot experiments, the protein concentration curves were linear in the presence of 0.04% (wt/vol) SDS, 0.1% (vol/vol) 2-mercaptoethanol and 0.1% (vol/vol) glycerol. Therefore, we diluted the cell extract 50-fold with water for protein quantification. The experiments were repeated at least three times, and representative results are shown in the figures. The values are expressed as the mean ± SE. Statistical analysis was performed using a one-way ANOVA plus Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

3. **Results**

3.1. Activation of MAPKAPK-2 by hydrogen peroxide

Our previous study strongly suggested that MAPKAPK-2 directly phosphorylated EGFR at Ser1047 after treatment of A549 cells with TNFα and flagellin (Noguchi et al., 2013). Therefore, we first examined whether or not treatment of the cells with hydrogen peroxide activated MAPKAPK-2. It has been reported that phosphorylation of any two of the residues Thr222, Ser272, and Thr334 was necessary and sufficient for maximal activation of MAPKAPK-2 (for review, see Ben-Levy et al., 1995). In the present experiment, we examined the phosphorylation of Thr222 and Thr334 using available antibodies (Fig. 1). Without hydrogen peroxide, no phosphorylation of Thr222 and Thr334 was observed. When we treated A549 cells for 30 min with various concentrations of hydrogen peroxide, both sites were robustly phosphorylated with 0.25 mM hydrogen peroxide and above (Fig. 1A). Immunoblotting analysis with an anti-MAPKAPK-2 antibody showed that the electrophoretic mobility of MAPKAPK-2 decreased concomitantly with 0.25 mM hydrogen peroxide or above, suggesting that phosphorylation of MAPKAPK-2 decreased the electrophoretic mobility of the protein. We reported that TNFα strongly activated MAPKAPK-2 (Noguchi et al., 2013), but hydrogen peroxide appeared to activate MAPKAPK-2 much more strongly than TNFα (Fig. 1A). When we treated the cells with 0.5 mM hydrogen peroxide for various periods, the phosphorylation of Thr222 and Thr334 was increased at 15 min, peaked at 30 min and declining thereafter (Fig. 1B).

3.2. Determination of cell viability

In order to determine the cell viability, we treated A549 cells with 0, 0.5, 2, and 5 mM hydrogen peroxide for 30 or 120 min. When we treated the cells with 5 mM hydrogen peroxide for 30 and 120 min, more than 94% and 87%, respectively, of the cells were alive.

3.3. Phosphorylation of EGFR by hydrogen peroxide

Because MAPKAPK-2 was strongly activated by hydrogen peroxide, we next examined the phosphorylation of EGFR at Ser1047 (Fig. 2). We first treated the cells with various concentrations of hydrogen peroxide for 30 min (Fig. 2A). We found that phosphorylation of Ser1047 was weaker with hydrogen peroxide than that with TNFα treatment. The level of phosphorylation of Ser1047 by TNFα was approximately 2.8-fold higher than that after treatment with 5 mM hydrogen peroxide. In contrast, phosphorylation of Tyr1173 was much more pronounced after 5 mM hydrogen peroxide treatment than after TNFα treatment. These results suggested that hydrogen peroxide activated EGFR more strongly than TNFα. EGFR was reportedly phosphorylated at Thr669 through activation of the MAPK pathway (Morrison et al., 1996). However, phosphorylation of Thr669 was not increased by the treatment of A549 cells with hydrogen peroxide or TNFα (Fig. 2A). We noticed that the decrease in electrophoretic mobility was more pronounced with TNFα treatment than with hydrogen peroxide (Fig. 2A). Phosphorylation of EGFR at Ser1047 was detected weakly at 5 min and peaked at 30 min using both 0.5 and 5 mM hydrogen peroxide (Figs. 2B and 2C). At 120 min, the phosphorylation declined following treatment with 0.5 mM hydrogen peroxide, whereas it was sustained using 5 mM hydrogen peroxide. The protein level was not changed even after 2-h treatment of the cells with 5 mM hydrogen peroxide (Fig. 2C). These results suggested that hydrogen peroxide did not induce the degradation of EGFR.

3.4. Effects of N-acetylcysteine on the phosphorylation of EGFR

Because hydrogen peroxide treatment induced the phosphorylation of EGFR, we considered the possibility that the effects of TNFα and flagellin might occur via generation of ROS in the cells. Therefore, we examined whether *N*-acetylcysteine (NAC), which increases intracellular glutathione levels, inhibited the phosphorylation of EGFR by TNFα or flagellin (Fig. 3). It has been reported that 10 mM NAC was effective in cells (Shah et al., 2006). We confirmed that 10 mM NAC inhibited both the activation of MAPKAPK-2 and phosphorylation of EGFR at Ser1047 and Tyr1173 after hydrogen peroxide treatment (Figs. 3A and 3B). In contrast, activation of MAPKAPK-2 and phosphorylation of EGFR by TNFα were not inhibited by NAC (Figs. 3A and 3B). In addition, NAC did not inhibit the flagellin-induced phosphorylation of EGFR at Ser1047 (Fig. 3C). These results indicated that ROS were not involved in the phosphorylation of EGFR by TNFα or flagellin. We have reported that flagellin treatment did not induce the phosphorylation of EGFR at Tyr1173 (Noguchi et al., 2013).

3.5. Effects of four inhibitors of protein kinases on the phosphorylation of EGFR

Figure 4 shows the effects of inhibitors for four protein kinases. Our previous study revealed that both flagellin and TNFα treatments induced the activation of MAPKAPK-2 and phosphorylation of EGFR at Ser1047 through the p38 MAPK pathway (Noguchi et al., 2013). SB203580, an inhibitor of p38 MAPK, and MK2ai, which binds to MAPKAPK-2 and inhibits its activation, inhibited both activation of MAPKAPK-2 and phosphorylation of EGFR at Ser1047 after hydrogen peroxide treatment, as expected (Fig. 4). ERK reportedly phosphorylated and activated MAPKAPK-2 *in vitro* (Ben-Levy et al., 1995), and we

confirmed these ERK effects (Noguchi et al., 2013). However, U0127, which inhibits ERK activation, did not clearly inhibit the activation of MAPKAPK-2 or phosphorylation of Ser1047 (Fig. 4). It has been reported that Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) phosphorylated EGFR at Ser1047 after the stimulation of EGFR (Countaway et al., 1992). We found that the TNFα-induced phosphorylation of EGFR at Ser1047 was not inhibited by KN93, an inhibitor of CaM kinase II (Noguchi et al., 2013). It was surprising that KN93 inhibited both activation of MAPKAPK-2 and phosphorylation of Ser1047 almost completely (Fig. 4). Phosphorylation of EGFR at Tyr1173 was not clearly inhibited by any of the protein kinase inhibitors. We found that mobility shifts of MAPKAPK-2 and EGFR after hydrogen peroxide treatment were strongly inhibited by KN93, as well as SB203580 and MK2ai (Fig. 4).

We next examined whether or not KN93 inhibited the activation of p38 MAPK (Fig. 5). We confirmed that treatment of A549 cells with 2 mM hydrogen peroxide activated p38 MAPK by approximately 16-fold. We noticed that p38 MAPK was slightly activated under the basal conditions, and that KN93 did not inhibit this basal activation. In contrast, the activation of p38 MAPK by hydrogen peroxide was inhibited by KN93 almost completely. When the anti-p38 MAPK antibody was used for immunoblotting, no significant changes in immunoreactivity were observed for any of the treatments (Fig. 5). It has been reported that MKK3 and MKK6 phosphorylate and activate p38 MAPK (for review, see Ambrosino et al., 2003). Therefore, we examined whether MKK3 or MKK6 was activated by hydrogen peroxide and inhibited by KN93 (Fig. 5). In our previous study, we reported that the treatment of A549 cells with flagellin activated both MKK3 and MKK6 (Kondo et al., 2012). In contrast, hydrogen peroxide activated MKK3 by approximately 6-fold but not MKK6.

Furthermore, we found that KN93 strongly inhibited the activation of MKK3. The levels of MKK3 and MKK6 proteins were not changed by any of the treatments (Fig. 5). These results strongly suggested that MKK3 was involved in the hydrogen peroxide-induced activation of p38 MAP kinase and that KN93 inhibited the MKK3 activation.

3.6. Internalization of EGFR by flagellin and TNFα

It has been reported that EGFR is internalized transiently after TNFα treatment through the p38 MAPK pathway (Singhirunnusorn et al., 2007). It was suggested that this internalization was induced through phosphorylation of Ser1047 in EGFR (Nishimura et al., 2009). Because hydrogen peroxide treatment and flagellin treatment induced the phosphorylation of Ser1047, we decided to examine whether or not internalization of EGFR was induced by these treatments.

We first examined the internalization by flow cytometry analysis with the fluorescent-labeled antibody against the extracellular domain of EGFR (anti-EGFR PeCP) (Fig. 6). A549 cells were treated without or with flagellin, TNFα, or hydrogen peroxide for 30 min. We found that treatment with flagellin and TNFα decreased labeling with the anti-EGFR antibody. It was of interest that the decrease in labeling of the cells was more remarkable after flagellin treatment than that after TNFα treatment. In contrast, no changes in labeling were observed after hydrogen peroxide treatment. The value of the mean fluorescence of the control cells was 49.0, whereas it was 30.6, 41.1, and 48.0, after treatment with flagellin, TNFα, and hydrogen peroxide, respectively.

The results of flow cytometry analysis are summarized in Fig. 7A. The values of mean fluorescence after flagellin and TNFα treatments were decreased to 62.8 ± 1.4% ($P < 0.01$)

(n = 4) and 82.7 ± 2.4% ($P < 0.05$) (n = 4), respectively, of the control value. However, the value was 94.2 ± 2.6% (n = 4) after hydrogen peroxide treatment, and no decrease was observed.

Next, the internalization of EGFR was investigated by cell surface biotinylation. After treatment of A549 cells with flagellin, TNFα, or hydrogen peroxide for 30 min, the cells were cooled to 4°C and cultured for 30 min with Sulfo-NHS-SS-biotin, a membrane-impermeable biotinylation reagent. When A549 cells were treated with flagellin, the level of biotinylated EGFR protein decreased to approximately 60% of that in untreated cells (Fig. 7B). In contrast, the level of biotinylated EGFR protein was not changed by TNFα or hydrogen peroxide treatment. In addition, the level of biotinylated E-cadherin protein was not changed by any of the treatments (Fig. 7B). We also confirmed that p38 MAPK, which is in the intracellular space, was mainly in a non-biotinylated form. The reasons why p38 MAPK was barely detected in the biotinylated form without any treatments were unknown but may be related to non-specific binding of non-biotinylated p38 MAPK to NeutrAvidin agarose. These results further supported our idea that flagellin, but not hydrogen peroxide, induced the internalization of EGFR.

We next examined the EGF-induced activation of ERK after pretreatment of A549 cells with flagellin, TNFα, and hydrogen peroxide (Fig. 7C). In the previous study, we found that the activation of ERK by flagellin peaked at 30 min and declined thereafter (Kondo et al., 2012). Therefore, we pretreated the cells with flagellin, TNFα, or hydrogen peroxide for 60 min. Without pretreatment, ERK was activated by 3.0 ± 0.6-fold by EGF treatment. In contrast, ERK was activated by EGF only 1.7 ± 0.1-fold ($P < 0.01$) and 1.7 ± 0.2-fold after pretreatment with flagellin and TNFα, respectively. Pretreatment with hydrogen peroxide

had no effect on the activation of ERK by EGF treatment, and ERK was activated 3.5 ± 0.6-fold. When the anti-ERK antibody was used for immunoblotting, no significant changes in immunoreactivity were observed for any of the treatments (data not shown). These results strongly suggested that flagellin induced desensitization of EGFR through its internalization.

3.7. Phosphorylation of HSP27 by hydrogen peroxide

It has been reported that HSP27 is a physiological substrate for MAPKAPK-2 (Rouse et al., 1994). Therefore, in addition to EGFR, we examined the phosphorylation of HSP27 after the hydrogen peroxide treatment (Fig. 8). We found that phosphorylation of HSP27 at Ser82 was increased by approximately 3.7-fold with 0.25 mM hydrogen peroxide, and that the phosphorylation level with hydrogen peroxide treatment was similar to that with TNFα (Fig. 8A). Phosphorylation of HSP27 was increased approximately 2.7-fold at 5 min and the increase was sustained until 60 min using 0.5 mM hydrogen peroxide (Fig. 8B). These results suggested that HSP27 was a good substrate for MAPKAPK-2 activated by hydrogen peroxide, as well as EGFR.

3.8. Phosphorylation of HSP27 by hydrogen peroxide in MLE12 cells

A549 cells have a *K-ras* mutation and tumorigenic features. We considered the possibility that these features might interfere with the results. Therefore, we decided to use another alveolar epithelial cell line, MLE12. This cell line was established from tumors in a mouse transgenic for the SV40 large T antigen under the control of the promoter of the surfactant protein C gene. We treated MLE12 cells with hydrogen peroxide and examined

the activation of p38 MAPK and MAPKAPK-2, and phosphorylation of HSP27. Treatment of the cells with hydrogen peroxide for 30 min activated p38 MAPK approximately 7- and 15-fold at 0.05 mM and 2 mM, respectively (Fig. 8C). Phosphorylation of MAPKAPK-2 at Thr222 and Thr334 was observed after treatment with 2 mM hydrogen peroxide. Hydrogen peroxide increased the phosphorylation of HSP27 approximately 2.6- and 4.6-fold at 0.05 mM and 2 mM, respectively. The levels of p38 MAPK, MAPKAPK-2, and HSP27 proteins were not changed by the treatments. These results indicated that the responses to hydrogen peroxide were features of alveolar epithelial cells but not that of the *K-ras* mutation.

4. Discussion

EGFR belongs to the ErbB protein family of receptor tyrosine kinases (for review, see Yamamoto et al., 2014). When EGF binds to its receptor, multiple tyrosine residues including Tyr1173 are autophosphorylated, resulting in the initiation of numerous cellular responses. We found that activation of EGFR in A549 cells by EGF stimulated cell growth (Maeda et al., unpublished observation). In the present study, treatment of A549 cells with hydrogen peroxide induced the phosphorylation of EGFR at Ser1047 and Tyr1173. Both amino acid residues were also phosphorylated after TNFα treatment (Fig. 3B), whereas only Ser1047 was phosphorylated after flagellin treatment (Noguchi et al., 2013). We found that Tyr1173 was phosphorylated more strongly by hydrogen peroxide treatment than by TNFα treatment, suggesting that EGFR was activated more strongly by hydrogen peroxide than TNFα. In contrast, Ser1047 was more strongly phosphorylated by TNFα than hydrogen peroxide. These results indicated that the phosphorylation of EGFR was different both in quality and in quantity among these treatments.

Flow cytometry with an antibody against the extracellular domain of EGFR and biotinylation experiments with a membrane-impermeable biotinylation reagent revealed that flagellin decreased the amount of EGFR in the cell membrane. In addition, ERK activation by EGF was markedly weakened after flagellin pretreatment, suggesting the desensitization of EGFR. Because the level of EGFR protein was not reduced by flagellin treatment (Fig. 3C), it is unlikely that proteolysis of EGFR caused the desensitization. To the best of our knowledge, the present study is the first report that flagellin induces the desensitization of EGFR. Although these results strongly suggested that flagellin induced the internalization of EGFR, this should be examined carefully by immunostaining of EGFR in future studies.

Hydrogen peroxide treatment induced phosphorylation of EGFR at Ser1047 as strongly as flagellin treatment. However, hydrogen peroxide treatment did not decrease the amount of EGFR in the cell membrane nor induced desensitization. In a previous study, we found that phosphorylation of Ser1047 by TNFα was approximately 5-fold higher than that with flagellin treatment (Noguchi et al., 2013). However, the desensitization of EGFR with flagellin was similar to that with TNFα, or even more pronounced. The reasons for these differences between phosphorylation of Ser1047 and desensitization of EGFR are not clear at present. We found that phosphorylation of EGFR at Tyr1173 was not increased after flagellin treatment, whereas it was increased after hydrogen peroxide and TNFα treatments (Fig. 2A and Noguchi et al., 2013). The phosphorylation level of Tyr1173 was higher after hydrogen peroxide treatment than after TNFα treatment. It is well known that phosphorylation of Tyr1173 provides a docking site for the Shc scaffold protein, followed by activation of the MAPK pathway. Therefore, binding of EGFR to the Shc scaffold protein after phosphorylation of Tyr1173 might inhibit the internalization and desensitization of EGFR. If this is the case, it makes sense that the internalization of EGFR was not observed after hydrogen peroxide treatment. Elucidation of the molecular mechanisms for the internalization after phosphorylation of Ser1047 and for the inhibition of the internalization by phosphorylation of Tyr1173 are extremely important for future studies.

Our data suggest that HSP27 is a physiological substrate of MAPKAPK-2 activated after hydrogen peroxide treatment. It has been reported that HSP27 is enriched at the leading edge of the fibroblasts, where HSP27 regulates actin polymerization (Miron et al., 1991). It was also reported that phosphorylation of HSP27 induced the accumulation of cortical F-actin at the leading edge (Lavoie et al., 1993). Therefore, reorganization of F-actin in lung

alveolar epithelial cells may occur after hydrogen peroxide treatment through the phosphorylation of HSP27. This change may contribute to cellular resistance to exposure to ROS.

We found that MKK3 was mainly phosphorylated after hydrogen peroxide treatment (Fig. 5). The MAPK kinase kinase (MAPKKK) that is activated and phosphorylates MKK3 after hydrogen peroxide treatment is not clear at present. In a previous study, we found that both MKK3 and MKK6 were activated after flagellin treatment (Kondo et al., 2012). Therefore, the MAPKKK involved may be different between treatments with hydrogen peroxide and flagellin. It was reported that transforming growth factor-β-activated kinase 1 (TAK1) was involved in the TNFα-induced phosphorylation of EGFR (Shinghirunnusorn et al., 2007). Therefore, TAK1 may also be involved in the response to hydrogen peroxide or flagellin. Another candidate protein kinase is apoptosis signal-regulating kinase 1 (ASK1) (for review see, Takeda et al., 2004). It was reported that ASK1 was activated by CaM kinase II after membrane depolarization of cultured neurons (Takeda et al., 2004). Therefore, it is intriguing to speculate that hydrogen peroxide activated ASK1 through activation of CaM kinase II in A549 cells. However, that study also found that hydrogen peroxide-induced activation of ASK1 was not inhibited by KN93 (Takeda et al., 2004). Recently, we confirmed that A549 cells expressed ASK1, but the phosphorylation of ASK1 at Thr845 in the activation loop was not increased after hydrogen peroxide treatment (Nishi et al., unpublished observations). Therefore, CaM kinase II may activate a MAPKKK other than ASK1. Because KN93 inhibits the CaM kinase family as well as CaM kinase II (for review, see Maeda et al., 2009), it is also possible that CaM kinases other than CaM kinase II are involved in the activation of the p38 MAPK pathway. Investigation of the activation

of the CaM kinase family and molecular mechanisms, by which a corresponding CaM kinase activates a corresponding MAPKKK, will be important for future studies.

The alveoli are always exposed to oxygen from the atmosphere, and lung alveolar epithelial cells are subjected to higher concentrations of oxygen compared with other epithelial cells. Therefore, it is easily assumed that lung alveolar epithelial cells are at a high risk of suffering from ROS. We found that the expression level of thioredoxin, an anti-oxidants, was high in A549 cells by DNA microarray analysis (Kondo et al., 2012). This may be one of the reasons why a relatively high concentration of hydrogen peroxide was necessary for activation of the p38 MAPK pathway in A549 cells and MLE12 cells. Examination of cell viability indicated some toxicity of hydrogen peroxide after 120-min treatment. Because we treated the cells within 30 min in many experiments, we believe that the toxicity of hydrogen peroxide would not interfere with our interpretations of the results.

Legionella pneumophila is a flagellated bacteria that causes Legionnaire pneumonia. It was suggested that stimulation of TLR5 by flagellin has a critical role in the onset of this disease (Newton et al., 2010). Therefore, the desensitization of EGFR by flagellin may be involved in the pathophysiology of Legionnaire pneumonia. It is worth examining whether or not phosphorylation and desensitization of EGFR occur *in vivo* after infection with *Legionella pneumophila*.

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References

Ambrosino, C., Mace, G., Galban, S., Fritsch, C., Vintersten, K., Black, E., Gorospe, M., Nebreda, A.R., 2003. Negative feedback regulation of MKK6 mRNA stability by p38α mitogen-activated protein kinase. Mol Cell Biol 23, 370-381.

Ben-Levy, R., Leighton, I.A., Doza, Y.N., Attwood, P., Morrice, N., Marshall, C.J., Cohen, P., 1995. Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2. EMBO J. 14, 5920-5930.

Countaway, J.L., Nairn, A.C., Davis, R.J., 1992. Mechanism of desensitization of the epidermal growth factor receptor protein-tyrosine kinase. J. Biol. Chem. 267, 1129-1140.

Finigan, J.H., Downey, G.P., Kern, J.A. 2012. Human epidermal growth factor receptor signaling in acute lung injury. Am. J. Respir. Cell Mol. Biol. 47, 395-404.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., Aderem, A. 2001. The innate immune response to bacteria flagellin is mediated by Toll-like receptor 5. Nature 410, 1099-1103.

Higa-Nakamine, S., Maeda, N., Toku, S., Yamamoto, T., Yingyuenyong, M., Kawahara, M., Yamamoto, H., 2011. Selective cleavage of ErbB4 by G-protein-coupled gonadotropin-releasing hormone receptor in cultured hypothalamic neurons. J. Cell. Physiol. 227, 2492-2501.

Kondo, Y., Higa-Nakamine, S., Noguchi, N., Maeda, N., Toku, S., Isohama, Y., Sugahara, K., Kukita, I., Yamamoto, H., 2012. Induction of epithelial-mesenchymal transition by flagellin in cultured lung epithelial cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 303, L1057-L1069.

Kuwahara, I., Lillehoj, E.P., Lu, W., Singh, I.S., Isohama, Y., Miyata, T., Kim, K.C., 2006. Neutrophil elastase induces IL-8 gene transcription and protein release through

p38/NF-κB activation via EGFR transactivation in a lung epithelial cell line. Am. J. Physiol. Lung Cell. Mol. Physiol. 291, L407-L416.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680-685.

Lavoie, J.N., Hickey, E., Weber, L.A., Landry, J., 1993, Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J. Biol. Chem. 268, 24210-24214.

Maeda, N., Toku, S., Naito, Y., Nishiura, H., Tanaka, T., Yamamoto, H., 2009. Phosphorylation of ribosomal protein S19 at Ser59 by CaM kinase Iα. J. Neurochem. 109, 393-402.

Miron, T., Vancompernolle, K., Vandekerckhove, J., Wilchek, M., Geiger, B., 1991, A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J. Cell. Biol. 114, 255-261.

Mizutani, A., Maeda, N., Toku, S., Higa-Nakamine, S., Isohama, Y., Sunakawa H., Sugahara, K., Yamamoto, H., 2010. Interaction of ethyl pyruvate *in vitro* with NF-κB subunits, RelA and p50. Eur. J. Pharmacol. 650, 151-156.

Morrison, P., Saltiel, A.R., Rosner, M.R., 1996. Role of mitogen-activated protein kinase kinase in regulation of the epidermal growth factor receptor by protein kinase C. J. Biol. Chem. 271, 12891-12896.

Newton, H.J., Ang, D.K., van Driel, I.R., Hartland, E.L., 2010 Molecular pathogenesis of infections caused by *Legionella pneumophila*. Clin. Microbiol. Rev. 23, 274-298.

Nishimura, M., Shin, M.S., Singhirunnusorn, P., Suzuki, S., Kawanishi, M., Koizumi, K., Saiki, I., Sakurai, H., 2009. TAK1-mediated serine/threonine phosphorylation of epidermal growth factor receptor via p38/extracellular signal-regulated kinase: NF-κB-independent survival pathways in tumor necrosis factor alpha signaling. Mol.

Cell. Biol. 29, 5529-5539.

Noguchi N., Kondo Y., Maeda N., Higa-Nakamine S., Toku S., Maruyama J., Isohama Y., Kukita I., Sugahara K., Yamamoto H., 2013 Phosphorylation of epidermal growth factor receptor at serine 1047 by MAP kinase-activated protein kinase-2 in cultured lung epithelial cells treated with flagellin. Arch. Biochem. Biophys. 529, 75-85.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Liamazares, A., Zamanillo, D., Hunt, T., Nebreda, A.R., 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78. 1027-1037.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., Ichijo, H., 2004. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. 17, 2596-2606.

Sarma, J.V., Ward, P.A., 2011. Oxidants and redox signaling in acute lung injury. Compr. Physiol. 1, 1365-1381.

Shah, B.H., Shah, F.B., Catt, K.J., 2006. Role of metalloproteinase-dependent EGF receptor activation in α_1-adrenoceptor-stimulated MAP kinase phosphorylation in GT1-7 neurons. J. Neurochem. 96, 520-532.

Singhirunnusorn, P., Ueno, Y., Matsuo, Y.M., Suzuki, S., Saiki, I., Sakurai, H., 2007. Transient suppression of ligand-mediated activation of epidermal growth factor receptor by tumor necrosis factor-α through the TAK1-p38 signaling pathway. J. Biol. Chem. 282, 12698-12706.

Sugahara, K., Tokumine, J., Teruya, K., Oshiro, T., 2006. Alveolar epithelial cells: differentiation and lung injury. Respirology. 11, S28-S31.

Takeda, K., Matsuzawa, A., Nishitoh, H., Tobiume, K., Kishida, S., Ninomiya-Tsuji, J., Matsumoto, K., Ichijo, H., 2004. Involvement of ASK1 in Ca^{2+}-induced p38 MAP ki-

nase activation. EMBO Rep. 5, 161-166.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.

Wu, D.G., Wang, L.H., Sato, G.H., West, K.A., Harris, W.R., Crabb, J.W., Sato, J.D., 1989. Human epidermal growth factor (EGF) receptor sequence recognized by EGF competitive monoclonal antibodies. Evidence for the localization of the EGF-binding site. J. Biol. Chem. 264, 17469-17475.

Yamamoto, H., Higa-Nakamine, S., Noguchi, N., Maeda, N., Kondo, Y., Toku, S., Kukita, I., Sugahara, K., 2014. Desensitization by different strategies of epidermal growth factor receptor and ErbB4. J. Pharmacol. Sci. 124, 287-293.

Yamanaka, A., Hiragami, Y., Maeda, N., Toku, S., Kawahara, M., Naito, Y., Yamamoto, H., 2007. Involvement of CaM kinase II in gonadotropin-releasing hormone-induced activation of MAP kinase in cultured hypothalamic neurons. Arch. Biochem. Biophys. 466, 234-241.

Legends for figures

Fig. 1. Activation of mitogen-activated protein kinase (MAPK)-activated protein kinase-2 (MAPKAPK-2) by hydrogen peroxide. A, A549 cells were treated with hydrogen peroxide at the indicated concentrations, or 100 ng/mL TNFα for 30 min. The cell extracts (33 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and immunoblotting analysis was performed with anti-P-Thr222 MAPKAPK-2 (1: 750) or an anti-P-Thr334 MAPKAPK-2 (1: 750) antibodies. After the anti-P-Thr334 MAPKAPK-2 antibody was stripped away, immunoblotting with an anti-MAPKAPK-2 antibody was performed at a dilution of 1: 750. B, A549 cells were treated with 0.5 mM hydrogen peroxide for the periods indicated. Immunoblotting analysis with P-Thr222 MAPKAPK-2 or P-Thr334 MAPKAPK-2 was performed using the cell extracts (61.5 μg) as described above. After the anti-P-Thr334 MAPKAPK-2 antibody was stripped away, immunoblotting with an anti-MAPKAPK-2 antibody was performed at a dilution of 1: 750. The bands for MAPKAPK-2 (P-Thr222 MAPKAPK2, P-Thr334 MAPKAPK2, P-MAPKAPK2, and MAPKAPK2) are indicated.

Fig. 2. Phosphorylation of epidermal growth factor receptor (EGFR) by hydrogen peroxide. A, A549 cells were treated with hydrogen peroxide at the indicated concentrations, or 100 ng/mL TNFα for 30 min. The cell extracts (33 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and immunoblotting analysis was performed with anti-P-Ser1047 EGFR (1: 100), anti-P-Tyr1173 EGFR (1: 300), or anti-P-Thr669 EGFR (1: 100) antibodies. After the anti-P-Thr669 EGFR antibody was stripped away, immunoblotting with anti-EGFR antibody was performed at a dilution of 1: 600. B, A549 cells were treated with

0.5 mM hydrogen peroxide for the periods indicated. Immunoblotting analysis with an anti-P-Ser1047 EGFR antibody and an anti-EGFR antibody was performed using the cell extracts (61.5 μg) as described above. C, A549 cells were treated with 5 mM hydrogen peroxide for the periods indicated. Immunoblotting analysis with an anti-P-Ser1047 EGFR antibody was performed using cell extracts (43.5 μg) as described above. After the anti-P-Ser1047 EGFR antibody was stripped away, immunoblotting with anti-EGFR antibody was performed at a dilution of 1: 600. The bands for EGFR (P-Ser1047 EGFR, P-Tyr1173 EGFR, P-Thr669 EGFR, and EGFR) are indicated.

Fig. 3. Effects of *N*-acetylcysteine (NAC) on activation of mitogen-activated protein kinase (MAPK)-activated protein kinase-2 (MAPKAPK-2) and phosphorylation of epidermal growth factor receptor (EGFR) by tumor necrosis factor α (TNFα), hydrogen peroxide, and flagellin. A, A549 cells were pretreated with or without 10 mM NAC for 60 min and treated with or without 100 ng/mL TNFα or 5 mM hydrogen peroxide for 15 min. The cell extracts (23.4 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and immunoblotting analysis was performed with an anti-P-Thr334 MAPKAPK-2 antibody (1: 750). After the anti-P-Thr334 MAPKAPK-2 antibody was stripped away, immunoblotting with an anti-MAPKAPK-2 antibody was performed at a dilution of 1: 750. The bands for MAPKAPK-2 (P-Thr334 MAPKAPK2, P-MAPKAPK2, and MAPKAPK2) are indicated. B, The cell extracts (23.4 μg) were also subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide and immunoblotting analysis was performed with anti-P-Ser1047 EGFR (1: 100), anti-P-Tyr1173 EGFR (1: 300), or anti-EGFR (1: 600) antibodies. C, A549 cells were pretreated with or without 10 mM NAC for 60 min and treated with or without 2 μg/mL

flagellin or 0.5 mM hydrogen peroxide for 15 min. Immunoblotting analysis with an anti-P-Ser1047 EGFR antibody was performed using the cell extracts (25.5 μg) as described above. After the anti-P-Ser1047 EGFR antibody was stripped away, immunoblotting with an anti-EGFR antibody was performed at a dilution of 1: 600. The bands for EGFR (P-Ser1047 EGFR, P-Tyr1173 EGFR, and EGFR) are indicated.

Fig. 4. Effects of protein kinase inhibitors on the activation of mitogen-activated protein kinase (MAPK)-activated protein kinase-2 (MAPKAPK-2) and phosphorylation of epidermal growth factor receptor (EGFR) by hydrogen peroxide. A, A549 cells were pretreated with or without 10 μM SB203580 (SB), 20 μM MK2a inhibitor (MK2ai), 10 μM U0127 (U01), or 20 μM KN93 for 30 min and treated with or without 5 mM hydrogen peroxide for 30 min. The cell extracts (40.5 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and immunoblotting analysis was performed with anti-P-Thr222 MAPKAPK-2 (1: 750), anti-P-Thr334 MAPKAPK-2 (1: 750), or anti-MAPKAPK-2 (1: 750) antibodies. The bands for MAPKAPK-2 (P-Thr222 MAPKAPK2, P-Thr334 MAPKAPK2, P-MAPKAPK2, and MAPKAPK2) are indicated. B, The cell extracts (40.5 μg) were also subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and immunoblotting analysis was performed with anti-P-Ser1047 EGFR (1: 100) or anti-P-Tyr1173 EGFR (1: 300) antibodies. After the anti-P-Ser1047 EGFR antibody was stripped away, immunoblotting with an anti-EGFR antibody was performed at a dilution of 1: 600. The bands for EGFR (P-Ser1047 EGFR, P-Tyr1173 EGFR, and EGFR) are indicated.

Fig. 5. Effects of KN93 on the activation of p38 mitogen-activated protein kinase (MAPK), MKK3, and MKK6 by hydrogen peroxide. A549 cells were pretreated with or without 20 μM KN93 for 30 min and treated with or without 2 mM hydrogen peroxide for 30 min. The cell extracts (20.1 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and immunoblotting analysis was performed with anti-phospho-p38 MAP kinase (1: 750) or anti-phospho-MKK3/MKK6 (1: 750) antibodies. After the anti-phospho-p38 MAP kinase antibody was stripped away, immunoblotting with an anti-p38 MAP kinase antibody was performed at a dilution of 1: 750. After the anti-phospho-MKK3/MKK6 antibody was stripped away, immunoblotting with an anti-MKK3 antibody (1: 750) or anti-MKK6 antibody (1: 750) was performed. The bands for p38 MAP kinase, MKK3, and MKK6 are indicated.

Fig. 6. Flow cytometry analysis after treatment of A549 cells with flagellin, tumor necrosis factor α (TNFα), or hydrogen peroxide. A549 cells were treated with 2 μg/mL flagellin, 100 ng/mL TNFα, or 2 mM hydrogen peroxide for 30 min. The cells were fixed and labeled with anti-EGFR-PerCP for 1 h without permeabilization of the cell membranes. After the cells were washed with Wash Buffer, flow cytometry analysis was performed.

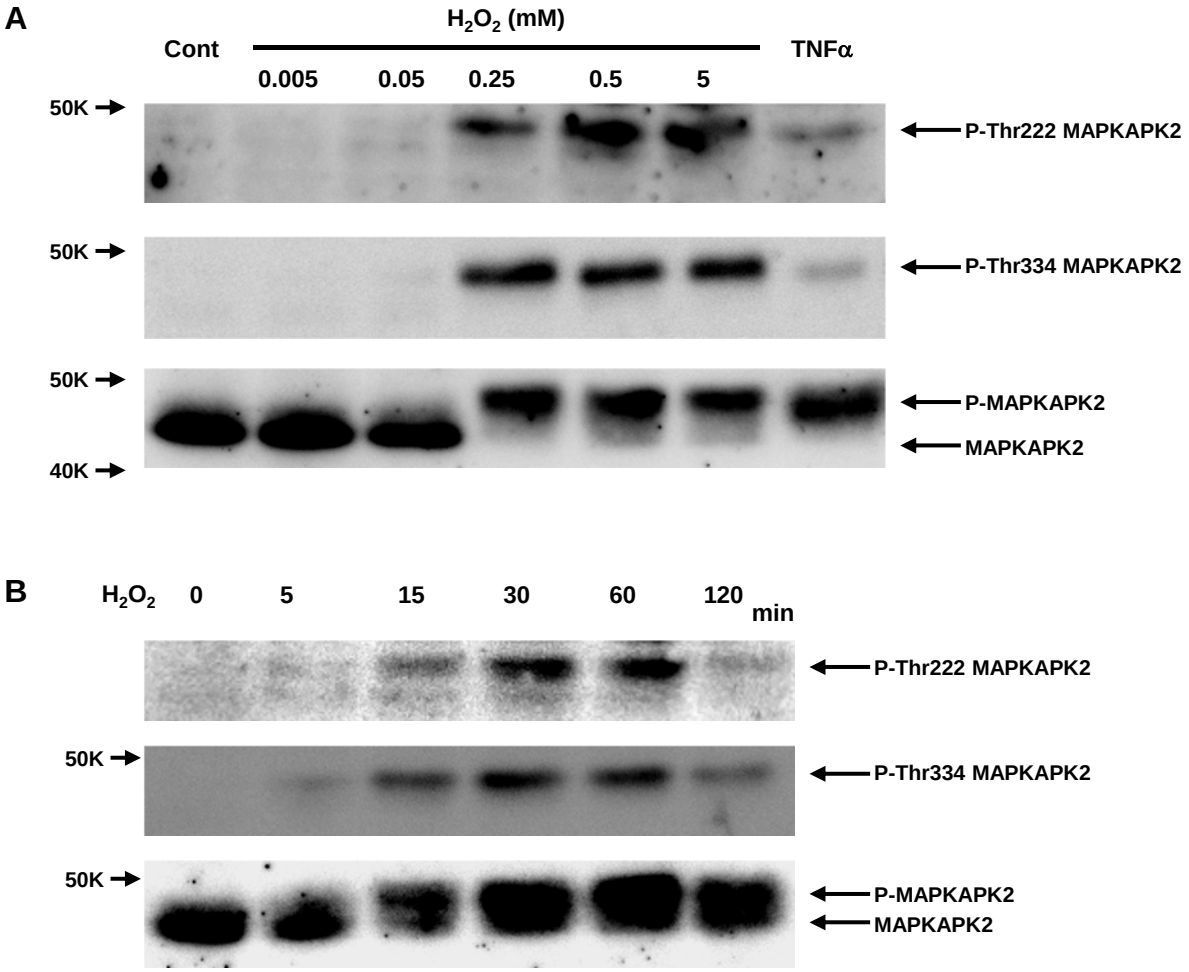
Fig. 7. Summary of flow cytometry analysis and detection of biotinylated epidermal growth factor receptor (EGFR), and epidermal growth factor (EGF)-induced activation of extracellular signal-regulated kinase (ERK), and after treatment of A549 cells with flagellin, tumor necrosis factor α (TNFα), or hydrogen peroxide. A, A549 cells were treated, fixed, and labeled with anti-EGFR-PerCP as described in Fig. 6. The mean fluorescence of the

control cells was taken as 100%, and from this value, other values were calculated. The values were expressed as mean ± standard error (SE) from four samples. We repeated the same experiments three times with reproducible results. Representative results are shown. B, Immunoblotting analysis of EGFR after cell surface biotinylation. After a 30-min treatment with 2 μg/mL flagellin, 100 ng/mL TNFα, or 2 mM hydrogen peroxide, A549 cells were treated with biotinylation reagent at 4°C for 30 min on a rocking platform. The flow-through (Non-biotinylated) and eluate (Biotinylated) from NeutrAvidin agarose gel, which were obtained from the same volume of cell lysate, were subjected to immunoblotting with anti-EGFR (1: 600), anti-E-cadherin (1: 750), or anti-p38 MAP kinase (1: 750) antibodies. The positions of EGFR, E-cadherin and p38 MAP kinase are indicated. C, A549 cells were pretreated with or without 2 μg/mL flagellin, 100 ng/mL TNFα, or 2 mM hydrogen peroxide for 60 min. Thereafter, the cells were treated with 10 ng/mL EGF for 30 min. The cell extracts (30 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and immunoblotting analysis was performed with an anti-active ERK antibody (1: 750). Phosphorylation of ERK without EGF treatment after 60-min pretreatment in each condition was taken as 100%, and from each value, activation levels are indicated by percentage. The mean ± standard error (SE) was determined from three independent experiments (*$P < 0.05$ *vs*, 60-min pretreatment without flagellin).

Fig. 8. Phosphorylation of HSP27 by hydrogen peroxide in A549 and MLE12 cells. A, A549 cells were treated with hydrogen peroxide at the indicated concentrations, or 100 ng/mL tumor necrosis factor α (TNFα) for 30 min. The cell extracts (33 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and immunoblotting analysis was performed with

an anti-phospho-HSP27 antibody (1: 750). After the anti-phospho-HSP27 antibody was stripped away, immunoblotting with an anti-HSP27 mouse monoclonal antibody was performed at a dilution of 1: 750. B, A549 cells were treated with 0.5 mM hydrogen peroxide for the periods indicated. Immunoblotting analysis with an anti-phospho-HSP27 antibody was performed using the cell extracts (61.5 μg) as described above. After the anti-phospho-HSP27 antibody was stripped away, immunoblotting with an anti-HSP27 antibody was performed at a dilution of 1: 750. C, MLE12 cells were treated without or with hydrogen peroxide at 0.05 or 2 mM for 30 min. The cell extracts (71 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and immunoblotting analysis was performed with anti-phospho-p38 mitogen-activated protein kinase (MAPK), anti-P-Thr222 MAPK-activated protein kinase-2 (MAPKAPK-2) (1: 750), anti-P-Thr334 MAPKAPK-2 (1: 750), or anti-phospho-HSP 27 (1: 750) antibodies. After the primary antibodies were stripped away, immunoblotting with anti-p38 MAP kinase (1: 750), anti-MAPKAPK-2 (1: 750), or anti-rodent HSP27 polyclonal (1: 750) antibodies, respectively, was performed. The bands for p38 MAP kinase (P-p38 MAP kinase and p38 MAP kinase), MAPKAPK-2 (P-Thr222 MAPKAPK2, P-Thr334 MAPKAPK2, and MAPKAPK2), and HSP27 (P-HSP27 and HSP27) are indicated.

Fig. 1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TNFα
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5
50K
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50K
P-Thr334 MAPKAPK2
50K
P-MAPKAPK2
MAPKAPK2
40K
B
H_2O_2
0
5
15
30
60
120
min
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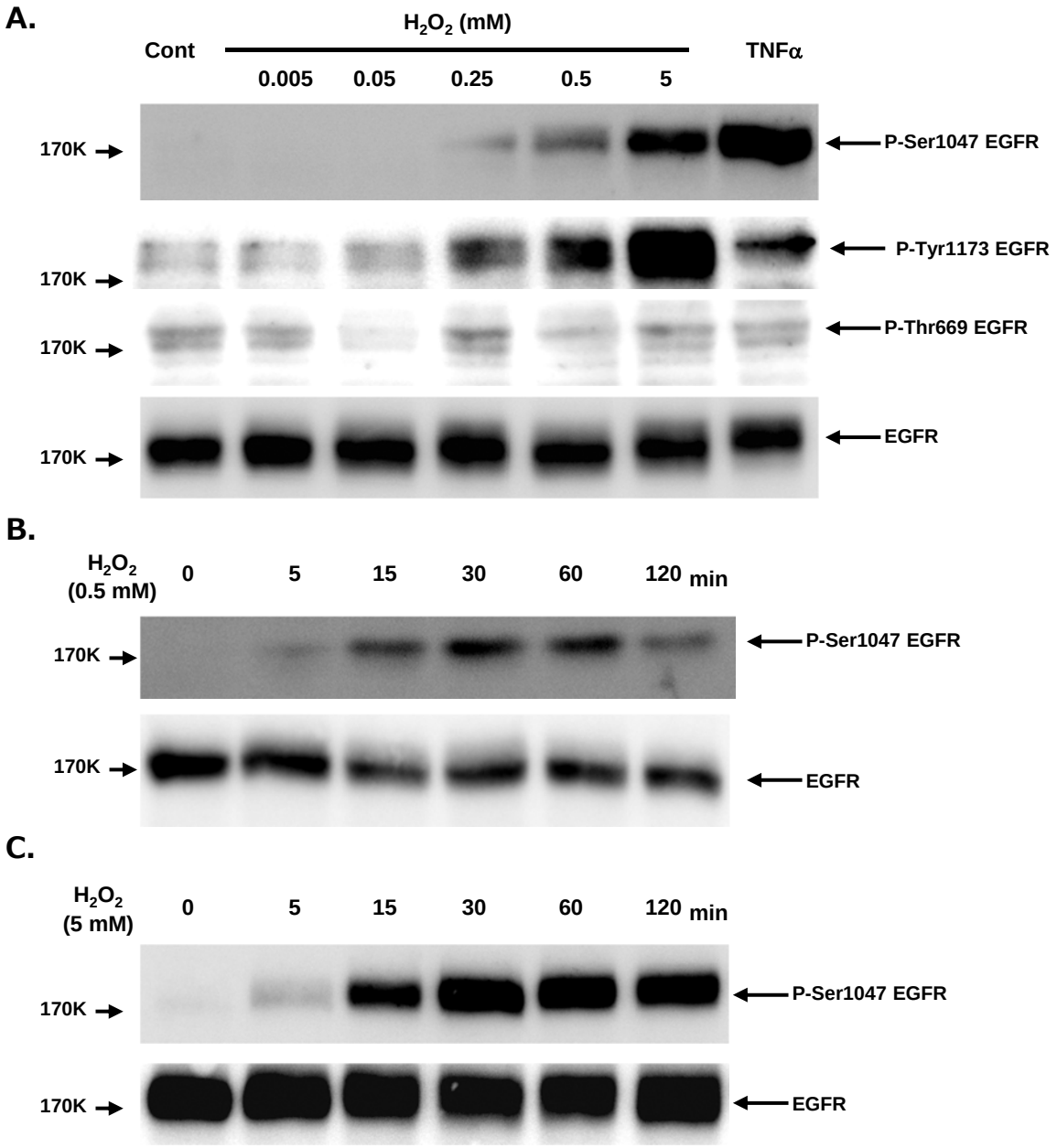
50K
P-MAPKAPK2
MAPKAPK2

Fig. 2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H2O2 (mM)
Cont
TNFα
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0.05
0.25
0.5
5
170K
P-Ser1047 EGFR
P-Tyr1173 EGFR
P-Thr669 EGFR
EGFR
B.
H2O2
(0.5 mM)
0
5
15
30
60
120 min
C.
H2O2
(5 mM)

Fig. 3

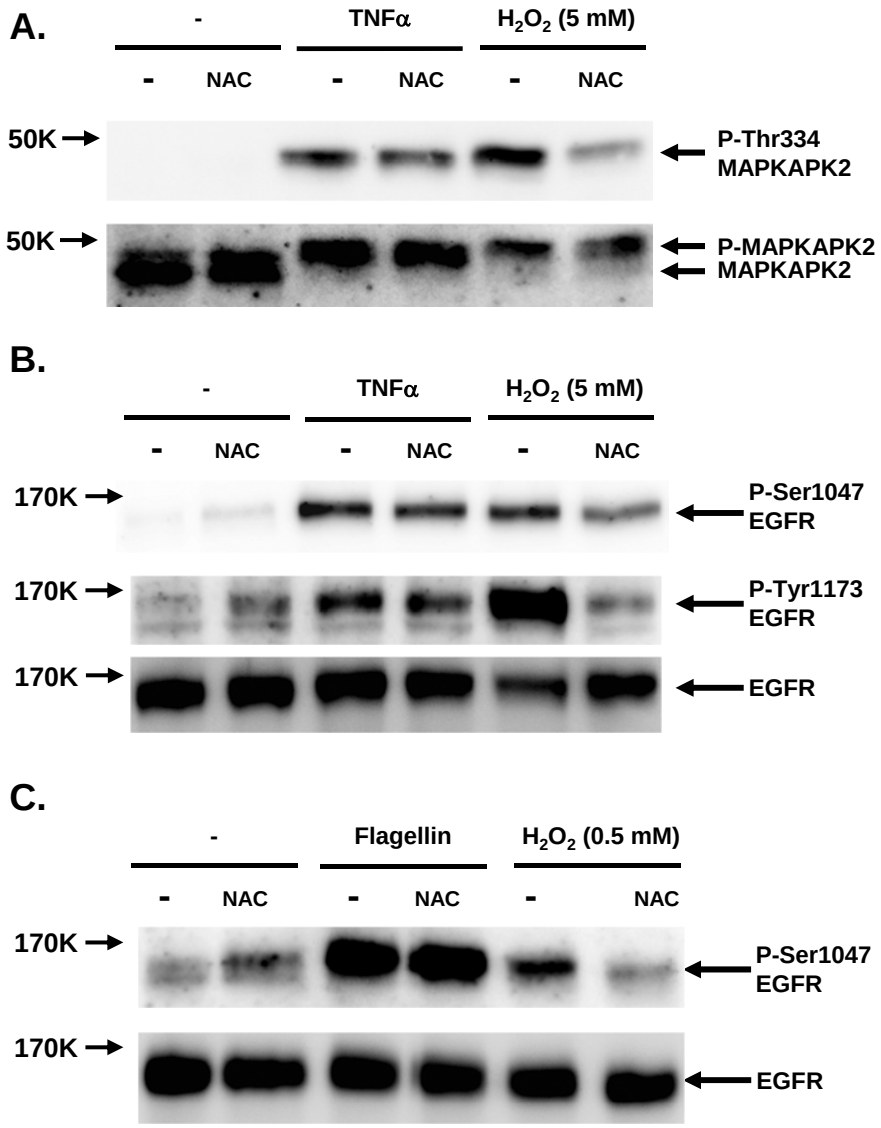
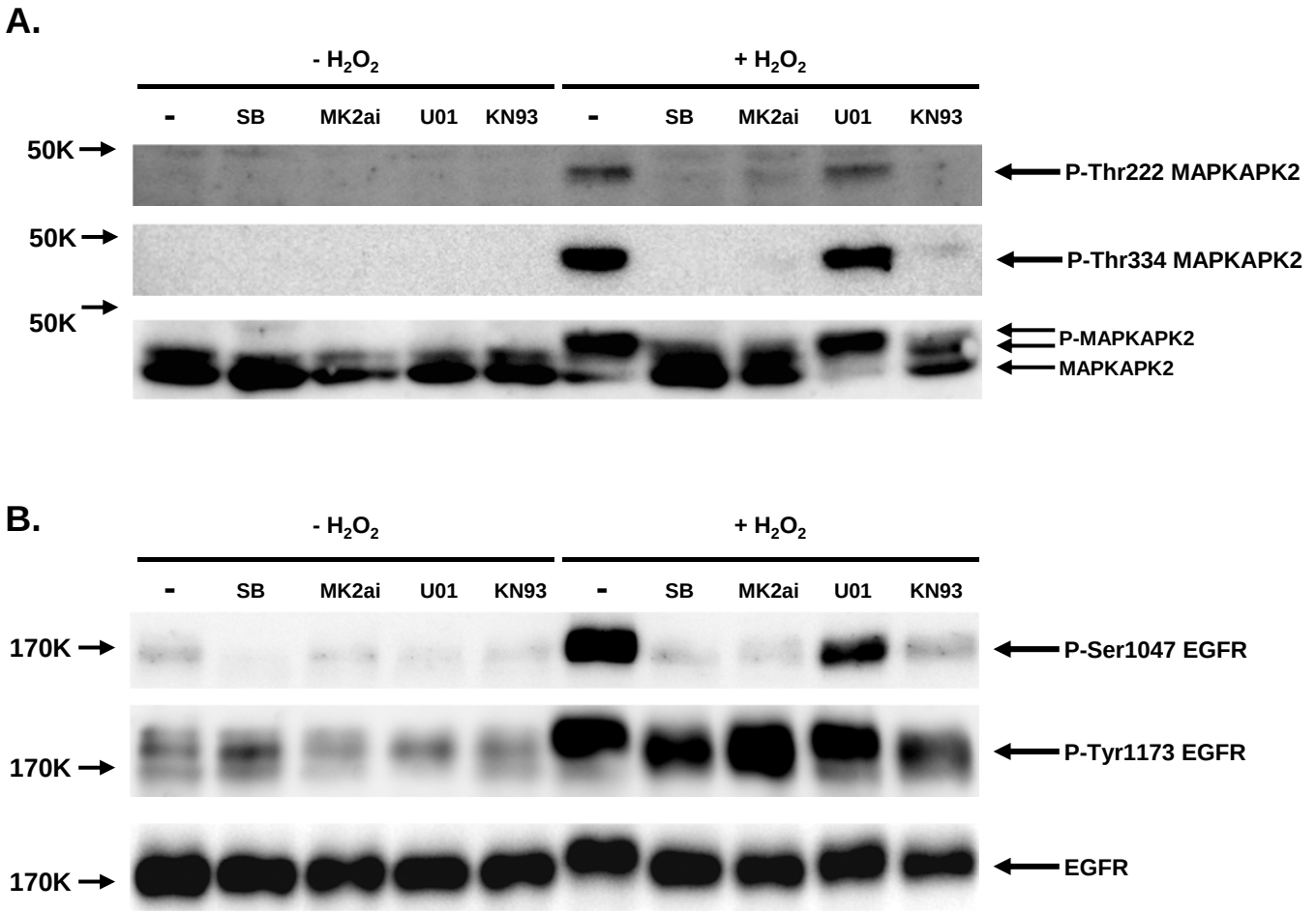

A.
-
TNFα
H2O2 (5 mM)
- NAC - NAC - NAC
50K
P-Thr334 MAPKAPK2
50K
P-MAPKAPK2
MAPKAPK2
B.
-
TNFα
H2O2 (5 mM)
- NAC - NAC - NAC
170K
P-Ser1047 EGFR
170K
P-Tyr1173 EGFR
170K
EGFR
C.
-
Flagellin
H2O2 (0.5 mM)
- NAC - NAC - NAC
170K
P-Ser1047 EGFR
170K
EGFR

Fig. 4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+ H_2O_2
-
SB
MK2ai
U01
KN93
-
SB
MK2ai
U01
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50K
50K
50K
P-Thr222 MAPKAPK2
P-Thr334 MAPKAPK2
P-MAPKAPK2
MAPKAPK2
B.
- H_2O_2
+ H_2O_2
-
SB
MK2ai
U01
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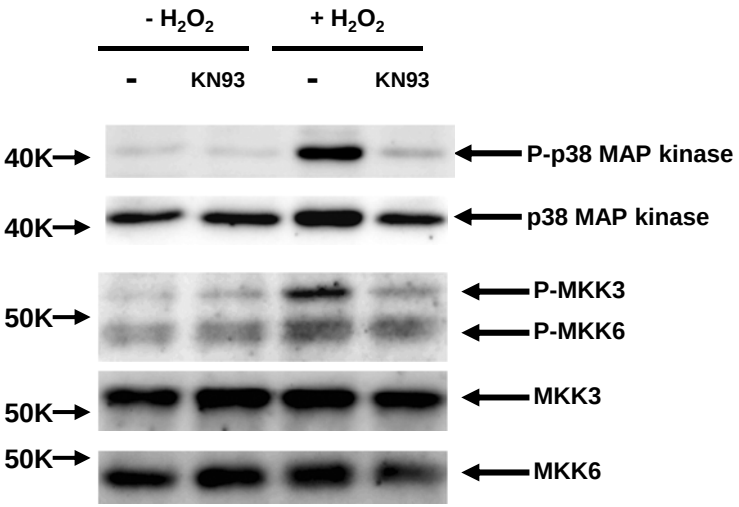
P-Ser1047 EGFR
P-Tyr1173 EGFR
EGFR

Fig. 5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+ H_2O_2
-
KN93
-
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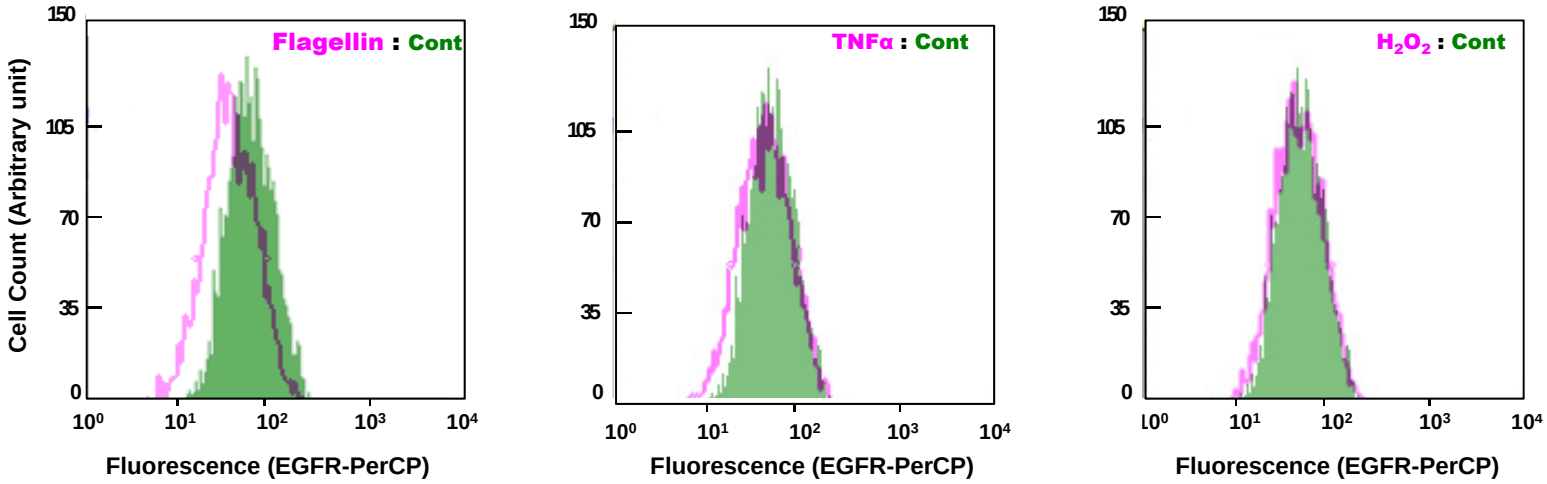
p38 MAP kinase
P-MKK3
P-MKK6
MKK3
MKK6

Fig. 6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TNFα : Cont
H_2O_2 : Cont
Cell Count (Arbitrary unit)
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105
70
35
0
10^0
10^1
10^2
10^3
10^4
Fluorescence (EGFR-PerCP)

Fig. 7

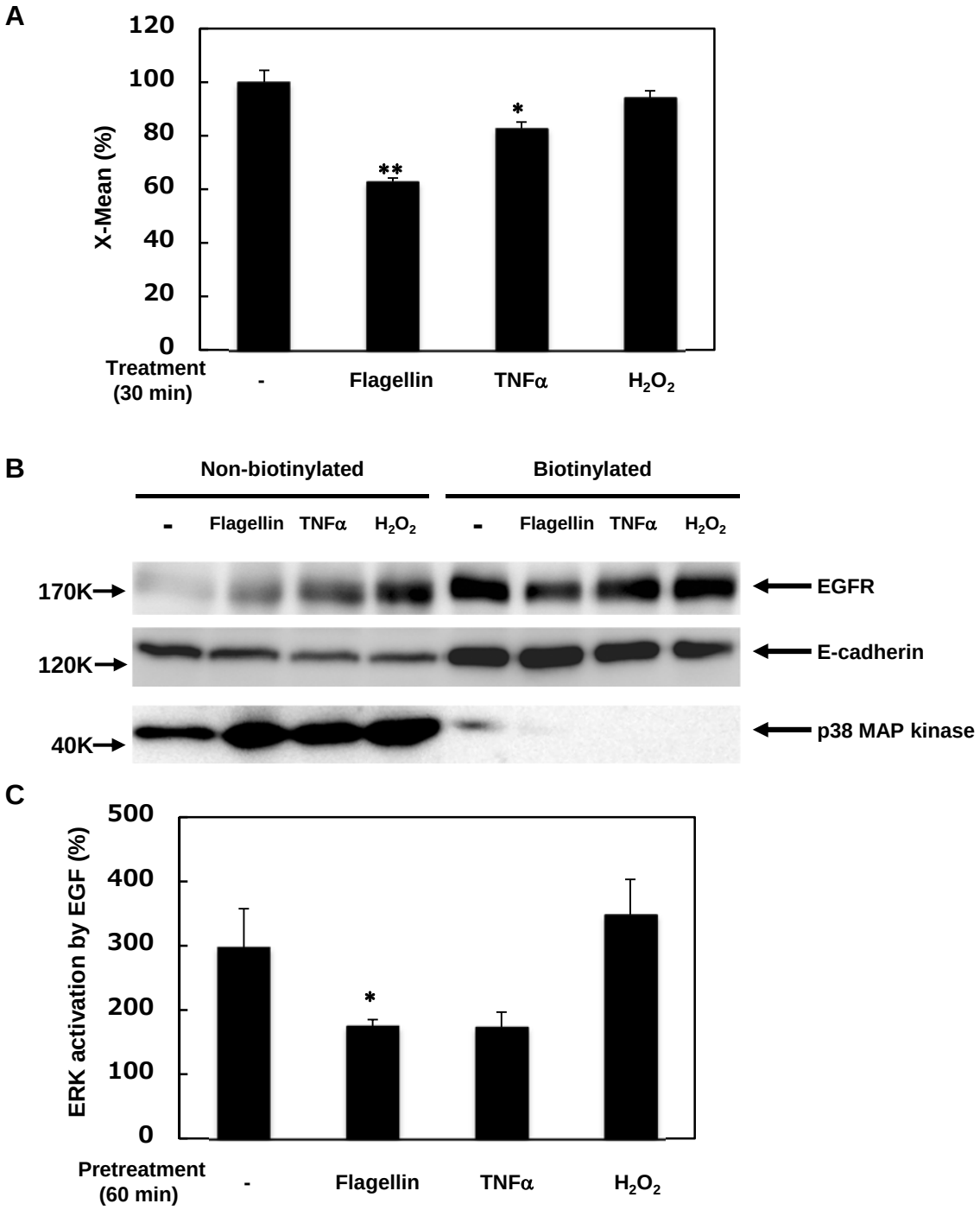
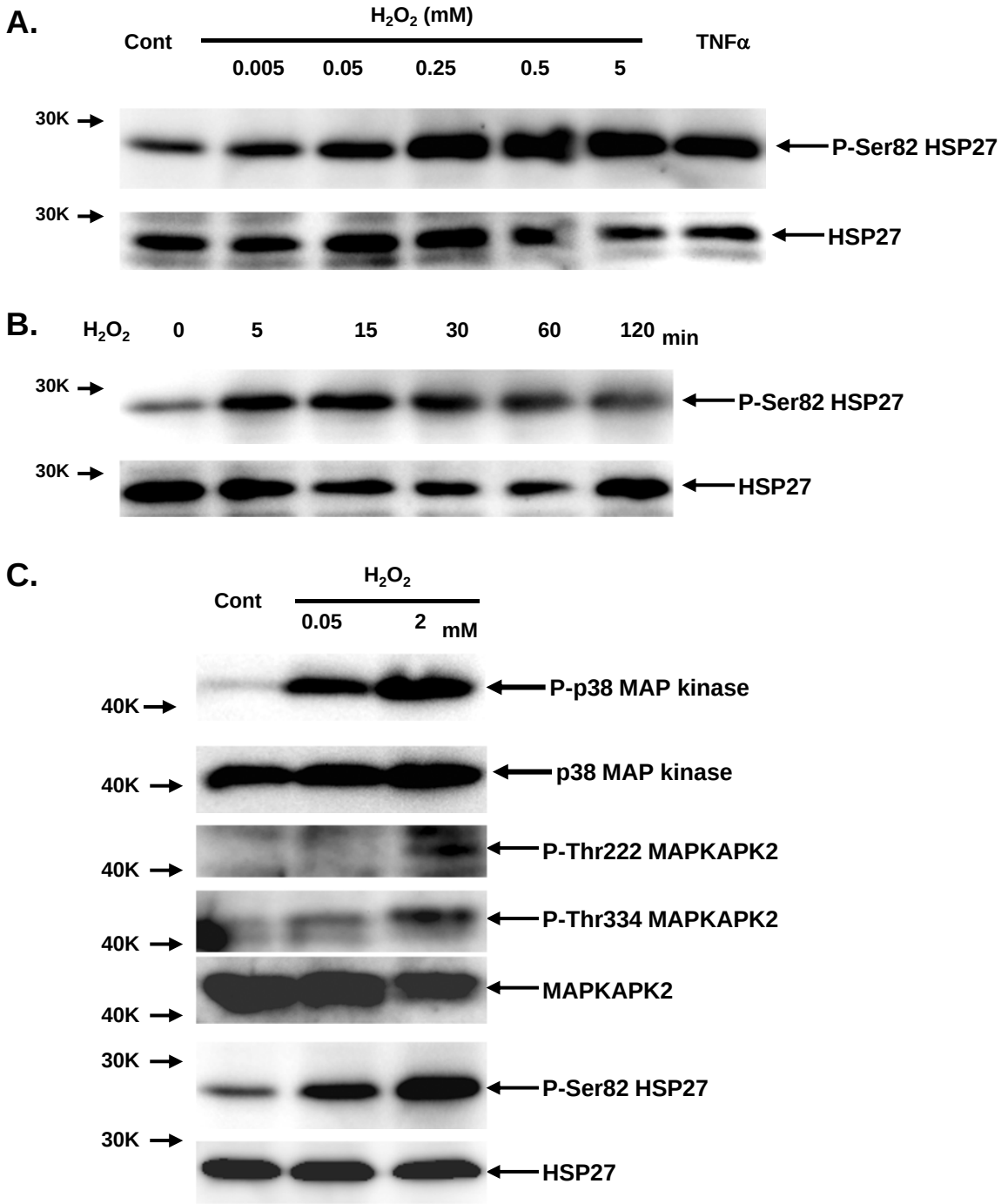

A
120
100
80
60
40
20
0
X-Mean (%)
**
*
Treatment (30 min)
-
Flagellin
TNFα
H2O2
B
Non-biotinylated
Biotinylated
-
Flagellin
TNFα
H2O2
-
Flagellin
TNFα
H2O2
170K
120K
40K
EGFR
E-cadherin
p38 MAP kinase
C
500
400
300
200
100
0
ERK activation by EGF (%)
*
Pretreatment (60 min)
-
Flagellin
TNFα
H2O2

Fig. 8


A.
Cont
H_2O_2 (mM)
0.005
0.05
0.25
0.5
5
TNFα
30K
P-Ser82 HSP27
30K
HSP27
B.
H_2O_2
0
5
15
30
60
120
min
30K
P-Ser82 HSP27
30K
HSP27
C.
Cont
H_2O_2
0.05
2
mM
40K
P-p38 MAP kinase
40K
p38 MAP kinase
40K
P-Thr222 MAPKAPK2
40K
P-Thr334 MAPKAPK2
40K
MAPKAPK2
30K
P-Ser82 HSP27
30K
HSP27