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Role of peptide transporter 2 and MAPK signaling pathways in the innate immune response induced by bacterial peptides in alveolar epithelial cells

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ABSTRACT

Aims: The innate immune response induced by bacterial peptidoglycan peptides, such as γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), is an important host defense system. However, little is known about the innate immune response in the lung alveolar region. In this study, we examined induction of the innate immune response by iE-DAP in human alveolar epithelial cell lines, NCI-H441 (H441) and A549.

Main methods: Induction of the innate immune response was evaluated by measuring the mRNA expression of cytokines and their release into the culture medium.

Key findings: iE-DAP treatment increased the mRNA expression of interleukin (IL)-6 and IL-8, and increased release of these pro-inflammatory cytokines into the culture medium in H441 cells, but not in A549 cells. Lack of release of these cytokines in A549 cells may have been due to lack of peptide transporter 2 (PEPT2) function. Intracellular nucleotide-binding oligomerization domain 1 (NOD1) recognizes iE-DAP and activates downstream signaling pathways to initiate the immune response. Therefore, the role of mitogen-activated protein kinase (MAPK) signaling pathways was examined in H441 cells. As a result of inhibition studies, receptor-interacting serine/threonine-protein kinase 2 and MAPK signaling pathways, such as p38 MAPK and extracellular signal-regulated kinase, but not c-Jun N-terminal kinase, were determined to be involved in the innate immune response in H441 cells. In addition, the nuclear factor κB pathway also played a role in the innate immune response.

Significance: These findings indicated that the innate immune response induced by bacterial peptides could occur in a PEPT2- and NOD1-dependent manner in alveolar epithelial cells.

1. Introduction

The lung is continuously exposed to the external environment, resulting in exposure to various pathogenic bacteria. Therefore, there are various mechanisms for protection from infection by pathogenic bacteria, such as mucociliary transport in the upper and lower airways and phagocytosis by alveolar macrophages [1]. The alveolar region of the lung is composed of type I and type II epithelial cells. Alveolar epithelial cells serve as the physical barrier against pathogen invasion and produce various cytokines, chemokines, and anti-microbial molecules, such as surfactant proteins. Recently, the importance of the innate immune response induced by recognition of pathogen-associated molecular patterns (PAMPs) presented by bacteria has been recognized in the lung [2,3]. Because respiratory infection and pneumonia are serious life-threatening diseases, it is important to elucidate detailed mechanisms of the innate immune response in alveolar epithelial cells.

The toll-like receptor family and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family have been shown to recognize PAMPs. NOD1 is a member of the NLR family of receptors, and is localized to the cytoplasm of cells. NOD1 recognizes dipeptide motif γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) which is a major component of the peptidoglycan cell wall of most gram-negative and some gram-positive bacteria [4,5]. Following ligand recognition, NOD1 binds to receptor-interacting-serine/threonine-protein kinase 2 (RIP2), which activates the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, resulting in production of pro-inflammatory cytokines to initiate the innate immune response [6]. The pathways involved in these responses vary between cell types. NOD1 is expressed in various organs such as the kidney, intestine, and liver [7]. In the lung, NOD1 is expressed in alveolar epithelial cells, alveolar macrophages, and bronchial epithelial cells [8,9]. NOD1 is involved in recognition and protection against pathogenic bacteria,

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such as *Legionella pneumophila* and *Pseudomonas aeruginosa* [10,11], and therefore has an important role in pulmonary defense against infection. However, the mechanisms underlying NOD1-dependent induction of the innate immune response in alveolar epithelial cells have not been characterized.

Influx of NOD1 ligands into cells is required for recognition of these ligands by intracellular NOD1. iE-DAP is a hydrophilic molecule and cannot permeate the cell membrane by passive diffusion. Therefore, iE-DAP influx requires facilitation by a transporter. Peptide transporter 2 (PEPT2), a proton-coupled oligopeptide transporter, has been suggested to mediate transport of NOD1 ligands such as iE-DAP and L-Ala-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP) into mouse spleen macrophages and airway epithelial cells [12,13]. However, the transporter for NOD1 ligands in alveolar epithelial cells has not been identified.

Previously, we reported that PEPT2 is functionally expressed in rat primary cultured alveolar type II epithelial cells [14] and in human-derived NCI-H441 (H441) cells, which display a phenotype similar to alveolar type II epithelial cells [15], suggesting that H441 cells may represent a useful *in vitro* model to study PEPT2-related signaling in type II cells. In the present study, we examined whether the innate immune response was induced by iE-DAP and Tri-DAP in H441 cells. Then, the roles of PEPT2 and associated signaling pathways for NOD1-dependent induction of the innate immune response were examined.

2. Materials and methods

2.1. Materials

RPMI-1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), MP Biomedicals (Solon, OH, USA), and GE healthcare Japan (Tokyo, Japan), respectively. Insulin-transferrin-sodium selenite (ITS) supplement and High Pure RNA Isolation Kit were purchased from Roche (Basel, Switzerland). D-Ala-(L)-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (D-Ala-Lys-AMCA), a fluorophore-conjugated dipeptide, was purchased from Biotrend Chemikalien GmbH (Köln, Deutschland). iE-DAP and Tri-DAP were purchased from InvivoGen (San Diego, CA, USA), and glycylsarcosine (Gly-Sar) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Gefitinib and U0126 were purchased from Selleck Chemicals (Osaka, Japan) and LC laboratory (Woburn, MA, USA), respectively. SB203580 and SC-514 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and SP600125 was purchased from Wako Pure Chemical, Inc. (Osaka, Japan). All other chemicals were of the highest purity commercially available.

2.2. Cell culture

H441 cells were cultured in RPMI-1640 medium containing 5% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1% sodium pyruvate. For experiments, the culture medium was replaced with RPMI-1640 medium containing dexamethasone (200 nM) and ITS supplement on day 1, and the cells were cultured until day 13. A549 cells were cultured in DMEM containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin until day 7. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Treatment of H441 and A549 cells with iE-DAP

H441 cells were seeded in 12 well plates. On day 13, H441 cells were preincubated with serum-free medium for 4 h, then treated with iE-DAP (10 μg/mL) for 24 h. To examine the interaction of iE-DAP with PEPT2, H441 cells were treated with iE-DAP with or without 5 mM Gly-Sar, a typical substrate of PEPT2, for 24 h. To examine the role of various signaling pathways, cells were treated with 10 μM gefitinib, a RIP2 inhibitor; 1 μM SB203580, a p38 MAPK inhibitor; 0.5 μM U0126, a MAP kinase kinase (MEK1/2) inhibitor, and therefore an inhibitor of its downstream effector extracellular signal-regulated kinase (ERK); 1 μM SP600125, a c-Jun N-terminal kinase (JNK) inhibitor; and 20 μM SC-514, an NF-κB pathway inhibitor. H441 cells were pretreated with serum-free medium for 4 h in the absence (control, iE-DAP) or presence of each inhibitor, then treated with iE-DAP with or without each inhibitor for 24 h. In some experiments, Tri-DAP was used instead of iE-DAP.

A549 cells were seeded in 12 well plates and were cultured until day 7. A549 cells were pretreated with serum-free medium for 4 h, then treated with iE-DAP (10 μg/mL) for 24 h on day 7.

2.4. Conventional RT-PCR and real-time PCR analyses

Total RNA was extracted from cells using High Pure RNA Isolation Kit. Reverse transcription of RNA was performed using ReverTra Ace (TOYOBO, Osaka, Japan) to produce cDNA. Polymerase chain reaction (PCR) products were separated by electrophoresis in 2.0% agarose gels, and bands were detected using a Typhoon FLA-7000 Imaging system (GE healthcare Japan, Tokyo, Japan). mRNA expression was analyzed using a CFX Connect™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), as described previously [16].

The primer sequences were as follows: human PEPT2 sense, 5′-AGGAAAATGGCTGTTGGTATGATC-3′ and antisense, 5′-CGCAACTG CAAATGCCAG-3′; human NOD1 sense, 5′-CTCGCAGATGCCTACGTG GAC-3′ and antisense, 5′-GGGCATAGCACAGCACGAAC-3′; human IL-6 sense, 5′-ATGAGGAGACTTGCCTGGTG-3′ and antisense, 5′-CAGGGGT GGTTATTGCATCT-3′; human IL-8 sense, 5′-AAGAAACCACCGGAAGG AAC-3′ and antisense, 5′-ATTTGGGGTGGAAAGGTTTG-3′; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-ACGGGA AGCTTGTCATCAAT-3′ and antisense, 5′-TGGACTCCACGACGTAC TCA-3′. The expression of each mRNA was normalized to that of GAPDH mRNA, a housekeeping gene.

2.5. Uptake of D-Ala-Lys-AMCA

H441 cells were preincubated with HEPES buffer (5 mM HEPES, 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) for 10 min at 37 °C. Cells were then incubated with D-Ala-Lys-AMCA (25 μM) in MES buffer (5 mM MES, 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM D-glucose, pH 6.5) for 60 min in the absence or presence of iE-DAP or Gly-Sar (10–1000 μM). After the cells were washed with ice-cold HEPES buffer, they were solubilized with 0.1% Triton X-100 in phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) for 30 min. Fluorescence of D-Ala-Lys-AMCA in cell lysates was measured using a Hitachi fluorescence spectrophotometer F-2700 (Tokyo, Japan) at Ex 350 nm/Em 455 nm. PEPT2-mediated uptake was calculated by subtracting D-Ala-Lys-AMCA uptake in the presence of cefadroxil (1 mM) from that in the absence of cefadroxil. Cellular protein content was determined by the Lowry method using bovine serum albumin as a standard.

2.6. Western blot analysis of phosphorylated ERK (p-ERK) in the nuclear fraction of H441 cells

H441 cells were treated with or without iE-DAP (10 μg/mL) for 24 h, and nuclear protein extracts were prepared according to the method reported by Milosevic et al. [17]. The protein levels of p-ERK1/2 and lamin A/C (a nuclear marker) in the nuclear fraction were measured by western blotting as described previously [14]. The primary antibodies used for the detection of p-ERK1/2 and lamin A/C were anti-phosphorylated ERK1/2 rabbit monoclonal antibody (#14474) (Cell Signaling Technology, Danvers, MA, USA) and anti-lamin A/C rabbit polyclonal antibody (GTX101127) (Gene Tex, Irvine, CA, USA), respectively.

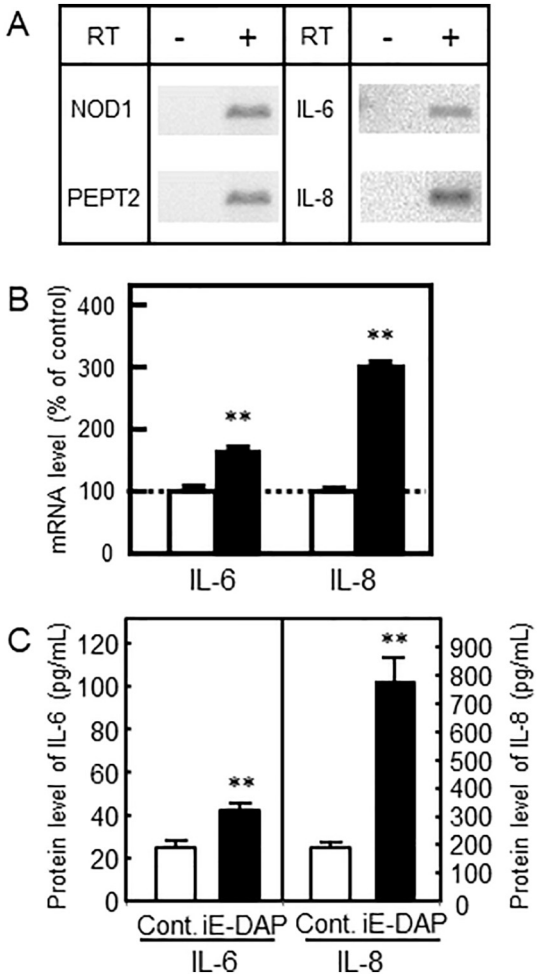

Fig. 1. Effect of iE-DAP treatment on the mRNA expression and secretion of pro-inflammatory cytokines.
(A) The mRNA expression of NOD1, PEPT2, IL-6, and IL-8 in H441 cells was determined using conventional RT-PCR. PCR products with (+) or without (−) reverse-transcription (RT) were separated by electrophoresis. (B, C) H441 cells were treated with (black columns) or without (control, white columns) iE-DAP (10 μg/mL) for 24 h. (B) The mRNA expression levels of IL-6 and IL-8 were analyzed by real-time PCR, which was normalized to GAPDH mRNA expression, and (C) secreted protein concentrations of IL-6 and IL-8 in the culture medium were measured using an enzyme-linked immunosorbent assay. Dashed line indicates control levels (iE-DAP (−)). $^{**}p < 0.01$; significantly different from each control (n = 3).

2.7. Statistical analysis

Data are expressed as mean ± standard error of the mean. To evaluate statistical significance, Student's *t*-test or one-way ANOVA followed by Tukey's test for multiple comparisons were performed. The level of significance was set at *or † $p < 0.05$ and ** or †† $p < 0.01$.

3. Results

3.1. The mRNA expression of PEPT2, NOD1, IL-6, and IL-8 in H441 cells

First, we examined the expression of mRNAs that are related to the induction of the innate immune response by iE-DAP. The expression of PEPT2 mRNA was confirmed in H441 cells, as reported previously [15]. The expression of NOD1 mRNA, an intracellular pattern recognition receptor which recognizes iE-DAP [4,5], and of IL-6 and IL-8 was detected in H441 cells (Fig. 1A).

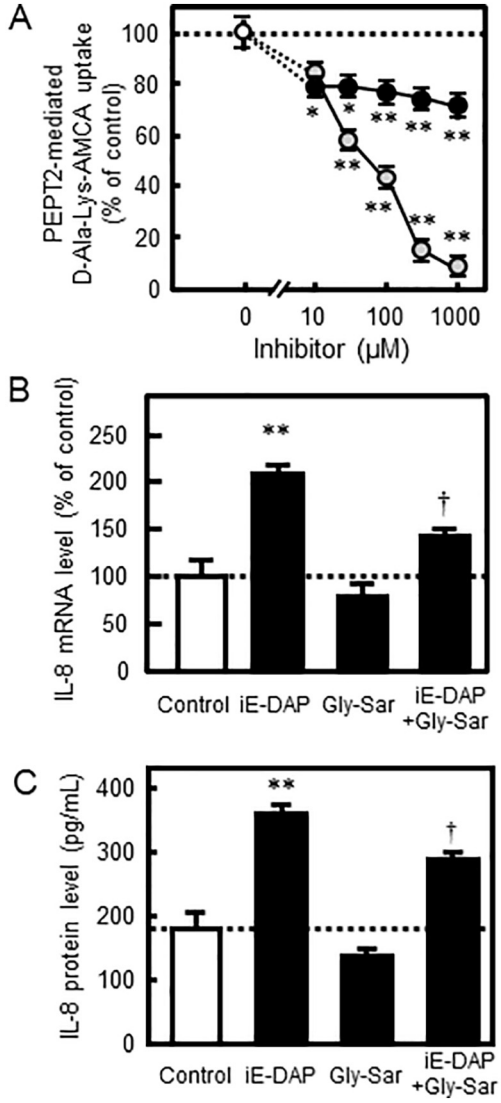

Fig. 2. Role of PEPT2 in the transport of iE-DAP in H441 cells.
(A) Effect of iE-DAP and Gly-Sar on PEPT2-mediated D-Ala-Lys-AMCA uptake in H441 cells. Cells were incubated with D-Ala-Lys-AMCA (25 μM) in the absence (open circle) or presence of various concentrations of iE-DAP (black circles) and Gly-Sar (gray circles). The control value of D-Ala-Lys-AMCA uptake was 180.6 ± 15.7 pmol/mg protein (n = 6). (B, C) Effect of Gly-Sar on (B) the mRNA expression level of IL-8 and (C) the secreted protein concentration of IL-8 in the culture medium. H441 cells were treated with 10 μg/mL iE-DAP with or without 5 mM Gly-Sar for 24 h. Dashed lines indicate control levels (iE-DAP (−) and Gly-Sar (−)). $^{*}p < 0.05$, $^{**}p < 0.01$; significantly different from each control, †$p < 0.05$; significantly different from each value observed in iE-DAP-treated cells (n = 3).

3.2. Effect of iE-DAP treatment on the mRNA expression of IL-6 and IL-8, and on the release of these cytokines into the culture medium of H441 cells

H441 cells were treated with iE-DAP for 24 h, and the mRNA expression of IL-6 and IL-8 was measured by real-time PCR. As shown in Fig. 1B, the expression of these mRNAs was significantly increased by iE-DAP treatment. In accordance with the change in mRNA expression, release of these cytokines into the culture medium was also increased by iE-DAP treatment (Fig. 1C), suggesting that iE-DAP induced the innate immune response in H441 cells. In both mRNA expression and protein release, IL-8 was more sensitively affected than IL-6 by iE-DAP treatment. In addition, the protein amount of IL-8 released into the culture medium was much higher than that of IL-6. Therefore, in the

following experiments, we measured IL-8 as a marker of iE-DAP-induced innate immune response.

3.3. Role of PEPT2 in the transport of iE-DAP and induction of innate immune response in H441 cells

D-Ala-Lys-AMCA was shown to be a substrate for PEPT2 in lung epithelial cells and renal LLC-PK1 cells [18,19]. Fig. 2A shows the effects of iE-DAP and Gly-Sar on PEPT2-mediated uptake of D-Ala-Lys-AMCA by H441 cells. Gly-Sar inhibited D-Ala-Lys-AMCA uptake in a concentration-dependent manner. Similarly, iE-DAP significantly inhibited D-Ala-Lys-AMCA uptake by H441 cells, though the inhibitory potency was weaker than that of Gly-Sar. When the cells were treated with iE-DAP in the presence of Gly-Sar, increased IL-8 mRNA expression was suppressed compared with that in the absence of Gly-Sar (Fig. 2B). Increased release of IL-8 into the culture medium by iE-DAP was also suppressed by the presence of Gly-Sar (Fig. 2C).

3.4. Effect of Tri-DAP treatment on IL-8 mRNA expression and role of PEPT2 in the induction of innate immune response in H441 cells

We also examined the effect of Tri-DAP, another peptidoglycan peptide, on IL-8 mRNA expression in H441 cells. The expression of IL-8 mRNA was significantly increased by Tri-DAP treatment in its concentration-dependent manner (Fig. 3A). In addition, increased IL-8 mRNA expression by Tri-DAP was significantly suppressed by co-treatment of the cells with Gly-Sar (Fig. 3B).

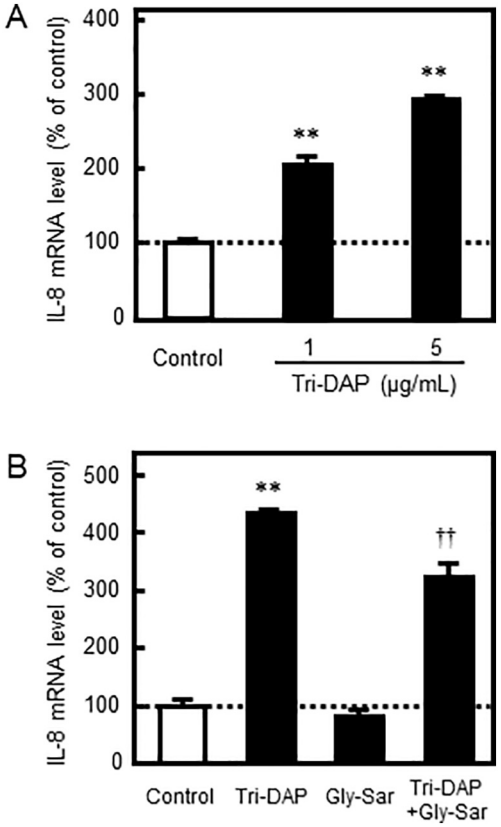

Fig. 3. Effect of Tri-DAP treatment on IL-8 mRNA expression (A) and role of PEPT2 in the induction of innate immune response (B) in H441 cells.
(A) H441 cells were treated with (black columns) or without (control, white column) Tri-DAP for 24 h. The mRNA expression level of IL-8 was analyzed by real-time PCR, which was normalized to GAPDH mRNA expression. (B) Effect of Gly-Sar on the mRNA expression level of IL-8. H441 cells were treated with 1 μg/mL Tri-DAP with or without 5 mM Gly-Sar for 24 h. Dashed lines indicate control level (Tri-DAP (−) and Gly-Sar (−)). $^{**}p < 0.01$; significantly different from each control, $\dagger\dagger p < 0.01$; significantly different from the value observed in Tri-DAP-treated cells (n = 3).

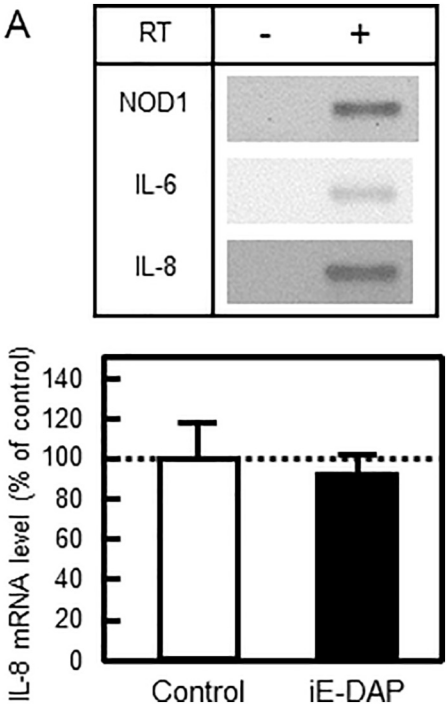

Fig. 4. The mRNA expression of NOD1, IL-6, and IL-8 (A), and the effect of iE-DAP on the mRNA expression level of IL-8 (B) in A549 cells.
(A) The mRNA expression of NOD1, IL-6, and IL-8 in A549 cells was analyzed by conventional RT-PCR. (B) A549 cells were treated with iE-DAP (10 μg/mL) for 24 h. Real-time PCR was performed to analyze the mRNA expression level of IL-8, which was normalized to GAPDH mRNA expression. The dashed line indicates the control level (iE-DAP (−)) (n = 3).

3.5. Effect of iE-DAP treatment on IL-8 mRNA expression in A549 cells

We previously reported that PEPT2 is functionally expressed in H441 cells, but not in A549 cells [15]. To further clarify the role of PEPT2, induction of the innate immune response by iE-DAP was examined in A549 cells. NOD1, IL-6, and IL-8 mRNAs were also detected in A549 cells (Fig. 4A). However, in contrast to H441 cells, treatment of A549 cells with iE-DAP did not increase the mRNA expression of IL-8 (Fig. 4B). These results suggested the important role of PEPT2 in uptake of iE-DAP into cells and subsequent induction of the innate immune response.

3.6. Role of intracellular signaling pathways in the innate immune response induced by iE-DAP and Tri-DAP in H441 cells

To characterize induction of the innate immune response by iE-DAP, the effects of various inhibitors on signaling pathways associated with IL-8 mRNA expression were examined. Gefitinib, an inhibitor of epidermal growth factor receptor tyrosine kinase, was shown to inhibit RIP2 tyrosine phosphorylation [20]. In H441 cells, gefitinib significantly inhibited iE-DAP-induced increases in IL-8 mRNA expression (Fig. 5A). Increased expression of IL-8 mRNA induced by Tri-DAP was also inhibited by gefitinib (Fig. 5B), suggesting involvement of NOD1/RIP2 and downstream signaling pathways in the innate immune response induced by these peptidoglycan peptides. We then examined the effects of MAPKK/MAPK inhibitors on iE-DAP-induced increases in IL-8 mRNA expression. SB203580, U0126, and SP600125 were used as inhibitors of the p38 MAPK, ERK (MEK1/2), and JNK signaling pathways, respectively. As shown in Fig. 5C and D, SB203580 and U0126 inhibited

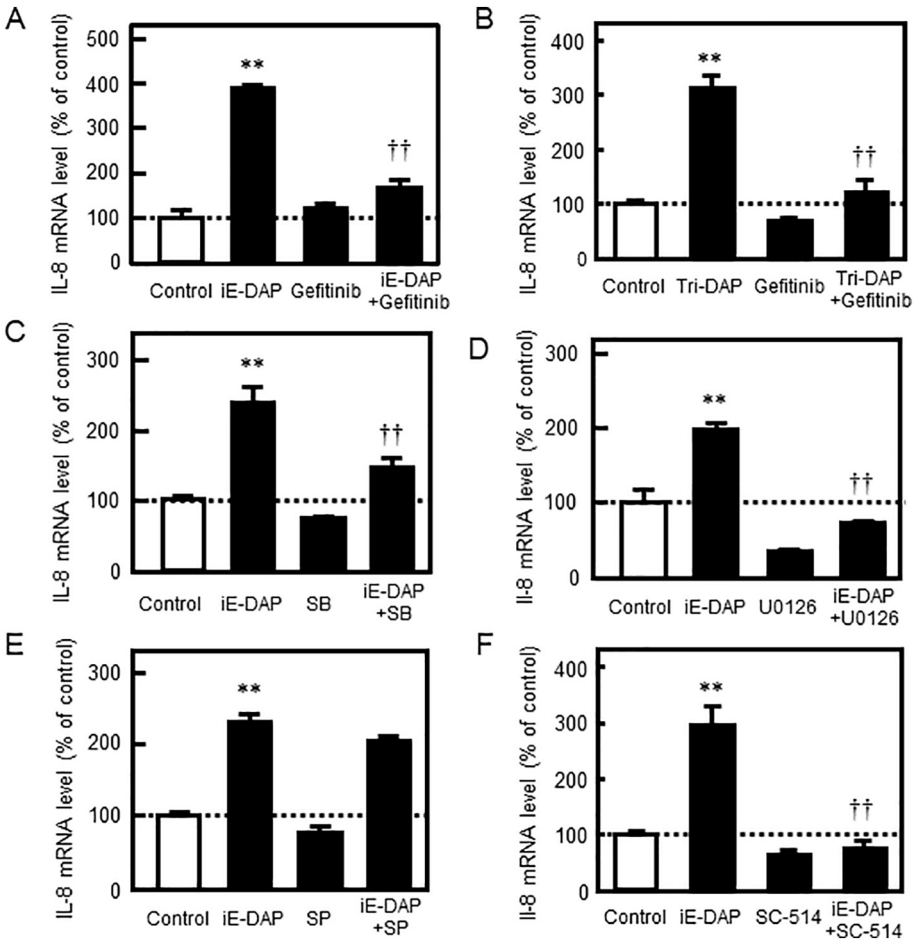

Fig. 5. Role of intracellular signaling pathways in the innate immune response induced by iE-DAP (A, C, D, E, F) and Tri-DAP (B) in H441 cells.
H441 cells were pretreated with serum-free medium for 4 h in the absence (control, iE-DAP, Tri-DAP) or presence of (A, B) the RIP2 inhibitor, gefitinib (10 μM); (C) the p38 MAPK inhibitor, SB203580 (SB, 1 μM); (D) the ERK (MEK1/2) inhibitor, U0126 (0.5 μM); (E) the JNK inhibitor, SP600125 (SP, 1 μM); and (F) the NF-κB inhibitor, SC-514 (20 μM). The cells were then treated with iE-DAP (10 μg/mL) or Tri-DAP (5 μg/mL) with or without each inhibitor for 24 h. Real-time PCR was performed to analyze the mRNA expression level of IL-8, which was normalized to GAPDH mRNA expression. Dashed lines indicate control levels (iE-DAP (−), Tri-DAP (−), inhibitors (−)). $^{**}p < 0.01$; significantly different from each control, $\dagger\dagger p < 0.01$; significantly different from each value observed in iE-DAP- or Tri-DAP-treated cells (n = 3).

iE-DAP-induced increases in IL-8 mRNA expression. In contrast, SP600125 did not inhibit iE-DAP-induced increases in IL-8 mRNA expression (Fig. 5E). SC-514, an inhibitor of the NF-κB pathway, also inhibited iE-DAP-induced increases in IL-8 mRNA expression (Fig. 5F).

In order to confirm the involvement of ERK pathway further, the effect of iE-DAP treatment on the protein level of p-ERK, a well-known key downstream factor in the MEK/ERK signaling pathway, in the nuclear fraction of H441 cells was measured by western blotting. As shown in Fig. 6, p-ERK1/2 level was markedly increased by iE-DAP treatment, and significant difference ($p < 0.01$) was observed between control and iE-DAP-treated cells by densitometric analysis (4,480.1 ± 747.8 *vs.* 12,375.0 ± 211.1 (arbitrary unit)). In contrast, no significant difference was observed in the protein level of lamin A/C (a nuclear marker/loading control) between these cells.

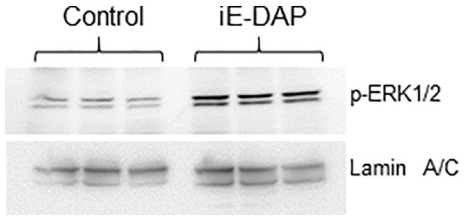

Fig. 6. Effect of iE-DAP treatment on the protein levels of p-ERK1/2 and lamin A/C in the nuclear fraction of H441 cells.
H441 cells were treated without (Control) or with iE-DAP (10 μg/mL) for 24 h. The protein levels of (A) p-ERK1/2 and (B) lamin A/C in the nuclear fraction were measured by western blotting.

4. Discussion

Recently, the important role of the innate immune system, especially the NOD1-dependent immune response, in protection against pulmonary infection has gained increasing attention. However, the NOD1-dependent innate immune response in alveolar epithelial cells has not been characterized. In this study, treatment of H441 cells, an alveolar type II epithelial cell model, with iE-DAP induced a NOD1-dependent immune response, as evidenced by increased production of pro-inflammatory cytokines such as IL-6 and IL-8. There are several pro-inflammatory cytokines including IL-6, IL-8, tumor necrosis factor-α, and interferon-γ that can be induced by immune activation. In this study, we measured IL-6 and IL-8, because they are often measured in iE-DAP-induced innate immune response [13]. In addition, the mechanism of uptake of iE-DAP into H441 cells was shown to be PEPT2-dependent. We also observed that the innate immune response was induced by Tri-DAP in H441 cells, likely in a PEPT2-dependent manner. In contrast, induction of this immune response by iE-DAP was not observed in A549 cells, in which no functional expression of PEPT2 was observed [15].

PEPT2 is an SLC transporter (SLC15A2) expressed in various organs such as the kidney, brain, lung, and mammary glands, and transports dipeptides, tripeptides, and peptide-like compounds such as β-lactam antibiotics [21–23]. We previously reported that PEPT2 is functionally expressed in alveolar type II epithelial cells, but expression decreases with transdifferentiation of type II cells into type I-like cells, and PEPT2 expression is almost completely absent in type I-like cells [14]. Moreover, we found that PEPT2 was functionally expressed in H441 cells, while PEPT2 activity was not detected in A549 cells, a widely used alveolar type II epithelial cell model [15]. H441 cells exhibit an

alveolar type II cell phenotype, confirmed by production of surfactant proteins and formation of lamellar bodies [24,25]. In addition, H441 cells can form confluent, electrically tight monolayers [26]. These findings indicated that H441 is a good *in vitro* model to study PEPT2 function in alveolar type II epithelial cells.

Some reports have suggested that PEPT2 plays a role in transport of PAMPs such as iE-DAP and Tri-DAP in alveolar and spleen macrophages, and in airway epithelial cells [7,11,12,27]. However, other transporters in the SLC15A transporter family such as peptide transporter 1 (PEPT1) and peptide/histidine transporters 1 and 2 (PHT1, PHT2), were suggested be involved in transport of PAMPs such as iE-DAP, Tri-DAP, and muramyl dipeptide in Caco-2, HEK293, and MDCK cells [28–31]. Therefore, the transporter responsible for transport of PAMPs may be cell-dependent. However, PEPT1 is not expressed in H441 cells [15]. In addition, when histidine was added as a competitive inhibitor of PHTs, the immune response induced by iE-DAP was not inhibited in H441 cells (data not shown). These findings strongly suggested that PEPT2, but not PEPT1 and PHTs, is responsible for uptake of iE-DAP and subsequent induction of the innate immune response in H441 cells.

Using the RIP2 kinase inhibitor gefitinib, we found that NOD1 activated downstream pathways *via* RIP2 kinase activation following treatment with iE-DAP. RIP2 kinase activates the NF-κB pathway and MAPK pathways (p38 MAPK, ERK, and JNK pathways). The role of the NF-κB pathway in the NOD1-dependent immune response has been reported in various cells such as murine macrophages [32]. We also observed that iE-DAP-induced increases in IL-8 mRNA expression were suppressed in the presence of an NF-κB inhibitor, suggesting that the NF-κB pathway is involved in the NOD1-dependent immune response in H441 cells. MAPK pathway involvement in the innate immune response has varied across reports. For example, cytokine production was induced by stimulating vascular endothelial cells with the NOD1 ligand, which activated the p38 MAPK pathway, but not the JNK and ERK pathways. In mesothelial cells, all MAPK pathways were involved in the NOD1 signaling pathway. This inconsistency may be due to different cells evaluated in these studies. Therefore, the role of these MAPK pathways was examined in H441 cells using inhibitors of each pathway. Our results showed that the p38 MAPK and ERK pathways, but not the JNK pathway, were involved in induction of the innate immune response by iE-DAP in H441 cells. The involvement of ERK pathway was also confirmed by the western blotting showing the increased level of p-ERK in the nuclear fraction of H441 cells treated with iE-DAP.

5. Conclusion

In this study, we found that PEPT2 played a crucial role in uptake of iE-DAP and induction of the innate immune response in H441 cells. In addition, the p38 MAPK, ERK, and NF-κB pathways, but not the JNK pathway, were involved in induction of the innate immune response. These results will contribute to understanding of the physiological role of PEPT2 and of the molecular mechanisms underlying the innate immune response in alveolar epithelial cells.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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