Role of the nucleotide-binding oligomerization domain-containing protein 1 pathway in the development of periodontitis

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ABSTRACT

Objective: During innate immune defense, pattern recognition receptors (PRRs) in the host can activate downstream pathways by recognizing the relevant molecular patterns (PAMPs) of pathogenic microorganisms, thereby triggering a host response. PRRs include Toll-like receptors and nucleotide-binding oligomerization domain-like receptors (NLRs). NOD1, an important cell membrane protein in the NLR-like receptor protein family, exerts anti-infective effects through iE-DAP recognition. Oral epithelial cells resist oral bacterial invasion through iE-DAP-induced interleukin (IL)-8 production, which recruits neutrophils to the site of inflammation in response to the threat posed by pathogenic bacteria to periodontal tissues. However, the regulatory mechanisms of iE-DAP in gingival epithelial cells (GECs) are poorly understood. Therefore, in this study, we aimed to investigate the role of the NOD1 pathway in the development of periodontitis by examining the effect of iE-DAP on the production of IL-8 in Ca9-22 cells.

Methods: IL-8 production of iE-DAP-stimulated-Ca9-22 cells was examined by enzyme-linked immune sorbent assay. The phosphorylation levels of intracellular signaling molecules were evaluated using western blot analysis.

Results: We found that iE-DAP upregulated IL-8 production. Moreover, iE-DAP may regulate IL-8 production by activating the mitogen-activated protein kinase (MAPK) signaling pathway. This pathway plays an important role in iE-DAP-enhanced IL-8 production in GECs.

Conclusions: Our results indicate that p38 MAPK and ER1/2, but not JNK, are involved in innate immune responses in GECs.

1. INTRODUCTION

The gingiva comprises epithelial and connective tissue that mediate defense mechanisms against microbial invasion. The oral epithelium is widely recognized as a primary immune and physical barrier [1]. Oral epithelial cells use various recognition receptors, such as the nucleotide oligomerization domain (NOD) and Toll-like receptors (NLRs and TLRs, respectively), to initiate innate immune responses [2–4]. The NOD protein-mediated recognition system may play a key role in tissues with low TLR expression [5]. NOD1 (also known as CARD4), a cytoplasmic protein that belongs to the NOD-like receptor protein family, is found in various body tissues. NOD1 triggers an innate immune response against bacterial infections by recognizing iE-DAP, a bacterial cell wall component [6].

iE-DAP induces interleukin (IL)-8 production [7,8]. IL-8, the first chemokine to be purified and sequenced in 1987 [9,10], specifically recruits and activates neutrophils in inflammatory tissues and regulates the expression of neutrophil adhesion molecules. IL-8 is secreted by multiple cell types, including monocytes, macrophages, fibroblasts, endothelial cells, and keratinocytes, and plays an important immunomodulatory role in periodontitis [11,12]. However, the effect of iE-DAP on the production of IL-8 and its regulatory mechanism in gingival epithelial cells (GECs) remains unknown.

NOD1 expression is upregulated in periodontitis [13,14]; thus, it may be involved in recognizing periodontal pathogens. Bacillaceae, Peptostreptococcaceae, and Proteobacteria species stimulate NOD1 activity [15]. In human periodontal ligament cells (hPDLCs), NOD1 senses microorganisms and induces IL-6 and IL-8 production through NF-κB and MAPK activation [16]. *Porphyromonas gingivalis* induces IL-6 and IL-8

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production in human gingival fibroblasts (hGFs) and hPDLCs through the NOD1-mediated activation of the NF-κB and ERK1 pathways [17]. The mitogen-activated protein kinase (MAPK) pathway transduces extracellular stimuli into various cellular responses and participates in different cellular processes, such as cell proliferation, differentiation, and apoptosis; it also plays a crucial role in disease regulation and development [18]. Therefore, in this study, we aimed to investigate and confirm the importance of the NOD1 pathway in the development of periodontitis by examining the effect of iE-DAP on the production of IL-8 in Ca9-22 cells. In addition, we aimed to determine the signaling pathways involved in these processes.

2. MATERIALS AND METHODS

2.1 Cell culture

Human gingival epithelial Ca9-22 cells were purchased from the JCRB Cell Bank (ID: JCRB0625; Tokyo, Japan). Cells were cultured in Dulbecco's modified Eagle's Medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal calf serum (Biowest, Nuaillé, France), 1% penicillin, and 1% streptomycin (Wako Pure Chemical Industries, Osaka, Japan). Ca9-22 cells were maintained at 37 °C, 5% CO_2, and 95% humidity.

2.2 Reagents, antibodies, and inhibitors

iE-DAP was purchased from Invitrogen (San Diego, CA, USA). Doramapimod was purchased from ChemScene (Monmouth Junction, NJ, USA). SB203580 was purchased from AdooQ BioScience (Irvine, CA, USA) and VX-745 from Cayman Chemicals (Ann Arbor, MI, USA). Primary anti-phospho-p38 MAPK, anti-p38α/β, anti-JNK, and anti-β-

actin antibodies, and horseradish peroxidase-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-phospho-p44/42 MAPK(ERK1/2) (Thr202/Tyr204), anti-p44/42 MAPK (ERK1/2), anti-phospho-NF-κB p65(Ser536), and anti-NF-κB p65 primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-JNK1/JNK2 (Thr183 and Tyr185) antibodies were purchased from Invitrogen (San Diego, CA, USA). Recombinant anti- IKBα inhibitor antibodies were purchased from Abcam (Cambridge, MA, USA).

2.3 Enzyme-linked immunosorbent assay (ELISA)

IL-8, IL-6, TNF-α, GMCSF, IL-1β production was quantified using the ELISA MAXTM Deluxe Set Human IL-8, IL-6, TNF-α, GMCSF, IL-1β Kits (Bio Legend, CA, USA).

2.4 Western blotting

The cells were lysed using a RIPA buffer (Nacalai Tesque, Kyoto, Japan) and total protein was extracted using a bisquinolinic acid kit (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. Total cell proteins (10 μg per lane) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel. The proteins were transferred onto polyvinylidene fluoride membranes using the wet transfer method. The membrane was then blocked for 1 h at room temperature. Blot images were visualized using ChemiDoc software (Bio-Rad). Whenever necessary, protein-bound primary and secondary antibodies on the membrane were removed using a WB stripping solution and probed with the corresponding antibodies. All experiments were performed in triplicate.

2.5 Statistical analysis

Student's t-test was used to determine the statistical significance of the differences between two groups and one-way analysis of variance (ANOVA) was used to determine the statistical significance of the differences between three or more groups. All analyses were performed using GraphPad software (San Diego, CA, USA). Values are expressed as mean ± standard deviation, and all experiments were performed at least thrice. P values < 0.05 were considered statistically significant.

3. RESULTS

3.1 iE-DAP enhanced IL-8 production in Ca9-22 cells

Various NOD1-expressing human epithelial cells produce antimicrobial factors but not proinflammatory cytokines [19]. However, in airway and alveolar epithelial cells, iE-DAP activation induces IL-8 production [7,20]. To determine whether iE-DAP affects the production of inflammatory cytokines in gingival epithelial cells, we stimulated Ca9-22 cells with iE-DAP (10 ng/ml) for 24 h. iE-DAP enhanced the production of IL-8; however, it had no effect on IL-6, TNF-α, GMCSF, and IL-1β production (Fig. 1a–1e).

3.2 iE-DAP phosphorylated MAPK in Ca9-22 cells

In airway and alveolar epithelial cells, both the NF-κB and MAPK signaling pathways are required for iE-DAP-stimulated IL-8 transcription [7,20]. To determine the signaling pathways involved in iE-DAP-stimulated IL-8 production in Ca9-22 cells, we measured MAPK phosphorylation (p38MAPK, ERK1/2, and JNK) using western blot analysis. At 5, 10, 30, 60, and 120 min, iE-DAP increased p38 MAPK and ERK1/2 phosphorylation,

peaking at 10 min. iE-DAP did not affect JNK phosphorylation. Moreover, p38 MAPK, ERK1/2, and JNK protein levels were not altered (Fig. 2). We also examined NF-κB p65 phosphorylation and IKB protein levels. NF-κB p65 protein levels were unchanged; however, IKB degradation occurred at 120 min. NF-κB p65 and β-actin protein levels were not altered (Fig. 3).

3.3 MAPK inhibitors suppressed IL-8 production in iE-DAP-stimulated Ca9-22 cells

To further confirm the involvement of the MAPK pathway in iE-DAP-induced IL-8 production, we determined the effects of MAPK pathway inhibitors [p38 MAPK inhibitors (doramapimod, a non-selective p38 MAPK inhibitor; SB203580, a p38α/β-selective inhibitor; and VX-745, a p38α-selective inhibitor) and an ERK1/2 inhibitor (U1206)] on the production of IL-8 in iE-DAP-induced Ca9-22 cells. The results showed that iE-DAP-induced IL-8 production was suppressed by MAPK pathway inhibitors (Fig 4). In contrast, iE-DAP-induced IL-8 production in Ca9-22 cells remained unchanged in the presence of a JNK inhibitor (SP600125) (Fig. 4).

4. DISCUSSION

When challenged with a bacterial infection, GECs and keratinocytes express inflammatory cytokines and growth factors, including IL-1β, IL-6, IL-8, TNF-α, and PDGF-BB. Gingival epithelial cells and keratinocytes have a high turnover rate, and the induction of epidermal growth factors is enhanced during inflammation. In the present study, we demonstrated that iE-DAP upregulated the production of IL-8 in human gingival epithelial Ca9-22 cells and did not affect IL-6, TNF-α, GMCSF, and IL-1β

production (Fig. 1). IL-8 is a chemokine that plays an important role in inflammation by recruiting and activating immune cells; it also elicits angiogenic responses in endothelial cells [21]. IL-8 is expressed in gingival keratinocytes stimulated by periodontal pathogenic microorganisms, which enhance neutrophil extravasation from the circulating vasculature and migration to the lesion site, thus acting as an innate immune response and protecting gingival cells [22,23]. Therefore, it is important to further investigate the specific signaling mechanism underlying the production of IL-8 in iE-DAP-stimulated gingival epithelial cells.

As a key protein involved in iE-DAP recognition, NOD1 plays an important role in innate immunity. NOD1 includes three characteristic domains: the ligand-recognition domain, leucine-rich repeats (LRRs), and the intermediate nucleotide-binding oligomerization domain (NOD, also known as the NACHT domain). The NOD induces protein self-oligomerization; the CARD domain recruits cysteine proteolytic enzymes and acts as a signal transmission site that activates downstream signaling pathways [24–26]. iE-DAP is a dipeptide found in all gram-negative and some gram-positive bacterial peptidoglycans (PGNs) and is the smallest motif recognized by NOD1. When the LRR domain of NOD1 recognizes the iE-DAP ligand, the intermediate NOD protein oligomerizes, and the CARD domain binds to and activates the downstream serine-threonine protein kinase receptor-interacting protein 2 (RIP2) [27]. Activated RIP2 then activates transcription factors through the NF-κB and MAPK pathways [25,26,28]. In the NF-κB pathway, the NOD-RIP2 complex promotes NF-κB kinase complex (IKK) aggregation, which phosphorylates and ubiquitinates the NF-κB inhibitor IKB, leading to its degradation. This ultimately leads to the dissociation of NF-κB from IKB, resulting in

NF-κB activation. NF-κB is then translocated to the nucleus, where it induces the transcriptional expression of a wide range of inflammatory factors associated with the natural immune response. In the MAPK pathway, RIP2 kinase activates TGF-β activated kinase-1 (TAK-1). Activated TAK-1 then activates MAPK (including p38MAPK, ERK1/2, and JNK), which in turn activates activator protein-1 (AP-1), which is also translocated to the nucleus and promotes the transcription and expression of multiple inflammatory factors [29,30]. In the present study, we found that iE-DAP induced p38 MAPK and ERK1/2 phosphorylation in Ca9-22 cells, which peaked at 10 min and caused IKB degradation at 120 min (Fig. 2). The phosphorylation of p38 MAKPK and ERK1/2 results in the recruitment of downstream effector proteins, including the IKK complex and TAK1 [31]. Our data showed that NOD1 stimulation promotes IKB degradation (Fig. 3).

We found that the p38 MAPK and ERK1/2 inhibitors suppressed iE-DAP-stimulated IL-8 production in Ca9-22 cells (Fig. 4). The NOD1 signaling complex activates JNK and IL-8 release. Treatment with the JNK inhibitor, SP600125, did not affect the iE-DAP-induced stimulation of IL-8 in Ca9-22 cells (Fig. 4). These results indicate that JNK is not involved in iE-DAP-induced IL-8 production, which is supported by previous studies [20].

Our results suggest that NOD1 may exert its innate immune role in periodontitis through the p38 MAPK and ERK1/2 pathways but not the JNK signaling pathway. However, this study has several limitations. First, the cells used in this study were derived from an epithelioid cell line of human gingival squamous cell carcinoma, and the exact mechanism of iE-DAP-induced IL-8 regulation in human GECs still needs to be

elucidated. Second, the upstream and downstream signaling pathways involved in iE-DAP-induced IL-8 regulation need to be further explored.

5. CONCLUSION

In conclusion, we found that the p38 MAPK and ERK1/2 pathways are involved in the iE-DAP-induced IL-8 production in GECs. Our results provide new insights into the innate defense mechanism initiated during periodontitis and highlight molecular targets for the treatment and prevention of periodontitis.

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mediator of NOD2 signaling in epidermal cells. J Biol Chem 2008;283:137–44.
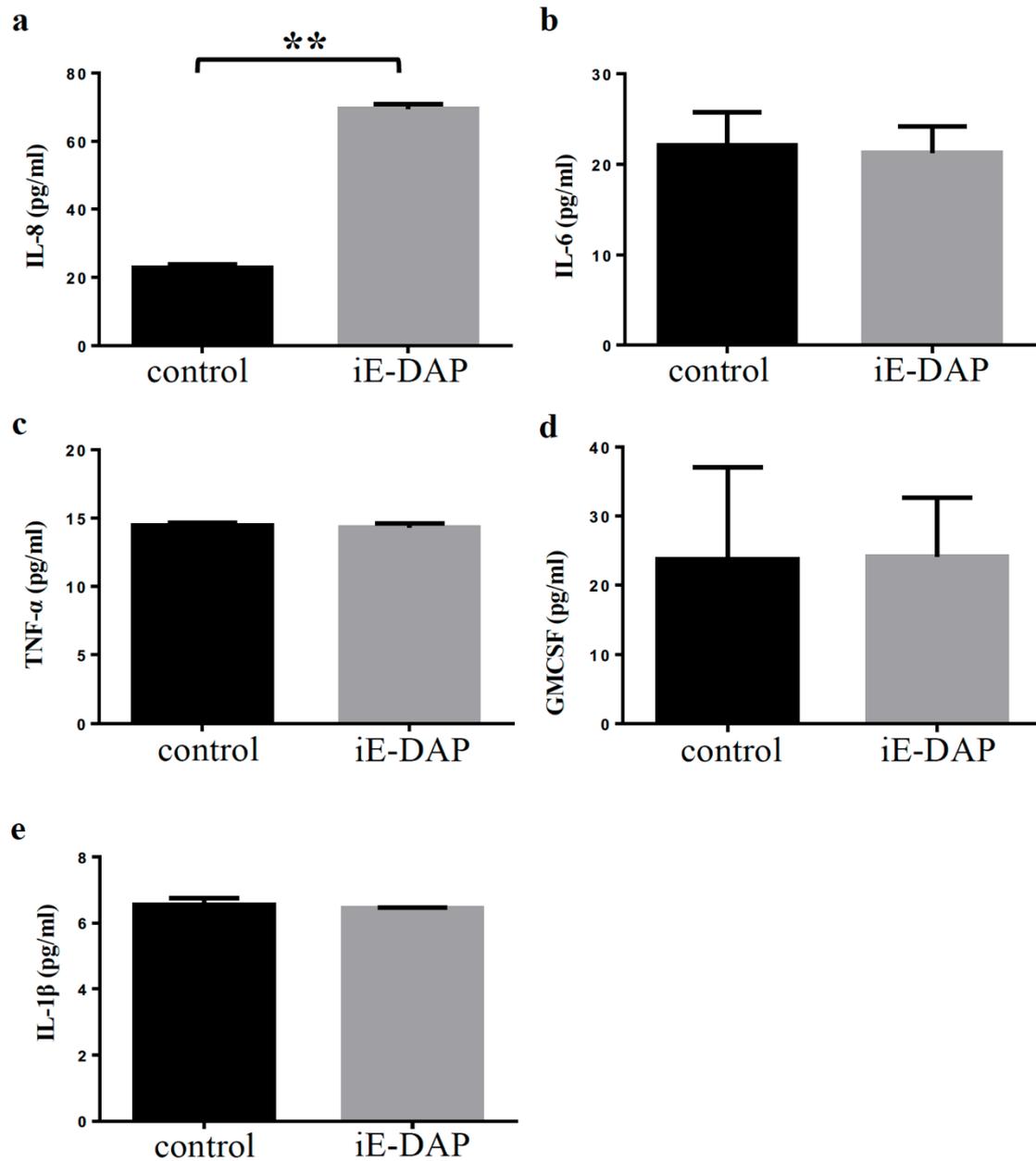
Figure 1. IL-8, IL-6, TNF-α, GMCSF, and IL-1β production in γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP)-stimulated (10 µg/mL) Ca9-22 cells (a–e). iE-DAP enhanced the production of IL-8 in Ca9-22 cells, but did not affect IL-6, TNF-α, GMCSF, IL-1β production. (**P < 0.01)
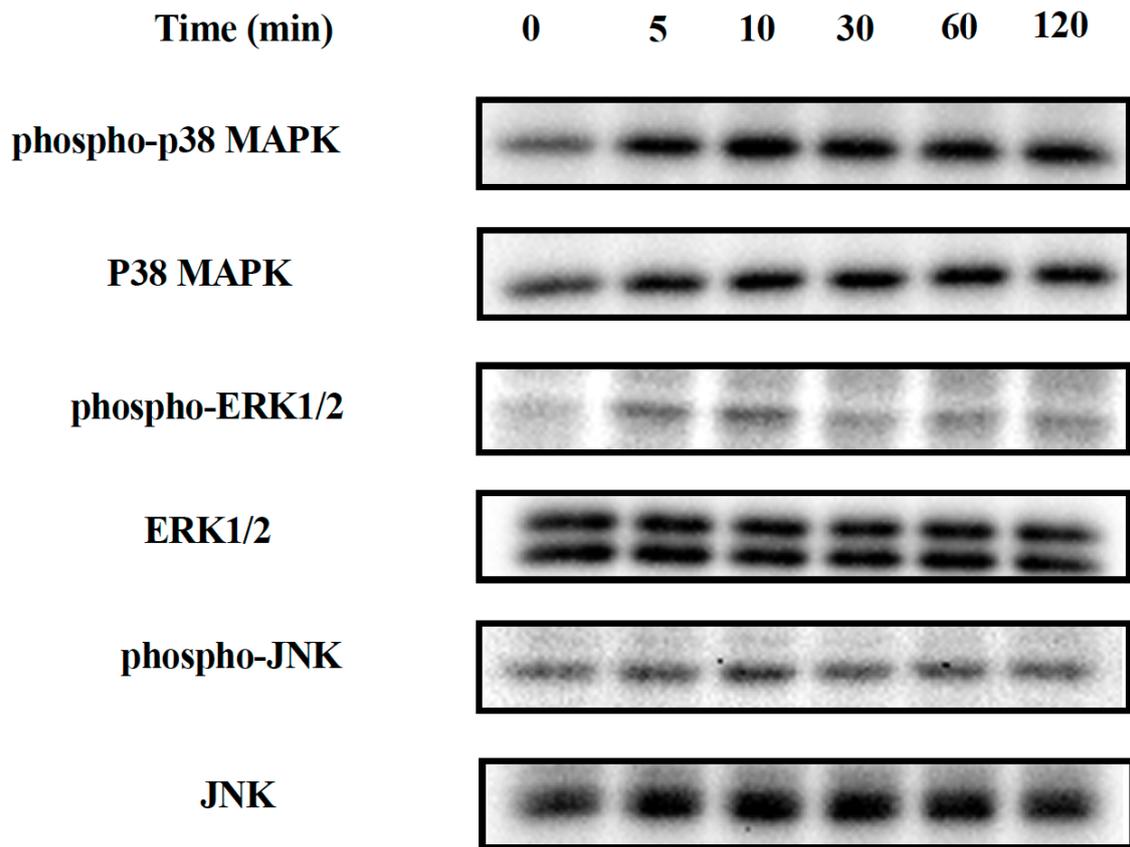
Figure 2. Western blot analysis of the phosphorylation of MAPK-related proteins (phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2, ERK1/2, phospho-JNK, and JNK) in γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP)-treated Ca9-22 cells. iE-DAP increased the phosphorylation levels of p38 MAPK and ERK1/2.
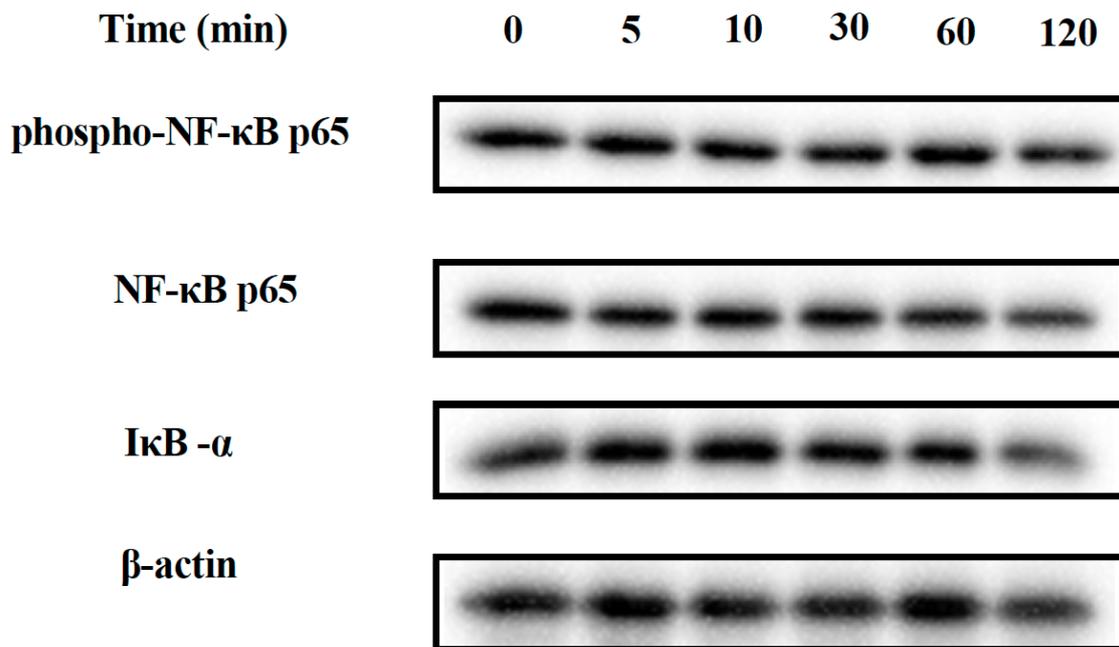
Figure 3. Western blot analysis of phospho-NF-κB p65, NF-κB p65, IKBα, and β-actin levels in γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP)-treated Ca9-22 cells. iE-DAP induced the degradation of IKBα at 120 min without altering the phosphorylation level of NF-κB p65.
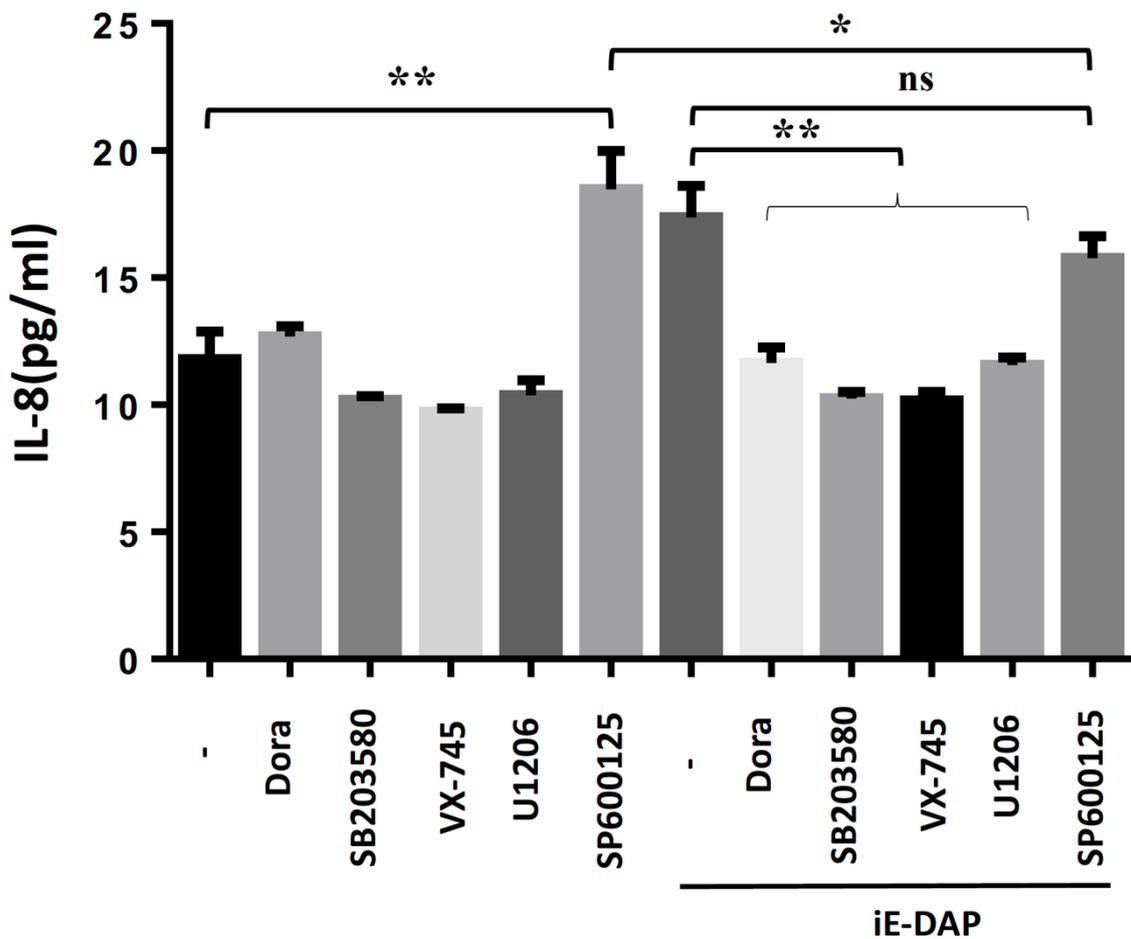
Figure 4. γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP)-induced IL-8 production in Ca9-22 cells in the presence of MAPK inhibitors. Inhibition of MAPK (p38 MAPK, ERK1/2, not JNK) resulted in the marked inhibition of iE-DAP-induced IL-8 production. IL-8 production in the supernatant was analyzed using enzyme-linked immunosorbent assay after pretreatment with p38 MAPK inhibitors (non-selective p38 MAPK inhibitor, doramapimod, 520 nM; p38α/β-selective inhibitor SB203580, 10 μM; or p38α-selective inhibitor, VX-745, 2 μM), ERK inhibitors (U1206, 10 μM), or JNK inhibitors (SP600125, 10 μM) for 1 h and treatment with 10 μg/mL iE-DAP for 24 h. (**P < 0.01)