Alkannin inhibits CCL3 and CCL5 production in human periodontal ligament cells

Yoshitaka Hosokawa[1], Ikuko Hosokawa[1], Satoru Shindo[1], Yoshihiro Ohta[2], Kazumi Ozaki[3], and Takashi Matsuo[1]

[1]Department of Conservative Dentistry, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Tokushima, Japan; [2]Graduate School of Mathematical Sciences, The University of Tokyo, Meguro-ku, Tokyo, Japan; [3]Department of Oral Health Care Promotion, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Tokushima, Japan;

Text pages: 16 (including 1 title page), Figures: 4

Short running title: Alkannin inhibits CCL3 and CCL5 production

Address correspondence and reprint requests to: Yoshitaka Hosokawa, Department of Conservative Dentistry, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto-cho, Tokushima, Tokushima, 770-8504, Japan

TEL: 81-886-33-7340

FAX: 81-886-33-7340

E-mail: hosokawa@tokushima-u.ac.jp

Keywords: Alkannin, CCL3, CCL5, NF-κB, periodontal ligament cells

Abbreviations

ANOVA: analysis of variance
CCL: CC chemokine ligand
CCR: CC chemokine receptor
ELISA: enzyme-linked immunosorbent assay

GAPDH: glyceraldehyde-3-phosphate dehydrogenase
HPDLC: human periodontal ligament cells
HRP: horseradish peroxidase
IL: interleukin
IκB: inhibitor of kappa B
ISHC: IL-1β-stimulated HPDLC
NF-κB: nuclear factor κB
PBS: phosphate-buffered saline
RANKL: receptor activator of nuclear factor κB ligand
TNF: tumor necrosis factor

Abstract

Alkannin, which is found in *Alkanna tinctoria*, a member of the borage family, is used as a food coloring. Alkannin has recently been reported to have certain biological functions, such as anti-microbial and anti-oxidant effects. It is known that CC chemokine receptor (CCR) 5-positive leukocytes contribute to alveolar bone resorption in periodontal lesions. The aim of this study was to examine whether alkannin inhibits the production of CC chemokine ligand (CCL) 3 and CCL5, which are CCR5 ligands, in human periodontal ligament cells (HPDLC). Interleukin (IL)-1β induced CCL3 and CCL5 production in HPDLC. Alkannin inhibited IL-1β-mediated CCL3 and CCL5 production in HPDLC in a dose-dependent manner. Moreover, we revealed that alkannin suppressed inhibitor of kappa B-α degradation in IL-1β-stimulated HPDLC. In addition, a nuclear factor (NF)-κB inhibitor significantly inhibited CCL3 and CCL5 production in IL-1β-stimulated HPDLC. These results demonstrate that alkannin inhibits CCR5 ligand production in IL-1β-stimulated HPDLC by attenuating the NF-κB signaling pathway.

1. Introduction

Periodontal disease is characterized by gingival inflammation, inflammatory cell infiltration, and alveolar bone resorption, which are induced in response to the bacteria present in biofilms. Previous studies have revealed that the immune responses seen in periodontal lesions are involved in the initiation and progression of periodontitis (Teng et al., 2003; Taubman et al., 2005). Recently, it has been found that CC chemokine receptor (CCR) 5+ leukocytes contribute to bone destruction in periodontal lesions (Ferreira et al., 2011). Ferreira et al. (2011) reported that CCR5+CD4+ cells express high levels of receptor activator of nuclear factor κB ligand (RANKL). In addition, the CCR5+F4/80 leukocytes that they detected in periodontal lesions were demonstrated to be active macrophages because they expressed interleukin (IL)-1β and tumor necrosis factor α. Therefore, the CCR5+ leukocytes found in tissues affected by periodontal disease are pro-osteoclastic or osteoclastogenic cell subsets (Ferreira et al., 2011). They also reported that the absence of CCR5 in mice reduced alveolar bone resorption and inflammatory cell accumulation in an experimental periodontal disease model (Ferreira et al., 2011). Therefore, CCR5+ leukocytes might be a target of periodontal disease treatment.

CC chemokine ligands (CCL) 3, 4, and 5 are ligands of CCR5 (Mueller et al., 2004). They are involved in the migration and accumulation of CCR5+ leukocytes in inflammatory lesions. There have been a number of reports about the expression of CCR5 ligands in human periodontal lesions. Thunell et al. (2010) reported that CCL3 and CCL5 were detected in

gingival crevicular fluid samples obtained from chronic periodontitis patients, and the initial treatment reduced the levels of CCL3 and CCL5 in gingival crevicular fluid. In an immunohistochemical study, Gemmell et al. (2000) revealed that CCL3+ cells and CCL5+ cells are present in human periodontal lesions. Therefore, CCL3 and CCL5 seem to be involved in the migration of CCR5+ cells in periodontal lesions. Recently, Nebel et al. (2010) reported that human periodontal ligament cells (HPDLC) that had been stimulated with lipopolysaccharide (LPS) from *Escherichia coli* produced CCL3 and CCL5. However, it is unclear if other molecules induce CCR5 ligand production in HPDLC.

Alkannin is a natural plant dye that is found in extracts of *Alkanna tinctoria*, a member of the borage family that grows in the south of France. Alkannin is used as a food coloring. It is known that alkannin has biological effects, such as anti-microbial (Rajbhandari et al., 2007) and anti-cancer effects (Deng et al., 2010). However, the effects of alkannin on chemokine production are uncertain.

Nuclear factor κB (NF-κB) plays an important role in inflammatory responses, such as cytokine and chemokine production. We previously reported that IL-1β activates the NF-κB pathway in HPDLC (Shindo et al., 2014), and the NF-κB pathway is positively associated with CCL20 and IL-6 production. However, it is unclear whether the NF-κB pathway is involved in CCL3 or CCL5 production in IL-1β-stimulated HPDLC (ISHC). Moreover, we do not know if alkannin suppresses NF-κB activation in ISHC.

In the present study, we examined the effects of alkannin on CCR5 ligand production in

ISHC. In addition, we investigated whether alkannin modulates the NF-κB pathway in order to explore which signal transduction pathways it affects.

2. Materials and Methods

2.1. Cell culture

HPDLC were obtained from Lonza Japan (Tokyo, Japan) and grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (penicillin G: 100 units/ml, streptomycin: 100 μg/ml) at 37°C in humidified air containing 5% CO_2. The cells were used between passage numbers 5 and 10.

2.2. CCL3 and CCL5 production in HPDLC

HPDLC were seeded at a density of 0.5×10^4 cells/cm^2 in a 24-well cell culture plate. After they had reached confluence, the HPDLC were stimulated with recombinant human IL-1β (Peprotech, Rocky Hill, NJ, USA) for 24 hours. The supernatants of the HPDLC cultures were collected, and the CCL3 and CCL5 concentrations of the culture supernatants were measured in triplicate with ELISA. DuoSet ELISA kits (R&D systems, Minneapolis, MN, USA) were used for each assay. All assays were performed according to the manufacturer's instructions, and chemokine levels were determined using the standard curve prepared for each assay. In selected experiments, the HPDLC were cultured for 1 hour in the presence or absence of alkannin (0.25, 0.5, 1, or 2 μM; Nagara Science Co., Ltd., Gifu, Japan) or SC514 (a selective NF-κB inhibitor, 10 μM; Santa Cruz Biotechnology, Inc., Dallas,

TX, USA) prior to their incubation with IL-1β.

2.3. Western blot analysis

Western blot analysis was performed to examine the IL-1β-induced phosphorylation or degradation of signal transduction molecules. HPDLC were seeded at a density of 0.5×10^4 cells/cm^2 in a 12-well cell culture plate. After reaching confluence, the HPDLC were subjected (or not) to alkannin (0.5 μM, 1 μM) pretreatment for 1 hour, stimulated with IL-1β (1 ng/ml), washed once with ice-cold PBS, and then incubated on ice for 30 min with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitor cocktail (Sigma). After the removal of debris by centrifugation, the protein concentrations of the lysates were quantified with the Bradford protein assay using IgG as a standard. A 20-μg protein sample was loaded onto a 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, before being electrotransferred to a polyvinylidene difluoride membrane. The phosphorylation of NF-κB p65 and the degradation of inhibitor of kappa B (IκB)-α were assessed using phospho-NF-κB p65 rabbit monoclonal antibody (Cell Signaling Technology), NF-κB p65 rabbit monoclonal antibody (Cell Signaling Technology), IκB-α mouse monoclonal antibody (Cell Signaling Technology), or GAPDH rabbit monoclonal antibody (Cell Signaling Technology) according to the manufacturer's instructions. The resultant protein bands were visualized via incubation with the horseradish peroxidase-conjugated secondary antibody (Sigma), followed by detection using the ECL prime Western blotting detection system (GE Healthcare, Uppsala, Sweden). The

quantitation of the chemiluminescent signals was performed using ImageJ (version 1.44).

2.4. Statistical analysis

Statistical significance was analyzed using one-way ANOVA. *P*-values of <0.05 were considered to be significant in the analyses shown in Figs. 1, 2, and 4.

3. Results

3.1. Effects of IL-1β on CCR5 ligand production in HPDLC

We first examined whether IL-1β induces CCR5 ligand production in HPDLC. Figure 1 shows that CCL3 and CCL5 were produced in ISHC in an IL-1β dose-dependent manner.

3.2. Effects of alkannin on CCL3 and CCL5 production in ISHC

Next, we examined whether alkannin inhibited CCL3 or CCL5 production in ISHC because we wanted to determine the anti-inflammatory effects of alkannin. Figure 2 shows that 0.5 μM of alkannin significantly inhibited CCL3 and CCL5 production in ISHC. The inhibitory effects of alkannin were dose-dependent.

3.3. Effects of alkannin on NF-κB p65 phosphorylation and IκB-α degradation in ISHC

Moreover, we examined the effects of alkannin on the NF-κB pathway because it is known that IL-1β activates the NF-κB pathway in HPDLC (Murayama et al., 2011; Shindo et al., 2014), and the NF-κB pathway is involved in the production of various chemokines (Smith et al., 1997; Roebuck et al., 1999). Figure 3 shows that alkannin inhibited IκB-α degradation, but did not affect NF-κB p65 phosphorylation, in ISHC.

3.4. Effects of an NF-κB inhibitor on CCL3 and CCL5 production in ISHC

Finally, we examined whether the NF-κB pathway is involved in CCL3 or CCL5 production in ISHC using SC514, an NF-κB inhibitor. Figure 4 shows that SC514 significantly inhibited CCL3 and CCL5 production. These results demonstrated that the NF-κB pathway is essential for CCL3 and CCL5 production in ISHC.

4. Discussion

We demonstrated that IL-1β induced CCL3 and CCL5 production in HPDLC. A previous study showed that HPDLC produced CCL3 and CCL5 after LPS stimulation (Nebel et al., 2010). Therefore, HPDLC might enhance CCR5+ leukocyte accumulation in order to increase CCL3 and CCL5 production. Moreover, it is reported that LPS upregulated the release of CCL3 or CCL5 from human epithelial cells (Ryu et al., 2007; Basso et al., 2015) and human gingival fibroblasts (Ryu et al., 2007; Morandini et al., 2010). Furthermore, Ryu et al. (2007) stated that IL-1β induced CCL3 production in human gingival epithelial cells. The findings of our and previous studies suggested that the release of CCL3 and CCL5 from LPS- or IL-1β-stimulated resident periodontal cells might be involved in CCR5+ leukocyte accumulation in periodontal lesions. Moreover, we found that alkannin inhibited IL-6, IL-8, and CCL20 production in ISHC (data not shown). So, we consider that alkannin could relieve inflammatory reactions in periodontal lesions by decreasing inflammatory cytokine expression.

There have been a few reports about the anti-inflammatory effects of alkannin. Wang et al. (2015) reported that alkannin suppressed IL-6, IL-12, IL-23, and IL-1β production in

dendritic cells. They also showed that alkannin treatment reduced IL-23 expression in skin lesions in a psoriasis mouse model (Wang et al., 2015). We found that alkannin inhibited CCL3 and CCL5 production in HPDLC. Next, we should examine whether alkannin is able to suppress CCL3 and CCL5 expression and alveolar bone resorption in the lesions of a periodontal disease mouse model.

In this study, we demonstrated that alkannin inhibited IκB-α degradation in HPDLC. IκB-α degradation is an important event in the activation of the NF-κB pathway because IκB-α binds to NF-κB and inhibits the translocation of NF-κB into the nucleus. We showed that 1 μM alkannin markedly inhibited IκB-α degradation in ISHC. On the other hand, 0.5 μM alkannin did not inhibit IκB-α degradation in ISHC even though CCL3 and CCL5 production were decreased. We consider that low doses of alkannin might also modulate signal transduction pathways other than NF-κB. In addition, we found that IκB-α degradation was strongly inhibited at 15 minutes after the addition of 1 μM alkannin, and the results obtained at 30 and 60 minutes indicated that the inhibitory effects of alkannin on IκB-α degradation decreased gradually over time. We consider that the molecules released from ISHC might modulate the effects of alkannin. Further studies are necessary to address this issue. A previous study demonstrated the effects of alkannin on NF-κB pathway activation. Yoshihisa et al. (2012) reported that alkannin pretreatment suppressed IκB-α degradation and NF-κB nuclear translocation in human keratinocytes after ultraviolet B (UVB) irradiation. They used 1 μM alkannin in their experiment, as did we. They also reported that alkannin treatment

reduced the mRNA expression levels of IL-1β, IL-6, and IL-8, which are regulated by NF-κB, in human keratinocytes after UVB irradiation (Yoshihisa et al., 2012). Their study and our study indicate that alkannin is able to suppress the activation of the NF-κB pathway in some types of cells and reduce inflammatory cytokine production in inflammatory lesions.

5. Conclusions

In conclusion, our results indicate that IL-1β induces CCL3 and CCL5 production in HPDLC, and alkannin suppresses CCL3 and CCL5 production in ISHC. However, we do not know whether alkannin is able to suppress CCR5 ligand production in other resident periodontal cells, such as gingival fibroblasts and gingival epithelial cells. Moreover, it is uncertain if alkannin is able to inhibit CCR5+ leukocyte migration and accumulation in periodontal lesions. We should conduct further investigations to address these questions.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (C) (25463219 and 15K11392) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflicts of interest

The authors confirm that they have no conflicts of interest.

Figure legends

Fig. 1. Effects of IL-1β on CCL3 and CCL5 production in HPDLC

HPDLC were stimulated with IL-1β (0.1, 1, or 10 ng/ml), and the supernatants were collected

after 24 hours. The levels of CCL3 (A) and CCL5 (B) in the supernatants were measured using ELISA. The results are shown as the mean and standard deviation (SD) of one representative experiment performed in triplicate. The error bars show SD values. * = $P<0.05$, significantly different from the non-stimulated HPDLC

Fig. 2. Effects of alkannin on CCL3 and CCL5 production in ISHC

HPDLC were pretreated with alkannin (0.25, 0.5, 1, or 2 μM) for 1 hour and then were stimulated with IL-1β (1 ng/ml). The supernatants were collected after 24 hours. The levels of CCL3 (A) and CCL5 (B) in the supernatants were measured using ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show SD values. * = $P<0.05$, significantly different from the IL-1β-stimulated HPDLC that were not treated with alkannin

Fig. 3. Effects of alkannin on IL-1β-induced NF-κB p65 phosphorylation and IκB-α degradation

The cultured cells were pretreated with alkannin (0.5 or 1 μM) for 60 min and then stimulated with IL-1β (1 ng/ml) for 15, 30, or 60 min. The cells were lysed in lysis buffer containing protease inhibitors, and the phosphorylation of NF-κB p65 and degradation of IκB-α were assessed using Western blot analysis. A representative Western blot showing the phospho-NF-κB p65, total-NF-κB p65, total-IκB-α, and GAPDH levels detected in HPDLC in three independent experiments.

Fig. 4. Effects of an NF-κB inhibitor on CCL3 and CCL5 production in ISHC

HPDLC were pretreated with SC514 (10 μM) for 1 hour, before being stimulated with IL-1β (1 ng/ml), and the supernatants were collected after 24 hours. The levels of CCL3 (A) and CCL5 (B) in the supernatants were measured using ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show SD values. * = $P<0.05$, significantly different from the ISHC that were not subjected to SC514 treatment

References

Basso FG, Soares DG, Pansani TN, Turrioni AP, Scheffel DL, de Souza Costa CA, Hebling J. (2015). Effect of LPS treatment on the viability and chemokine synthesis by epithelial cells and gingival fibroblasts. Arch Oral Biol; 60: 1117-1121.

Deng R, Tang J, Xie BF, Feng GK, Huang YH, Liu ZC, Zhu XF. (2010). SYUNZ-16, a newly synthesized alkannin derivative, induces tumor cells apoptosis and suppresses tumor growth through inhibition of PKB/AKT kinase activity and blockade of AKT/FOXO signal pathway. Int J Cancer; 127: 220-229.

Ferreira SB Jr, Repeke CE, Raimundo FM, Nunes IS, Avila-Campos MJ, Ferreira BR, Santana da Silva J, Campanelli AP, Garlet GP. (2011). CCR5 mediates pro-osteoclastic and osteoclastogenic leukocyte chemoattraction. J Dent Res; 90: 632-637.

Gemmell E, Grieco DA, Seymour GJ. (2000). Chemokine expression in Porphyromonas gingivalis-specific T-cell lines. Oral Microbiol Immunol; 15: 166-171.

Morandini AC, Sipert CR, Gasparoto TH, Greghi SL, Passanezi E, Rezende ML, Sant'ana AP, Campanelli AP, Garlet GP, Santos CF. (2010). Differential production of macrophage inflammatory protein-1alpha, stromal-derived factor-1, and IL-6 by human cultured periodontal ligament and gingival fibroblasts challenged with lipopolysaccharide from P. gingivalis. J Periodontol; 81: 310-317.

Mueller A, Strange PG. (2004). The chemokine receptor, CCR5. Int J Biochem Cell Biol; 36: 35-38.

Murayama R, Kobayashi M, Takeshita A, Yasui T, Yamamoto M. (2011). MAPKs, activator protein-1 and nuclear factor-κB mediate production of interleukin-1β-stimulated cytokines, prostaglandin E_2 and MMP-1 in human periodontal ligament cells. J Periodontal Res; 46: 568-575.

Nebel D, Jönsson D, Norderyd O, Bratthall G, Nilsson BO. (2010). Differential regulation of chemokine expression by estrogen in human periodontal ligament cells. J Periodontal Res; 45: 796-802.

Rajbhandari M, Schoepke TH, Mentel R, Lindequist U. (2007). Antibacterial and antiviral naphthazarins from Maharanga bicolor. Pharmazie; 62: 633-635.

Roebuck KA, Carpenter LR, Lakshminarayanan V, Page SM, Moy JN, Thomas LL. (1999). Stimulus-specific regulation of chemokine expression involves differential activation of the redox-responsive transcription factors AP-1 and NF-kappaB. J Leukoc Biol; 65: 291-298.

Ryu OH, Choi SJ, Linares AM, Song IS, Kim YJ, Jang KT, Hart TC. (2007). Gingival epithelial cell expression of macrophage inflammatory protein-1alpha induced by interleukin-1beta and lipopolysaccharide. J Periodontol; 78: 1627-1634.

Shindo S, Hosokawa Y, Hosokawa I, Ozaki K, Matsuo T. (2014). Genipin inhibits IL-1β-induced CCL20 and IL-6 production from human periodontal ligament cells. Cell Physiol Biochem; 33: 357-364.

Smith RS, Smith TJ, Blieden TM, Phipps RP. (1997). Fibroblasts as sentinel cells Synthesis of chemokines and regulation of inflammation. Am J Pathol; 151: 317-322.

Taubman MA, Valverde P, Han X, Kawai T. (2005). Immune response: the key to bone resorption in periodontal disease. J Periodontol; 76: 2033-2041.

Teng YT. (2003). The role of acquired immunity and periodontal disease progression. Crit Rev Oral Biol Med; 14: 237-252

Thunell DH, Tymkiw KD, Johnson GK, Joly S, Burnell KK, Cavanaugh JE, Brogden KA, Guthmiller JM. (2010). A multiplex immunoassay demonstrates reductions in gingival crevicular fluid cytokines following initial periodontal therapy. J

Periodontal Res; 45 :148-152.

Wang Y, Zhao J, Zhang L, Di T, Liu X, Lin Y, Zeng Z, Li P. (2015). Suppressive effect of β, β-dimethylacryloyl alkannin on activated dendritic cells in an imiquimod-induced psoriasis mouse model. Int J Clin Exp Pathol; 8: 6665-6673.

Yoshihisa Y, Hassan MA, Furusawa Y, Tabuchi Y, Kondo T, Shimizu T. (2012). Alkannin, HSP70 inducer, protects against UVB-induced apoptosis in human keratinocytes. PLoS One; 7: e47903.

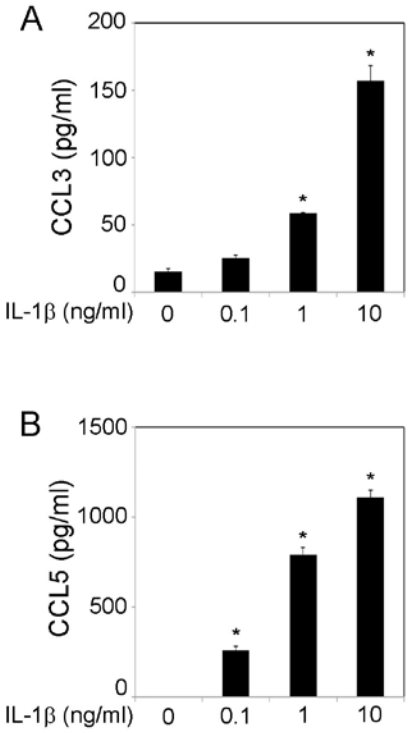
A
200
150
100
50
0
CCL3 (pg/ml)
*
*
*
IL-1β (ng/ml) 0 0.1 1 10
B
1500
1000
500
0
CCL5 (pg/ml)
*
*
*
IL-1β (ng/ml) 0 0.1 1 10

Fig. 1

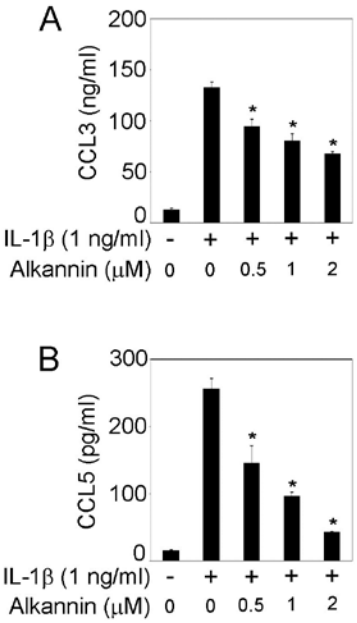
A
CCL3 (ng/ml)
200
150
100
50
0
IL-1β (1 ng/ml) - + + + +
Alkannin (μM) 0 0 0.5 1 2
B
CCL5 (pg/ml)
300
200
100
0
IL-1β (1 ng/ml) - + + + +
Alkannin (μM) 0 0 0.5 1 2

Fig. 2

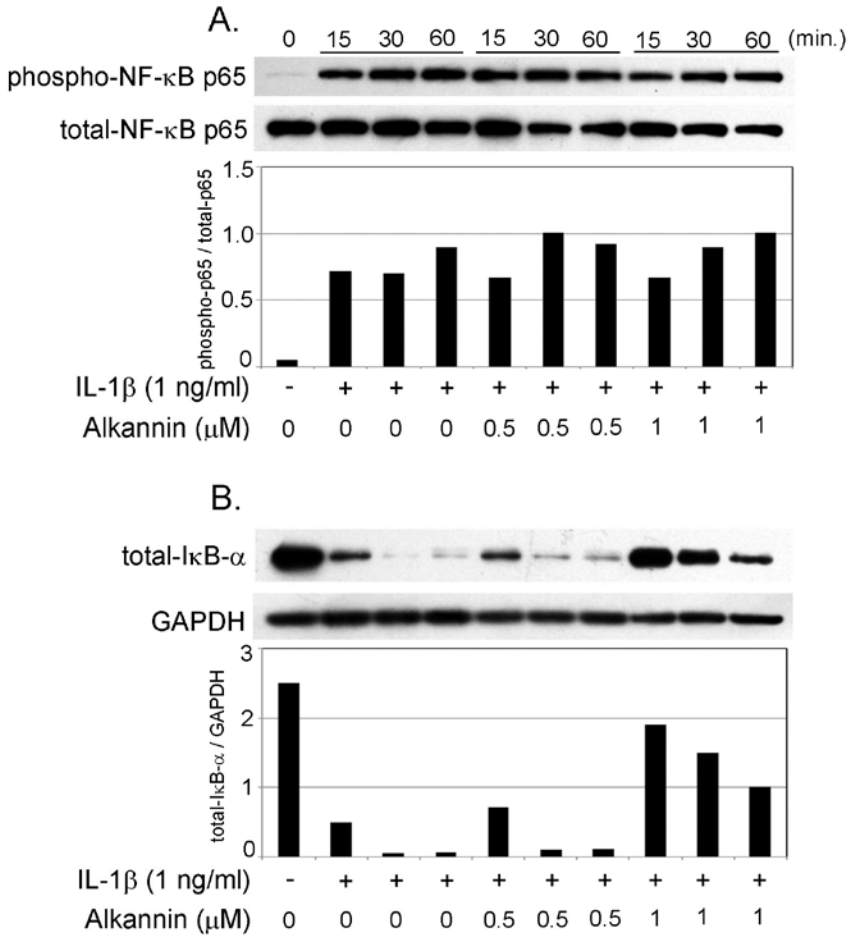
A.
0 15 30 60 15 30 60 15 30 60 (min.)
phospho-NF-κB p65
total-NF-κB p65
phospho-p65 / total-p65
1.5
1.0
0.5
0
IL-1β (1 ng/ml) - + + + + + + + + +
Alkannin (μM) 0 0 0 0 0.5 0.5 0.5 1 1 1
B.
total-IκB-α
GAPDH
total-IκB-α / GAPDH
3
2
1
0
IL-1β (1 ng/ml) - + + + + + + + + +
Alkannin (μM) 0 0 0 0 0.5 0.5 0.5 1 1 1

Fig. 3

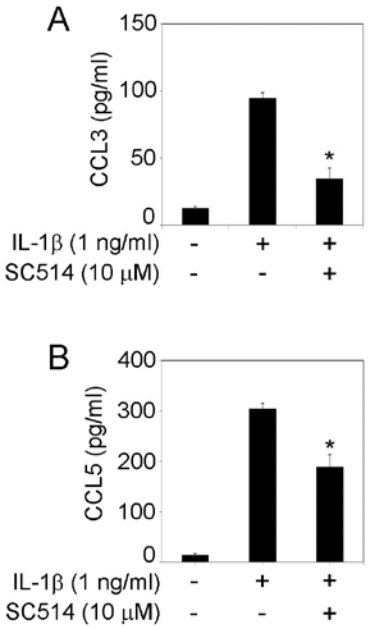
A
150
100
50
0
CCL3 (pg/ml)
*
IL-1β (1 ng/ml) - + +
SC514 (10 μM) - - +
B
400
300
200
100
0
CCL5 (pg/ml)
*
IL-1β (1 ng/ml) - + +
SC514 (10 μM) - - +

Fig. 4