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RESEARCH NOTE

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Anti-inflammatory Mechanism of Seaweeds in Murine Macrophage

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Abstract The effect of 4 seaweed extracts (*Desmarestia viridis, Dictyopteris divaricata, Scytosiphon lomentaria,* and *Ishige okamurae*) on pro-inflammatory mediators as well as nuclear factor (NF)-κB in the stimulated Raw 264.7 cells was investigated. They reduced iNOS and interlukin (IL)-1β expressions at transcription level. Of those, 3 extracts (*D. divaricata*, *I. okamurae*, and *S. lomentaria*) inhibited the COX-2 expression at translation level. IκB-α degradation was inhibited by *D. divaricata* and *S. lomentaria* extracts. Therefore, we concluded that the extracts from *D. divaricata* and *S. lomentaria* could inhibit the activation of murine macrophage through the blocking of NF-κB activation.

Keywords *Desmarestia viridis, Dictyopteris divaricata, Scytosiphon lomentaria, Ishige okamurae*, inducible nitric oxide synthase (iNOS), interlukin-1β, cyclooxygenase (COX)-2, IκB, proinflammatory cytokine, anti-inflammation

Introduction

Seaweeds, primary producers of the oceans, have been consumed as food, medicine, manure, and fodder since ancient times. Korean also has eaten either raw or cooked seaweeds. A couple of reviews focused on the nutritional aspects of seaweeds as food and their associated health benefits (1-3). There are a number of papers dealing with pharmaceutical and medicinal aspects of seaweeds such as antioxidant (4,5), anti-inflammatory (6-9), antidiabetic (5,10), and antiviral (11) activity, as well. In case of anti-inflammatory properties of seaweeds, the active components were sulfated polysaccharides (6,9), ω-3 polyunsaturated fatty acids (7), and dichloromethane or ethanol extract (8). However, little is known about the secondary metabolites of seaweed showing anti-inflammatory effects.

During the evaluation of the cancer chemopreventive effects of Korean seaweed extracts by measuring the induction of phase II detoxification enzymes and the inhibition of inflammatory responses *in vitro*, 7 seaweed extracts showed the promising anti-inflammatory effects through the inhibition of nitric oxide (NO) and prostaglandin E_2 (PGE_2) production (12). Both NO and PGE_2 which are synthesized by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively, have been known to be important mediators of acute and chronic inflammation (13,14).

Inflammation and infection up-regulate the expression of several kinds of immune-related proteins in the affected cells. Pro-inflammatory enzymes such as iNOS and COX-2 are the part of them. Pro-inflammatory cytokines are important mediators of inflammation and autoimmune disease as well. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are major pro-inflammatory cytokines produced in macrophages activated by lipopolysaccharides (LPS) or other bacterial products (15-17). Transcription of pro-inflammatory mediators such as iNOS, COX-2, TNF-α, and IL-1β is increased by bacterial endotoxin via the nuclear factor-κB (NF-κB) activation (18,19). NF-κB is one of multipotent transcriptional factors and plays a key role in regulating the immune response including cancer, inflammatory, and autoimmune diseases (20-23). In resting cells, NF-κB is localized in cytosol as a homodimer or heterodimer, which is associated with inhibitor κB protein (IκB). NF-κB activation is mediated by IκB degradation. The resulting free NF-κB is translocated into the nucleus and induces gene transcription through binding to the *cis*-acting κB element (24).

In this study, to clarify the mechanism for anti-inflammatory activities of seaweed extracts, we investigated the anti-inflammatory effects on the production of pro-inflammatory mediators as well as the inhibitory effect on NF-κB activation of the selected seaweed extracts in the stimulated Raw 264.7 cells.

Materials and Methods

Seaweed extracts The seaweed extracts used for this study were prepared according to the previous report (12). Briefly, 7 dried seaweed powders, *Desmarestia viridis, Dictyopteris divaricata, Scytosiphon lomentaria, Ishige okamurae*, *Desmarestia ligulata*, *Dictyota coriaceum*, and *Sargassum yezoense*, were extracted 3 times with 95% ethanol at room temperature. The ethanol extract was obtained after evaporation of solvent and the each weight was measured.

Cell culture Raw 264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained at subconfluence in 95% air and 5% CO_2 humidified atmosphere at 37°C. Dulbecco's modified Eagle's Medium (DMEM, Hyclone, Logan, UT, USA) was used for Raw 264.7 cells cultivation. The medium was supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin (100 units/mL), and streptomycin (100 μg/mL).

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Cell viability The cytotoxicity of 7 seaweed extracts was evaluated using the WST-1-based colorimetric assay system (Takara Bio Inc., Shiga, Japan). Raw 264.7 cells (5×10^4 cells/well) were plated into 96-well plates in triplicate and stabilized at 37°C for 4 hr. Cells were then treated with 2 concentrations (20 and 40 μg/mL) of extracts and incubated for 2 hr. Then, the stimulants [10 μg/mL LPS and 100 units/mL interferon (IFN)-γ] were added if necessary and incubated for an additional 24 hr. At that point, the WST-1 reagent was added to the wells and incubation was continued for another 3 hr. The level of dye formed was then measured using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at the wavelength of 450 nm. The blank value without cells was subtracted from each experimental value as background.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) Raw 264.7 cells were cultured in the presence of each extract (20 μg/mL) in 6-well plates (1×10^6 cells/mL) for 2 hr and the stimulants (10 μg/mL LPS and 100 units/mL IFN-γ) were put together. After 4 (iNOS) or 6 hr (TNF-α and IL-1β) incubation, total cellular RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Total RNA (1 mg) was reverse-transcribed into cDNA with AccuPower RT/PCR Premix (Bioneer Co., Daejeon, Korea). PCR primers used in this study were listed below and were purchased from Bioneer: sense strand iNOS 5'-CTGCAGC ACTTGGATCAGGAACCTG-3', anti-sense strand iNOS 5'-GGGAGTAGCCTGTGTGCACCTGGAA-3'; sense strand TNF-α 5'-GGCAGGTTCTGTCCCTTTCACTC-3', anti-sense strand TNF-α 5'-CACTTGGTGGTTTGCTACGAC G-3'; sense strand IL-1β 5'-GCTACCTGTGTCTTTCCCG TGG-3', anti-sense strand IL-1β 5'-TTGTCGTTGCTTGGT TCTCCTTG-3'; sense strand β-actin 5'-AGGCTGTGCTG TCCCTGTATGC-3', ant-sense strand β-actin 5'-ACCCAA GAAGGAAGGCTGGAAA-3'. For each PCR, the following sequence was used: preheating at 94°C for 5 min, 94°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec, with a final extension phase at 72°C for 7 min. A variable number of cycles were used to ensure that amplification occurred in the linear phase. PCR amplification employed β-actin as the internal control.

Western blot analysis Raw 264.7 cells were treated with various concentrations (10 and 20 μg/mL for COX-2 Western blot; 20 μg/mL for IκB-α Western blot) of extracts in 6 well plates (1×10^6 cells/mL) for 2 hr. The culture continued for 18 hr (COX-2) or for 30 min (IκB-α) after adding the stimulants (10 μg/mL LPS and 100 units/mL IFN-γ). The cells were harvested and lysed directly in lysis buffer (0.5% Triton, 50 mM β-glycerophosphate/pH 7.2, 0.1 mM sodium vanadate, 2 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl urea, 2 mg/mL leupeptin, and 4 mg/mL aprotinin). The lysates were resolved by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. The membranes were blocked in Tris-buffered saline (pH 7.4) containing 0.5% Tween 20, and 5% nonfat dry milk, incubated with the first specific antibody in blocking solution for 5 hr, washed, and incubated for 1 hr with the developing second antibody. The protein bands were detected by chemiluminescence (Amersham Pharmacia Biotech, New Brunswick, NJ, USA).

Statistics Statistical analyses of data were performed by the Student's *t*-test to determine statistical significance. Values are given as mean±standard deviation (SD).

Results and Discussion

Cytotoxicity of 7 different seaweed extracts In the previous study, 7 seaweed extracts showed the promising anti-inflammatory effects through the inhibition of NO and PGE_2 production (12). Apparently, the cytotoxicity by sample treatment can show the same result as such inhibitory activities. To exclude the case, the cytotoxicity of seaweed extracts against Raw 264.7 cells was evaluated. Sometimes, there is a discrepancy between resting (non-stimulated) and differentiating (stimulated) cells in cytotoxicity, which may be due to the physiological change during the differentiation of cells. For the reason, we have performed the cell viability assay divided into 2 groups LPS/IFN-γ-stimulated and non-stimulated cells. As shown in Fig. 1, every seaweed extract was hardly cytotoxic to normal cells up to the concentration of 40 μg/mL. However, the extract of *D. ligulata* (Dl), *D. coriaceum* (Dc), and *S. yezoense* (Sy) showed a serious cytotoxicity to the LPS/IFN-γ-stimulated cells at the concentration of 40 μg/mL (Fig. 1). That is why we went forward the further study with 4 seaweed extracts *D. viridis* (Dv), *D. divaricata* (Dd), *S. lomentaria* (Sl), and *I. okamurae* (Io).

Anti-inflammatory activities of 4 different seaweed extracts To clarify the mechanism for anti-inflammatory activities of 4 seaweed extracts, we investigated the production of pro-inflammatory mediators as well as the inhibitory effect on NF-κB activation of the seaweed extracts in the stimulated Raw 264.7 cells by RT-PCR and Western blot analysis. As shown in Fig. 2A, non-activated cells (untreated) did not express any detectable level of iNOS, whereas the treatment of LPS/IFN-κ induced high levels of iNOS transcription. All seaweed extracts reduced iNOS expression dramatically in the stimulated Raw 264.7 cells, which could explain the marked decrease in NO production by the 4 seaweed extracts in the previous study (12) because iNOS is a NO-producing enzyme. However, all of them did not inhibit the COX-2 expression induced by LPS/IFN-κ. As shown in Fig. 3A, the effectiveness is Dd>Io>Sl. Interestingly, Dv did seldom affect the COX-2 expression, although it decreased the PGE_2 production by more than 40% at the concentration of 20 μg/mL in the previous report (3). In other extracts, the reduced level of COX-2 expression coincided with the decrease in PGE_2 production (12) because COX-2 is a PGE_2-producing enzyme.

In case of pro-inflammatory cytokines (Fig. 2B), 4 seaweed extracts significantly inhibited the LPS/IFN-γ-induced IL-1β production in Raw 264.7 cells at mRNA level. However, they did not affect TNF-α transcription at all. IL-1 is an important part of the inflammatory diseases such as bacterial infections, autoimmune disorders, noninfectious hepatitis, asthma, and graft-versus-host

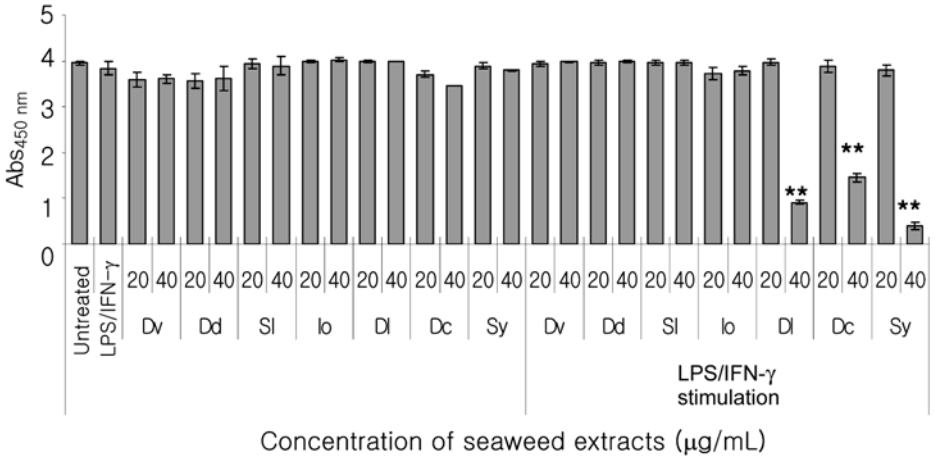

Fig. 1. Cytotoxicity of each seaweed extract on Raw 264.7 cells. Values shown are means±SD of 3 independent experiments. $^{**}p<0.01$, compared to cells treated with lipopolysaccharides (LPS).

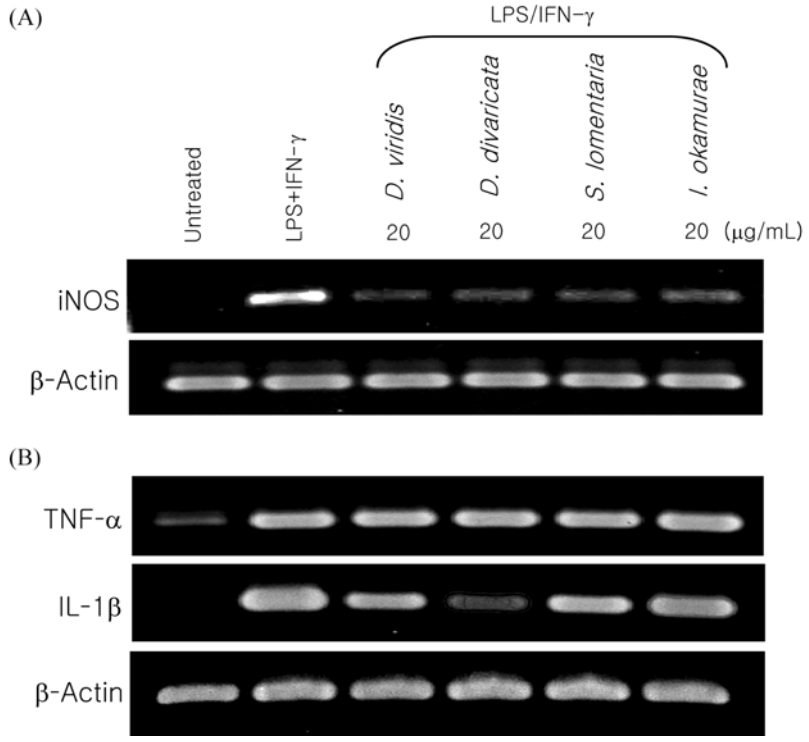

Fig. 2. RT-PCR analysis showing the effect of each seaweed extract on (A) iNOS and (B) pro-inflammatory cytokines transcription in Raw 264.7 cells.

disease (25). Moreover, IL-1 consistently induces COX-2 gene expression and PGE_2 synthesis in several cell lines and in primary human blood monocytes (26,27). IL-1β stimulated NO release and iNOS expression as well (28, 29). Taken together, IL-1β has a powerful activity in the inflammation and therefore specific inhibition activity of seaweed extracts on IL-1β production, not on TNF-α production, was considered as an important meaning in medicinal food.

The expression and production of iNOS, NO, COX-2, and pro-inflammatory cytokines in macrophages require the activation of NF-κB (30,31). NF-κB is a mammalian transcriptional factor regulating various genes that are important for LPS- and cytokine-induced immunity and inflammation (32,33). In the present study, we demonstrated that the extracts from Dd and Sl inhibited LPS/IFN-γ-induced activation of NF-κB as a consequence of the inhibition of IκB-α degradation (Fig. 3B). The other extracts from Dv and Io did not show the inhibition of IκB-a degradation, although they decreased the transcription level of iNOS as well as IL-1β (Fig. 2) and especially the extract of Io inhibited the COX-2 expression as well (Fig. 3 (A)).

We have used 2 stimulants, LPS and IFN-γ, for the activation of murine macrophage Raw 264.7 cells. LPS which is recognized by toll-like receptor 4 (TLR4)

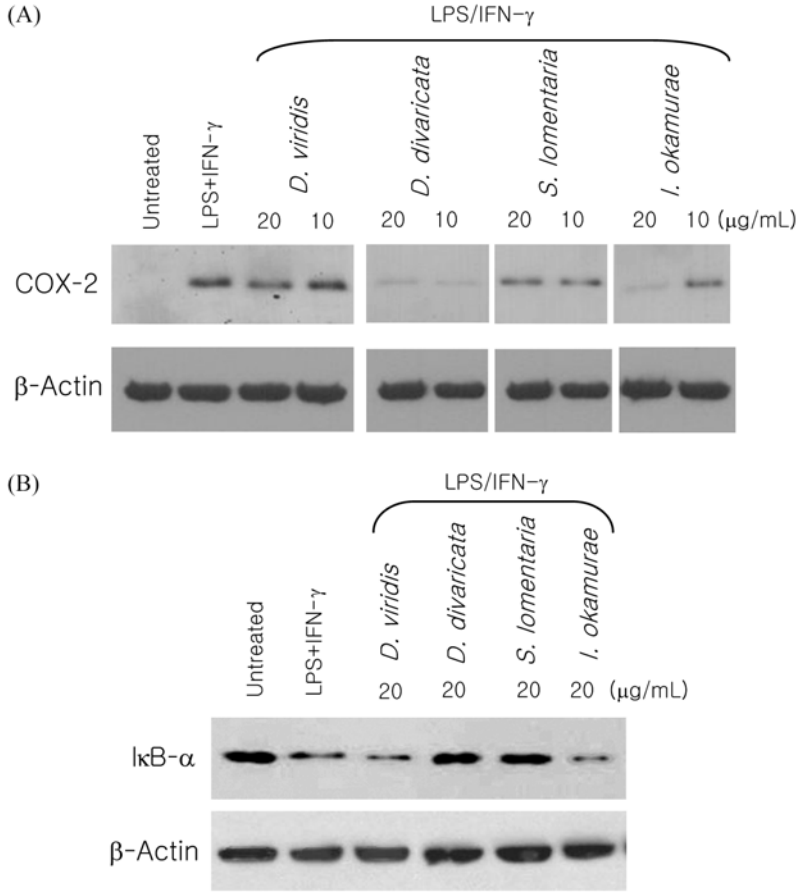

Fig. 3. Western blot analysis showing the effect of each seaweed extract on (A) COX-2 and (B) IκB-α degradation in Raw 264.7 cells.

activates NF-κB signaling (34), while IFN-γ binds to its receptor and activates STAT1 via the phosphorylation by janus kinase Jak. Activated STAT1 transfers into the nucleus and induces iNOS transcription (35). In our case, Dd and Sl inhibited IκB-α degradation suggesting that they should block the activation pathway by LPS resulting in the reduced expression of iNOS, COX-2, and IL-1β. In case of Dv and Io, they may decrease the iNOS expression via the inhibition of Jak-Stat pathway activated by IFN-γ that we have not examined yet. To elucidate the action mechanism of each seaweed extract in detail, we need to isolate the active component from the extract and investigate the upstream signal of IκB-α under the stimulation by LPS alone as well as the Jak-Stat pathways under the stimulation by IFN-γ alone.

In summary, our results showed that the extracts of Dv, Dd, Sl, and Io have anti-inflammatory activities in the activated macrophages. That is, we found that each extract is a potent inhibitor of the LPS/IFN-γ-induced iNOS, COX-2, and IL-1β production. We concluded that these 4 seaweeds should be good candidates for the development of functional food to prevent inflammation.

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References

1. Ito K, Hori K. Seaweed: Chemical composition and potential food uses. Food Rev. Int. 5: 101-144 (1989)
2. Darcy-Vrillon B. Nutritional aspects of the developing use of marine macroalgae for the human food industry. Int. J. Food Sci. Nutr. 44: S23-S35 (1993)
3. MacArtain P, Gill CI, Brooks M, Campbell R, Rowland IR. Nutritional value of edible seaweeds. Nutr. Rev. 65: 535-543 (2007)
4. Takamatsu S, Hodges TW, Rajbhandari I, Gerwick WH, Hamann MT, Nagle DG. Marine natural products as novel antioxidant prototypes. J. Nat. Prod. 66: 605-608 (2003)
5. Iwai K. Antidiabetic and antioxidant effects of polyphenols in brown alga *Ecklonia stolonifera* in genetically diabetic KK-A^{y} mice. Plant Food Hum. Nutr. 63: 163-169 (2008)
6. Ushakova NA, Preobrazhenskaya ME, D'Incecco A, Piccoli A, Totani L, Tinari N, Morozevich GE, Berman AE, Bilan MI, Usov AI, Ustyuzhanina NE, Grachev AA, Sanderson CJ, Kelly M, Rabinovich GA, Iacobelli S, Nifantiev NE. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of 9 different fucoidans from brown seaweeds. Glycobiology 17: 541-552 (2007)
7. Khan MN, Cho JY, Lee MC, Kang JY, Park NG, Fujii H, Hong YK. Isolation of two anti-inflammatory and one pro-inflammatory polyunsaturated fatty acids from the brown seaweed *Undaria pinnatifida*. J. Agr. Food Chem. 55: 6984-6988 (2007)
8. Kang JY, Khan MN, Park NH, Cho JY, Lee MC, Fujii H, Hong YK. Antipyretic, analgesic, and anti-inflammatory activities of the seaweed *Sargassum fulvellum* and *Sargassum thunbergii* in mice. J. Ethnopharmacol. 116: 187-190 (2008)

9. Medeiros VP, Queiroz KC, Cardoso ML, Monteiro GR, Oliveira FW, Chavante SF, Guimaraes LA, Rocha HA, Leite EL. Sulfated galactofucan from *Lobophora variegata*: Anticoagulant and anti-inflammatory properties. Biochemistry -Moscow+ 73: 1018-1024 (2008)
10. Kim MS, Kim JY, Choi WH, Lee SS. Effects of seaweed supplementation on blood glucose concentration, lipid profile, and antioxidant enzyme activities in patients with type 2 diabetes mellitus. Nutr. Rep. Rev. 2: 62-67 (2008)
11. Wang H, Ooi EV, Ang PO. Antiviral activities of extracts from Hong Kong seaweeds. J. Zhejiang Univ. Sci. B. 9: 969-976 (2008)
12. Lee SB, Lee JY, Song DG, Pan CH, Nho CW, Kim MC, Lee EH, Jung SH, Kim HS, Kim YS, Um BH. Cancer chemopreventive effects of Korean seaweed extracts. Food Sci. Biotechnol. 17: 613-622 (2008)
13. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. 126: 131-138 (1982)
14. Wang HQ, Smart RC. Overexpression of protein kinase C-alpha in the epidermis of transgenic mice results in striking alterations in phorbol ester-induced inflammation and COX-2, MIP-2, and TNF-alpha expression but not tumor promotion. J. Cell Sci. 112: 3497-3506 (1999)
15. Arend WP, Dayer JM. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor alpha in rheumatoid arthritis. Arthritis Rheum. 38: 151-160 (1995)
16. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu. Rev. Immunol. 14: 397-440 (1996)
17. Brennan FM, Maini RN, Feldmann M. Role of pro-inflammatory cytokines in rheumatoid arthritis. Springer Semin. Immun. 20: 133-147 (1998)
18. Hewett JA, Roth RA. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. Pharmacol. Rev. 45: 382-411 (1993)
19. Kubes P, McCafferty DM. Nitric oxide and intestinal inflammation. Am. J. Med. 109: 150-158 (2000)
20. Donadelli R, Abbate M, Zanchi C, Corna D, Tomasoni S, Benigni A, Remuzzi G, Zoja C. Protein traffic activates NF-κB gene signaling and promotes MCP-1-dependent interstitial inflammation. Am. J. Kidney Dis. 36: 1226-1241 (2000)
21. Ray A, Siegel MD, Prefontaine KE, Ray P. Anti-inflammation: Direct physical association and functional antagonism between transcription factor NF-κB and the glucocorticoid receptor. Chest 107: 139S (1995)
22. Giuliani C, Napolitano G, Bucci I, Montani V, Monaco F. NF-κB transcription factor: Role in the pathogenesis of inflammatory, autoimmune, and neoplastic diseases and therapy implications. Clin. Ther. 152: 249-253 (2001)
23. Notarbartolo M, Poma P, Perri D, Dusonchet L, Cervello M, D'Alessandro N. Antitumor effects of curcumin, alone or in combination with cisplatin or doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-κB activation levels and in IAP gene expression. Cancer Lett. 224: 53-65 (2005)
24. Karin M. The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. J. Biol. Chem. 274: 27339-27342 (1999)
25. Dinarello CA. Biologic basis for interleukin-1 in disease. Blood 87: 2095-2147 (1996)
26. Dinarello CA, Thompson RC. Blocking IL-1: interleukin 1 receptor antagonist *in vivo* and *in vitro*. Immunol. Today 12: 404-410 (1991)
27. Reznikov LL, Kim SH, Westcott JY, Frishman J, Fantuzzi G, Novick D, Rubinstein M, Dinarello CA. IL-18 binding protein increases spontaneous and IL-1-induced prostaglandin production via inhibition of IFN-gamma. P. Natl. Acad. Sci. USA 97: 2174-2179 (2000)
28. Ahsan S, Lacey M, Whitehead SA. Interactions between interleukin-1 beta, nitric oxide, and prostaglandin E2 in the rat ovary: Effects on steroidogenesis. Eur. J. Endocrinol. 137: 293-300 (1997)
29. Ellman C, Corbett JA, Misko TP, McDaniel M, Beckerman KP. Nitric oxide mediates interleukin-1-induced cellular cytotoxicity in the rat ovary. A potential role for nitric oxide in the ovulatory process. J. Clin. Invest. 92: 3053-3056 (1993)
30. Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA. Possible new role for NF-kappaB in the resolution of inflammation. Nat. Med. 7: 1291-1297 (2001)
31. Renard P, Raes M. The proinflammatory transcription factor NFkappaB: A potential target for novel therapeutical strategies. Cell. Biol. Toxicol. 15: 341-344 (1999)
32. Barnes PJ, Karin M. Nuclear factor-kappaB: A pivotal transcription factor in chronic inflammatory diseases. New Engl. J. Med. 336: 1066-1071 (1997)
33. Makarov SS. NF-kappaB as a therapeutic target in chronic inflammation: Recent advances. Mol. Med. Today 6: 441-448 (2000)
34. Zhang G, Ghosh S. Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. J. Endotoxin Res. 6: 453-457 (2000)
35. Takaoka A, Yanai H. Interferon signalling network in innate defence. Cell Microbiol. 8: 907-922 (2006)