3-Deoxysappanchalcone Inhibits Tumor Necrosis Factor-α-Induced Matrix Metalloproteinase-9 Expression in Human Keratinocytes through Activated Protein-1 Inhibition and Nuclear Factor-Kappa B DNA Binding Activity

Ui Joung YOUN,[a] Kung-Woo NAM,[b] Hui-Seong KIM,[c,d] Goya CHOI,[e] Woo Seok JEONG,[e] Mi Young LEE,[e] and Sungwook CHAE*,[e]

[a] *Natural Product Chemistry Laboratory, College of Pharmacy, Ewha Womans University; Seoul 120–750, Korea:* [b] *Natural Products Research Institute, College of Pharmacy, Seoul National University; Seoul 151–742, Korea:* [c] *Biomolecular Science, University of Science and Technology; Daejon 305–350, Korea:* [d] *Immune Modulator Research Center, Korea Research Institute of Bioscience and Biotechnology; Daejon 363–883, Korea: and* [e] *Aging Research Center, Korea Institute of Oriental Medicine; Daejeon 305–811, Korea.*

Received October 22, 2010; accepted March 3, 2011; published online March 10, 2011

Tumor necrosis factor α (TNF-α), which is a primary cytokine responsible for inflammatory responses in skin, induces the synthesis of matrix metalloproteinase-9 (MMP-9), which causes skin aging. The protective effects of 3-deoxysappanchalcone against TNF-α-induced damage was investigated using human skin keratinocytes. The results showed that 3-deoxysappanchalcone inhibited MMP-9 expression at the protein and mRNA level, by blocking the activation of activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB). Taken together, the inhibitory activity of 3-deoxysappanchalcone on MMP-9 expression and production in TNF-α-treated cells was found to be mediated by the suppression of AP-1 and NF-κB activation.

Key words 3-deoxysappanchalcone; matrix metalloproteinase-9; activator protein-1; nuclear factor kappa B; skin aging

Tumor necrosis factor-α (TNF-α) is a well-known modulator of matrix metalloproteinase (MMP) gene expression and it is a primary cytokine responsible for eliciting inflammatory responses in the skin. Chronic exposure of epidermal cells to TNF-α resulted in an unbalanced MMP production, which may further result in irreversible damage to the inflamed epidermis. A relationship between TNF-α treatment and increased MMP-9 production was consistently seen in the TNF-α exposed epidermal cells.[1)] It is important to inhibit the production of MMP-9 in order to prevent skin damage and allow skin repair. Transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) might be involved in the production of MMP-9.[2)] Therefore, many studies have described pharmacological and cosmetic agents that inhibit MMP-9 or block its transcriptional regulation, leading to skin renewal.

In the course of screening MMP-9 inhibitors of bioactive compounds, we found that 3-deoxysappanchalcone effectively inhibited TNF-α-induced MMP-9 activation through AP-1 and NF-κB inhibition. In recent years, studies on pharmacological characterizations of 3-deoxysappanchalcone have concluded that it has several biological properties, including apoptosis-inducing activity,[3)] anti-allergic activity,[4)] anti-influenza viral activity,[5)] anti-inflammatory activity,[6)] and antioxidant activity.[7)] However, to the best of our knowledge, the MMP-9 inhibitory activity of 3-deoxysappanchalcone has not yet been investigated.

Here, we demonstrated the ability of 3-deoxysappanchalcone to reduce MMP-9 production via AP-1 and NF-κB inhibition.

MATERIALS AND METHODS

Reagents 3-Deoxysappanchalcone was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Recombinant TNF-α was purchased from R&D Co. (Minneapolis, MN, U.S.A.). Pyrrolidine dithiocarbamate (PDTC), AP-1 antibody (A5968), and β-actin (A1978) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). p-IκBα and IβBα antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The primary antibody against MMP-9 (#2270) was obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). The NF-κB luciferase reporter gene (pNifty-Luc) was obtained from InvivoGen (San Diego, CA, U.S.A.). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell Culture Human keratinocytes were purchased from Invitrogen (Carlsbad, CA, U.S.A.) and were cultured in Epilife® medium (Gibco-BRL) (Burlington, ON, U.S.A.), which contained the MEPI500CA medium supplemented with bovine pituitary extract (0.2% v/v), bovine insulin (5 μg/ml), hydrocortisone (0.18 μg/ml), bovine transferrin (5 μg/ml), human epidermal growth factor (0.2 ng/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Cells were incubated with or without TNF-α, an inducer, for the indicated time periods.

Western Blot and Reverse Transcription Polymerase Chain Reation (RT-PCR) For Western blot analysis, cells were harvested at the indicated times, and washed twice with phosphate buffer saline (PBS). The harvested cells were then lysed on ice for 30 min in 100 μl of lysis buffer (120 mM NaCl, 40 mM Tris [pH 8], 0.1% NP 40) and centrifuged at 13000×g for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, U.S.A.), which were then incubated with the primary antibody. The membranes were further incubated with secondary im-

* To whom correspondence should be addressed. e-mail: kendall@kiom.re.kr

munoglobulin G-horseradish peroxidase conjugates (Pierce, Rockland, IL, U.S.A.) and then exposed to X-rays. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, U.K.).

Total RNA was isolated using the TRIzol reagent kit. A total of 1 μg of RNA was used for the reverse transcription (RT) reaction, which also contained the AccuPower RT premix and random hexamers. Total cDNA corresponding to 1 μg of RNA was used in PCR reactions. The following PCR primers were used in this study: MMP-9, 5′-CCC GGA CCA AGG ATA CAG-3′ (sense) and 5′-GGC TTT CTC TCG GTA CTG-3′ (antisense) and GADPH, 5′-ATT GTT GCC ATC AAT GAC CC-3′ (sense) and 5′-AGT AGA GGC AGG GAT GAT GT-3′ (antisense). Amplification products were resolved by 1.5% agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed under ultraviolet light. All the primers were purchased from Bioneer (Daejeon, Korea).

Determination of MMP-9 Activity The measurement of the secreted active MMP-9 was performed using the Quantikine® human MMP-9 kit (R&D Systems Inc., Minneapolis, MN, U.S.A.) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA) For electrophoretic mobility shift assay (EMSA), 2 μg of nuclear protein was incubated with 10×Gel Shift Binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM dithiothreitol (DTT), 250 mM NaCl, and 50 mM Tris–HCl), 0.25 mg/ml poly(dI)–poly(dC), and IRDye 700-labeled NF-κB oligonucleotide, or AP-1 oligonucleotide (LI-COR Inc., Lincoln, NE, U.S.A.). After incubation at room temperature for 30 min, the samples were loaded on a pre-run 8% polyacrylamide gel and electrophoresis was continued at 30 mA for 90 min. The signal was then detected and quantified with Odyssey infrared imaging system (LI-COR Inc., Lincoln, NE, U.S.A.).

Transient Transfection and Luciferase Assay A day before transfection, cells were sub-cultured at a density of 1×10^5 cells in 24-well dish to maintain approximately 60—80% confluent. The cells were transiently co-transfected with the plasmid harboring the NF-κB promoter and Renilla luciferase expression using the transfection reagent FuGeneHD according to the manufacturer's instructions (Roche, Mannheim, Germany). After overnight transfection, cells were treated with various concentrations of 3-deoxysappanchalcone followed by TNF-α treatment. Cells were then washed twice with PBS and lysed with cell lysis buffer. After vortex-mixing and centrifugation at 12000×g for 1 min at 4 °C, the supernatant was stored at −70 °C for the luciferase assay. After 20 μl of the cell extract was mixed with 50 μl of the luciferase assay reagent at room temperature, the mixture was placed in a luminometer to measure the light produced. The luciferase activity was normalized to transfection efficiency monitored by renilla expression.

Statistical Analysis All measurements were made in triplicate and all values were represented as means±standard error (S.E.). The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the differences. $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

In the course of screening MMP-9 inhibitors, we found that 3-deoxysappanchalcone effectively inhibited TNF-α-induced MMP-9 activation by inducing AP-1 and NF-κB inhibition. The chemical structure (Fig. 1) was confirmed by the

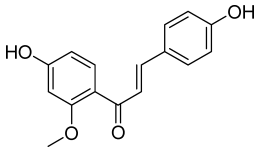

Fig. 1. Chemical Structure of 3-Deoxysappanchalcone

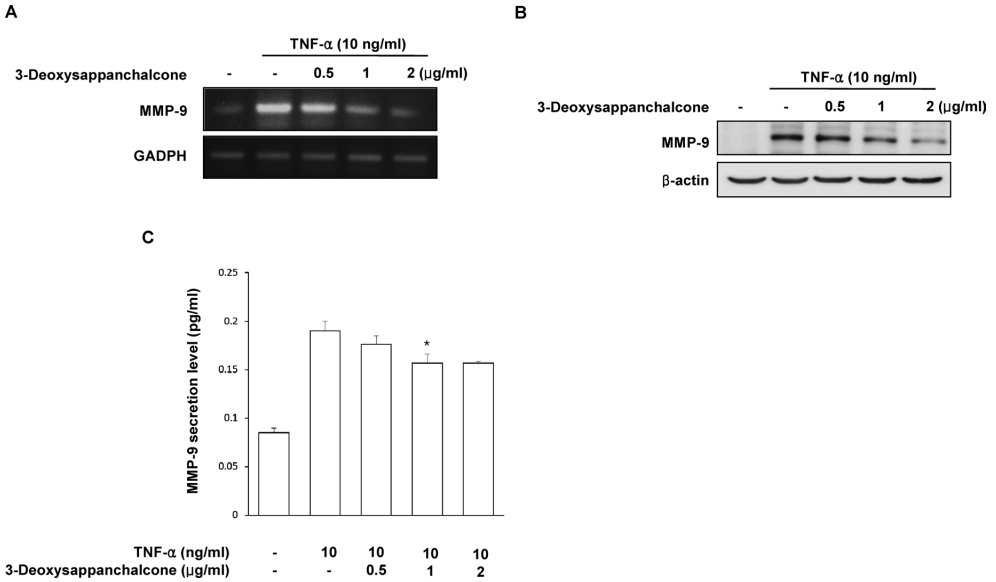

Fig. 2. Inhibitory Activity of 3-Deoxysappanchalcone on TNF-α-Induced MMP-9 mRNA Level, Protein Expression, and Activity in HEKa Cells

(A) HEKa cells were pretreated with the indicated concentration of 3-deoxysappanchalcone for 1 h and then treated with 10 ng/ml TNF-α for 18 h. The levels of MMP-9 were determined by RT-PCR. (B) Inhibition of TNF-α-induced MMP-9 protein expression by 3-deoxysappanchalcone. Cells were pretreated with the indicated concentrations of 3-deoxysappanchalcone for 1 h and then treated with TNF-α for 36 h. The levels of MMP-9 were determined by Western blotting analysis. (C) Inhibition of MMP-9 activity measured by ELISA. * Significantly different from TNF-α treatment ($p<0.05$).

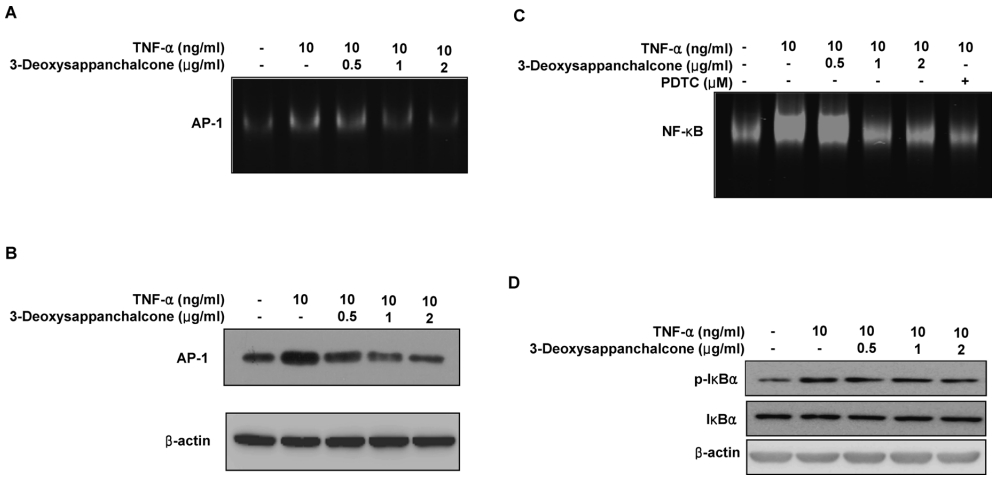

Fig. 3. Suppression of TNF-α-Induced AP-1 and NF-κB Activation by 3-Deoxysappanchalcone in HEKa Cells

(A) HEKa cells were pretreated with various concentrations of 3-deoxysappanchalcone for 1 h and then stimulated with TNF-α for 2 h. The DNA-binding activities of AP-1 were determined by EMSA, and (B) AP-1 level was determined by Western blot analysis. (C) HEKa cells were pretreated with various concentrations of 3-deoxysappanchalcone for 1 h and then stimulated with TNF-α for 2 h. The DNA-binding activities of NF-κB were determined by EMSA, and (D) the IκBα level was determined by Western blot analysis.

direct comparison of spectral data reported in the literature.[8)]

Chalcones, like 3-deoxysappanchalcone, are a group of flavonoids known to possess antioxidant and anti-inflammatory activities.[9,10)] During inflammation, MMP-9 is upregulated in response to various inflammatory signals.[11)] Therefore, anti-inflammatory agents are expected to reduce MMP-9 activity.

We found that TNF-α treatment markedly increased the MMP-9 mRNA levels, as evidenced by RT-PCR analysis, whereas 3-deoxysappanchalcone blocked the expression of MMP-9 mRNA in a dose-dependent manner (Fig. 2A). The results of Western blot analysis were consistent with that of RT-PCR, and revealed that 3-deoxysappanchalcone inhibited the TNF-α-induced expression of the MMP-9 protein (Fig. 2B). Moreover, the decreased MMP-9 expression produced by 3-deoxysappanchalcone correlated with suppression of MMP-9 activity (Fig. 2C). As transcriptional regulation of MMP genes involves the activation of the transcriptional factor, AP-1,[12)] the DNA binding activity of 3-deoxysappanchalcone on AP-1 was measured. As shown in Fig. 3A, incubation of cells with TNF-α increased the DNA binding activity of AP-1, whereas 3-deoxysappanchalcone was found to inhibit AP-1 activity. To measure the inhibition of AP-1 activity by 3-deoxysappanchalcone in terms of protein expression, Western blot analysis was performed. As shown in Fig. 3B, the decrease in AP-1 protein expression was correlated with treatment with increasing concentrations of 3-deoxysappanchalcone.

As previously mentioned, NF-κB and AP-1 activation are closely related. In order to evaluate the selectivity of 3-deoxysappanchalcone on NF-κB and AP-1, we further studied the activation of NF-κB in nuclear and cytosolic extracts of HEKa cells stimulated with TNF-α 1 h after treatment with 3-deoxysappanchalcone. Nuclear extracts obtained after 1 h of TNF-α stimulation were incubated with pyrrolidine dithiocarbamate (PDTC), a specific NF-κB inhibitor and then a gel shift assay was performed. While the intensity of the NF-κB band was reduced after treatment with 3-deoxysappanchalcone, the NF-κB band obtained with PDTC treatment remained unchanged, as compared to control, and TNF-

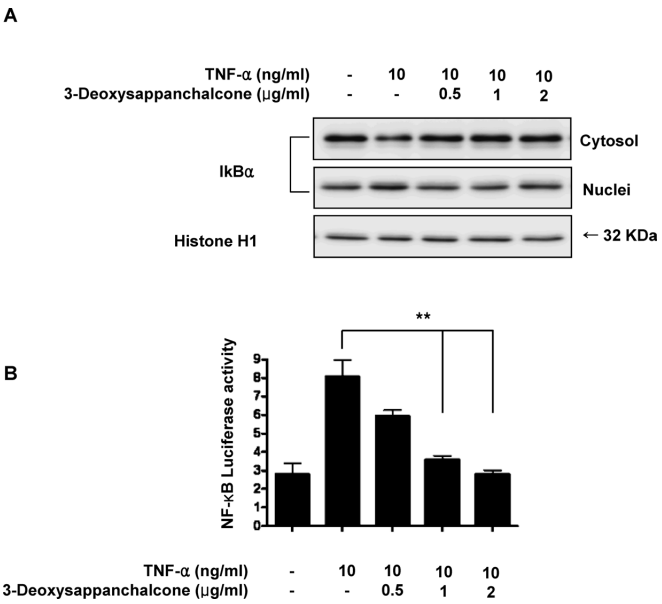

Fig. 4. The Inhibition of NF-κB by 3-Deoxysappanchalcone in HEKa Cells

(A) HEKa cells were pretreated with various concentrations of 3-deoxysappanchalcone for 1 h and then stimulated with TNF-α for 1 h. The IκBα level was determined by Western blot analysis. (B) HEKa cells were transfected with the NF-κB-luciferase construct (1 μg per well). After overnight, cells were treated with 3-deoxysappanchalcone, cell lysates were mixed with a luciferase substrate, and the luciferase activity was measured by the luminometer and normalized to transfection efficiency monitored by renilla expression. Data are expressed as the means$\pm$S.E. in triplicate experiments. ** Significantly different from TNF-α treated cells ($p<0.01$).

α-induced NF-κB was directly inhibited by 3-deoxysappanchalcone (Fig. 3C). As translocation of NF-κB to the nucleus is processed by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα,[13)] we then examined whether the inhibition of TNF-induced NF-κB activation was due to the inhibition of IκBα degradation by using Western blot analysis. As shown in Fig. 3D, IκBα degradation and phosphorylation was not affected by 3-deoxysappanchalcone. 3-Deoxysappanchalcone might have directly inhibited NF-κB activity, which was not related to the inhibition of IκBα phosphorylation. The inhibition of TNF-α-induced NF-κB

activity was not affected by the level of IκBα phosphorylation. For further investigation on the inhibition of IκBα phosphorylation, IκBα expression level between nuclei and cytosol fractions and NF-κB luciferase activity were measured. As shown in Fig. 4A, the expressions of IκBα in nuclei and cytosol remained unchanged after treatment of TNF-α, meaning IκBα degradation and phosphorylation was not affected by 3-deoxysappanchalcone. The result in NF-κB luciferase assay in Fig. 4B showed that NF-κB activity was directly inhibited by 3-deoxysappanchalcone, which was consistent as Fig. 3C. Taken together, 3-deoxysappanchalcone specifically inhibited the activation of AP-1 without affecting IκBα phosphorylation, thereby suggesting that the MMP-9 inhibition by 3-deoxysappanchalcone is related to the inhibition of AP-1 and NF-κB.

Acknowledgements This study was supported by Grants (C10010 and K11101) received from Korea Institute of Oriental Medicine.

REFERENCES

1) Bahar-Shany K., Ravid A., Koren R., *J. Cell. Physiol.*, **222**, 729—737 (2010).
2) Holvoet S., Vincent C., Schmitt D., Serres M., *Exp. Cell Res.*, **290**, 108—119 (2003).
3) Lee Y. M., Jeong G. S., Lim H. D., An R. B., Kim Y. C., Kim E. C., *Toxicol. In Vitro*, **24**, 776—782 (2010).
4) Yodsaoue O., Cheenpracha S., Karalai C., Ponglimanont C., Tewtrakul S., *Phytother. Res.*, **23**, 1028—1031 (2009).
5) Liu A. L., Shu S. H., Qin H. L., Lee S. M., Wang Y. T., Du G. H., *Planta Med.*, **75**, 337—339 (2009).
6) Liu Y. C., Hsieh C. W., Wu C. C., Wung B. S., *Life Sci.*, **80**, 1420—1430 (2007).
7) Anto R. J., Sukumaran K., Kuttan G., Rao M. N., Subbaraju V., Kuttan R., *Cancer Lett.*, **97**, 33—37 (1995).
8) Yahara S., Ogata T., Saijo R., Konishi R., Yamahara J., Miyahara K., Nohara T., *Chem. Pharm. Bull.*, **37**, 979—987 (1989).
9) Ban H. S., Suzuki K., Lim S. S., Jung S. H., Lee S., Ji J., Lee H. S., Lee Y. S., Shin K. H., Ohuchi K., *Biochem. Pharmacol.*, **67**, 1549—1557 (2004).
10) Herencia F., Ferrándiz M. L., Ubeda A., Guillén I., Dominguez J. N., Charris J. E., Lobo G. M., Alcaraz M. J., *FEBS Lett.*, **453**, 129—134 (1999).
11) Saja K., Babu M. S., Karunagaran D., Sudhakaran P. R., *Int. Immunopharmacol.*, **7**, 1659—1667 (2007).
12) Crawford H. C., Matrisian L. M., *Enzyme Protein*, **49**, 20—37 (1996).
13) Pandey M. K., Sandur S. K., Sung B., Sethi G., Kunnumakkara A. B., Aggarwal B. B., *J. Biol. Chem.*, **282**, 17340—17350 (2007).