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Cinnamon Extract Promotes Type I Collagen Biosynthesis via Activation of IGF-I Signaling in Human Dermal Fibroblasts

Naoko Takasao,[†,§] Kentaro Tsuji-Naito,[‡,§] Seiko Ishikura,[‡] Azusa Tamura,[‡] and Mitsugu Akagawa*[,†]

[†]Department of Biological Chemistry, Division of Applied Life Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai 599-8531, Japan

[‡]DHC Corporation Laboratories, Division 2, 2-42 Hamada, Mihama-ku, Chiba 261-0025, Japan

ABSTRACT: The breakdown of collagenous networks with aging results in hypoactive changes in the skin. Accordingly, reviving stagnant collagen synthesis can help protect dermal homeostasis against aging. We searched for type I collagen biosynthesis-inducing substances in various foods using human dermal fibroblasts and found that cinnamon extract facilitates collagen biosynthesis. Cinnamon extract potently up-regulated both mRNA and protein expression levels of type I collagen without cytotoxicity. We identified cinnamaldehyde as a major active component promoting the expression of collagen by HPLC and NMR analysis. Since insulin-like growth factor-I (IGF-I) is the most potent stimulator of collagen biosynthesis in fibroblasts, we examined the effect of cinnamaldehyde on IGF-I signaling. Treatment with cinnamaldehyde significantly increased the phosphorylation levels of the IGF-I receptor and its downstream signaling molecules such as insulin receptor substrate-1 and Erk1/2 in an IGF-I-independent manner. These results suggested that cinnamon extract is useful in antiaging treatment of skin.

KEYWORDS: *cinnamaldehyde, cinnamon, collagen, insulin-like growth factor, fibroblast*

INTRODUCTION

A well-balanced array of extracellular matrix (ECM) components gives skin its healthy structural and functional conditions. The dermal ECM is synthesized from fibroblasts and primarily comprises fibrillar collagen bundles and elastic fibers, which provide skin with its mechanical strength and resiliency, respectively.[1] Of the many types of collagen, type I is the most abundant in normal skin, comprising more than 90% of its dry weight.[2] Collagen precursor molecules (i.e., procollagen molecules) are synthesized in the endoplasmic reticulum and are transported to the Golgi apparatus for secretion into the extracellular spaces, where they are enzymatically processed to form mature collagen.[3] Both intrinsic and extrinsic aged skin (e.g., the emergence of wrinkles and elastosis and loss of skin tone) can be caused by reductions in the amount and organization of the ECM in the dermis,[4,5] suggesting that the promotion of collagen synthesis could help protect dermal homeostasis against aging.[6]

Insulin-like growth factor-I (IGF-I) is a single-chain polypeptide homologous to proinsulin (49%).[7] IGF-I is involved in cell proliferation, survival, migration, and production of ECM,[8] including collagen fibrils, in dermal fibroblasts.[9] IGF-I signaling pathways in various cell systems have been well documented,[10] demonstrating that IGF-I action results primarily from the activation of the IGF-I receptor and its satellites, which include Src homology 2 domain containing transforming protein 1 and insulin receptor substrate-1 (IRS-1). Subsequent pathways activated include the phosphoinositide 3-kinase and/or mitogen-activated protein (MAP) kinase pathways.[11] IGF-I is considered to be the major growth factor responsible for the induction of collagen synthesis in dermal fibroblasts.[12]

Cinnamon bark or cortex has been used as a natural spice and as a component of traditional Chinese medicine for centuries to treat gastritis, blood circulation disturbances, and inflammatory disease.[13,14] In addition, in recent years, its prophylactic and therapeutic effects against diabetes have attracted wide attention, implicating their beneficial actions mimicking insulin.[15] We previously reported that thioctic acid potentiates collagen biosynthesis through transforming growth factor-β (TGF-β) signaling in normal human dermal fibroblasts.[16] Our study implicates that bioactive molecules could be growth factor-mimics to promote collagen synthesis in the dermis, but little work has been done to evaluate the effect of natural plant extracts on collagen synthesis in a growth factor-independent manner. In this study, we searched for type I collagen biosynthesis-inducing medicinal food by using normal human dermal fibroblasts and demonstrated for the first time that cinnamon extract strongly facilitates type I collagen biosynthesis. Furthermore, we identified cinnamaldehyde as a major active component, and revealed that its mechanism of action could be attributable to the activation of IGF-I signaling in an IGF-I-independent manner. Our results suggested that cinnamon extract may be useful in preventing skin aging.

MATERIALS AND METHODS

Materials. Hybond-ECL membrane was obtained from GE Healthcare UK, Ltd. (Buckinghamshire, UK). The protease inhibitor cocktail was obtained from Roche Applied Science (Mannheim, Germany). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). The EZ-ECL chemiluminescence detection kit for chemiluminescence detection was from Biological Industries (Kibbutz Beit Haemek, Israel). Ethylenediamine tetraacetic acid (EDTA), skim milk powder, Tween 20, and dithiothreitol (DTT) were purchased from Wako Pure Chemical (Osaka, Japan). DMEM

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medium, penicillin, streptomycin, cinnamyl alcohol, HEPES, phosphatase inhibitor cocktail, and Triton X-100 were purchased from Nacalai Tesque (Kyoto, Japan). Cinnamaldehyde, 2-methoxycinnamaldehyde, cinnamic acid, cinnamyl acetate, and α-methylcinnamaldehyde were from Sigma-Aldrich (St. Louis, MO, USA). IGF-1 was purchased from PeproTech (Rocky Hill, NJ, USA). A variety of powdered spices were purchased at local supermarkets. Spice extracts were prepared by the extraction of spice powder (10 mg) with 1.0 mL of dimethyl sulfoxide (DMSO) at room temperature for 60 min using a rotator. The resultant solution was centrifuged at 16,000*g* for 10 min, and the supernatant was removed and used as a 10 mg/mL spice extract. The extracts were stored at −20 °C until use.

Cell Culture. Normal human skin fibroblast NB1RGB cells (RCB0222) were provided by the RIKEN Bio-Resource Center (Tsukuba, Japan) and maintained in a 5% CO_2 humidified atmosphere at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were seeded into 10-cm Petri dishes and cultured until they reached confluence. The cells were starved in serum-free DMEM for 24 h before they were treated with spice extracts in the serum-free medium.

Cell Growth Assay. NB1RGB cells were seeded in 96-well plates (3,000 cells/well). After 24 h of culture in 10% FBS-DMEM, the medium in the 96-well plate was exchanged with 100 μL of serum-free DMEM. Twenty-four hours later, the cells were treated with Ceylon cinnamon extract (0−100 μg/mL) in serum-free medium (100 μL) for 24 h. After the treatment, cell viability was determined by the WST-8 reduction assay using a Cell Count Reagent SF kit (Nacalai Tesque) according to the manufacturer's instructions.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblot Analysis. After treatment, the cells were lysed with cold RIPA buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) containing 1× protease inhibitor cocktail (Nacalai Tesque) and then sonicated briefly. The protein concentration of each lysate was measured with BCA Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA, USA) or Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA). After reduction with 50 mM DTT, the proteins were run on 8% SDS−polyacrylamide gels, transferred to a nitrocellulose membrane, incubated with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 1 h for blocking, washed three times in TBS-T for 10 min, and treated overnight with the primary antibody at 4 °C. After washing three times with TBS-T, the blots were further incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated IgG antibody in TBS-T. To enhance the immunoreactions for phosphorylated proteins, we used an immunoreaction enhancer solution, Can Get Signal solutions 1 and 2 (Toyobo, Osaka, Japan) to dilute primary and secondary antibodies, respectively. Blots were then washed three times in TBS-T before visualization. An ECL kit and LAS-4000 system (Fujifilm, Tokyo, Japan) were used for chemiluminescence detection. The following antibodies were used: anti-β-actin mouse monoclonal IgG (AC-15) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:4,000), donkey antigoat IgG-HRP (Santa Cruz Biotechnology; 1:4,000), goat antimouse IgG-HRP (Santa Cruz Biotechnology; 1:4,000), mouse anti-IGF-I receptor monoclonal IgG (Santa Cruz Biotechnology; 1:4,000), rabbit anti-IRS-1 polyclonal IgG (Santa Cruz Biotechnology; 1:4,000), goat antirabbit IgG-HRP (Cell Signaling Technology, Danvers, MA, USA; 1:4,000), rabbit antiphospho-IGF-I receptor β (Tyr1131) polyclonal IgG (Cell Signaling Technology; 1:2,000), rabbit antiphospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) polyclonal IgG (Cell Signaling Technology; 1:4,000), rabbit anti-p44/42 MAPK (Erk1/2) monoclonal IgG (Cell Signaling Technology; 1:4,000), goat anticollagen type I polyclonal antibody (Millipore, Billerica, MA, USA), and rabbit antiphospho-IRS-1 (Tyr612) polyclonal IgG (Invitrogen; 1:4,000).

Real-Time PCR for Type I Collagen α1 Subunit. Type I collagen α1 subunit (COL1A1) mRNA induction was determined using real-time PCR. NB1RGB cells were plated onto 6-well plates and cultured until they reached confluence. The cells were starved in serum-free DMEM for 24 h before they were treated with cinnamon extract or components in the serum-free medium. After cells were treated as indicated, total RNA was collected using the RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA, USA). RNA was eluted into RNase-free water, quantified by UV absorbance, and aliquoted at −80 °C until use. Reverse transcription (RT)-PCR was performed on equal amounts of total RNA (500 ng) using the Primescript II High RT-PCR kit (Takara Bio, Shiga, Japan). Real time-PCR was performed using TaqMan gene expression assay (Applied Biosystems) for COL1A1 (Hs00164004_m1) and eukaryotic 18S rRNA (Hs99999901_s1), according to the manufacturer's instructions on the Applied Biosystems 7500 Real-Time PCR System. Samples were run in triplicate, and relative quantification was performed by comparing the values obtained at the fractional cycle number at which the amount of amplified target reaches a fixed threshold (threshold cycle).

Immunofluorescence Microscopy. NB1RGB cells were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min, washed three times with PBS, and permeabilized with 1% Triton X-100-containing PBS for 20 min. After washing three times with PBS, cells were blocked with 5% skim milk in TBS-T. After washing with TBS-T, cultures were incubated with anticollagen type I polyclonal antibody (Millipore) overnight at 4 °C. They were washed three times with TBS-T and exposed to Alexa Fluor 488 goat antirabbit IgG antibody (Invitrogen) for 1 h. After rinsing three times with TBS-T, the cells were incubated with Hoechst 33342 (Wako Pure Chemical) for 1 h at room temperature. After encapsulation, each sample was examined under a Leica confocal fluorescence microscope (Leica Microsystems Japan, Tokyo, Japan).

Sequential Solvent Extraction of Cinnamon. Ceylon cinnamon powder (10 mg) was sequentially extracted with 1.0 mL of *n*-hexane, chloroform, methanol, and water at room temperature for 60 min using a rotator. Each extract was evaporated to dryness under reduced pressure with a centrifugal concentrator at room temperature and then reconstituted with 1.0 mL of DMSO.

High-Performance Liquid Chromatography (HPLC). For HPLC, we used Pump L-2310 (Hitachi, Tokyo, Japan), Rheodyne (Cotati, CA, USA) Model 7725i equipped with a 5 μL sample loop, and an L-4000 UV detector (Hitachi). Two mobile phase solvents were employed. Solvent A was prepared by adding concentrated acetic acid (0.1%) to ultrapure water. Solvent B was prepared by adding acetic acid (0.1%) to HPLC grade acetonitrile. For analysis, we used a reverse phase column (Cosmosil $5C_{18}$-MS-II, 2.0 × 150 mm, Nacalai Tesque) and the following mobile gradient: 0−30 min, linear gradient from 80% solvent A to 100% solvent A. The flow rate was constant at 300 μL/min. For isolation, HPLC employed a reverse phase column (Cosmosil $5C_{18}$-AR-II, 10 × 250 mm, Nacalai Tesque) under the same chromatographic conditions. The flow rate was constant at 2.0 mL/min.

^{1}H NMR Analysis of Cinnamaldehyde. Cinnamaldehyde was isolated from methanol extract of Ceylon cinnamon by HPLC as described above. The solution eluted under the chromatographic peak was collected and evaporated under reduced pressure with a rotary evaporator at 30 °C. The structure of purified cinnamaldehyde was confirmed by ^{1}H NMR and two-dimensional correlated spectroscopy experiments (JNM-AL 400 NMR spectrometer system, a JEOL, Ltd., Tokyo, Japan) and identified by comparison against spectra for authentic cinnamaldehyde. ^{1}H NMR (400 MHz, chloroform-*d*) spectrum of isolated cinnamaldehyde was assigned as follows: δ 6.71 (*dd*, 1H, J = 16.4, 8.0 Hz, β-CH), 7.47 (*d*, 1H, J = 16.4 Hz, γ-CH), 7.42−7.43 (*m*, 3H, Ar−H), 7.56 (*dd*, 2H, J = 6.0, 3.6 Hz, Ar−H), 9.70 (*d*, 1H, J = 8.0 Hz, CHO).

RESULTS

Screening for Medicinal Food Inducing Type I Collagen Expression in Normal Human Skin Fibroblasts. We initially searched for type I collagen expression-inducing medicinal food in normal human skin fibroblasts NB1RGB cells. The fibroblasts were exposed to each extract with DMSO for 6 h, and then the level of pro-type I collagen was determined by immunoblot analysis with antitype I collagen antibody. We investigated the effects of extracts from 3 teas, 12 spices, and

7 herbs on the expression level of type I collagen and found that some spice extracts apparently stimulate collagen production. As shown in a representative result for spice extracts (Figure 1), mace,

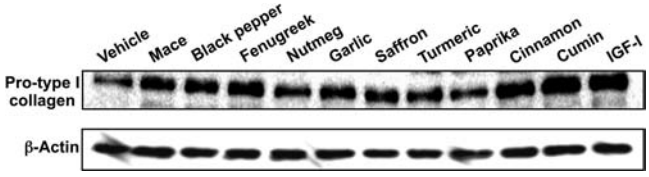

Figure 1. Effects of spice extracts on the expression of type I collagen in NB1RGB fibroblasts. Indonesian mace, Indian black pepper, Indian fenugreek, Indonesian nutmeg, Chinese garlic, Spanish saffron, Indian turmeric, Spanish paprika, Ceylon cinnamon, and Iranian cumin were extracted with DMSO as described in the Materials and Methods section. NB1RGB cells were treated with spice extracts (10 μg/mL) or 100 ng/mL IGF-I (positive control), in serum-free medium for 6 h. Then, the level of pro-type I collagen was determined by immunoblotting with antitype I collagen antibody. β-Actin was also determined and used as a loading control.

black pepper, fenugreek, cinnamon, and cumin markedly increased the levels of pro-type I collagen. Among these extracts, we focused on cinnamon extract as a potent inducer of type I collagen synthesis.

Several *Cinnamomum* species are often sold as cinnamon worldwide, and the contents of their chemical constituents are reported to vary from species to species.[17,18] Therefore, we evaluated the activity of three cinnamon powders, namely, cassia cinnamon (*Cinnamomum aromaticum*), unknown cinnamon, and Ceylon cinnamon (*Cinnamomum verum*), from Vietnam, Malaysia, and Sri Lanka, respectively, by immunoblot analysis. As shown in Figure 2A, all extracts significantly enhanced the production of pro-type I collagen, implying that general constituent(s) in *Cinnamomum* species are active compounds. Furthermore, we assessed the expression level of type I collagen α1 subunit mRNA in NB1RGB cells exposed to cinnamon extract by real-time PCR (Figure 2B). The treatment of NB1RGB cells with the extract from cassia and that from Ceylon cinnamon for 6 h significantly potentiated the mRNA expression by approximately 2.4- to 2.8-fold compared with the vehicle control, suggesting that cinnamon constituents stimulate the signaling pathways for type I collagen gene expression. As shown in Figure 2C, the exposure to Ceylon cinnamon extract at concentrations from 1 to 20 μg/mL for 6 h significantly increased in the pro-type I collagen level in a dose-dependent manner. The pro-type I collagen level was increased for 6 h after the treatment with cinnamon extract and thereafter decreased gradually (Figure 2D), whereas there were no change in the level for up to 24 h after the treatment with vehicle (data not shown). We also confirmed that cinnamon extract showed no cytotoxicity at a concentration up to 100 μg/mL over a period of 24 h (data not shown).

Mature type I collagen synthesis involves inter- and intracellular modifications in multiple posttranslational stages.[19] Before the formation of the procollagen triple helix, type I collagens are modified by the hydroxylation of prolyl and lysyl residues, and glycosylation of certain hydroxylysyl residues. The procollagen is excreted and is converted extracellularly into collagen by cleaving the propeptides. Subsequently, collagen molecules assemble into ordered fibrils, which are finally cross-linked by lysyl oxidase. To further evaluate the accumulation of type I collagen, we examined total levels of newly synthesized

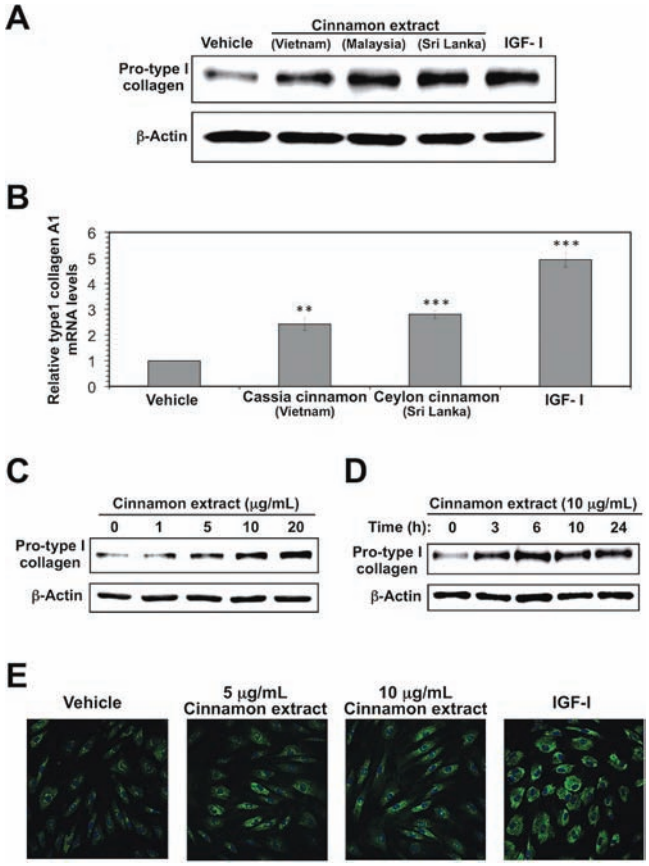

Figure 2. Upregulation of type I collagen expression in NB1RGB fibroblasts by the treatment of cinnamon extracts. (A and B) NB1RGB cells were treated with several species of cinnamon extracts (10 μg/mL) or 100 ng/mL IGF-I in serum-free medium for 6 h. (A) Immunoblott analysis of the levels of pro-type I collagen. (B) Analysis of mRNA expression of type I collagen A1 by real-time PCR. Total RNA was collected from treated cells and subjected to real-time PCR using type I collagen A1- and 18S rRNA-specific primers. 18S rRNA was used as the internal control. Values represent the mean ± SD of triplicate determinations. The significance of differences was evaluated using Student's *t* test. ** $p < 0.01$, *** $p < 0.001$ versus the vehicle control group. (C) Concentration-dependent expression of pro-type I collagen by the treatment of cinnamon extract. NB1RGB cells were exposed to the indicated concentrations of Ceylon cinnamon extracts for 6 h. Then, the level of pro-type I collagen was determined by immunoblotting. (D) Time-dependent expression of pro-type I collagen by the treatment of cinnamon extract. NB1RGB cells were exposed to Ceylon cinnamon extract (10 μg/mL) for 0–24 h. Then, the level of pro-type I collagen was determined by immunoblotting. (E) Immunofluorescent microscopic analysis of of type I collagen in cinnamon extract-treated NB1RGB cells. The cells were exposed to the indicated concentrations of Ceylon cinnamon extracts or 100 ng/mL IGF-I for 24 h, and then were stained with antitype I collagen antibody (green) and Hoechst 33342 (blue) as described in the Materials and Methods section.

and posttranslationally modified type I collagens in cinnamon extract-treated NB1RGB cells by immunofluorostaining with antitype I collagen antibody. As shown in Figure 2E, immunofluorescent microscopic analysis revealed that type I collagen was significantly deposited by the treatment with cinnamon extract for 24 h as compared with the vehicle control. Taken together, these data indicate that cinnamon extract potentiates the expression of type I collagen and promotes its accumulation.

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Determination of Active Components That Stimulate Type I Collagen Expression from Cinnamon Extract. To elucidate the active components in cinnamon extract that promote type I collagen expression, we next investigated the activity of sequential extracts with four solvents with increasing polarity from cinnamon powder. After NB1RGB cells were treated with each extract for 6 h, the level of pro-type I collagen was determined by immunoblotting. As shown in Figure 3A,

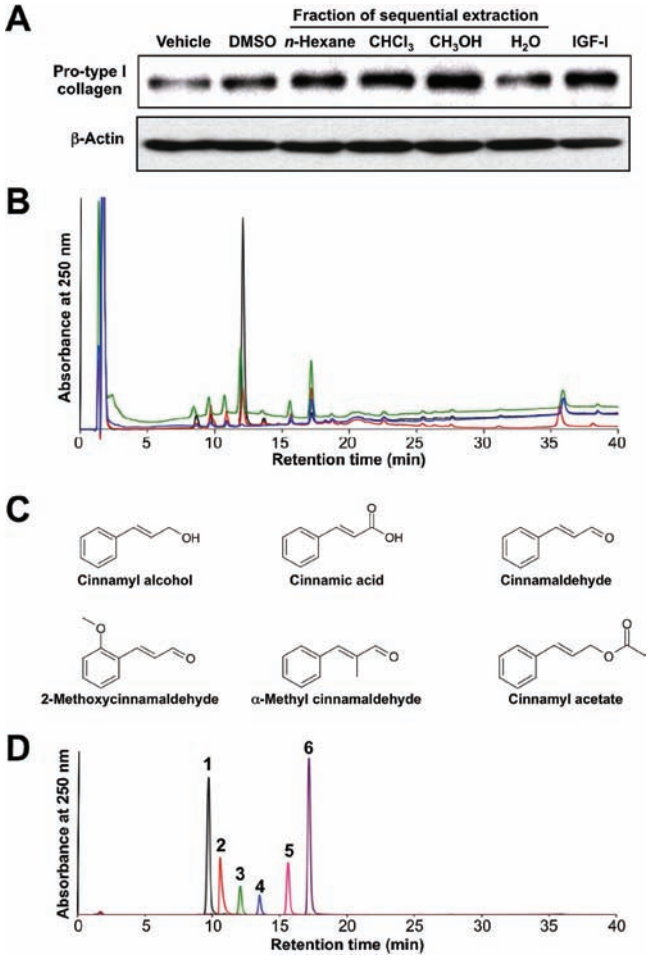

Figure 3. Searching for the active constituent promoting type I collagen expression from cinnamon extracts. (A) Immunoblott analysis of the levels of pro-type I collagen in NB1RGB cells exposed to sequential extracts. Ceylon cinnamon powder was sequentially extracted with *n*-hexane, chloroform, methanol, and water as described in the Materials and Methods section. NB1RGB cells were treated with each extract in serum-free medium for 6 h. Then, the level of pro-type I collagen was determined by immunoblotting. (B) HPLC profiles of the sequential extracts from Ceylon cinnamon. Black line, *n*-hexane extract; red line, chloroform extract; green line, methanol extract; blue line, water extract. (C) Structures of cinnamaldehyde derivatives in cinnamon. (D) HPLC profiles of cinnamaldehyde derivatives. Peak 1, cinnamyl alcohol; peak 2, cinnamic acid; peak 3, cinnamaldehyde; peak 4, 2-methoxycinnamaldehyde; peak 5, α-methyl cinnamaldehyde; peak 6, cinnamyl acetate.

organic extracts with *n*-hexane, chloroform, and methanol intensely up-regulated the level of pro-type I collagen, but not with water, suggesting that the active components are lipophilic. Figure 3B shows HPLC chromatograms for the sequential extracts from cinnamon powder. We observed a major peak with a retention time of 12.5 min in all organic extracts. Therefore, the fraction of the major peak was collected from cinnamon extract by preparative HPLC. Structural analysis of this compound isolated was characterized as cinnamaldehyde (Figure 3C) by ^{1}H NMR and 2D-COSY and confirmed by comparison against the ^{1}H NMR spectra of the authentic standard. Cinnamaldehyde is one of the major components of *Cinnamomum* species[20] and has been known to exert several pharmacological effects such as anti-inflammatory,[21] antitumor,[22] and antidiabetic activities.[23] In addition to cinnamaldehyde, *Cinnamomum* species contain several cinnamaldehyde derivatives. The chemical components of *Cinnamomum* species have been clearly defined by HPLC analysis in previous studies.[24,25] Therefore, we determined the HPLC profile for well-defined cinnamaldehyde derivatives. Consequently, the peaks of cinnamyl alcohol (peak 1), cinnamic acid (peak 2), cinnamaldehyde (peak 3), 2-methoxycinnamaldehyde (peak 4), α-methyl cinnamaldehyde (peak 5), and cinnamyl acetate (peak 6), shown in Figure 3D, were determined from cinnamon extract by comparing their retention times from individual authentic standards (Figure 3C). Considering these observations, we evaluated the activities of six cinnamaldehyde derivatives for collagen production using immunoblot analysis. As shown in Figure 4A, cinnamaldehyde, α-methyl cinnamaldehyde, and

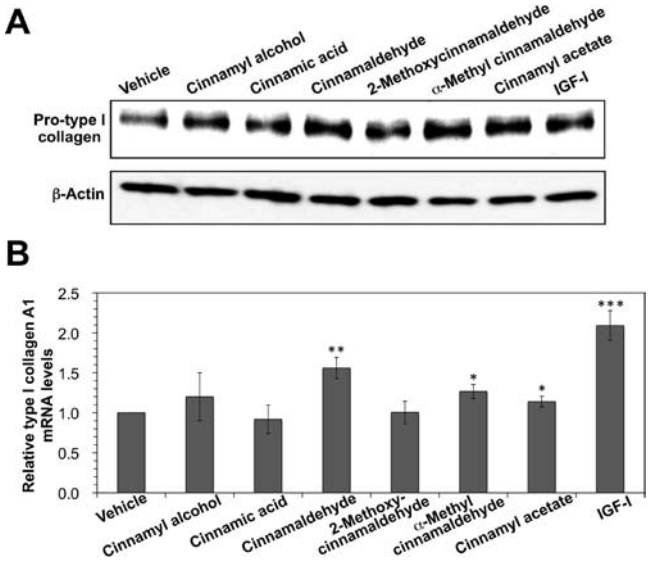

Figure 4. Effects of the constituents of cinnamon on the expression of type I collagen in NB1RGB fibroblasts. (A and B) NB1RGB cells were treated with 10 μM constituents of cinnamon or 100 ng/mL IGF-I in serum-free medium for 6 h. (A) Immunoblott analysis of the levels of pro-type I collagen. (B) Analysis of mRNA expression of type I collagen A1 by real-time PCR. Total RNA was collected from treated cells and subjected to real-time PCR using type I collagen A1- and 18S rRNA-specific primers. 18S rRNA was used as the internal control. Values represent the mean ± SD of triplicate determinations. The significance of differences was evaluated using Student's *t* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the vehicle control group.

cinnamyl acetate significantly enhanced the production of pro-type I collagen during incubation for 6 h. Furthermore, we assessed the expression level of type I collagen α1 subunit mRNA in NB1RGB cells exposed to cinnamaldehyde derivatives by real-time PCR (Figure 4B). Consistent with immunoblot analysis, cinnamaldehyde, α-methyl cinnamaldehyde, and cinnamyl acetate significantly increased the mRNA levels for

the type I collagen α1 subunit. The treatment of NB1RGB cells with cinnamaldehyde for 6 h most strongly potentiated the mRNA expression by approximately 1.6-fold compared with the vehicle control. These data indicate that these compounds are major active components in cinnamon extract that stimulate the signaling pathways for type I collagen gene expression.

To further characterize the action of cinnamaldehyde, we examined the dose- and exposure time-dependent expression of pro-type I collagen in cinnamaldehyde-exposed NB1RGB cells. As shown in Figure 5A, the treatment with cinnamaldehyde at

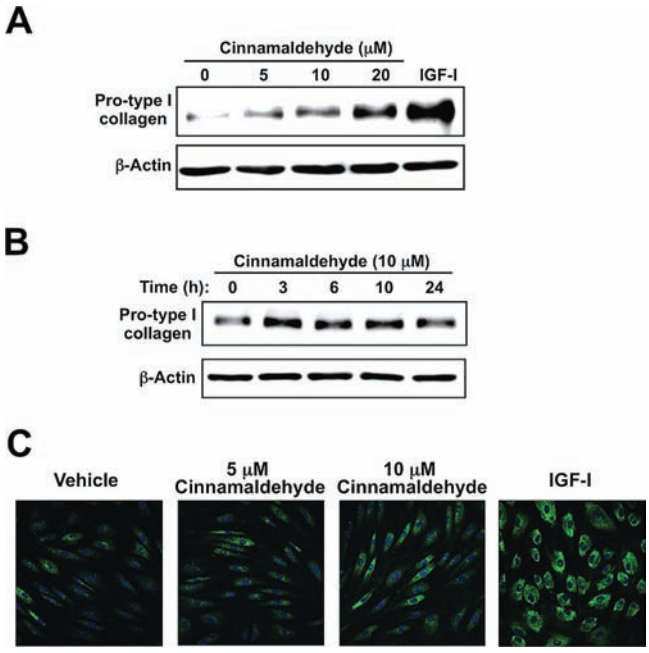

Figure 5. Effect of cinnamaldehyde on the expression of type I collagen in NB1RGB fibroblasts. (A) Concentration-dependent expression of pro-type I collagen by the treatment of cinnamaldehyde. NB1RGB cells were exposed to the indicated concentrations of cinnamaldehyde for 6 h. Then, the level of pro-type I collagen was determined by immunoblotting. (B) Time-dependent expression of pro-type I collagen by the treatment of cinnamaldehyde. NB1RGB cells were exposed to 10 μM cinnamaldehyde for 0−24 h. Then, the level of pro-type I collagen was determined by immunoblotting. (C) Immunofluorescent microscopic analysis of type I collagen in cinnamaldehyde-treated NB1RGB cells. The cells were exposed to the indicated concentrations of cinnamaldehyde or 100 ng/mL IGF-I for 24 h and then were stained with antitype I collagen antibody (green) and Hoechst 33342 (blue) as described in the Materials and Methods section.

concentrations from 5 to 20 μM for 6 h significantly increased the pro-type I collagen level, and the level was increased for 6 h after the treatment of cinnamaldehyde and thereafter decreased gradually (Figure 5B). We also confirmed that multiple type I collagens significantly accumulated after the treatment of NB1RGB cells with cinnamaldehyde for 24 h by immunofluorostaining (Figure 5C).

Activation of IGF-I Signaling by Cinnamaldehyde Treatment. We further investigated the molecular mechanism underlying the induction of type I collagen expression by treatment with cinnamaldehyde. The activation of extracellular signal-regulated kinase 1/2 (Erk1/2) is one of the major downstream signaling events that participate in type I collagen gene expression.[26,27] Therefore, we examined whether Erk1/2 activation is involved in the cinnamaldehyde-induced up-regulation of type I collagen by immunoblot analysis. As shown in Figure 6A, the treatment of NB1RGB cells with

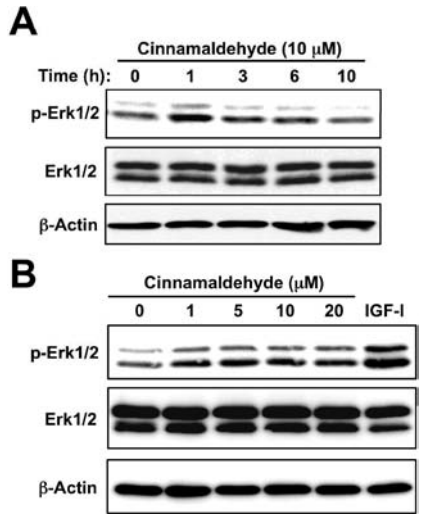

Figure 6. Activation of Erk1/2 during the exposure of NB1RGB cells to cinnamaldehyde. (A) Time-dependent phosphorylation of Erk1/2 by the treatment of cinnamaldehyde. NB1RGB cells were exposed to 10 μM cinnamaldehyde for 0−10 h. Then, phosphorylation of Erk1/2 was determined by immunoblotting. (B) Concentration-dependent phosphorylation of Erk1/2 by the treatment of cinnamaldehyde. NB1RGB cells were exposed to the indicated concentrations of cinnamaldehyde or 100 ng/mL IGF-I for 30 min. Then, phosphorylation of Erk1/2 was determined by immunoblotting.

10 μM cinnamaldehyde induced the phosphorylation of Erk1/2 with maximum induction at 1 h. The phosphorylation then gradually diminished in a time-dependent manner. We also detected the activation of Erk1/2 after the exposure to cinnamaldehyde at concentrations from 1 to 20 μM for 30 min as well as IGF-I (Figure 6B), suggesting that cinnamaldehyde induces the intracellular Erk signaling cascade and thereby enhances the expression of type I collagen. IGF-I is the most potent inducer of type I collagen biogenesis by an Erk phosphorylation-dependent mechanism in fibroblasts.[12,26] Binding of IGF-I to the IGF-I receptor results in autophosphorylation of the receptor β subunits at Tyr1131, Tyr1135, and Tyr1136, and increased receptor tyrosine kinase activity and tyrosine phosphorylation of IRS-1.[8,11] Phosphorylated IRS-1 plays a key role in transmitting signals from the IGF-I receptor to intracellular Erk pathways. Hence, we further examined whether IGF-I signaling is involved in the cinnamaldehyde-triggered activation of Erk1/2 by immunoblot analysis. As shown in Figure 7A, the treatment of NB1RGB cells with 10 μM cinnamaldehyde significantly induced the phosphorylation of both the IRS-1 and IGF-I receptors after the incubation for 15 min. We also observed that the exposure to cinnamaldehyde at concentrations from 5 to 20 μM for 30 min caused the activation of both IRS-1 and IGF-I receptors (Figure 7B). These data suggest that cinnamaldehyde-dependent stimulation of type I collagen biosynthesis in NB1RGB fibroblasts can be attributed to the activation of IGF-I signaling.

DISCUSSION

The maintenance of skin texture is largely dependent on the activities of dermal fibroblasts, and type I collagen plays a

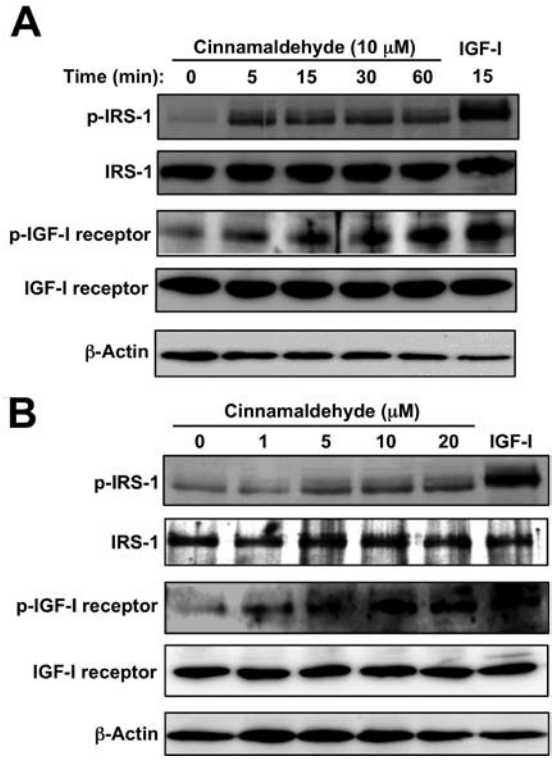

Figure 7. Activation of IGF-I signaling during the exposure of NB1RGB cells to cinnamaldehyde. (A) Time-dependent activation of IGF-I signaling by the treatment of cinnamaldehyde. NB1RGB cells were exposed to 10 μM cinnamaldehyde or 100 ng/mL IGF-I for 0−60 min. Then, phosphorylation of the IRS-1 and IGF-I receptors was determined by immunoblotting. (B) Concentration-dependent activation of IGF-I signaling by the treatment of cinnamaldehyde. NB1RGB cells were exposed to the indicated concentrations of cinnamaldehyde for 30 min or 100 ng/mL IGF-I for 15 min. Then, phosphorylation of the IRS-1 and IGF-I receptors was determined.

crucial role in maintaining of the tensile strength and elasticity of skin. Reduction and degradation of type I collagen in the dermis is a prominent feature of aged and photodamaged skin.[28] This notion is clearly supported by the fact that biosynthesis of type I collagen by fibroblasts is markedly diminished in elderly persons and photodamaged skin, resulting in delayed wound healing and loss of dermal elasticity.[29] Therefore, an approach that enhances type I collagen production and thereby strengthens skin texture is an attractive strategy against the aging of skin such as wrinkle formation and sagging of skin. In this study, as a preliminary test, we initially performed a molecular target-based screening assay to identify type I collagen expression-inducing medicinal food among 22 plant extracts by using normal human dermal fibroblasts. Interestingly, the spice series promoted collagen production more strongly than the others. Therefore, we narrowed the search to the spice series and then identified cinnamon extract as the most potent inducer of dermal collagen synthesis (Figure 1). Regardless of the origin of *Cinnamomum* species, the cinnamon extract up-regulated both mRNA and protein expression levels of pro-type I collagen (Figure 2A and B). Furthermore, we revealed that cinnamaldehyde is the major active component in cinnamon extract that stimulates type I collagen biosynthesis (Figure 4) and that its action mechanism could be attributed to the IGF-I receptor-activating pathway (Figures 6 and 7). It is noteworthy that cinnamaldehyde has been shown to penetrate human skin over a period of 24 h following its topical application.[20,30] These facts suggest that cinnamon extract could be utilized as a therapeutic against skin aging.

We previously reported that thioctic acid, an antioxidant found in almost all foods, functions as a TGF-β mimic to promote collagen production through the activation of TGF-β receptor in normal human dermal fibroblasts.[16] Moreover, some food constituents such as polyphenols have been recently shown to mimic insulin and IGF-I signaling.[31−33] Interestingly, recent studies on cinnamon have also shown that cinnamon extract and its constituents could mimic insulin by activating the insulin receptor and exhibit antidiabetic effects independently from insulin in vitro and in vivo.[14,15,34] These findings imply that food-derived small molecules including cinnamaldehyde could latently be growth factor-mimics. Since IGF-I is the most potent stimulator for collagen biosynthesis in dermal fibroblasts,[12,26] we investigated the effect of cinnamaldehyde on IGF-I signaling. In the present study, we found that the exposure of the dermal fibroblasts to cinnamaldehyde contributed to a definite activation of IGF-I receptor and its downstream signaling molecules such as IRS-1 and MAP-kinases (Erk1/2) in the absence of IGF-I (Figures 6 and 7), in other words, cinnamaldehyde could mimic the actions of IGF-I. These events can consequently lead to the up-regulation of type I collagen expression in the fibroblast. Synthesis of IGF-I, which occurs predominantly in the liver and partly in the muscles, brain, and kidneys, is regulated primarily by growth hormone secretion.[35] Aging decreases the biological activities of dermal fibroblasts both in vivo and in vitro, in part, due to the decline in IGF-I responsiveness.[36] In addition, the acceleration of aging is related to the decline of IGF-I blood levels in such a manner that aging rates progress as growth hormone secretion diminishes.[37] Thus, our findings suggest that the potentiating effects of cinnamon extract on dermal IGF-I responsiveness are beneficial in the treatment and prevention of the visible signs of aging.

The ligand-independent activation of IGF-I signaling evoked by cinnamaldehyde may be indirectly attributed to the phosphorylation−dephosphorylation reaction of the IGF-I receptor. Cinnamon extract has been previously shown to stimulate autophosphorylation of insulin receptor and inhibit protein tyrosine phosphatase-1B (PTP-1B), which is a negative regulator of insulin signal transduction.[34] Recently, Fu et al. reported that *p*-carboxycinnamaldehyde and its tripeptide-substituted derivatives act as a reversible covalent inhibitor against PTP-1B.[38] The mechanism of inhibition has been proposed to involve the formation of an imine/enamine adduct between the aldehyde group of the cinnamaldehyde derivatives and the guanidine group of Arg-221 in the PTP-1B active site. Furthermore, Moran et al. have previously shown that tripeptide-substituted cinnamic acids potently inhibit PTP-1B.[39] In the insulin signaling pathway, PTP-1B dephosphorylates several substrates such as the tyrosine residues 1146, 1150, and 1151 of the phosphorylated insulin receptor β subunit, causing termination of receptor tyrosine kinases cascade initiated when insulin binds to the insulin receptor, and the phosphorylated IRS-1, resulting in down-regulation of insulin signaling. Recent studies have demonstrated that PTP-1B inhibitors could lead to the activation of the insulin receptor and its downstream signaling molecules in an insulin-independent manner and exhibit insulin-mimetic effects in vitro and in vivo.[40] Therefore, PTP-1B has recently emerged as a promising therapeutic molecular target in the effective management of type 2 diabetes. In addition, PTP-1B has also been shown to dephosphorylate and

inactivate IGF-I receptor, which has 70% homology to the insulin receptor. On the basis of these facts, we speculate that cinnamaldehyde and its derivatives can inhibit PTP-1B and thereby induce the activation of IGF-I receptor and its downstream signaling molecules in the fibroblasts, resulting in the upregulation of type I collagen expression. In the present study, the series of cinnamaldehyde derivatives varied in their effectiveness in type I collagen expression (Figure 4), suggesting that their modest structural differences may exert influence on the inhibition of PTP1B in the fibroblast. Further studies are needed to elucidate the exact molecular mechanism underlying cinnamaldehyde-triggered activation of IGF-I signaling.

In conclusion, we demonstrated for the first time that the cinnamon extract significantly potentiates type I collagen biosynthesis within dermal fibroblasts. Furthermore, we revealed that cinnamaldehyde is the major active component in cinnamon extract that induces type I collagen biosynthesis. The underlying molecular mechanism was considered to involve the activation of IGF-I signaling via the direct IGF-I receptor-activating pathway. Our findings could be helpful in improving the signs and symptoms of aging skin since a reduction in dermal collagen production is considered to be its principal cause. The mechanism by which cinnamaldehyde induces the ligand-independent activation of IGF-I receptor will be addressed further in the near future.

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-72-254-9460. Fax: +81-72-254-9460. E-mail: akagawa@biochem.osakafu-u.ac.jp.

Author Contributions

§These authors contributed equally to this work.

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ABBREVIATIONS USED

ECM, extracellular matrix; ECL, enhanced chemiluminescence; Erk1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; HRP, horseradish peroxidase; IGF-I, insulin-like growth factor-I; IRS-1, insulin receptor substrate-1; MAP, mitogen-activated protein; PTP-1B, protein tyrosine phosphatase-1B; RIPA, radio-immunoprecipitation assay; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing Tween 20; TGF-β, transforming growth factor-β

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