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Simulation of the Elastin and Fibrillin in Non-Irradiated or UVA Radiated Fibroblasts, and Direct Inhibition of Elastase or Matrix Metalloptoteinases Activity by Nicotinamide or Its Derivatives

NEENA PHILIPS, JOVINNA CHALENSOUK-KHAOSAAT, and SALVADOR GONZALEZ, *Department of Biology, Fairleigh Dickinson University, Teaneck, NJ (N.P., J.C-K.) and Department of Dermatology, Medicine and Medical Specialties, Alcala University, Madrid, Spain (S.G.).*

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Synopsis

Skin aging/photoaging is associated with altered the structure of collagen and elastin fibers, and increased activity of matrix metalloproteinases (MMP) and elastase. Nicotinamide and its derivatives, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide, and 3-hydroxypicolinamide (collectively niacin derivatives) stimulate fibrillar collagen and heat shock proteins in dermal fibroblasts. The goal of this research was to extend the understanding of the anti–skin aging mechanism of these niacin derivatives through the stimulation of elastin (at the protein and promoter levels), fibrillin (1 and 2) in nonirradiated or ultraviolet (UVA) radiated dermal fibroblasts, and through the direct inhibition of MMP (1, 3, and 9) and elastase activities. UVA radiation stimulated elastin and inhibited fibrillin-1 and fibrillin-2 in dermal fibroblasts. The niacin derivatives significantly stimulated the expression of elastin (transcriptionally), fibrillin-1 and fibrillin-2 in nonirradiated and UVA radiated fibroblasts, and directly inhibited MMP or elastase activity. Overall, the niacin derivatives, more so nicotinamide and 2,6-dihydroxynicotinamide, have anti–skin aging potential through the stimulation of elastin and fibrillin, and the direct inhibition of the extracellular matrix proteolytic enzymes.

INTRODUCTION

The structural integrity of the extracellular matrix (ECM), composed predominantly of collagen and elastin fibers, is essential to skin structure and function (1–8). The structure of the ECM deteriorates with intrinsic aging, and exposure to environmental factors such as ultraviolet (UV) radiation (1–27). UVA radiation can penetrate the dermis and damage ECM through the generation of oxidative stress and inflammation, and direct damage of biomolecules (9–14). The dermal fibroblasts are the primary synthesizers of the ECM proteins (1–4,8).

Address all correspondence to Neena Philips at nphilips@fdu.edu and neenaphilips@optonline.net.

Polyphenols with their antioxidative and anti-inflammatory properties have been identified to beneficially regulate the ECM, and thereby, prevent skin aging or cancer (1–8,27). The structure of the phenolic components includes at least one aromatic ring with one or more hydroxyl groups (5). The activity of the phenolic compounds is dependent on the number and location of these hydroxyl groups, in addition to their structure (5,28–34). The structure of nicotinamide or its derivatives, such as 3-hydroxypicolimanide, has photophysical and UV radiation absorptive properties (28–34). Skin aging or photoaging reduces cellular antioxidant defense, ECM structure, and NAD content (1–8,35). Nicotinamide serves as a precursor for several cellular coenzymes that are essential to metabolism and counteracts inhibition of ATP/glycolysis, oxidative DNA damage, oxidative stress, and inflammation (35–41). Nicotinamide improves skin appearance, independently or in combination with retinoids, by reducing hyperpigmentation and wrinkles (42–46). We recently reported an anti–skin aging mechanism of nicotinamide through the stimulation of fibrillar collagen and heat shock proteins in dermal fibroblasts (27).

The goal of this research was to determine the anti–skin aging mechanism of nicotinamide and three of its derivatives, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide, and 3-hydroxypicolinamide (collectively niacin derivatives), through the beneficial regulation of elastin and fibrillin (1 and 2) in nonirradiated or UVA-radiated dermal fibroblasts, and direct inhibition of on matrix metalloproteinases (MMP) (1, 3, and 9) and elastase activities. The hypothesis of this research was that the niacin derivatives would stimulate expression of elastin and fibrillin, and directly inhibit ECM proteolytic activity.

METHODS

CELL CULTURE AND DOSING

Human neonatal dermal fibroblasts from two donors (Cascade Biologics, part of Thermofischer Scientific, Waltham, MA) were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 1% penicillin/streptomycin (P/S), and 1% L-glutamine (Sigma, St. Louis, MO). The cells were rinsed with Hank's Balanced Salt solution (Sigma) and nonirradiated (control) or radiated with 2.5 J/cm^2 (minimal toxicity) of UVA radiation using a four-tube UVA lamp with irradiance of 1E−03 W/cm^2 for a sum of wavelengths from 320 to 400 nm (2,4,27). The nonirradiated or UVA-radiated cells were then not exposed (control or UVA-radiated control) or exposed to 0.01% (0.05 mM), 0.1% (0.5 mM), or 1% (5 mM) of each of the niacin derivatives in experimental media (DMEM containing 1% serum replacement and 1% P/S) for 24 h (2,4,27). Four independent experiments, in replicates of 3–4, were performed with dermal fibroblasts at passages 4–12. The cells were examined for cell viability by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (Promega, Madison, WI). The cell viability was not altered at these concentrations of UVA radiation or niacin derivatives.

ELASTIN, AND FIBRILLIN (-1, -2) PROTEIN LEVELS

The elastin and fibrillin proteins levels in the media of nonirradiated or UVA radiated fibroblasts ± niacin derivatives were determined by using enzyme linked immunosorbant assay (ELISA) (Kirkguaard and Perry Laboratories Inc., Milford, MA; Elastin Products Co.)

(1–4,8,27). One hundred microliter aliquots of media or cells from each sample, or respective standards were added to independent wells of 96 well plates for 24 h. The wells were blocked with bovine serum albumin, and then incubated with respective antibodies (Elastin Products Co.) for 1 h. The plates were washed with wash buffer, incubated with respective secondary antibodies linked to peroxidase for 1 h, washed, and subsequently incubated with peroxidase substrate until color development, which was measured spectrophotometrically at 405 nm and quantitated from standard curves.

ELASTIN PROMOTER ACTIVITY

Fibroblasts were cotransfected with elastin promoter-firefly luciferase (Pgl4 vector) and TK-Renilla luciferase plasmids (for normalization of transfection efficiency) using Escort (Sigma) for 24 h before dosing with or without UVA-radiation ± niacin derivatives for 24 h (2,4,8). The cells were measured for luminescence from firefly or renilla luciferase with specific substrates and quantitated using recombinant luciferase as standard (Promega).

MMP-1, -9, AND -9, AND ELASTASE ACTIVITIES

The inhibition of ECM proteolytic enzymes (MMP-1, MMP-2, MMP-3, Elastase) (Biomol, Torrance, CA; Enzo Life Sciences, Farmingdale, NY; Elastin products Co.) was performed as previously reported (1,3). Each of the enzymes at optimal concentration was incubated with the niacin derivatives at 0%, 0.01%, 0.1%, or 1% for 10 min followed by the addition of its respective substrate (Bachem). The reaction kinetics were measured flourometrically (355 excitation/450 emission) every 10 min for a total of 60 min. The initial reading (0 time) was subtracted from the final reading (60 min) and the data converted to percentage of control.

DATA ANALYSIS

The data were analyzed for significant difference by analysis of variance and student *t*-tests at 95% confidence interval. The effects of UVA radiation on dermal fibroblasts were analyzed relative to nonirradiated control cells. The effects of the niacin derivatives on nonirradiated cells were analyzed relative to nonirradiated cells (control). The effects of each of the niacin derivatives on UVA-radiated fibroblasts were analyzed relative to UVA radiation effect alone (UVA-radiated respective control). The direct MMP or elastase inhibitory activity of the niacin derivatives was analyzed relative to control.

RESULTS

STIMULATION OF EXPRESSION OF ELASTIN, ELASTIN PROMOTER, FIBRILLIN-1, AND FIBRILLIN-2 BY NICOTINAMIDE, 2,6-DIHYDROXYNICOTINAMIDE, 2,4,5,6-TETRAHYDROXYNICOTINAMIDE, AND 3-HYDROXYPICOLINAMIDE IN NONIRRADIATED FIBROBLASTS

Niacin or its derivatives stimulated the expression of elastin and fibrillin in dermal fibroblasts. The niacin derivatives significantly stimulated elastin expression (protein and promoter) and fibrillin-1 and fibrillin-2 at 0.1% and 1% in nonirradiated fibroblasts ($p < 0.05$,

relative to control) (Figure 1A–D). In addition, fibrillin-1 and fibrillin-2 were stimulated by nicotinamide and 2,6-dihydroxynicotinamide at 0.01% in nonirradiated fibroblasts ($p < 0.05$, relative to control) (Figure 1C and D).

In nonirradiated fibroblasts, the elastin protein levels (70 ng/ml as 100%)/elastin promoter activity (400 pg/ml as 100%) were stimulated by nicotinamide, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide, and 3-hydroxypicolinamide upto 308/222%, 413/719%, 243/358%, and 224/183% of nonirradiated controls, respectively ($p < 0.05$) (Figure 1A and B). The expression of fibrillin-1 (0.5 µg/ml as 100%)/fibrillin-2 (35 ng/ml as 100%) was stimulated by nicotinamide, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide, and 3-hydroxypicolinamide upto 614/578%, 601/533%, 402/234%, and 336/290% of nonirradiated controls, respectively ($p < 0.05$) (Figure 1C and D).

STIMULATION OF EXPRESSION OF ELASTIN, ELASTIN PROMOTER, FIBRILLIN-1, AND FIBRILLIN-2 BY NICOTINAMIDE, 2,6-DIHYDROXYNICOTINAMIDE, 2,4,5,6-TETRAHYDROXYNICOTINAMIDE, AND 3-HYDROXYPICOLINAMIDE IN UVA RADIATED FIBROBLASTS

UVA-radiation stimulated elastin protein level to 185% (±14%) of control, and elastin promoter activity to 125% (±8%) of control ($p < 0.05$). UVA-radiation significantly inhibited fibrillin-1 and fibrillin-2 to 65% (±4%) of control and 73% (±7%) of control ($p < 0.05$). The effects of the niacin derivatives on UVA-radiated fibroblasts were similar

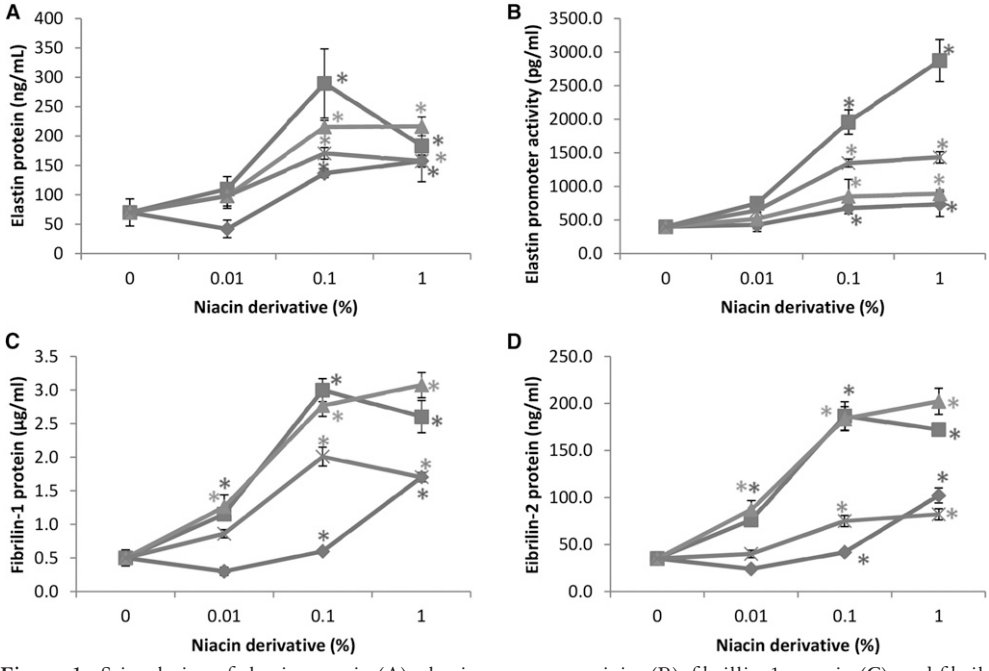

Figure 1. Stimulation of elastin protein (A), elastin promoter activity (B), fibrillin-1 protein (C), and fibrillin-2 protein (D) by nicotinamide (green line), 2,6-dihydroxynicotinamide (red line), 2,4,5,6-tetrahydroxynicotinamide (violet line), and 3-hydroxypicolinamide (blue line) in non-irradiated dermal fibroblasts; * = $p < 0.05$, relative to control, error bars (A–D) represent standard deviation, $n = 4$.

to those on non-irradiated fibroblasts, except for the lack of regulation of fibrillin expression by 0.01% nicotinamide and 2,6-dihydroxynicotinamide.

Relative to UVA-radiated controls, the expression of elastin protein/promoter was stimulated by nicotinamide, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide, and 3-hydroxypicolinamide upto 348/268%, 365/708%, 230/547%, and 221/214%, respectively ($p < 0.05$) (Figure 2A and B). The expression of fibrillin-1/fibrillin-2 protein levels was stimulated by nicotinamide, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide, and 3-hydroxypicolinamide upto 524/482%, 859/563%, 347/312%, and 253/227% of UVA-radiated controls, respectively ($p < 0.05$) (Figure 2C and D).

DIRECT INHIBITION OF MMP-1, MMP-3, MMP-9, AND ELASTASE ACTIVITIES BY NICOTINAMIDE, 2,6-DIHYDROXYNICOTINAMIDE, 2,4,5,6-TETRAHYDROXYNICOTINAMIDE, AND 3-HYDROXYPICOLINAMIDE

The MMP-1 and MMP-3 activities were significantly inhibited by nicotinamide and 2,6-dihydroxynicotinamide; MMP-9 activity by nicotinamide, and elastase activity by nicotinamide, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide, and 3-hydroxypicolinamide ($p < 0.05$) (Figure 3).

The MMP-1/MMP-3 activities were significantly inhibited by nicotinamide upto 43/36%, and by 2,6-dihydroxynicotinamide upto 70/57%, of respective controls ($p < 0.05$)

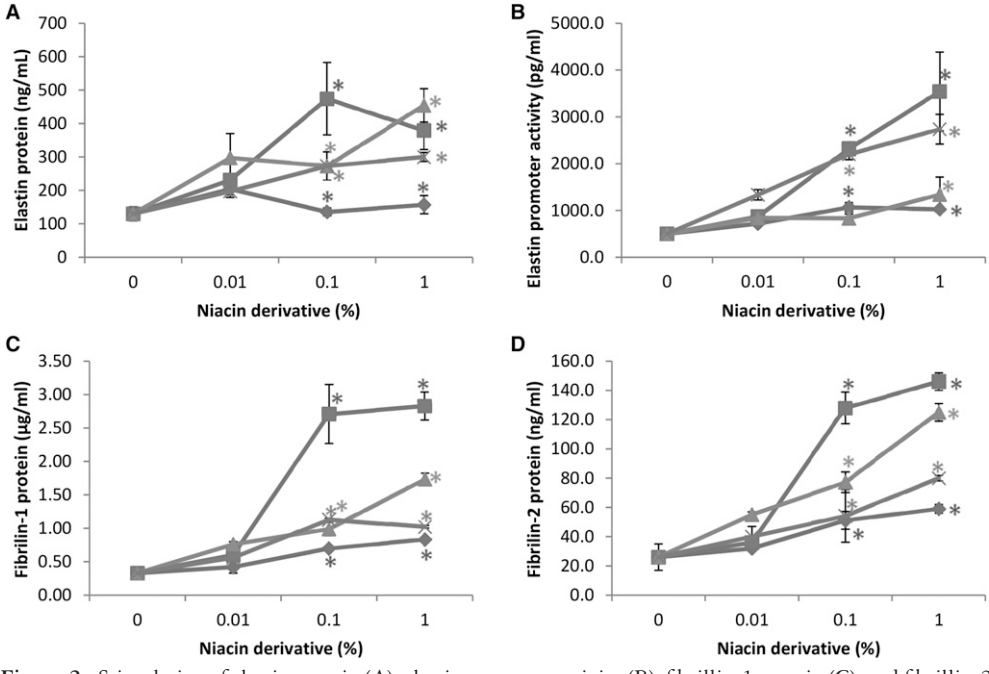

Figure 2. Stimulation of elastin protein (A), elastin promoter activity (B), fibrillin-1 protein (C), and fibrillin-2 protein (D) by nicotinamide (green line), 2,6-dihydroxynicotinamide (red line), 2,4,5,6-tetrahydroxynicotinamide (violet line), and 3-hydroxypicolinamide (blue line) in UVA-radiated dermal fibroblasts; * = $p < 0.05$, relative to UVA radiated control cells, error bars (A–D) represent standard deviation, $n = 4$.

(Figure 3A). There was direct stimulation of MMP-3 activity by 3-hydroxypicolinamide upto 329% of control, respectively ($p < 0.05$) (Figure 3B).

The MMP-9 activity was significantly inhibited only by nicotinamide, upto 44% of control ($p < 0.05$) (Figure 3C).

The elastase activity was inhibited by nicotinamide, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide and 3-hydroxypicolinamide at 1–40%, 72%, 70%, and 66% of control, respectively ($p < 0.05$) (Figure 3D).

DISCUSSION

Nicotinamide, with an amide linked to an aromatic ring has UV absorptive, anti-inflammatory properties, cellular metabolic, and antiapoptotic properties. The hypothesis of this research was that nicotinamide and its derivatives, 2,6-dihydroxynicotinamide, 2,4,5,6-tetrahydroxynicotinamide, and 3-hydroxypicolinamide would stimulate elastin and fibrillin in nonirradiated, and UVA radiated dermal fibroblasts, and exhibit direct antiproteolytic activity on the ECM proteins.

The elastin fibers, composed of elastin and fibrillin microfibrils, give skin its firmness and elasticity (1–4,15–18). The formation of the elastin fibers in the dermis is directed by the microfibrils, composed primarily of fibrillin-1 and fibrillin-2. The microfibrils also form at the epidermal–dermal junction (15). There is loss of elastin and fibrillin with intrinsic aging, and in addition elastotic deposits occur in photoaged skin (1–4,15–18). Nicotinamide

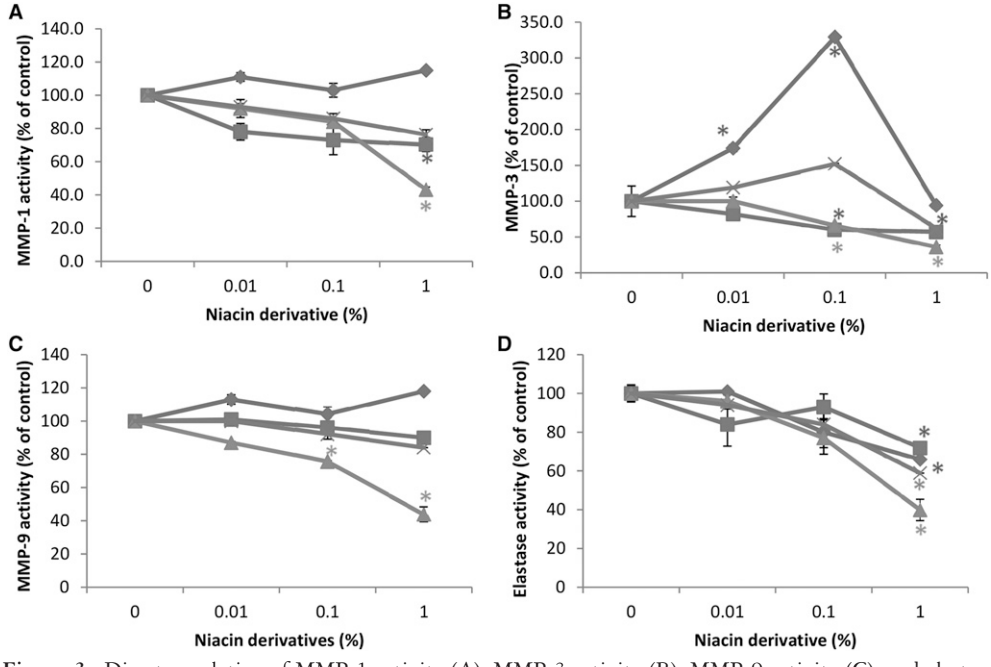

Figure 3. Direct regulation of MMP-1 activity (A), MMP-3 activity (B), MMP-9 activity (C), and elastase activity (D) by nicotinamide (green line), 2,6-dihydroxynicotinamide (red line), 2,4,5,6-tetrahydroxynicotinamide (violet line), and 3-hydroxypicolinamide (blue line); * = $p < 0.05$, relative to control, error bars (A–D) represent standard deviation, $n = 4$.

(4–5%), independently or in combination with peptide/retinyl propionate, reduces skin wrinkles and increases skin elasticity in subjects; and is better tolerated than tretinoin (42–44). The niacin derivatives stimulated the expression of elastin and fibrillin in nonirradiated and UVA radiated fibroblasts, with nicotinamide and 2,6-dihydroxynicotinamide having a more potent effect. It is inferred that niacin or its derivatives improve skin elasticity by stimulating the expression of elastin and fibrillin in dermal fibroblasts.

There is loss of elastin and fibrillin with intrinsic aging, and in addition elastotic deposits occur in photoaged skin (1–4,15–18). In dermal fibroblasts, UVA radiation stimulates elastin expression whereas UVB-radiation inhibits it (2). Topical 5% nicotinamide counteracts UV-radiation-induced immunosuppression in human subjects (37). Nicotinamide at 50 μm prevents UV-radiation-induced oxidative damage in HaCat keratinocytes (40). UVA radiation stimulated the expression of elastin, and inhibited fibrillin-1 and fibrillin-2 protein levels in dermal fibroblasts. The expression of elastin, fibrillin-1, and fibrillin-2 were significantly stimulated by 0.1% and 1% each of the four niacin derivatives in UVA radiated fibroblasts, which suggests that the mechanism to nicotinamide's counteraction of the clinical signs of photoaging is through the stimulation and formation of proper elastin fibers. The increased expression of fibrillin-1 and fibrillin-2 may allow for deposition of well-formed elastic fibers instead of elastotic deposits.

The ECM is remodeled by MMPs and elastases (19–26). The proteolytic enzymes of collagen and elastin fibers include MMPs-1, -3, -9 and elastase (18–26). A mechanism to the damage to the ECM with skin aging is the increased activity of these ECM remodeling enzymes. The niacin derivatives inhibited elastase activity, and nicotinamide and 2,6-dihydroxynicotinamide inhibited the activities of MMPs, which suggests protective effects on the dermal collagen and elastin fibers.

CONCLUSION

The clinical signs of intrinsic aging and photoaging result from increased oxidative stress, which activates mitogen activated protein kinase pathway and activator protein (AP)-1 transcription factor, and inflammation, which activates nuclear factor-kappa B (NF-κB) transcription factor (5–7,27). Nicotinamide reduces wrinkles and improves the appearance of skin (35–41). This research reports for the first time the anti–skin aging mechanism of nicotinamide and its derivatives; 2,6-dihydroxynicotinamide; 2,4,5,6-tetrahydroxynicotinamide; and 3-hydroxypicolinamide through the stimulation of elastin transcription and fibrillin expression in nonirradiated and UVA radiated fibroblasts; and the direct inhibition of ECM proteolytic enzymes. The transcription of elastin is inhibited by AP-1 and NF-kB transcription factors (47,48). It is inferred that the niacin derivatives reduce oxidative stress and inflammation, and thereby AP-1 and NF-kB transcription factors; to facilitate the increased transcription of the elastin gene. The formation of organized elastin fibers *in vivo* would need to be investigated to validate the ECM effect of nicotinamide in skin.

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