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ORIGINAL RESEARCH

Topical effects of *N*-acetyl-L-hydroxyproline on ceramide synthesis and alleviation of pruritus

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Purpose: *N*-acetyl-L-hydroxyproline (AHYP) is an acetylated form of L-hydroxyproline that is used to treat skin ulcers and porphyria cutanea tarda. Its other biological and physiological effects on the skin have not been elucidated. We investigated the effects of AHYP on the skin-barrier function, focusing on ceramide synthesis and the effects of topical AHYP on atopic dermatitis.

Materials and methods: AHYP was applied to a three-dimensional cultured skin model. Ceramides were quantified by high-performance thin-layer chromatography. Serine palmitoyltransferase (SPT) is the rate-limiting enzyme in de novo ceramide synthesis, and the mRNA of its long-chain base subunit 1 (*SPTLC1*) was evaluated by quantitative reverse-transcription polymerase chain reaction. A clinical trial in the form of an intraindividual, comparative, double-blind, randomized, vehicle-controlled test involving 15 female subjects suffering from slight atopic dermatitis was performed. Subjects applied 1% (w/w) AHYP cream to one forearm and a control cream to the other forearm twice daily for 4 weeks. Skin condition was evaluated by measuring transepidermal water loss (TEWL). Dermatological observations were made by a dermatologist, and subjects evaluated their own pruritus intensity before beginning treatment and 4 weeks after the start of treatment.

Results: *SPTLC1* expression and ceramide synthesis were significantly increased in an AHYP-treated skin model ($P < 0.05$). In the clinical trial, no adverse effects were observed in any subjects. TEWL was increased in the control-treated region of the forearm ($P < 0.05$) after 4 weeks' application, whereas there was no change in the AHYP-treated region of the forearm. Pruritus intensity declined in the AHYP-treated forearms between 0 and 4 weeks ($P < 0.05$), but there was no change in the control-treated forearms.

Conclusion: AHYP increased ceramide synthesis by upregulating *SPTLC1* in a three-dimensional cultured skin model, and it prevented TEWL increase and alleviated pruritus in human subjects with slight atopic dermatitis.

Keywords: skin barrier, ceramide, pruritus, *N*-acetyl-L-hydroxyproline, amino acids

Introduction

N-acetyl-L-hydroxyproline (AHYP) (Figure 1) is an acetylated form of L-hydroxyproline that has been used orally or topically for a long time as oxaceprol (CAS 33996-33-7) to treat rheumatoid arthritis and osteoarthritis.[1] Orally administered AHYP increased the synthesis of collagen in burn-injured skin in rabbits;[2] healing effects have been reported in skin-ulcer patients[3] and in porphyria cutanea tarda patients.[4] However, the other effects of AHYP have not yet been elucidated. We examined the effects of AHYP on ceramide synthesis in a three-dimensional cultured

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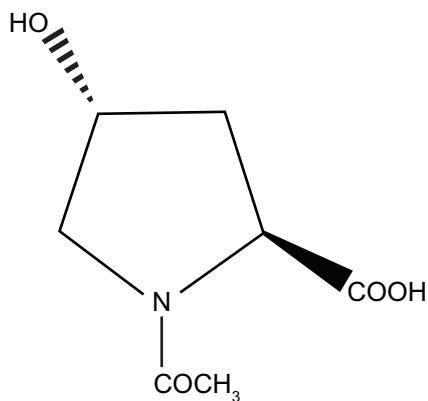
Figure 1 Structure of *N*-acetyl-L-hydroxyproline.

skin model, as well as the effects of topical AHYP cream in patients with atopic dermatitis.

Materials and methods
In vitro study in a three-dimensional cultured skin model
Model and tissue cultivation
Three-dimensional cultured skin models (Testskin living skin equivalent-high; Toyobo, Tokyo, Japan) were cultured at 37°C in living skin equivalent-assay medium in a 5% CO_2 humidified incubator for 48 hours to evaluate gene expression and for 7 days to analyze ceramide synthesis. Some three-dimensional cultured skin models were described to provide a promising means for both fundamental and applied skin research,[5] and Testskin was used for evaluation of stimulating ceramide production.[6] Test samples were prepared as follows. Vehicle control samples consisted of phosphate-buffered saline (PBS) (−). Test samples consisted of AHYP (Kyowa Hakko Bio, Tokyo, Japan) dissolved in PBS (−) and then adjusted to pH 7.0 with 4 N NaOH. AHYP samples were prepared at 1 or 3 mg/mL. Each sample (0.2 mL) was applied to the top of the three-dimensional cultured skin model twice a day for the times mentioned above.

Lipid extraction and HPTLC assay
Epidermal layers were detached from the cultured three-dimensional skin model, then rinsed with PBS (−) and freeze-dried. Lipids containing ceramide were extracted in chloroform:methanol (2:1) by sonication for 5 minutes. The pooled lipid extracts were dried under N_2, and then the pellets were dissolved in 250 μL of chloroform:methanol (2:1). Ceramide standards of Cer[NS], Cer[AP], and Cer[EOP] (Cosmoferm, Delft, Netherlands) were also dissolved in chloroform:methanol (2:1).

The lipid extracts were fractionated by high-performance thin-layer chromatography (HPTLC) on a silica gel 60-glass plate (Merck, Darmstadt, Germany) using chloroform:methanol:acetate (190:90:1).[7] The plates were liberally sprayed with 10% (w/v) cupric sulfate and 8% (w/v) orthophosphoric acid aqueous solution and then heated in an oven at 180°C for 15 minutes. The fractionated lipids were identified from the rate-of-flow values of the ceramide standards and quantified by density measurements with a CS Analyzer version 2.0 (Atto, Tokyo, Japan).

Total RNA preparation and RT-PCR assay
Epidermal layers detached from the cultured three-dimensional skin model were washed with PBS (−) and treated in 1 mL TRIzol reagent. Total RNA was isolated by using a TRIzol Plus RNA Purification Kit (Life Technologies, Carlsbad, CA, USA). One microgram of RNA was provided for cDNA synthesis by using the superscript first-strand synthesis system according to the manufacturer's protocol (Life Technologies). The resulting first-strand cDNA from each RNA sample was amplified with TaqDNA Polymerase (Roche Applied Science, Penzberg, Germany), with primers designed with DNASIS Mac version 3.5 (Hitachi Software Engineering, Tokyo, Japan), namely forward 5′-GTGACCACAACCCGAATG-3′ and reverse 5′-GATTACAGGCATCCCGTAG-3′. Reverse transcription polymerase chain reaction (RT-PCR) was performed with initial denaturation at 95°C for 10 minutes followed by 35 cycles of 15 seconds at 95°C and 1 minute at 50°C. The PCR products were electrophoresed and stained with SYBR Gold Nucleic Acid Gel Stain (Life Technologies), and quantified by scanning densitometry with the CS Analyzer version 2.0. The data were normalized against the housekeeping gene cyclophilin and converted to ratios (%) relative to the control value.

Cytotoxicity assay
The cytotoxicity of AHYP was tested by using the Alamar Blue method according to the manufacturer's instructions (Alamar Biosciences, Sacramento, CA, USA). After 7 days of cultivation, three-dimensional cultured skin models were cultured at 37°C for 2 hours in Alamar Blue-containing medium. The fluorescence intensities of the supernatants were calculated with a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA) with Ex/Em = 560/590 nm.

Statistical analysis
Results are presented as means ± standard deviation. Data were analyzed for statistical significance by using a two-sided

paired *t*-test (PASW Statistics 18; IBM, Armonk, NY, USA). A *P*-value of < 0.05 was considered statistically significant.

Clinical trial in human subjects with atopic dermatitis

Subjects

Fourteen female volunteer subjects (20–49 years old, mean 30.3 years) were chosen by pre-examination. They had been diagnosed with slight atopic dermatitis by a dermatologist in accordance with the atopic dermatitis treatment guidelines of the Japanese Dermatological Association (Table 1).[8] Pregnant or breast-feeding women were excluded, as were those with any type of internal disease that could influence the study outcome.

Test cream

Both the test cream and the control cream were provided by CS Lab (Tokyo, Japan). The test-cream components, listed according to the International Nomenclature of Cosmetic Ingredients, were: aqua, squalane, glycerin, glyceryl stearate, stearic acid, steareth-20, cetyl alcohol, arginine, 1% (w/w) AHYP, dimethicone, sorbitan stearate, 1,2-hexanediol, caprylyl glycol, and phenoxyethanol. The control cream did not contain AHYP, but was otherwise the same as the test cream.

Study design

We performed an intraindividual, comparative, double-blind, randomized, vehicle-controlled study under the dermatological direction of a contract research organization (Inforward, Tokyo, Japan) in winter in Tokyo, Japan. Subjects applied a 1% (w/w) AHYP cream to one forearm and a control cream to the other forearm twice daily for 4 weeks. Subjects were instructed not to use any other new medicines to treat their skin, apart from their usual treatment, and not to change or add any kind of emollient or medicine during the study. Before beginning treatment and at 2 and 4 weeks after the start for treatment,

transepidermal water loss (TEWL) was evaluated on the treated region of each forearm. Dermatological observations and self-evaluation of pruritus intensity were made before the start of treatment and 4 weeks after the start.

Transepidermal water loss

All subjects rested in a room controlled at 45% ± 5% humidity and 21°C ± 2°C temperature for 20 minutes before assessment. TEWL was measured with a VapoMeter SWL-2 (Delfin Technologies, Kuopio, Finland). Two measurements per site were performed, and the means were used for statistical analysis. Data were expressed as $g/m^2/hour$ (means ± standard error of mean).

Pruritus change

The pruritus intensity on each of the subjects' forearms was evaluated by using a 100 mm visual analog scale on an interview sheet (0 mm = no itch, 100 mm = maximum itch) before and after the 4-week application. Visual analog scale scores were analyzed statistically.

Statistical analysis

Statistical differences were determined by two-sided paired *t*-test or Wilcoxon signed-rank test, using PASW Statistics 18. All differences were considered significant at $P < 0.05$.

Ethical approval

The study was approved by the local ethics committee (Medical Board of Shinjuku Minamiguchi Clinic, Tokyo, Japan). Individual subjects agreed to an informed consent contract. The contract confirmed their willingness to participate in the test, their freedom to opt out at any time, and their willingness to use the agent under the control of a doctor. The confidentiality of each participant's information was also safeguarded under this contract.
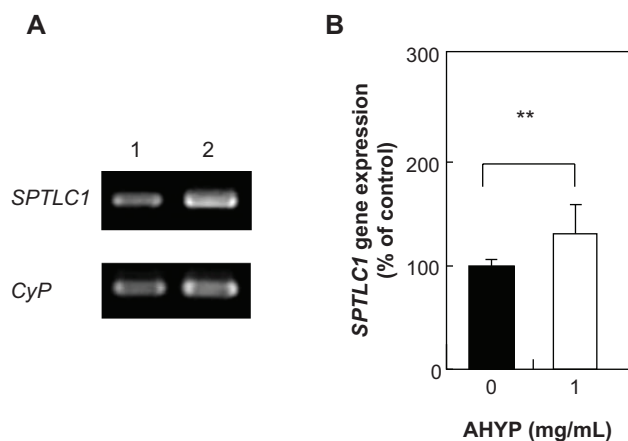
Results

Three-dimensional cultured skin model and tissue cultivation

RT-PCR analysis

SPTLC1 expression in the epidermal layer treated with 1 mg/mL AHYP was significantly increased to 128% of that with the control ($P < 0.01$) (Figure 2).

HPTLC detection of ceramides

Synthesis of Cer[NS] in the epidermal layer of the three-dimensional cultured skin models was significantly

Table 1 Severity of atopic dermatitis (from the atopic dermatitis treatment guidelines of the Japanese dermatological association)

Severity	Eruption
Severe	Primarily severe swelling/edema/infiltration or erythema with lichenification, multiple papules, severe scales, crusts, vesicles, erosion, multiple excoriations, and pruriginous nodules
Moderate	Primarily moderate erythema, scales, a few papules and excoriations
Mild	Primarily dryness, mild erythema, and scales
Slight	Primarily dryness with negligible inflammation

A

1   2

SPTLC1

CyP

B

Figure 2 (**A** and **B**) Effects of *N*-acetyl-L-hydroxyproline (AHYP) treatment on gene expression in a three-dimensional cultured skin model. (**A**) Gene expression in the three-dimensional cultured skin model. Vehicle, lane 1; 1 mg/mL AHYP, lane 2. (**B**) Bar graph representing a densitometric analysis of the results in A.
Notes: **P < 0.01 vs control. *P*-values were calculated by paired *t*-test. Data were normalized against the expression of the housekeeping gene *CyP* and converted into ratios compared with the control (0 mg/mL AHYP) results; they are expressed as means ± standard deviation (n = 3).
Abbreviations: *SPTLC1*, serine palmityl transferase; *CyP*, cyclophilin.

increased with 1 or 3 mg/mL AHYP (128% and 115%, respectively, of that in the control; $P < 0.01$). Synthesis of Cer[AP] was significantly increased with 1 mg/mL AHYP (174%, $P < 0.01$) but not with 3 mg/mL. Cer[EOP] synthesis in the model treated with 3 mg/mL AHYP was

significantly greater than in the control (108%, $P < 0.05$); 1 mg/mL AHYP treatment nonsignificantly increased the synthesis of this subfraction. Concentration dependence of AHYP was not found with the synthesis of these ceramides (Figure 3).

Cytotoxicity assay
Supernatant fluorescence intensity was measured as an indicator of cell viability. Tissues treated with 1 mg/mL AHYP showed 103.2% ± 5.1% cell viability compared with the control; those treated with 3 mg/mL showed 103.5% ± 5.2% cell viability. There were no significant differences in cell viability between the AHYP treatments and the control (Table 2).
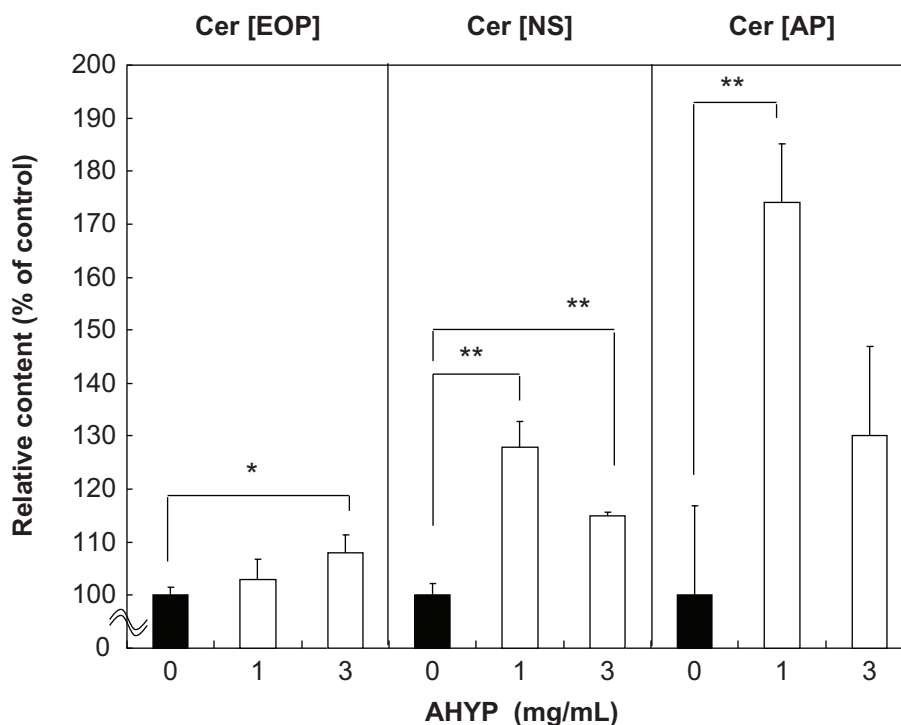
Clinical trial in atopic dermatitis patients
Withdrawals
Data were available from 14 of the 15 patients who began the trial. One participant opted out for personal reasons.

Safety
Dermatological evaluation revealed no adverse side effects in the test; that is, there was no inflammation, irritation, or allergic reactions of the skin in any of the subjects.

Figure 3 Effects of *N*-acetyl-L-hydroxyproline (AHYP) treatment on ceramide (CER) contents in the epidermal layer of three-dimensional cultured skin models.
Notes: *P < 0.05; **P < 0.01 vs control. *P*-values were calculated by paired *t*-test. Bar graphs represent the results of a densitometric analysis with high-performance thin-layer chromatography. Values are ratios compared with control (0 mg/mL AHYP) results and are expressed as means ± standard deviation (n = 3).

Table 2 Cytotoxicity assay of *N*-acetyl-L-hydroxyproline (AHYP) in the three-dimensional cultured skin model

Concentration of AHYP	Supernatant fluorescence intensity (% of control)
1 mg/mL	103.2 ± 5.1
3 mg/mL	103.5 ± 5.2

Efficacy

Transepidermal water loss

The TEWL of the region of the forearm treated with control cream was significantly greater at 4 weeks (9.7 ± 0.5 g/m^2/hour) than at 0 weeks (9.0 ± 2.5 g/m^2/hour) ($P < 0.05$). In contrast, the TEWL of the region of the forearm treated with AHYP showed no change (0 weeks, 9.4 ± 0.6 g/m^2/hour; 4 weeks, 9.4 ± 0.4 g/m^2/hour) (Figure 4A).
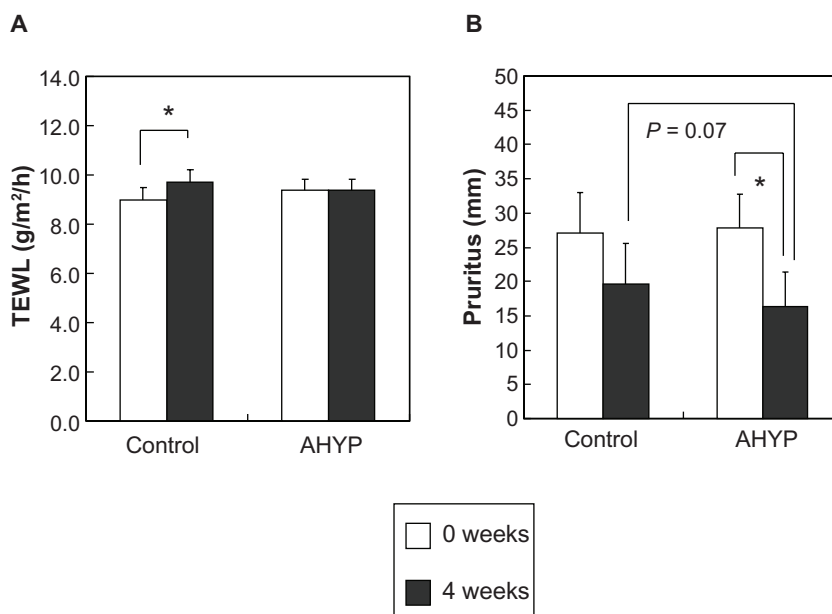
Pruritus change

No significant change in pruritus intensity was observed in the control-treated forearms (0 weeks, 27.1 ± 5.9 mm; 4 weeks, 19.6 ± 6.0 mm, $P > 0.1$). In contrast, topical application of AHYP cream resulted in a significant reduction in pruritus intensity at 4 weeks compared with at 0 weeks (0 weeks, 27.8 ± 5.9 mm; 4 weeks, 16.4 ± 5.0 mm, $P < 0.05$) (Figure 4B).

Discussion

AHYP increased the expression of *SPTLC1*, which encodes the long-chain base subunit 1 of serine palmitoyltransferase

(SPT), in the three-dimensional cultured skin model. Synthesis of ceramide is regulated by several enzymes, such as sphingomyelinase, β-glucocerebrosidase, ceramidase,[9,10] and SPT. SPT catalyzes the initial rate-limiting step in ceramide synthesis.[11] AHYP also significantly promoted synthesis of the ceramide subfractions Cer[NS], Cer[AP], and Cer[EOP] in the epidermal layer of the three-dimensional cultured skin models. Although SPT activity has not been measured in atopic dermatitis,[12] SPT is the key enzyme in ceramide synthesis, and ceramide was shown to be deficient in stratum corneum of atopic dermatitis,[7,13] so that it may play a important role in skin. Considering from above, it is suggested that AHYP increases ceramide synthesis by upregulation of SPT.

The skin-barrier function is important in maintaining skin health, and skin-barrier dysfunction is one factor in skin disease.[14] A correlation between skin-barrier function and disease severity has been reported in atopic dermatitis,[15] and TEWL is increased in both dry skin and clinically normal skin in subjects with atopic dermatitis.[16] Stratum corneum intercellular lipids, such as ceramides, cholesterol esters, and free fatty acids, influence skin-barrier function.[17] Almost half of the intercellular lipids of the stratum corneum are ceramides,[18,19] and stratum corneum ceramide levels are decreased in skin with barrier dysfunction[13] and in atopic dermatitis patients.[7,21] The ceramides of the stratum corneum are therefore very important components for maintenance of skin barrier function.[22]

Figure 4 (**A** and **B**) Effects of *N*-acetyl-L-hydroxyproline (AHYP) on transepidermal water loss (TEWL) and pruritus intensity. (**A**) Changes in TEWL; (**B**) changes in pruritus intensity.
Notes: *$P < 0.05$, difference considered significant. Values are means ± standard error of mean (n = 14). Each measurement was taken twice, and the average was used for statistical analysis. *P*-values were calculated for differences within treatments and between treatments by two-sided paired *t*-test.

Application of nicotinamide increases ceramide biosynthesis and decreases TEWL in dry skin.[23] The TEWL value shows TEWL from the skin surface and is used as an index of skin-barrier function.[24] When the barrier function declines or is destroyed, the skin surface becomes sensitive to stimulation and itches.[25] The clinical association between itching and dry skin is well established, and the clinical conditions involving dry skin and itch include common skin diseases, such as atopic dermatitis, psoriasis, xerosis, and pruritus in the elderly.[26]

Our study was performed in winter (November–December), when the air in Tokyo is getting drier and colder. After 4 weeks' application, TEWL was increased on control-treated forearms but unchanged on AHYP-treated forearms. Since both test creams contained such ingredients as water, glycerin, squalane, etc, which could act as moisturizers, it was difficult to show a significant difference between control and AHYP cream, while pruritus intensity significantly declined only in the AHYP-treated forearms between 0 and 4 weeks. Relative levels of Cer[EOS-O] (ceramide 1 linoleate) are depleted in winter.[27] Ceramides themselves are not considered to maintain skin hydration, but they help to prevent water loss.[28] Although we did not measure ceramide contents in the human study, our data strongly suggest that AHYP application potentiates the barrier function of the skin by promoting ceramide synthesis and suppressing TEWL increase. This could be the mechanism by which AHYP alleviates pruritus.

AHYP affected Cer[EOP] content dose-dependently, however Cer[NS] and Cer[AP] content changes did not correspond with it. It is suggested that there are optimum AHYP concentrations for each ceramide synthesis promotion. In our study, AHYP concentrations were limited, and further study will be necessary. We observed no cytotoxicity in the three-dimensional cultured skin models at the test concentrations of AHYP (1 or 3 mg/mL), and there were no adverse effects in the clinical trial using 1% (w/w) AHYP. These results, together with the fact that AHYP has been used as a medicament for a long time, support the safety of AHYP as a topical ingredient.

Conclusion

To our knowledge, this study is the first to demonstrate that AHYP, which is an acetylated derivative of an amino acid, increases ceramide synthesis by upregulating *SPTLC1* expression in three-dimensional cultured skin models. Topical application also alleviated pruritus in atopic dermatitis patients. Our data support the safety of AHYP

and present a new function for this compound as a useful ingredient for improving the skin barrier in dry skin and atopic dermatitis.

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