Pyridoxine Stimulates Filaggrin Production in Human Epidermal Keratinocytes

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Abstract

Pyridoxine (PN), one of the vitamers of vitamin B6, plays an important role in the maintenance of epidermal function and is used to treat acne and rough skin. Clinical studies have revealed that PN deficiency causes skin problems such as seborrheic dermatitis and stomatitis. However, the detailed effects of PN and its mechanism of action in epidermal function are poorly understood. In this study, we examined the effects of PN on epidermal function in normal human epidermal keratinocytes and found that PN specifically causes an increase in the expression of profilaggrin mRNA, among marker genes of terminal epidermal differentiation. In addition, PN treatment caused an increase in the production of filaggrin protein in a concentration-dependent manner. Treatment with P_{2x} purinoceptor antagonists, namely, pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate and TNP-ATP hydrate, induced an increase in the filaggrin protein levels. Moreover, we showed that elevated filaggrin production induced upon PN treatment was suppressed by ATP (known as P_{2x} purinoceptor agonist). This study is the first to report that PN causes an increase in filaggrin transcription and production, and these results suggest that PN-induced filaggrin production may be a useful target as a daily care component in atopic dermatitis, wherein filaggrin levels are specifically reduced.

Introduction

Vitamin B6 is a multifunctional micronutrient that mediates numerous metabolic processes, including amino acid metabolism, gluconeogenesis, and lipid metabolism. Vitamin B6 includes six water-soluble vitamers: pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), and their phosphorylated forms [1]. Previous studies have suggested that PN deficiency causes skin problems such as seborrheic dermatitis [2] and stomatitis [3], and transcutaneous administration of PN improved these symptoms [4]. Pyridoxal-5-phosphate, a metabolite of PN, has been reported to function as a P_{2X} purinoceptor antagonist in the vagus nerve and vas deferens isolated from rats [5]. Although clinical findings suggest that PN plays an important role in the maintenance of epidermal function, detailed mechanisms have not yet been elucidated. Filaggrin was characterized as a protein involved in the aggregation of keratin fibers in corneocytes during the terminal differentiation of epidermal cells [6]. During the differentiation of granule cells to corneocytes, profilaggrin is dephosphorylated and proteolyzed to filaggrin monomers [7–9]. Filaggrin is degraded into free amino acids, and the amino acids are further metabolized into urocanic and pyrrolidone carboxylic acids in corneocytes. These are called natural moisturizing factors, and are largely responsible for the ability of the stratum corneum of the skin to remain hydrated at low environmental humidity [10]. In skin diseases such as epidermolytic hyperkeratosis and lamelliform ichthyosis, profilaggrin was reported to accumulate in the skin without being degraded to filaggrin, suggesting that filaggrin is beneficial in maintaining epidermal function [11]. Moreover, it has been reported that filaggrin expression levels are lower in the skin of individuals with atopic dermatitis [12].

As described above, filaggrin is expressed in granular cells during its final stage of differentiation and aggregates keratin filaments [6], [9]. During terminal differentiation, lipid processing enzymes are

expressed, and several proteins required for the formation of the cornefied envelope are produced simultaneously [13]. This indicates that filaggrin is expressed along with multiple genes involved in the final stage of differentiation [14, 15]. Among these, the functions and regulatory mechanisms of several transcriptional factors have been elucidated. For instance, it has been shown that the skin of p63-deficient mice does not progress past an early developmental stage, lacks stratification and does not express differentiation markers [16]. In addition, Notch signaling is implicated in the late stage of differentiation [17], and is upregulated in embryonic keratinocytes and epidermis [17, 18]. However, the regulatory mechanisms of profilaggrin transcription are not fully understood.

To identify the mechanisms of PN underlying the maintenance of epidermal function, we first evaluated the effects of PN on the expression of keratinocyte differentiation markers, including filaggrin. We then focused on the effects of PN on profilaggrin expression and production in normal human epidermal keratinocytes (NHEKs). In addition, the involvement of P_{2X} purinoceptor in profilaggrin production was examined.

Materials And Methods

Cell culture

NHEKs (normal human epidermal keratinocytes) and MCDB153-modified specific medium HuMedia KG-2 were obtained from Kurabo (Osaka, Japan). NHEKs were cultured in HuMedia KG2 (Kurabo) at 37 °C with 5% CO_2. Reagents used in this study were dissolved in HuMedia KG-2 without bovine pituitary extract (KG2(-BPE)).

Reagents

Pyridoxine hydrochloride (PH), adenosine 5′-triphosphate disodium salt hydrate (ATP), pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS), TNP-ATP hydrate, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). Triton X-100 was purchased from Wako, Tokyo, Japan. Ambion® Cells-to-CT™ TaqMan® kits, the TaqMan® Fast Universal PCR Master Mix, and Pierce™ BCA Protein Assay kit were purchased from Thermo Fisher Scientific (Kanagawa, Japan). Anti-filaggrin antibody (sd-80609, Lot. C2817) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), horseradish peroxidase-conjugated polyclonal anti-IgG was purchased from Nichirei (Tokyo, Japan), and ElectroChemiLuminescence (Lumi-Light Western blotting substrate) was purchased from Roche Diagnostics (Indianapolis, IA, USA).

Effect of PN on the expression of filaggrin mRNA and other keratinocyte-specific markers in NHEKs.

NHEKs were seeded in 96-well plates at a seeding density of 15,000 cells per well and cultured in KG2 medium for 24 h. Then, the culture medium was replaced with KG2 (-BPE) medium containing 200 μM PN and the cells were further cultured for 6, 12, 24, or 48 h. Total RNA was extracted and real-time

polymerase chain reaction (PCR) for profilaggrin (FLG), serine palmitoyltransferase (SPT), keratin 10 (K10), involucrin (INV), corneodesmosin (CDSN), and loricrin (LOR) was performed.

Effect of PN on filaggrin protein levels in NHEKs.

NHEKs were seeded in 96-well plates at a seeding density of 10,000 cells per well and cultured in KG2 medium for 24 h. Then, the culture medium was replaced with KG2 (-BPE) medium containing a predetermined concentration of PN, PPADS, or TNP-ATP. Then, the cells were cultured for 72 h. Subsequently, cells were lysed using PBS containing 0.5% Triton X-100, and dot blotting for filaggrin protein was performed.

Effect of ATP (P_{2X} purinoceptor agonist) on filaggrin protein levels in NHEKs.

NHEKs were seeded in 96-well plates at a seeding density of 10,000 cells per well and cultured in KG2 medium for 24 h. Then, the culture medium was replaced with KG2 (-BPE) medium containing 200 μM PN and the cells were cultured for 72 h. Next, the culture medium was replaced with KG2 (-BPE) medium containing a predetermined concentration of ATP and the cells were cultured for 24 h. Subsequently, dot blotting for filaggrin protein was performed.

NHEKs were seeded in 96-well plates at a seeding density of 10,000 cells per well and cultured in KG2 medium for 24 h. The culture medium was then replaced with KG2 (-BPE) medium containing a predetermined concentration of ATP and cultured for 72 h. Subsequently, dot blotting against filaggrin protein was performed.

Dot blotting

NHEKs were lysed with PBS containing 0.5% Triton X-100 and 2 mM PMSF (100 μL/well), and the lysates were blotted onto a nitrocellulose membrane (2 μL/spot) and dried overnight at room temperature. The blots were incubated in a blocking solution (PBS containing 1% bovine serum albumin) for 1 h at room temperature. Filaggrin protein was detected with an anti-human filaggrin antibody diluted 1:4,000 for 1 h at room temperature, horseradish peroxidase-conjugated polyclonal anti-IgG diluted 1:100 for 1 h at room temperature, and ECL. Filaggrin protein levels were then quantified by image analysis of the dot-blots. Protein concentrations of NHEK lysates were determined using a BCA protein assay kit. Filaggrin protein levels were expressed as luminescence intensity per protein.

Real-time reverse transcription–PCR (RT-PCR)

Total RNA was extracted from NHEKs using DNase, and 2 μg cDNA was synthesized using the Ambion® Cells-to- CT™ TaqMan® Kits. Real-time RT-PCR was performed using TaqMan® Fast Universal PCR Master Mix and the Step One Plus™ Real-time PCR system (Thermo Fisher Scientific). The holding stage was set at 20 s at 95 °C and the cycling stage was set as follows: 1 s at 95 °C and 20 s at 62 °C, and 40 cycles were performed. The following probes were purchased from Thermo Fisher Scientific and used to amplify selected genes: KRT10 (Hs00166289_m1), FLG (Hs00856927_g1), SPT (Hs00272311_m1), IVN

(Hs00902520_m1), CDSN (Hs00169911_m1), LOR (Hs01894962_s1), and cyclophillin (PPIA control mix). Expression analysis was performed using the ΔΔCT method.

Statistical analysis

All data are indicated as mean ± SEM. GraphPad Prism 5 software (GraphPad Software Inc., CA, USA) was used to analyze data. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or Student's *t* test. $p < 0.05$ was considered statistically significant.

Results

Effect of PN on the expression of keratinocyte-specific markers in NHEKs.

In order to examine the effects of PN on skin function, we first evaluated the effect of PN on the expression of terminal epidermal differentiation markers in NHEKs using real-time PCR. PN caused a significant increase in profilaggrin mRNA expression (by 1.24-fold) upon treatment with 200 μM PN for 24 h. Conversely, the same concentrations of PN exhibited no effect on SPT, K10, INV, CDSN, and LOR mRNA expression levels (Fig. 1A).

Effect of PN on the synthesis of filaggrin mRNA and protein levels in NHEKs.

As treatment with 200 μM PN for 24 h specifically increased profilaggrin mRNA levels, the effects of PN on the synthesis of profilaggrin mRNA and filaggrin protein were evaluated using real-time PCR and dot-blotting, respectively. Treatment for 24 h with PN significantly increased profilaggrin mRNA expression; however, treatment for 6, 12, and 48 h did not induce increased profilaggrin expression (Fig. 1B). In addition, PN treatment caused an increase in the production of filaggrin protein in a concentration-dependent manner (Fig. 1C). Filaggrin protein levels after treatment with 100 μM PN were 1.31-fold higher than those observed upon vehicle treatment (0 μM PN) (Fig. 1C). We confirmed that PN-treated-NHEKs (200 μM PN for 72 h) proliferated at the same level as the untreated control. Moreover, Ca^{2+}-treated NHEKs (1.8 mM Ca^{2+} for 72 h) inhibited cell proliferation (Supplemental Fig. S1A). We also confirmed that the treatment with 200 μM PN did not affect cell morphology. Conversely, treatment with 1.8 mM Ca^{2+} induced the differentiated morphology of NHEKs (Supplemental Fig. S1B).

Effect of P_{2X} purinoceptor antagonists on filaggrin protein levels in NHEKs.

Effects of P_{2X} purinoceptor antagonists on filaggrin protein levels were evaluated using dot blotting. PPADS treatment resulted in an increased filaggrin protein production in a concentration-dependent manner. Results revealed a significant (1.27-fold) increase in filaggrin protein levels upon treatment with 160 μM PPADS compared to those in untreated control (Fig. 2A). Increased production of filaggrin protein in a TNP-ATP concentration-dependent manner was also observed. TNP-ATP treatment (12 μM) caused a

significant increase in filaggrin protein levels compared to those of untreated control (1.24-fold increase) (Fig. 2B).

Inhibitory effect of ATP (P_{2X} purinoceptor agonist) on filaggrin protein levels after treatment with PN in NHEKs.

The inhibitory effect of ATP on filaggrin protein levels upon treatment with PN was evaluated using dot blotting. Treatment with 200 μM PN significantly increased filaggrin protein levels in the absence of ATP or with 25 μM ATP. However, ATP treatment (50 and 100 μM) prevented the PN (200 μM)-induced filaggrin production (Fig. 3A). Treatment with ATP did not alter filaggrin production in non-stimulated NHEKs (Fig. 3B).

Discussion

In order to evaluate the effect of PN on epidermal function, we first examined the effect of PN on the expression of terminal epidermal differentiation markers. Previous studies have reported that profilaggrin mRNA is expressed along with other epidermal differentiation markers during terminal differentiation of epidermal tissues [16,17]. It has also been shown that the production of various differentiation marker proteins is promoted at the gene expression level by differentiation-inducing stimuli such as calcium ions in an *in vitro* experiment [19]. In the present study, we showed that PN specifically induces an increase in the expression of profilaggrin mRNA. However, similar effects were not observed in other terminal epidermal differentiation markers, suggesting that PN is involved in intracellular functions rather than differentiation. We also demonstrated that PN induced an increase in the filaggrin protein levels through using smaller concentration for the dot blotting experiment because the incubation periods of pyridoxine treatment for conducting dot blotting was 72 h to detect the expression level of filaggrin protein. Also, we confirmed that PN-treated-NHEKs proliferated at the same level as the untreated control, and that PN treatment did not affect the cell morphology. These results also support the hypothesis that PN is involved in intracellular functions rather than differentiation.

Direct evidence has been discovered linking nonsense mutations in the filaggrin gene and atopic dermatitis [20]. In addition, decreased filaggrin protein levels have been observed in almost all cases of moderate-to-severe atopic dermatitis, even in the absence of genetic mutations of filaggrin [21]. Given these findings, regulating filaggrin expression is considered to be a prospective strategy for the treatment and prevention of atopic dermatitis, and many drugs have been developed from this point of view. We showed that PN specifically caused an increase in profilaggrin expression and production, suggesting that PN is a beneficial compound that can be used in daily care for the prevention of aggravation and recurrences of atopic skin, and that PN can also be used as an ingredient in non-medical skin care products.

P_2 purinoceptors are classified as P_{2X} receptors for inion-gated channels and P_{2Y} receptors that form G-protein-coupled receptors. ATP, a ligand for P_2 purinoceptors, is released from many tissues [22–24] and

has been reported to function as one of the signaling mediators connecting the central and peripheral nervous systems [25, 26]. In epidermal cells, extracellular ATP has been reported to regulate cell growth and differentiation [27–32]. In addition, eight sub-types of P_{2Y} purinoceptors have been identified and are known to contribute to cell growth [28, 30]. On the other hand, seven subtypes of P_{2x} purinoceptors have been identified [33] and have been reported to progress epidermal differentiation [29, 32].We evaluated the effects of P_{2X} purinoceptor antagonists on filaggrin protein production and found that both PN and P_{2X} purinoceptor antagonists resulted in an increase in the filaggrin protein levels. In addition, PN induced-filaggrin production was inhibited by ATP. In general, PN is metabolized to pyridoxal-5-phosphate in the body and to be activated, and pyridoxal-5-phosphate has been reported to function as a P_{2X} purinoceptor antagonist in the vagus nerve and vas deferens isolated from rats [5]. Although further studies are necessary to verify these findings, our results suggest that PN may enhance filaggrin production via P_{2x} purinoceptors.

It has been reported that ATP was released from HaCaT keratinocytes upon changing the culture medium [34]. Furthermore, it has been shown that ATP released from keratinocytes increases cytokine and chemokine secretion and induces inflammation [35, 36]. It was also indicated that inflammatory condition suppresses filaggrin expression and eventually impairs the skin barrier function in atopic dermatitis [37]. We consider that NHEKs may release a certain amount of ATP, and then, P_{2X} receptors might be activated, and that the production of cytokines and chemokines in NHEKs might be increased, thereby resulting in inflammation. In such a situation, P_{2X} receptor antagonists might be used to inhibit P_{2X} receptor activity and suppress the production of cytokines and chemokines, leading to an increase in filaggrin expression. To confirm this, further studies are required to be conducted to clarify the mechanism of P_{2X} receptor function on filaggrin expression.

In summary, this is the first study to report that PN increases filaggrin production in NHEKs. Adequate skin care post medical treatment is considered important for the prevention of recurrence of atopic dermatitis. In addition, it is necessary to provide a non-pharmaceutical formulation that the patients can use for daily skin care as cosmetics and quasi drugs. As PN specifically causes an increase in filaggrin levels in NHEKs, PN would be a putative candidate in daily care formulations for atopic dermatitis, and will contribute to significant improvement of patient quality of life.

Declarations

Author contribution

MF collected the data and wrote the manuscript. SY collected the data. ST wrote the manuscript. TS and IS involved in direction of experiment and writing the manuscript.

Ethics declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

The authors declare that they consent for publication of this study.

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Figures

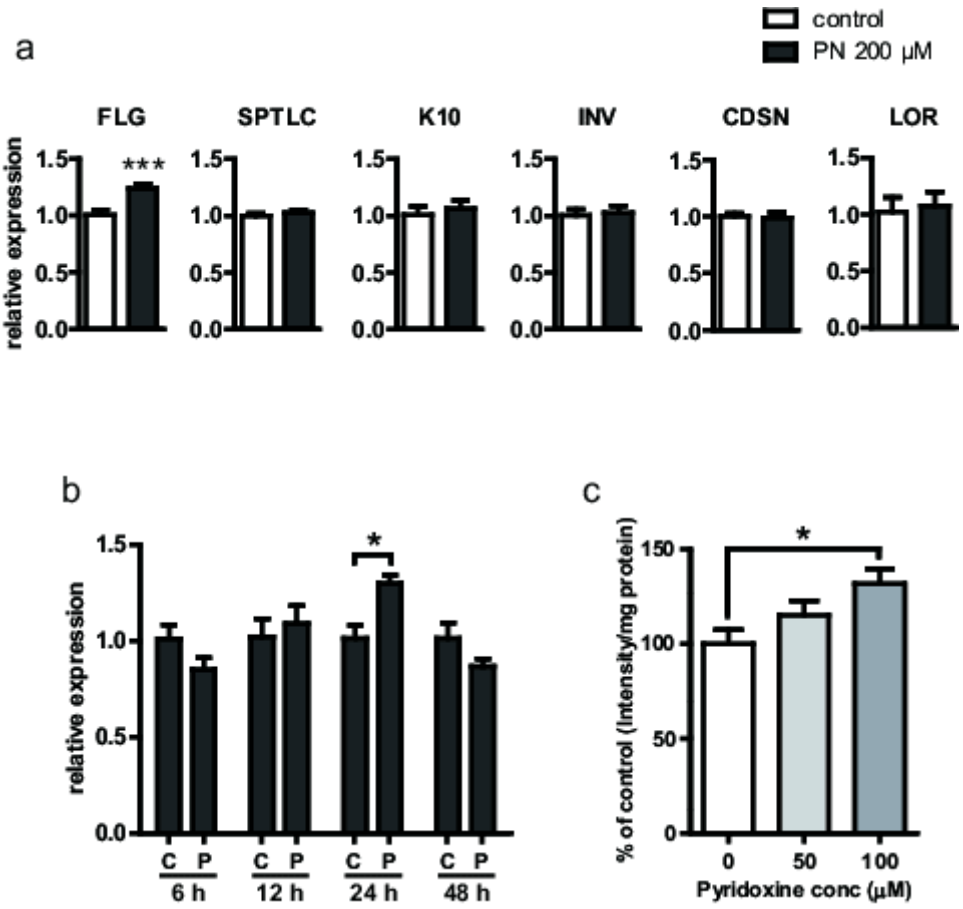

Figure 1

Effect of PN on the expression of keratinocyte-specific markers, the synthesis of filaggrin mRNA and its protein levels in NHEKs. (A)Real-time PCR was performed for the determination of profilaggrin (FLG), serine palmitoyltransferase (SPT), keratin 10 (K10), and involcrin (INV), corneodesmosin (CDSN), and loricrin (LOR) expression. Only FLG mRNA expression was found to increase upon PN treatment. Each value represents the mean ± SEM. n = 4; ***P < 0.001. (B) Although 24 h-treatment of PN caused a significant increase in profilaggrin mRNA expression, 6, 12, and 48 h-treatment did not cause an increase in profilaggrin expression. Each value represents the mean ± SEM. n = 6; *P < 0.05. C; Control. P; PN treatment. (C) Filaggrin protein expression levels were found to be increased after PN treatment in a concentration-dependent manner, and a significant increase was found only upon 100 μM PN treatment. Each value represents the mean ± SEM. n = 10; *P < 0.05 vs 0 μM.

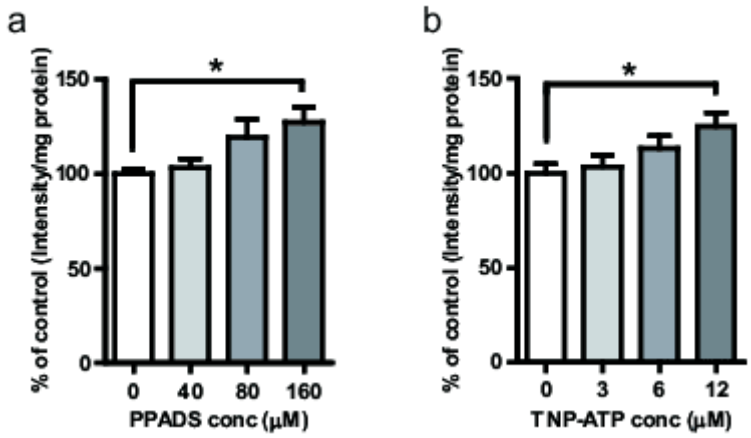

Figure 2

Effect of P2X purinoceptor antagonists on filaggrin protein levels in NHEKs. Both 160 μM PPADS (A) and 12 μM TNP-ATP (B) induced a significant increase in filaggrin protein levels. Each value represents the mean ± SEM. n = 5-10; * P< 0.05 vs 0 μM.

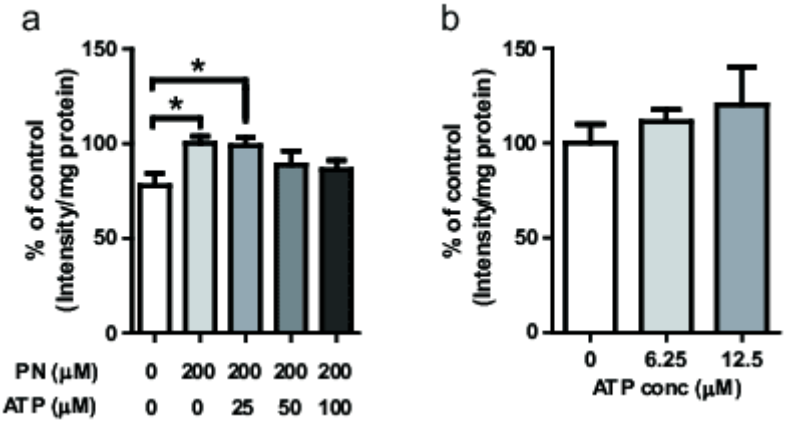

Figure 3

Inhibitory effect of ATP (P2X purinoceptor agonist) on filaggrin protein levels after the treatment of NHEKs with PN. (A) Filaggrin protein levels in the absence of ATP or with 25 μM ATP were found to be significantly increased upon 200 μM PN, however, higher concentrations (50 and 100 μM) of ATP suppressed the increase in filaggrin protein levels induced upon treatment with 200 μM PN. Each value represents the mean ± SEM. n = 10; *P < 0.05 vs 0 μM PN and 0 μM ATP. (B) ATP has no effect on filaggrin protein levels in NHEKs. Each value represents the mean ± SEM. n = 5.

Supplementary Files

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