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 P2x 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 P2x 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.

Keywords Pyridoxine · Filaggrin · P2x purinoceptor · ATP · Epidermal keratinocytes

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 P2x 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 during the transition from a granular cell to a cornified cell [6]. During the differentiation of granular 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 constituents of 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 cornified 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 activated 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 P2X 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% CO2. 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 profilaggrin 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 (P2X 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 1:4000 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 cyclophilin (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 in the result of dot-blotting (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, Ca²⁺-treated NHEKs (1.8 mM Ca²⁺ 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 Ca²⁺ induced the differentiated morphology of NHEKs (Supplemental Fig. S1B).

**Effect of P₂X purinoceptor antagonists on filaggrin protein levels in NHEKs**

Effects of P₂X 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 (P₂X 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 [14, 15]. 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. In a previous study, Otsuka et al. found that JTC801 (a selective antagonist against nociceptin receptor) showed a stimulatory effect on filaggrin mRNA expression without causing epidermal differentiation; AP-1 has been reported to be the main transcription factor for JTC801-dependent filaggrin expression [20]. Although further detailed studies are required to elucidate the mechanism underlying the effect of PN on filaggrin transcription, AP-1 might be a possible candidate regulator of PN-induced filaggrin expression. 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 linking the nonsense and frameshift mutations in the filaggrin gene and atopic dermatitis has been discovered [21, 22]. 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 [23]. 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.

P2 purinoceptors are classified as P2X receptors for ion-gated channels and P2Y receptors that form G-protein-coupled receptors. ATP, a ligand for P2 purinoceptors, is released from many tissues [24–26] and has been reported to function as one of the signaling mediators connecting the central and peripheral nervous systems [27, 28]. In epidermal cells, extracellular ATP has been reported to regulate cell growth and differentiation [29–34]. In addition, eight sub-types of P2Y purinoceptors have been identified and are known to contribute to cell growth [30, 32]. Seven subtypes of P2x purinoceptors have been identified [35] and have been reported to progress epidermal differentiation [31, 34]. On the other hand, pyridoxal-5-phosphate, which is a metabolite of PN, has been reported to function as a P2X purinoceptor antagonist in the vagus nerve and vas deferens isolated from rats [5], we therefore speculated that P2X receptor antagonists could increase filaggrin protein production. We found that PPADS and TNP-ATP (P2X receptor antagonists) increased the filaggrin protein production, similar to that observed for PN. In addition, PN induced-filaggrin production was inhibited by ATP. Although further studies are necessary to verify these findings, our results suggest that PN may enhance filaggrin production via P2X purinoceptors.

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.

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

**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.

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

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