Abstract. The most common adverse effects that are related to all-trans retinoic acid (atRA) treatment are irritation and dryness of the skin. atRA therapy is reported to impair barrier function as achieved by trans-epidermal water loss (TEWL). Treatment with nicotinamide prior to initiation of atRA therapy provides additional barrier protection and thus reduces susceptibility of retinoic acid. Our previous studies showed that atRA upregulates aquaporin 3 (AQP3) in cultured human skin keratinocytes and fibroblasts. Others have demonstrated that in atopic dermatitis, overexpression of AQP3 is linked to elevated TEWL and that nicotinamide treatment reduces skin TEWL. In this study, we observed that while atRA upregulates AQP3 expression in cultured human skin keratinocytes (HaCaT cells), nicotinamide attenuates the effect of atRA in a concentration-dependent manner. atRA treatment induces EGFR and ERK activation. PD153035, an EGFR inhibitor, and U0126, an ERK inhibitor, inhibit atRA-induced upregulation of AQP3. Nicotinamide also inhibits atRA-induced activation of EGFR/ERK signal transduction and decreases water permeability by downregulating AQP3 expression. Collectively, our results indicate that the effect of atRA on AQP3 expression is at least partly mediated by EGFR/ERK signaling in cultured human skin keratinocytes. Nicotinamide attenuates atRA-induced AQP3 expression through inhibition of EGFR/ERK signal transduction and eventually decreases water permeability and water loss. Our study provides insights into the molecular mechanism through which nicotinamide reverses the side effects of dryness in human skin after treatment with atRA.

Introduction

All-trans retinoic acid (atRA), a major biologically active retinoid, remains one of the most important regulators of skin function. Topical treatment with atRA improves the clinical and histological appearance of long-term photo-damaged skin (1). Although retinoic acid provides benefits to sun-exposed skin, it is frequently accompanied by significant skin irritation which limits its clinical application. The most common side effects of retinoid acid treatment are irritation and dryness of the skin (2). The mechanisms leading to retinoid adverse effects have not been fully elucidated, but retinoic acid therapy has been demonstrated to impair barrier function as assessed by trans-epidermal water loss (TEWL) measurement (3-5).

Skin water transport has a close relationship with the expression of aquaporin 3 (AQP3) in skin keratinocytes. Recent studies have shown that AQP3-deficient mice show delayed skin barrier recovery, suggesting a possible involvement of AQP3 in the regulation of keratinocyte differentiation and proliferation (6). Moreover, inhibition of AQP3 activity significantly decreases epidermal water flux (7). In contrast, higher AQP3 expression is observed in young rat skin, which is thought to be associated with increased TEWL in young rats (8). Evidence also shows that higher expression of AQP3 is linked to increased TEWL in atopic eczema (AE), leading to skin dryness in AE (9). It has been demonstrated that atRA increases expression of AQP3 in normal human skin (10), suggesting that AQP3 is involved in the impairment of skin barrier function induced by atRA.

Clinically, some moisturizers have been proven to improve the skin barrier function of atopic dry skin. Nicotinamide, also known as vitamin B3, has been demonstrated to have beneficial effects on certain skin conditions, such as photo...
damage and cutaneous hyperpigmentation (11-14). Studies have shown that topical nicotinamide application on dry human skin results in increased levels of ceramide and free fatty acids in the epidermis, and decreased levels of TEWL, suggesting that nicotinamide improves the epidermal permeability barrier (15). Furthermore, myristyl nicotinate reduces TEWL and provides an additional barrier protection and tolerability of retinoic acid (16). However, the question whether nicotinamide retains skin barrier function by attenuating atRA-induced AQP3 overexpression remains to be answered.

The promotion of keratinocyte proliferation and differentiation by retinoids is partly mediated by the epidermal growth factor receptor (EGFR) signal pathway. EGFR activation plays a central role in retinoid-induced epidermal hyperplasia and EGFR inhibitors mitigate retinoid-induced scaling (17). The involvement of the EGFR pathway in atRA signaling was further demonstrated by increased phosphorylation of EGFR and its downstream signaling component ERK1/2 in our previous study (18). The evidence that ERK1/2 activation by atRA is abolished by EGFR inhibitor AG1478 strongly suggests that the EGFR and ERK1/2 signal pathway is involved in atRA actions in many cell types in vitro and in human skin epidermis in vivo (18-20). However, the question whether nicotinamide antagonizes atRA-induced AQP3 overexpression by inhibiting EGFR and ERK1/2 activation needs to be further addressed.

Thus, the aim of this study was to investigate whether nicotinamide treatment prior to atRA attenuates water permeability mediated by AQP3 in cultured human keratinocytes. Furthermore, we undertook this study to investigate whether the EGFR/ERK signal pathway is involved in AQP3 expression and whether nicotinamide decreases AQP3 expression by inhibiting EGFR/ERK activation.

Materials and methods

Cell culture. As described previously (21,22), spontaneously immortalized human keratinocytes (HaCaT cells) and human skin fibroblasts were maintained in a DMEM medium (Sigma, St. Louis, MO) supplemented with a 10% fetal bovine serum (FBS) and 1% Pen/Strep and 0.49 mM L-glutamine (Sigma), in a CO2 incubator (5% CO2, 95% air) at 37°C. For Western blotting, cells were reseeded in 6-well plates at a density of 1x10^5 cells/ml with fresh complete medium that did not contain antibiotics, for 4 days. Cells (1x10^5) were seeded into a 6-well plate 1 day prior to transfection and cultured to 70-80% confluence the following day. For RNAi experiments, 12 μl of FuGene6 (Roche Diagnostics, Indianapolis, IN) was diluted in 88 μl of DMEM for 5 min in room temperature. Then 10 μl of 20 μM double-stranded RNAs for AQP3 RNAi was mixed with DMEM containing FuGene6 and incubated for 30 min at room temperature for complex formation. Finally the complex was added to the well containing 2 ml medium with the final AQP3 siRNA concentration of 100 nM. AQP3 protein expression and water permeability was determined 24 h after treatment.

Measurement of water permeability. The water permeability was measured using a well established method as described previously (23-25). The coverslips with the HaCaT cells were mounted in a closed perfusion chamber on the stage of a Zeiss fluorescence microscope. HaCaT cells were loaded with calcine and the water permeability measurement was performed. Cells were loaded with calcine by a 5-min exposure to 20 μM calcine-acetoxyxymethyl ester (calcine-AM; Molecular Probes) in 300 mosM PBS (in mM: 137 NaCl, 0.9 CaCl₂, 0.49 MgCl₂, 2.7 KCl, 1.5 KH₂PO₄, 8.1 Na₂HPO₄, pH 7.35). The loading of the cells with calcine and incubation with the drugs was performed at 30°C for 10 min. The subsequent water permeability measurements were carried out at 10°C to decrease water diffusion through the lipid bilayer of plasma membrane. To measure water permeability, the cells were perfused with cold 300 mosM PBS for 1 min, and then the perfusate was switched to 150 mosM PBS at the same temperature (in mM: 62 NaCl, 0.9 CaCl₂, 0.49 MgCl₂,
2.7 KCl, 1.5 KH₂PO₄, 8.1 Na₂HPO₄, pH 7.35). Fluorescence images were recorded every 30 sec with excitation at 488 nm and emission at 515-525 nm (green) before and after the solution switch. The series of images was analyzed off-line by measuring the time course of the calcein fluorescence in cytoplasmic regions of individual cells. The half-life of deduction of fluorescence intensity was used as a comparable indicator of water permeability.

**Statistical analysis.** The values in the figures are expressed as the means ± standard error (SE). The figures in this study are representative of 3 different experiments. Statistical analysis of the data between the control and treated groups was performed by a Student's t-test. Values of p<0.05 were considered as statistically significant.

**Results**

Nicotinamide inhibits all-trans retinoic acid-induced upregulation of AQP3 in cultured human skin keratinocytes. First, we examined whether atRA treatment induces AQP3 upregulation in cultured keratinocytes. HaCaT cells were treated with different concentrations of atRA (0.1, 0.5 and 1.0 μM, shown in A) for 24 h or treated with atRA (1.0 μM) for different time periods (6, 12 and 24 h, shown in B). AQP3 expression was measured by Western blotting and quantified and normalized to β-actin. HaCaT cells were treated with lower concentrations of Nico (10, 50 and 100 μM, shown in D) alone or followed by atRA (1.0 μM, shown in C) treatment for 24 h; AQP3 was measured by Western blotting and quantified and normalized to β-actin. HaCaT cells were treated with higher concentrations of Nico (1, 5 and 10 mM, shown in F) alone or followed by atRA (1.0 μM, shown in E) treatment for 24 h; AQP3 was measured by Western blotting and quantified and normalized to β-actin. *P<0.05 vs untreated (UNTR) group. #P<0.05 vs 1.0 μM atRA-treated group.
atRA induces AQP3 upregulation in a concentration-dependent manner (Fig. 1A). A greater than five-fold increase in AQP3 expression was observed in 1.0 μM atRA-treated cells, compared to untreated cells. atRA treatment also induced AQP3 upregulation in a time-dependent manner and the increased expression of AQP3 was observed as early as 6 h after atRA (1.0 μM) treatment (Fig. 1B). We then investigated whether nicotinamide inhibited atRA-induced upregulation of AQP3. HaCaT cells were pretreated with different concentrations of nicotinamide (10 μM-10 mM) followed by treatment with atRA (1.0 μM) for 24 h. Results showed that nicotinamide at all concentrations (10 μM-10 mM) inhibited AQP3 expression induced by atRA (Fig. 1C and E). Furthermore, nicotinamide treatment alone decreased AQP3 expression at high concentrations (1-10 mM, Fig. 1F), but the expression of AQP3 was not altered when cells were treated with nicotinamide alone at low concentrations (10 μM-100 μM) (Fig. 1D).

All-trans retinoic acid induces EGFR/ERK activation in cultured keratinocytes. Above data show that atRA treatment induced AQP3 upregulation in cultured human skin keratinocytes and our previous study showed that EGFR and ERK activation by atRA is mediated through the EGFR/ERK pathway. The EGFR/ERK pathway is involved in the regulation of AQP3 expression in keratinocytes. Therefore, we investigated whether atRA-induced EGFR/ERK activation in cultured human skin keratinocytes (HaCaT cells). HaCaT cells were treated with atRA (1 μM) for different time periods (5, 15, 30, 60 and 90 min, shown in A) or treated with different concentrations of atRA (0.1, 0.5 and 1.0 μM, shown in B) for 30 min; phosphorylation EGFR (p-EGFR, Y1068) was analyzed by Western blotting and quantified and normalized to β-actin. HaCaT cells were treated with atRA (1.0 μM) for different time periods (15, 30, 60, 90 and 120 min, shown in C) or treated with different concentrations of atRA (0.1, 0.5 and 1.0 μM, shown in D) for 60 min; phosphorylation ERK (p-ERK) was analyzed by Western blotting and quantified and normalized to β-actin. HaCaT cells were pretreated with PD153035 (PD, 1.0 μM) or U0126 (U, 10 μM) for 1 h followed by atRA (1.0 μM) treatment; phosphorylation EGFR (p-EGFR, Y1068) was analyzed by Western blotting 30 min after atRA treatment and quantified in E. Phosphorylation ERK (p-ERK) was analyzed by Western blotting 60 min after atRA treatment and quantified in F. *P<0.05 vs untreated (UNTR) group. #P<0.05 vs 1.0 μM atRA-treated group.
were involved in AQP3 expression (22). In this study, we further investigated the signal transduction pathway of atRA-induced AQP3 expression. As shown in Fig. 2A and C, phosphorylation EGFR (p-EGFR, Y1068) and phosphorylation ERK (p-ERK) were induced by atRA (1.0 μM) in a time-dependent manner. When HaCaT cells were treated with various concentrations of atRA (0.1, 0.5, and 1.0 μM), the activation levels of p-EGFR and p-ERK were in an atRA concentration-dependent manner (Fig. 2B and D). To further verify the involvement of the EGFR/ERK pathway, cells were pretreated with different concentrations of nicotinamide (10, 50, and 100 μM) with less than 30% of p-EGFR and 50% of p-ERK observed in cells pretreated with nicotinamide (100 μM) compared with cells treated with atRA (1.0 μM) alone (Fig. 3A and B). EGFR and ERK inhibitors block AQP3 upregulation induced by all-trans retinoic acid (atRA) in cultured human skin keratinocytes. Our data above demonstrated that nicotinamide inhibits EGFR/ERK activation and AQP3 expression induced by atRA treatment in HaCaT cells. However, whether EGFR/ERK activation is

Figure 3. Nicotinamide (Nico) inhibits all-trans retinoic acid (atRA)-induced EGFR/ERK activation in cultured human skin keratinocytes. HaCaT cells were pretreated with different concentrations of Nico (10, 50 and 100 μM) for 1 h followed by atRA (1.0 μM) treatment; phosphorylation EGFR (p-EGFR, Y1068) was analyzed by Western blotting 30 min after atRA treatment and quantified in A. Phosphorylation ERK (p-ERK) was analyzed by Western blotting 60 min after atRA treatment and quantified in B. *P<0.05 vs untreated (UNT) group. #P<0.05 vs 1.0 μM atRA-treated group.

Figure 4. EGFR and ERK inhibitors block AQP3 upregulation induced by all-trans retinoic acid (atRA) in cultured human skin keratinocytes. HaCaT cells were pretreated with PD153035 (PD, 1.0 μM) or U0126 (U, 10 μM) for 1 h followed by atRA (1.0 μM) treatment or not for 24 h; AQP3 expression was analyzed by Western blotting and quantified in A. HaCaT cells were pretreated with siRNA (100 nM) followed by atRA (1.0 μM) treatment or not for 24 h; AQP3 expression was analyzed by Western blotting and quantified in B. *P<0.05 vs untreated (UNT) group. #P<0.05 vs 1.0 μM atRA-treated group.

Nicotinamide inhibits all-trans retinoic acid-induced EGFR/ERK activation in cultured keratinocytes. Above data show that nicotinamide inhibits atRA-induced upregulation of AQP3 at various concentrations and EGFR/ERK signal transduction pathway is involved in atRA-treated HaCaT cells. However, the question whether nicotinamide inhibits EGFR/ERK activation induced by atRA remains unanswered. To further examine the inhibitory role of nicotinamide in EGFR/ERK activation, HaCaT cells were pretreated with different concentrations of nicotinamide for 1 h followed by atRA (1.0 μM) treatment. Results from Western blot analysis showed that EGFR and ERK activation was inhibited by nicotinamide at various concentrations (10, 50, and 100 μM) with less than 30% of p-EGFR and 50% of p-ERK observed in cells pretreated with nicotinamide (100 μM) compared with cells treated with atRA (1.0 μM) alone (Fig. 3A and B).
involved in the regulation of AQP3 expression has not been studied. To investigate this, HaCaT cells were pretreated with PD153035 and U0126 for 1 h followed by atRA (1.0 μM) treatment for 24 h, and AQP3 expression was analyzed by Western blotting. Results showed that PD153035 and U0126, which block activation of EGFR and ERK, inhibit AQP3 expression induced by atRA (Fig. 4A). These data support the notion that the EGFR/ERK pathway is involved in upregulation of AQP3 by atRA. As expected, AQP3 siRNA attenuates AQP3 expression induced by atRA (1.0 μM) treatment (Fig. 4B).

Nicotinamide inhibits all-trans retinoic acid induced water permeability mediated by AQP3 in cultured keratinocytes. Previous studies have shown that AQP3 is involved in water permeability. We tested whether atRA-induced AQP3 up-regulation leads to increased water permeability in cultured keratinocytes and whether nicotinamide has inhibitory effect. HaCaT cells were treated with atRA (1.0 μM) for 24 h with or without nicotinamide (100 μM) pretreatment for 1 h. Cell water permeability was analyzed by the method described above. Results showed that atRA treatment increased water permeability and nicotinamide pretreatment decreased water permeability induced by atRA. Moreover, the water permeability of HaCaT cells transfected with AQP3 siRNA is decreased significantly, which supports the notion that AQP3 is involved in the regulation of cell water permeability (Fig. 5A-C).

Discussion

atRA and its derivatives, commonly called retinoids, are widely used in cosmetics for the prevention of skin aging. atRA is capable of regulating keratinocyte proliferation and differentiation and increasing stratum granulosum thickness (26,27). However, retinoid treatment leads to skin dryness, irritation and peeling, which are caused by the impairment of skin barrier function (28,29). These side effects limit the application of retinoid therapy. It would therefore be beneficial to develop certain moisturizers to attenuate the adverse effects of retinoids and preserve the skin barrier function. Nicotinamide, an essential dietary vitamin whose deficiency leads to pellagra, is commonly used to slow skin aging (30,31). However, whether nicotinamide attenuates atRA-induced skin dryness and prevents water loss in the epidermis remains to be determined. Also, little is known about the underlying molecular mechanism of nicotinamide in skin physiology. Our study demonstrated that nicotinamide attenuates atRA-induced overexpression of AQP3 in cultured human skin keratinocytes and decreases water permeability. We also provided evidence that nicotinamide inhibits atRA-induced EGFR and ERK activation. This novel finding...
provides an insight into the molecular mechanism of nicotinamide's beneficial effect in preventing water loss due to the application of atRA.

AQ3 is the predominant aquaporin in the skin. Moreover, the expression of AQ3 is far higher in skin than in other tissues, indicating a vital role of AQ3 in skin water and glycerol transport (7,32). Recent studies have indicated that atRA induces AQ3 overexpression at both gene and protein levels when applied topically on human skin, and elevated AQ3 expression leads to a burst of glycerol uptake (10,32). Our previous study also showed that atRA attenuates UV radiation-induced AQ3 downregulation in cultured human skin keratinocytes, and that upregulation of AQ3 by atRA is accompanied by an increase of water transport (18). We confirmed in this study that atRA treatment increases the expression of AQ3 in a concentration-dependent manner, which leads to increased water permeability. The data that AQ3 siRNA treatment reduces water permeability support the notion that AQ3 plays an important role in cellular water transport in keratinocytes.

There are contradictory viewpoints about whether overexpression of AQ3, at least in part, accounts for skin dryness. Phenotypical studies in AQ3 null mice indicate that AQ3 plays an important role in epidermal glycerol and water transport, and upregulation of AQ3 leads to more water movement from dermis to epidermis (6,7). In AQ3-deficient mice, skin barrier recovery and wound healing is significantly delayed, while upregulation of AQ3 facilitates epidermal cell migration during wound healing (33,34). However, hyperexpression of AQ3 is also associated with the increase of TELW. If the water-holding capacity of the stratum corneum is decreased, the increased water transport to the stratum corneum may lead to more water loss and skin dryness. This viewpoint is supported by a study in atopic eczema which is characterized by dry skin (35-37). Also, increased AQ3 expression is found in the epidermis of patients with atopic eczema (AE) in contrast with that of healthy skin. Patients with AE show defective skin barrier function and reduced water-holding capacity in stratum corneum which may contribute to increased water loss and dry skin in AE (9). In addition, published data also show that AQ3 expression influences skin hydration and contributes to increased water loss through rat immature skin (8). These findings demonstrate that increased expression of AQ3 may lead to increased water loss in AE. Our data show that atRA upregulates the expression of AQ3. This may further explain why atRA therapy induces skin dryness when it is used topically.

In this study, we found that nicotinamide decreased the expression of AQ3 and water permeability induced by atRA in a concentration-dependent manner. Our findings suggest that nicotinamide may be used as a moisturizer by downregulating the expression of AQ3 in keratinocytes. Previous studies have shown that topical nicotinamide improves skin barrier function, and nicotinamide cream is an effective moisturizer on atopic dry skin and may be used as an auxiliary medicine to treat atopic dermatitis (38,39). Administration with myristyl nicotinate for one month reduces skin TELW and provides additional barrier protection and tolerability of retinoic acid without interfering with improving efficacy (16). In addition, multiple clinical studies have shown that topical nicotinamide treatment is well tolerated by skin and the appearance of aging facial skin, such as wrinkles, yellowing and elasticity, are all significantly improved (30,40). Moreover, nicotinamide treatment increases the levels of ceramide and free fatty acids in the epidermis, suggesting that nicotinamide improves the epidermal permeability barrier (15). Collectively, this suggests that nicotinamide attenuates the dryness of skin induced by atRA by inhibiting the expression of AQ3.

The regulation of AQ3 expression by nicotinamide treatment remains unknown. Our data showed that atRA upregulates AQ3 expression through the activation of EGFR and ERK. Nicotinamide treatment inhibits the activation of EGFR/ERK and the expression of AQ3. In order to confirm that the EGFR/ERK signal pathway is involved in regulating AQ3 expression, we pretreated keratinocytes with EGFR and ERK inhibitors, PD153035 and U0126, followed by atRA treatment. Results showed that AQ3 was downregulated by PD153035 and U0126, which blocks the activation of EGFR and ERK. The involvement of the EGFR pathway in atRA signaling has been also demonstrated by the increased phosphorylation of EGFR and its downstream signaling component ERK1/2 in previous studies (17-19). Combined with our present study, we conclude that nicotinamide decreases water permeability mediated by AQ3. The downregulation of AQ3 by nicotinamide is through the inhibition of EGFR/ERK signal transduction which is activated by atRA. However, our data only showed that nicotinamide reduced atRA-induced high water permeability in cultured human skin keratinocytes in vitro. They do not fully elucidate the protective mechanism of nicotinamide when it is used topically on human skin. Further study will be carried out focusing on the protective effects of nicotinamide in vivo and in AE patients.

In conclusion, nicotinamide decreased the expression of AQ3 induced by atRA in a concentration-dependent manner in cultured human skin keratinocytes. atRA upregulated AQ3 expression through the activation of EGFR and ERK which was inhibited by nicotinamide. The water permeability decrease was accompanied by downregulation of AQ3. Our results demonstrate that nicotinamide downregulates atRA-induced AQ3 expression by inhibiting EGFR/ERK signal transduction with a result of decrease in water permeability. This study provides insight into the molecular mechanism through which nicotinamide attenuates the side effects of skin dryness due to topical application of atRA. As atRA and nicotinamide are both used in cosmetic products, the protective effects of nicotinamide against the side effects of atRA may enhance the anti-aging efficacy of atRA on skin.

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