Effects of Cyclooxygenase-1 or -2 Inhibitors on Expressions of ATP2A2 and ATP2C1 in Cultured Normal Human Keratinocytes

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Background: Darier’s disease (DD) and Hailey–Hailey disease (HHD) are autosomal dominantly inherited skin disorders characterized by the loss of adhesion between epidermal cells and by abnormal keratinization. DD is caused by mutations in ATP2A2, encoding the sarco/endoplasmic reticulum Ca²⁺ ATPase type 2 isoform (SERCA2), and HHD arises from mutations in ATP2C1, a homologue of Golgi secretory pathway Ca²⁺ ATPase (SPCA1). Clinically and histologically, these diseases have common features. Exposure to UVB irradiation exacerbates the skin lesions of both diseases and expressions of ATP2A2 and ATP2C1 mRNA are suppressed by UVB irradiation in cultured normal human keratinocytes. UVB irradiation also induces Cyclooxygenase-2 (COX-2) mRNA expression.

Objectives: To examine the effects of COX-1 and COX-2 on ATP2A2 and ATP2C1 mRNA expression.

Methods: We used quantitative reverse transcriptase–polymerase chain reactions to examine the effects of celecoxib (a COX-2 selective inhibitor), SC-560 (COX-1 selective), and apigenin, (which is known to prevent UVB-induced COX-2 expression) on UVB-induced suppression of ATP2A2 and ATP2C1 mRNA in vitro.

Results: Celecoxib and apigenin increased and SC-560 decreased the levels of UVB-induced suppression of ATP2A2 and ATP2C1 mRNA.

Conclusions: These findings suggest that COX-2 suppresses the expression of ATP2A2 and ATP2C1 mRNA, while COX-1 might play an important role in the gene-regulating mechanism of ATP2A2 and ATP2C1 and is required to maintain the homeostasis of Ca²⁺ in keratinocytes.

Key words: cyclooxygenase, Darier’s disease, Hailey–Hailey disease, gene expression, reverse transcriptase–polymerase chain reaction

Introduction

Darier’s disease (DD; MIM no. 124200) and Hailey–Hailey disease (HHD; MIM no. 16960) are autosomal dominantly inherited skin disorders characterized by loss of adhesion between epidermal cells and by abnormal keratinization. DD is a skin disorder characterized by warty papules and plaques, in the seborrheic area, palmo-planter pits, and distinctive nail abnormalities. The typical histological features are loss of adhesion between epidermal cells (acantholysis) and abnormal keratinization (dyskeratosis), occasionally accompanied by a wide range of neuropsychiatric problems including epilepsy and depression. HHD is a rare blistering disorder in which recurrent vesicles and erosions develop predominantly at flexural and intertriginous areas. The pathological features in HHD are acantholysis, suprabasal cleavage and intracellular oedema, resulting in an appearance sometimes described as resembling a ‘dilapidated brick wall’.

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ing the sarcoplasmic reticulum Ca\(^{2+}\) ATPase type 2 isoform (SERCA2), and HHD arises from mutations in ATP2C1, a homologue of Golgi secretory pathway Ca\(^{2+}\) ATPase (SPCA1)\(^{1,2}\). Both of these calcium ATPases are expressed in human epidermal keratinocytes. The mechanism underlying autosomal dominant inheritance of DD and HHD is thought to be haploinsufficiency.

Ultraviolet (UV) irradiation, heat and secondary infection frequently cause exacerbation of skin eruptions in DD, especially in the summer\(^{3}\). UV radiation can also provoke blistering and erosions in unaffected skin on HHD patients.\(^{4}\) In an earlier study\(^{5}\) we examined the expression of ATP2A2 and ATP2C1 in cultured normal human keratinocytes and found that UVB irradiation obviously suppressed gene expression, which remained depressed for up to 6h afterwards. UV irradiation is also demonstrated to induce cyclooxygenase-2 (COX-2) mRNA expression, while cyclooxygenase-1 (COX-1) mRNA expression remains unchanged in human keratinocytes.\(^{6}\) Based on these facts, we hypothesized that cyclooxygenases may influence the gene expression of ATP2A2 and ATP2C1.

In this study, we examined the effect of celecoxib (a COX-2 selective inhibitor), SC-560 (COX-1 selective), and apigenin, which is known to prevent UVB-induced COX-2 expression, on UVB-induced suppression of ATP2A2 and ATP2C1 mRNA in vitro.

**Materials and Methods**

**Cell culture**

Normal human keratinocytes (derived from normal newborn foreskin) were obtained from KURABO (Osaka, Japan) and maintained in a serum-free standard medium containing 0.08mmol/l \(\text{Ca}^{2+}\) (Epilife-KGM® KURABO), supplemented with 10 \(\mu\text{g/ml}\) insulin, 0.1ng/ml human epidermal growth factor, 0.5 \(\mu\text{g/ml}\) hydrocortisone, 50 \(\mu\text{g/ml}\) gentamicin, 50ng/ml amphotericin B and 4% bovine pituitary extract, and grown at 37°C with 5% CO\(_2\) in 100-mm tissue culture dishes. Third-passage keratinocytes in monolayer culture were used for experiments.

**Drug treatment and ultraviolet B irradiation**

The cultured keratinocytes were grown in standard medium (Epilife-KGM®) until 90% confluence and then washed in phosphate-buffered saline (PBS), the keratinocytes were exposed to 50mJ cm\(^{-2}\) from two fluorescent bulbs with an emission peak at 312nm (AB-1500/HP-15M: ATTO Co., Tokyo, Japan). UVB output was measured at 312nm using an ATV-3W radiometer (ATTO Co.). At this UVB irradiation dose, the viability of cultured human keratinocytes does not decrease significantly.\(^{6}\) After irradiation, medium was replaced with fresh standard medium to which several drugs were added at various concentrations as previously described\(^{10}-^{12}\): 10 \(\mu\text{mol/l}\) celecoxib (Toronto Research Chemicals, North York, Canada), 50 \(\mu\text{mol/l}\) apigenin (Sigma, St. Louis, MO), or 10 \(\mu\text{mol/l}\) SC-560 (Sigma, St. Louis, MO), and the keratinocytes were incubated for 6h.

**Quantification of ATP2A2 and ATP2C1 mRNA levels**

Total RNA from the keratinocytes was extracted by the guanidine-isothiocyanate-phenol-chloroform method using ISOGEN® (Wako Pure Chemical, Osaka, Japan) according to the manufacturer’s instructions. The RT reaction was performed with an RNA PCR Kit® (Takara Shuzo, Otsu, Japan). The expression levels of ATP2A2 and ATP2C1 were analysed by using an online fluorescence PCR detection system (Light-Cycler®; Roche Diagnostics Co., Indianapolis, IN, USA). Briefly, real-time PCR was performed using the appropriate Taq DNA polymerase: SYBR Green I. For relative quantification, each levels of mRNA was normalized to that of the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH), which is not modulated by UVB. The sequences of PCR primers were used as follows: ATP2A2 mRNA exon 5, 5’-TTGATGACGAGAAAATG-3’ and 5’-TTGTCACCACACAGCAATTTCT-3’; exon15, 5’-TATACATTGTGTGGTCTG-3’ and 5’-CAGGAAATACAGACAACTTC-3’; ATP2C1 mRNA exon 5, 5’-TTTTGTTCCAGAAATATCATTCT-3’ and 5’-ATGACAAACACCCCTTGCTT-3’; exon 15, 5’-AACACAGCGAGCACACAAC-3’ and 5’-AGACGACTGGCAGTATTGCAACT-3’.

**PCR conditions**

begin with 2 min of denaturation at
95℃, annealing at 59℃ (GAPDH: 65℃) for 10 s and extension at 72℃ for 10 s. After PCR amplification, melting curve analysis and quantification analysis were performed to confirm PCR amplification specificity. The levels of mRNA expression of both genes were compared with those of nonirradiated normal keratinocyte controls (=1.0). All experiments were repeated five times using five different commercially available cell lines. Respective cell lines were established every week, and sent to us every week upon request. We also used one nonirradiated control cell line. Statistical analysis was done by t-test.

Results

Effect of celecoxib on UVB-induced suppression of ATP2A2 and ATP2C1 expression

As shown in Figure–1, UVB irradiation resulted in decreased levels of both ATP2A2 mRNA and ATP2C1 mRNA in normal human keratinocytes, relative to levels detected in unirradiated cells. Transcript levels in UVB-irradiated cells relative to levels in unirradiated cells (in each case standardized to GAPDH transcripts) were 0.167 ± 0.027 and 0.316 ± 0.118 for ATP2A2 and ATP2C1, respectively.

The addition of 10μmol/l celecoxib increased the levels of ATP2A2 mRNA to 0.316 ± 0.074 and the levels of ATP2C1 to 0.475 ± 0.242.

Effect of apigenin on UVB-induced suppression of ATP2A2 and ATP2C1 expression

Transcript levels in UVB-irradiated cells relative to levels in unirradiated cells (in each case standardized to GAPDH transcripts) were 0.164 ± 0.026 and 0.424 ± 0.126 for ATP2A2 and ATP2C1, respectively.

The addition of 50μmol/l apigenin increased the levels of ATP2A2 mRNA to 0.424 ± 0.096, and the levels of ATP2C1 to 0.568 ± 0.108.

Effect of SC-560 on UVB-induced suppression of ATP2A2 and ATP2C1 expression

Transcript levels in UVB-irradiated cells relative to levels in unirradiated cells (in each case standardized to GAPDH transcripts) were 0.194 ± 0.034 and 0.492 ± 0.062 for ATP2A2 and ATP2C1, respectively.

The addition of 10μmol/l SC-560 decreased the levels of ATP2A2 mRNA to 0.118 ± 0.018, and the levels of ATP2C1 to 0.364 ± 0.114.

Discussion

The sarco/endoplasmic reticulum Ca^{2+} ATPase gene (ATP2A2) encodes a calcium transport pump.
(SERCA2), the function of which is to maintain low cytosolic Ca$^{2+}$ concentrations by actively transporting Ca$^{2+}$ from the cytosol into the lumen of the ER$^9$. A homologue of Golgi secretory pathway Ca$^{2+}$ ATPase (SPCA1), ATP powered pump that sequesters calcium into the Golgi apparatus$^2$, is also highly expressed in human keratinocytes, where the golgi Ca$^{2+}$ pump accumulates Ca$^{2+}$ and releases it in response to inositol 1, 4, 5-trisphosphate (IP3)$^{13}$. The Golgi apparatus shares with the ER not only the property of accumulating Ca$^{2+}$ in the keratinocytes, but also that of rapidly releasing it upon agonist-dependent IP3 generation. Changes in cytoplasmic free Ca$^{2+}$ concentration ([Ca$^{2+}$]c) in the epidermal cells play an important role in the adhesion of one epidermal keratinocyte to another, as well as in the proliferation of these cells.

Past studies have established that DD and HHD result from autosomal dominant mutations in the ATP2A2 and ATP2C1 genes, respectively. Mutations in ATP2A2 and ATP2C1 are thought to cause DD and HHD through the mechanism of haploinsufficiency (not dominant negative nor gain of function)$^{11,12}$. Suppression of the expression from the intact allele is thought to be enough to induce skin lesions, because proteins from the mutated allele might be nonfunctional. This theory might be furthered by the discovery of the complete loss of a single allele in a kindred with HHD and by the fact that keratinocytes from this kindred showed less response to an increase in extracellular Ca$^{2+}$ levels, elevated [Ca$^{2+}$]c levels and abnormally low [Ca$^{2+}$] Golgi level$^2$. On the other hand, Ca$^{2+}$ uptake and storage by the ER were impeded in normal human keratinocytes cultured with thapsigargin (inhibitor for all SERCA family members)$^{14}$. Moreover, the addition of thapsigargin or antisense oligonucleotides to induce morphological changes similar to those in DD. These findings also support the hypothesis that the pathomechanism underlying both diseases is haploinsufficiency.

Both DD and HHD clinical symptoms are frequently aggravated by Ultraviolet (UV) irradiation, heat and secondary infection$^{3,4}$. These factors might induce to suppress the expression of ATP2A2 and ATPC1 from the intact allele. In previous study$^3$, we reported that the expression of ATP2A2 and ATP2C1 mRNA was suppressed after exposure to UVB irradiation in cultured normal human keratinocytes and the results supports the appropriateness of the hypothesis. UVB irradiation is also established to induce Cyclooxygenase-2 (COX-2) mRNA expression$^6$. The enhanced COX-2 expression in turn may lead to exacerbation of DD and HHD symptoms by downregulating the expression of the responsible genes. Bearing in mind this possibility, in the present study, we examined the effect of celecoxib (a COX-2 selective inhibitor), and apigenin (a nonmutagenic, naturally occurring flavonoid found in a variety of fruits and leafy vegetables, shown to inhibit UVB-induced COX-2 expression in mouse keratinocytes). As shown in Figure-1, 2, celecoxib and apigenin increased the levels of ATP2A2 mRNA and ATPC1 mRNA.

Cyclooxygenases are key enzymes in the conversion of arachidonic acid to prostaglandinG2 and H2 in sequence. PGH2 is subsequently converted to a variety of eicosanoids that include PGE2, PGD2, PGF2α, PGJ2, and thromboxane (TX) A2$^{15}$. Two isoforms of this enzyme, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), have been identified. Both of them are bound to the luminal surface of the ER and contiguous outer membrane of the nuclear envelope. Moreover, they also appear to be present on the luminal surface of the inner membrane of the nuclear envelope$^{16}$. Despite the structural similarity between the two isoforms, COX-1 and COX-2 differ substantially in the regulation of their expression, and their roles in tissue biology and disease. The reason for the existence of the two isoforms is unknown. Both isoforms have been reported to be expressed in keratinocytes and in human skin$^9$. COX-1 is constitutively expressed in many tissues and plays roles in tissue homeostasis. COX-1 gene lacks a TATA box and behaves as a housekeeping gene$^{17,18}$. COX-1 occurs as part of an ER prostanoid biosynthetic system, which forms prostanoids that act extracellularly as local hormones functioning through cell surface, G protein-linked receptors to mediate acute housekeeping responses to circulating hormones (e.g. in the regulation of renal, gastrointestinal, and platelet functions)$^{19}$. On the other hand, in most cells and tissues, COX-2 is an
keratinocytes. In an earlier study, we reported that the addition of corticosteroids to the cell culture inhibited the UVB–induced suppression of both ATP2A2 and ATP2C1 mRNA levels. Corticosteroids inhibit stimulated expression of COX–2 mRNA and protein via transcriptional and post–transcriptional mechanisms. Furthermore, it was reported that corticosteroids induce COX–1 expression in rat cardiomyocytes. Consequently, corticosteroids’ inhibition of UVB–induced suppression of ATP2A2 and ATP2C1 mRNA levels might be induced by the suppression of COX–2 and elevated expression of COX–1.

In clinical management of DD, the systemic therapy most frequently used is treatment with oral retinoids. Topical retinoids, topical corticosteroids and oral ciclosporin are also used for treatment of DD, but evidence for efficiency with these treatments has not been established. Topical corticosteroids are effective for treatment of HHD, and oral retinoids and diaphenylsulphone have also been used for the treatment of HHD in severe cases. Based on the present study, topical or oral COX–2 inhibitors might be a new choice in clinical management of DD and HHD, especially in lesions aggravated by UV irradiation or inflammation caused by other factors. Furthermore, the use of apigenin, a natural component of vegetables might

**Figure 2** The effects of apigenin on expression of (a) ATP2A2 and (b) ATP2C1 mRNA. Normal human keratinocytes were exposed to 50 mJ cm⁻² UVB. The cells were cultured with apigenin for 6 h after the irradiation. Total RNA was extracted and ATP2A2 and ATP2C1 mRNA levels were analyzed by reverse transcriptase–polymerase chain reaction. For relative quantification, each mRNA level was normalized to that of GAPDH. Results are expressed as mean ± SD. *p < 0.05 compared with control.
be a potential chemopreventive agent of the exacerbation of DD and HHD. The mechanism how COX-1 and COX-2 regulate the expression of the both genes is unknown. The products in the downstream of arachidonic acid cascade, such as PGE2 might play a role in the regulation. In mouse keratinocytes, UVB irradiation stimulates production of PGE2, associated with increased expression of COX-2 mRNA and protein\textsuperscript{39}. Further detailed investigation is required to reveal the mechanism of the regulation of ATP2A2 and ATP2C1.

References


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抄録

背景: ダリエ病 (DD) とヘイリー-ヘイリー病 (HHD) は、共に表皮細胞間の解離と異常角化を特徴とする常染色体優性遺伝疾患である。DDは小胞体に局在するCaポンプ蛋白であるSERCA2 (sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase type 2 isoform) をコードするATP2A2遺伝子の変異によって、またHHDはゴルジ体に局在するCaポンプ蛋白であるSPCA1 (a homologue of Golgi secretory pathway Ca\(^{2+}\) ATPase) をコードするATP2C1遺伝子の変異によって引き起こされる。DDおよびHHDの臨床症状はUVB照射によって悪化し、正常培養ヒト表皮角化細胞においてATP2A2およびATP2C1のmRNAの発現量は、UVB照射によって抑制される。一方で、UVB照射はシクロオキシゲナーゼ (COX) -2のmRNA発現を誘導する。

目的: ATP2A2、ATP2C1の各遺伝子のmRNA発現に対するCOX-1、2の及ぼす影響を調べる。

方法: UVB照射によってATP2A2、ATP2C1のmRNA発現を抑制した培養ヒト表皮角化細胞に、セレコキシピブ (選択的COX-2阻害薬)、SC-560 (選択的COX-1阻害薬)、アピゲニン (UVB照射によるCOX-2発現抑制を抑制する物質) を加え、両遺伝子のmRNA発現量をリアルタイムPCR法にて定量した。

結果: セレコキシピブとアピゲニンはUVB照射によって抑制されたATP2A2、ATP2C1のmRNA発現量を有意に増加させたが、SC-560はmRNA発現量をさらに減少させた。

結論: COX-2はATP2A2、ATP2C1のmRNA発現を抑制し、一方でCOX-1は、両遺伝子の発現の制御に重要な役割を果たし、表皮角化細胞におけるカルシウム濃度の恒常性の維持に必要である可能性が示唆された。

キーワード: シクロオキシゲナーゼ、ダリエ病、ヘイリー-ヘイリー病、遺伝子発現、RT-PCR