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Nobiletin, a polymethoxylated flavone from citrus peels, induces differentiation of normal human epidermal keratinocytes

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Nobiletin, a polymethoxylated flavone from citrus peels, is known to have a wide range of pharmacological activities. In this study, we examined the effects of nobiletin on differentiation of normal human epidermal keratinocytes (NHEKs). Treatment of NHEKs with nobiletin was found to cause marked increases in the expression level of keratin 10 (K10) and involucrin, differentiation makers of keratinocytes.

Key words: Nobiletin, polymethoxylated flavone, citrus, keratinocyte, keratin.

INTRODUCTION

The epidermis consists of several cell layers, each of which contains keratinocytes at distinct stages of differentiation. The deepest or basal layer located at the dermal-epidermal junction is composed of undifferentiated keratinocytes that continuously proliferate (Regnier et al., 1986). While migrating upward through the epidermis, keratinocytes undergo extensive differentiation that is essential for the skin to function as a protective barrier (Proksch et al., 1993). Keratinocyte differentiation initiates in the spinous layer (Roop et al., 1983), which is characterized by growth arrest and expression of the keratins 1 (K1) and 10 (K10) proteins. This early differentiation in the spinous layer is followed by late differentiation in the granular layer, which is accompanied by expression of proteins such as involucrin (Eckert et al., 1993). After terminal differentiation, keratinocytes undergo an epidermal-specific programmed cell death to form the cornified envelope that serves as a barrier to water loss and microbial invasion (Nemes et al., 1999). The envelope contains many proteins, among which involucrin was first discovered and shown to become cross-linked to a cellular transglutaminase (Simon et al., 1985).

However, abnormal differentiation of keratinocytes in epidermis has lead to epidermal dysfunction, such as epidermal thinning, barrier dysfunction, and delayed wound healing (Nuccitelli et al., 2011). Therefore, the inducer of keratinocyte differentiation may serve as dermatological agent by normalization of epidermal turnover.

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Nobiletin is one of the most abundant polymethoxylated flavones present in citrus peels. This compound shows various biological and pharmacological activities such as anti-inflammatory (Murakami et al., 2000; Lin et al., 2003), carcinogenic (Morley et al., 2007; Walle et al., 2007; Akao et al., 2008) and allergic effects (Itoh et al., 2008). However, it is not yet known whether nobiletin affects the keratinocyte differentiation. In the present study, we investigated the effects of nobiletin on differentiation of normal human epidermal keratinocytes (NHEKs).

**MATERIALS AND METHODS**

**Materials**

Nobiletin, sinensetin, 5-demethyl sinensetin and tangeretin were purified from *Citrus reticulata* and their purities were greater than 98% (Iinuma et al., 1980). Each compound was dissolved in DMSO and added to the cell culture medium with a final DMSO concentration of 0.1% v/v.

**Cell culture**

NHEKs were purchased from Kurabo (Osaka, Japan). Cells were cultured in a serum-free keratinocyte growth medium, HuMedia-KB2 (Kurabo, Osaka and Japan), supplemented with bovine pituitary extract (0.4% v/v), human recombinant epidermal growth factor (0.1 ng/ml), insulin (10 µg/ml), hydrocortisone (0.5 µg/ml), gentamicin (50 µg/ml) and amphotericin-B (50 µg/ml), at 37°C in a humidified, CO₂-controlled (5%) incubator.

**Western blot analysis**

The expression levels of keratinocyte differentiation-specific markers in NHEKs were analyzed by Western blot analysis. NHEKs were lysed by incubating at 4°C for 30 min in lysis buffer (10 mM Tris-HCl pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) containing the protease inhibitor mixture (Complete™). After centrifugation of the cell lysates, the supernatant was isolated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred electrophoretically into a Microporous membrane (Polyvinylidene difluoride (PVDF) membrane. After blocking in 5% skim milk and 0.05% Tween-20, blots were incubated with either anti-K10 (Lab vision) or -involucrin (Lab vision) antibody, and then further incubated with a horseradish peroxidase-conjugated secondary antibody (GE healthcare). Proteins were visualized using the Enhanced chemiluminescence (ECL) Western blotting detection system and gel images were obtained with the LAS 4000 imaging system (Fuji Film, Tokyo, Japan).

**RESULTS AND DISCUSSION**

To examine the effects of nobiletin on epidermal keratinocyte differentiation, we analyzed by Western blot analysis changes in protein expression of K10, an early-stage differentiation marker, as well as involucrin, a late-stage differentiation marker. Calcium is known to be a major factor for triggering the differentiation of cultured keratinocytes (Yuspa et al., 1989). Calcium chloride was used as a positive control to induce differentiation. As shown in Figure 1A, nobiletin treatment at 10 µM for 72 h markedly enhanced the expression of K10 protein (27.4-fold), while K10 induction by the presence of high extracellular calcium (1.2 mM) was only 5.3-fold. Nobiletin also induced involucrin protein expression. However, the involucrin induction by nobiletin (2.8-fold) was lower than that by calcium treatment (8.7-fold). The cornified envelope precursor proteins such as involucrin are expressed later in the keratinocyte differentiation in granular layers of the epidermis. It has been reported that high calcium may propel cultured keratinocytes past early differentiation steps to a later differentiation stage, resulting in a slight reduction in K10 promoter activity (Yuspa et al., 1989). This and our present findings suggest that nobiletin induces keratinocyte differentiation especially early phase differentiation. Nobiletin increased the expression level of K10 in a concentration-dependent manner with a maximum induction at 10 µM (Figure 1B). In addition, the levels of K10 protein increased with increasing incubation time, and maximum induction was seen at 72 h after nobiletin treatment (Figure 1C).

Finally, we examined the effects of other three polymethoxylated flavones of *Citrus* on expression of K10 protein, including sinensetin, 5-demethyl sinensetin and tangeretin. Sinensetin and 5-demethyl sinensetin significantly increased expression levels of K10, but the effects were less than that seen for nobiletin (Figure 2). Intriguingly, tangeretin that differs from nobiletin only by the absence of a methoxyl group on the B-ring exhibited much less effect on induction of K10 protein. The presence of two methoxyl groups on the B ring appears to be critical for the differentiation-inducing effect. Several intracellular signaling pathways have been identified as regulators of keratinocyte differentiation. Phosphatidylinositol 3-kinase (PI3K), nuclear factor kappa B (NF-κB), and extracellular signal-regulated kinase (ERK) are implicated in the early phase of differentiation (Sayama et al., 2002; Liu et al., 2009; Schmidt et al., 2000). It is also known that transcription of K10 gene is regulated by the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) (Zhu et al., 1999). It is thus likely that nobiletin induces differentiation by affecting these signaling pathways. Further studies are required to elucidate the exact mechanisms underlying the effects of nobiletin on keratinocyte differentiation, which are in progress in our laboratory.

**Conclusion**

In summary, the present study demonstrated that the ability of nobiletin to induce differentiation of NHEKs suggests a dermatological and cosmetic agent for normalizing epidermal turnover.

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Figure 1. Induction of differentiation markers by nobiletin in human epidermal keratinocytes. The levels of K10, involucrin and β-actin as an internal loading control in total cell lysates were analyzed by Western blot analysis. A representative blot of three independent experiments is shown.
Figure 2. Effects of various polymethoxylated flavones on expression of K10 in human epidermal keratinocytes. The levels of K10 and β-actin as an internal loading control in total cell lysates were analyzed by Western blot analysis. A representative blot of three independent experiments is shown. Data represent the mean ± S.D. of three independent experiments. Asterisks indicate statistical significance as determined by Student’s t test (* p < 0.05, ** p < 0.01 vs. Cont).
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Conflicts of Interest

All authors report no conflict of interest.

REFERENCES


