Expression of Uncoupling Proteins in Human Skin and Skin-Derived Cells

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Uncoupling protein (UCP) is a mitochondrial membrane protein that uncouples oxidative phosphorylation. The physiological function of major isoforms of UCPs is related to the control of body temperature and reactive oxygen species production. Although skin is an important organ for heat radiation and protection against stress, the expression and function of UCPs in the skin have remained unclear. The expression of UCPs in human skin and its derived cells was researched at the mRNA and protein levels. The effects of norepinephrine (NE) and 9-*cis* retinoic acid (RA) on UCP expression were also investigated. The expression of UCP1 mRNA was found in the human epidermis and was upregulated in differentiated keratinocytes. UCP1 expression in keratinocytes was synergistically upregulated by NE and RA treatment. Significant expression of UCP2 and UCP3 was observed also in cultured keratinocytes and fibroblasts. By immunohistochemistry, localization of UCP1 was found in the granular layer of the epidermis, sweat glands, hair follicles, and sebaceous glands of various sites in the human body. UCP3 was widely found in the dermis. This showed that UCPs exist in human skin, with their expression being under hormonal control. These findings are in stark contrast with the well-accepted view of UCP1 expression being exclusive to brown adipose tissue.

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INTRODUCTION

Uncoupling protein (UCP), as the name suggests, is a molecule that uncouples mitochondrial oxidative phosphorylation by bypassing the electrochemical gradient across the inner membrane from the F1-ATPase, thereby dissipating energy as heat (Jezek, 1999). Among the several isoforms of the UCP family reported so far (Ricquier and Bouillaud, 2000), UCP1 is expressed exclusively in brown adipose tissue (BAT), which is the major site of regulatory thermogenesis in small rodents during cold acclimation, arousal from hibernation, and recovery from anesthetic hyperthermia (Shimizu and Saito, 1991). Since UCP1 is also activated during voluntary overfeeding, this isoform is believed to be a key molecule in diet-induced thermogenesis as well as cold-induced thermogenesis and thereby in the autonomic control of energy expenditure and whole-body energy balance (Cannon *et al.*, 1982; Nedergaard and Cannon, 1985; Klaus *et al.*, 1998; Baumruk *et al.*, 1999). However, unlike small rodents, larger mammals, including humans, have only minute amounts of BAT, and the physiological significance of UCP1 in these species has been debatable. In contrast to UCP1, UCP2 is expressed ubiquitously in various tissues, and UCP3 is expressed abundantly in the skeletal muscle and BAT. Two forms of UCP3 are found in human tissue, the long form (UCP3L) and the short form (UCP3S). They are produced from the same gene by alternative splicing (Boss *et al.*, 1997; Fleury *et al.*, 1997; Solanes *et al.*, 1997). Since these UCP isoforms have uncoupling activity when expressed in yeast, they have been suggested to be involved in the regulation of energy expenditure. UCPs have also been suggested to play a role in diminishing reactive oxygen species (Nagase *et al.*, 1994, 2001; Tsukazaki *et al.*, 1995; Boss *et al.*, 1998; Hinz *et al.*, 1999; Clapham *et al.*, 2000; Vidal-Puig *et al.*, 1997, 2000).

The mammalian skin is a critical site for regulating the entry and release of heat, and acts as a barrier against water and other substances. Heat produced in the body is dispersed from the skin surface by radiation or perspiration, whereas subcutaneous adipose tissue under the dermis acts as an insulator (Cassard *et al.*, 1990; Irie *et al.*, 1999). Heat production and radiation via skin, including subcutaneous tissue, are interesting with regard not only to thermoregulation, but also to energy metabolism; however, there have been no reports about the expression of UCPs in the skin, except for that in subcutaneous fat tissue, including BAT.

In this study, expression of the three UCP isoforms was examined at the mRNA and protein levels in human skin and skin-derived cells. The effects of norepinephrine (NE) and retinoid treatment were also investigated.

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*Abbreviations: BAT, brown adipose tissue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NE, norepinephrine; RA, 9-*cis* retinoic acid; UCP, uncoupling protein*

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RESULTS

UCP mRNA in human skin-derived cells

Total RNA of fetal foreskin-derived keratinocytes and fibroblasts, the major cell types of the epidermis and dermis, respectively, was analyzed by reverse transcriptase (RT)–PCR (Figure 1). UCP1 mRNA was detected in primary cultured keratinocytes but not in fibroblasts. The mRNA of UCP2, UCP3L, and UCP3S was observed in primary cultured keratinocytes and fibroblasts at levels comparable to those in skeletal muscle. The size of the PCR products for each isoform of the UCPs and their nucleotide sequences were completely identical to those for human UCPs (Moschella *et al.*, 1975).

UCP1, UCP2, and UCP3L expression was examined in keratinocytes during differentiation. As shown in Figure 2a, all UCP signals, including UCP1, were observed in keratinocytes during differentiation. The quantity of each UCP by real-time PCR is shown in Figure 2b. UCP1 expression was almost stable, but gradually increased up to 2.5-fold depending on the differentiation time. UCP2 was upregulated transiently but reverted to basal level. The expression of UCP3 was stable for 8 days of differentiation. Similar UCP expression patterns were confirmed in the other four lots of keratinocytes derived from fetal foreskin and adult breast skin (data not shown).

The effects of 9-*cis* retinoic acid (RA) and NE treatment on UCP1 expression were investigated and quantified by RT–PCR and real-time PCR (Figure 3a and b). When keratinocytes were treated with 1 μM RA for 24 hours, UCP1 mRNA expression increased about 2.6-fold. Treatment with 1 μM NE also slightly increased UCP1 mRNA levels about 1.8-fold. Moreover, co-treatment with RA and NE synergistically enhanced UCP1 mRNA levels about 4.1-fold. In contrast, UCP2 and UCP3L expression was slightly decreased by these treatments.

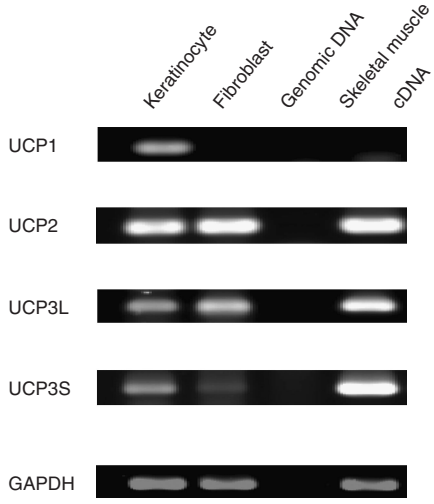

Figure 1. Expression of UCP1, UCP2, and two forms of UCP3 mRNA in cultured keratinocytes and fibroblasts derived from human skin. Genomic DNA, as negative control, was not found at the same size as mRNA. Skeletal muscle cDNA was used as positive control for UCP2 and UCP3.

UCP mRNA in human skin

The expression of UCP mRNA was evaluated in the epidermis obtained from human skin, which did not contain dermis, subcutaneous tissue, or any blood cells (Figure 4). In epidermis samples from the forearm and abdomen, clear signals corresponding to UCP2, UCP3L, and UCP3S were observed. Furthermore, a UCP1 signal was detected in both epidermal samples. The nucleotide sequence of this PCR product was completely identical to that of human UCPs. No mRNA signal for the adipocyte-specific molecule aP2 or the myoblast-specific molecule myogenin was detected in these samples. In the commercially available human skeletal muscle cDNA library, which was examined as a positive control, strong signals for UCP2 and two forms of UCP3 were found, but no signal corresponding to UCP1 was detected. In contrast, in the commercially available human skin cDNA library, a clear UCP1 signal, as well as those for UCP2, UCP3L, and UCP3S, was detected (data not shown).

Immunohistochemistry of human skin and keratinocytes

The localization of UCP1 and UCP3 was studied in sections of human skin. Weak positive signals for UCP1 were widely observed in the epidermis, and strong signals were observed in the granular layer, the epidermal second outer layer composed of flattened living keratinocytes, and all skin samples obtained from four volunteers (Figure 5a). Sweat glands of the forearm, abdomen, and cheek; sebaceous glands of the cheek; upper thigh and abdomen; and hair follicles of the cheek were also stained (Figure 5b).

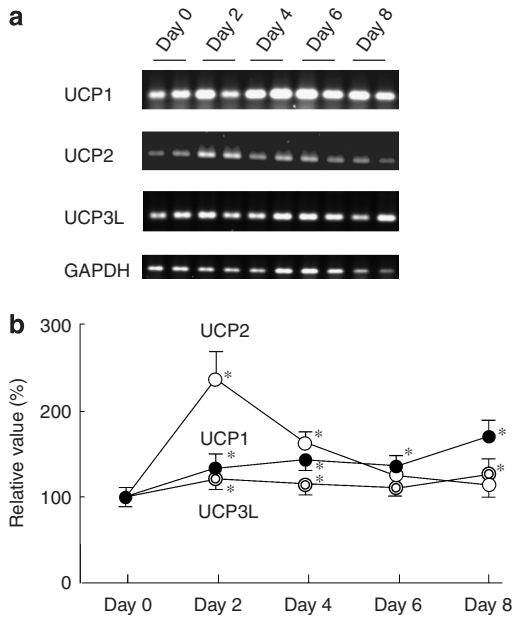

Figure 2. Time course of UCP1, UCP2, and UCP3L mRNA expression during differentiation of human keratinocytes. (**a**) UCP signals were shown by RT-PCR. (**b**) UCP signals quantified by real-time PCR were normalized to GAPDH signals. Each value represents the mean ± SEM of four incubation wells. *$P<0.05$ compared with the value of the control (before high-calcium condition for differentiation).

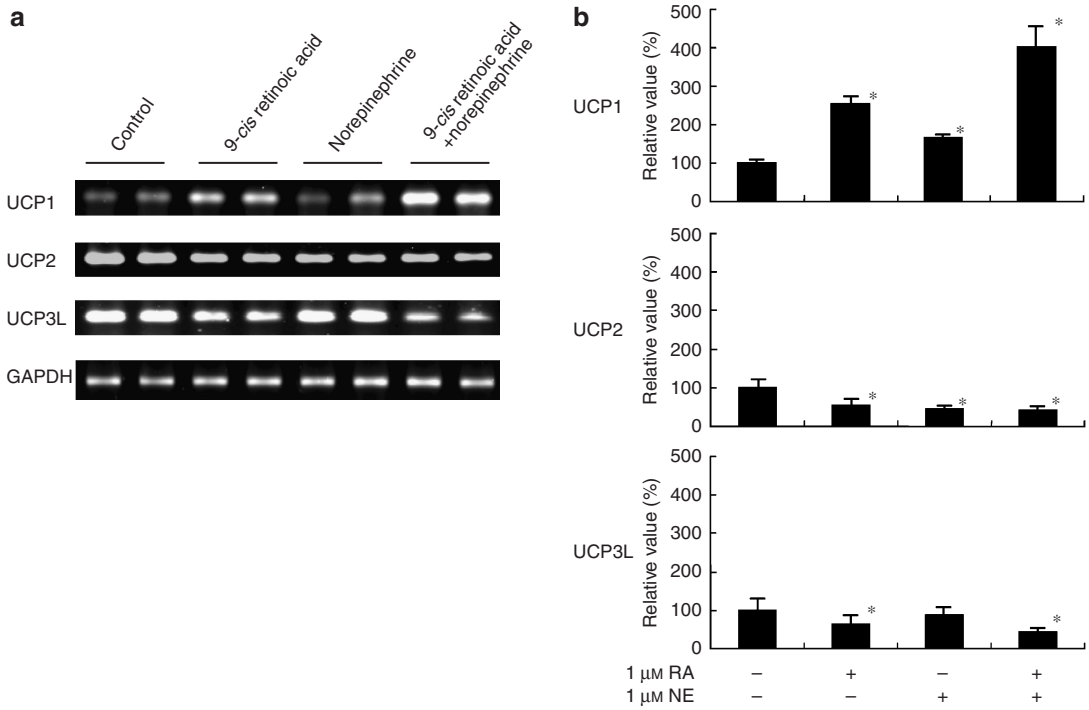

Figure 3. Effects of RA and NE treatments on UCP1, UCP2, and UCP3L mRNA expression. Human keratinocytes were incubated with or without 1 μm NE and/or 1 μm RA for 24 hours. (**a**) UCP signals were shown by RT-PCR. (**b**) UCP signals quantified by real-time PCR were normalized to GAPDH signals. Each value represents the mean ± SEM of four incubation wells. *$P<0.05$ compared with the value of the control (without RA and NE).

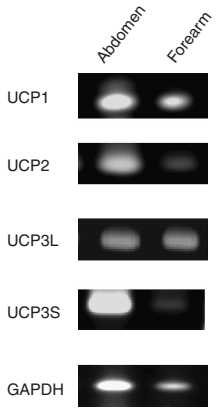

Figure 4. Expression of UCP1, UCP2, and two forms of UCP3 mRNA in human epidermis. GAPDH was used as control.

Stronger positive signals for UCP3 were observed in the epidermis than in the dermis, the major component of which was fibrous extracellular matrix. Intense staining of the cytoplasm was noted particularly in the stratum basale of the epidermis, the major area for active proliferation of keratinocytes (Figure 6). Furthermore, positive staining was detected in the sweat glands and epithelium of the hair follicle (Figure 7).

Expression of UCP1 in incubated human keratinocytes was also investigated. Intense positive staining of the cytoplasm was confirmed in most cells. Signals were dispersed throughout the cytoplasm and absent from the nucleus.

Localization of UCP1 in the human epidermis and in the keratinocytes was compared with that of porin, a mitochondrial marker (Figure 8). The epidermis and the incubated keratinocytes were uniformly stained by an anti-porin antibody with densely dyed spots. UCP1 was densely localized in the granular layer and widely in incubated keratinocytes with condensed spots. These results showed colocalization of UCP1 with the mitochondrial marker in keratinocytes, and the former was highly expressed in the granular layer of the epidermis.

DISCUSSION

In this study, we examined the expression of UCP1, UCP2, and UCP3 in human skin, including the epidermis and dermis with accessory organs. While UCP1 has been considered as being expressed exclusively in BAT, the present RT–PCR analysis revealed UCP1 expression in a cDNA library of human skin and cultured keratinocytes, the major component of the epidermis, but not in a cDNA library of human skeletal muscle and cultured fibroblasts. UCP1 signals were also detected in human skin, that is, UCP1 mRNA was detected in two sites of the human body. The size and nucleotide sequence of the PCR products were completely identical to those expected from human UCP1.

Furthermore, we established UCP1 expression at the protein level and its localization not only in the granular layer of the epidermis, but also in sweat glands, sebum

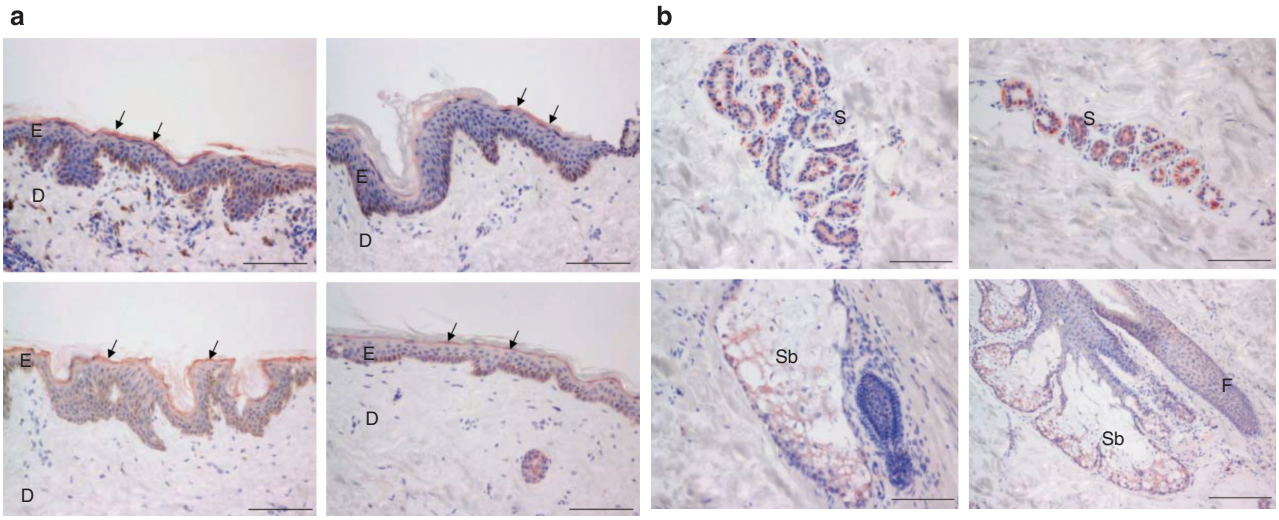

Figure 5. Immunohistochemistry for UCP1. (**a**) UCP1 expression in human epidermis at various sites. The upper left figure is the forearm, upper right is upper thigh, lower left is abdomen, and lower right is cheek. Arrows show positively stained area (red). E, epidermis; D, dermis; Bar = 100 μm. (**b**) UCP1 expression in the accessory organs of the skin. The upper left figure is a sweat gland of the forearm, upper right is a sweat gland of abdomen, lower left is a sebaceous gland of upper thigh, and lower right is a sebaceous gland of cheek. S, sweat gland; Sb, sebaceous gland; F, hair follicle. Bars = 100 and 200 μm (only in the lower right figures).

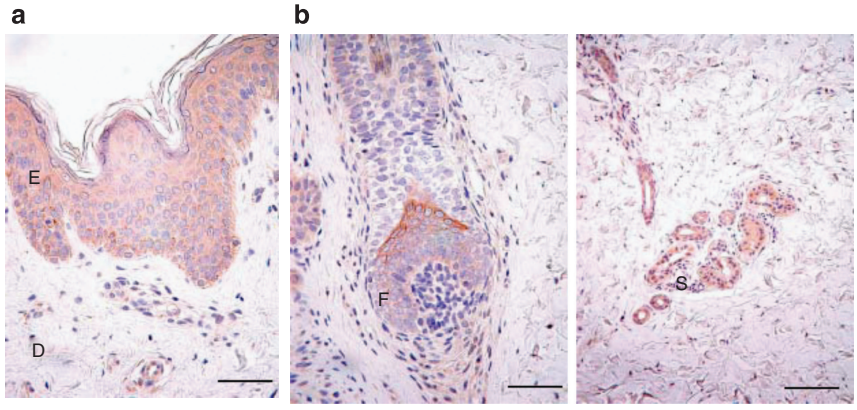

Figure 6. Immunohistochemistry for UCP3. (**a**) UCP3 expression in human epidermis. Positively stained area is brown. E, epidermis; D, dermis. Bar = 100 μm. (**b**) UCP3 expression in the accessory organs of the skin. F, hair follicle; S, sweat gland. Bars = 100 μm.

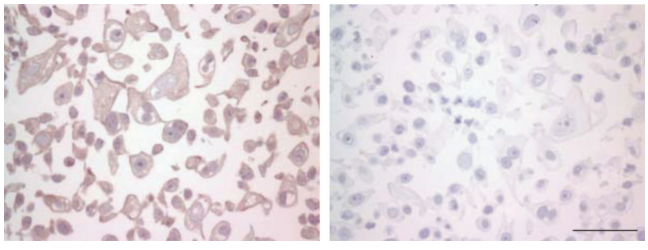

Figure 7. Immunohistochemistry for UCP1 in human keratinocytes. The left figure is stained by anti-UCP, and the right figure by control IgG. Positively stained area is red. Bar = 50 μm.

glands, and hair follicles of various sites. *In vitro* study showed high expression of UCP1 in differentiated keratinocytes. These results showed significant expression of UCP1 in human skin, particularly in the epithelium, including keratinocytes of the epidermis and exocrine glands, and its upregulation in a differentiated stage.

UCP1 expression in BAT is known to be upregulated by NE or a β-adrenergic agonist and RA through activation of the β-adrenergic receptor and retinoid X-receptor, respectively (Chuong *et al.*, 2002). Similarly, UCP1 mRNA levels in cultured keratinocytes were increased after stimulation with NE and RA synergistically, suggesting nutritional and hormonal mechanisms controlling keratinocyte UCP1 expression similar to those in BAT. More investigation at the protein level is necessary, but these results are indirect evidence suggesting some physiological function under hormonal control in the skin.

The mRNA expression of UCP2 and UCP3 was detected not only in the epidermis and skin-derived keratinocytes, but also in the fibroblasts. While UCP3 expression has been

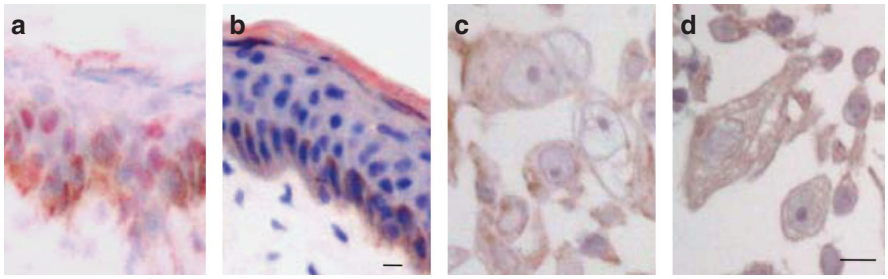

Figure 8. Immunohistochemical analysis of porin expression compared with UCP1 in human epidermis and incubated keratinocytes. (**a** and **b**) Epidermis; (**c** and **d**) keratinocytes. The left figures are stained by anti-porin, and the right figures by anti-UCP1. Positively stained area is red. Bar = 10 μm.

considered to be specific to the skeletal muscle, heart, and adipose tissue, including BAT, the present results revealed UCP3 expression in human skin at levels comparable to muscle tissue. In particular, UCP3 protein was detected in the stratum basale of the epidermis and also in sweat glands and hair follicles, suggesting UCP3 expression in the epithelium of the skin, including fibroblasts.

Although the roles of UCPs in the skin are not known, it seems interesting that UCP2 might have the ability to modify the production of reactive oxygen species. In this regard, skin is frequently exposed to various chemical, physical, and biological stresses, such as microbial infection, ultraviolet ray, and temperature change, which accelerate the secretion and peroxidation of sebum lipid and protein degeneration with the inflammatory process or immune response. Since reactive oxygen species are produced in these protective processes, UCP2 in the skin may be involved in regulating the production of skin reactive oxygen species, being controlled via the β-adrenergic receptor and retinoid receptor (Solanki and Murray, 1978, 1982; Schallreuter *et al.*, 1992; Mao-Qiang *et al.*, 2004; Schmuth *et al.*, 2004).

There are various reports about non-adipose UCP1 expression in the rodent brain at the protein level and in the thymus at the mRNA level, but the latter is controversial (Carroll *et al.*, 2004, 2005; Lengacher *et al.*, 2004). Our report includes the first findings of human UCP1 at the mRNA level and protein level in non-adipose tissues. Considering the significant role of UCP1 in BAT for metabolic thermogenesis, UCP1 in human skin may also be involved in thermoregulation in the skin. Analytical methods to detect UCPs should be established. Alternatively, UCP1, as well as UCP2, may participate in the protective function against oxidative stress in the skin. Further studies are needed to make these intriguing ideas more convincing.

MATERIALS AND METHODS

Cell culture

Human keratinocytes (NHEK-B) and fibroblasts (NHDF-NB) were obtained from KURABO (Osaka, Japan) and cultured individually in a proliferation medium (HuMedia-KG2; KURABO) or DMEM at 37 °C in a 5% CO_2 atmosphere until confluence, the medium being renewed twice a week. In the first mRNA analysis experiment, cells were collected and total RNA was extracted with Isogen (Nippon Gene Co. Ltd, Tokyo, Japan). In the second experiment, keratinocytes were differentiated in the presence of a high concentration of calcium under an air–liquid boundary condition. Briefly, keratinocytes from fetal foreskin were seeded on the inserts with a collagen type I-coated semi-permeable membrane (Biocoat Cell Culture Inserts; BD Biosciences, San Jose, CA) in a differentiation medium (mixture of DMEM and Ham's F-12 medium with 10% fetal calf serum, 10 μg ml^{-1} insulin, 1 mM hydrocortisone, 0.3 mM L-ascorbic acid, and 1.3 mM calcium chloride). The next day, the medium on the membrane was removed and its upper surface was exposed to air. On the first day and every two days, cells were collected and total RNA was extracted in the same manner. In the third experiment, keratinocytes were cultured until confluence in the proliferation medium and treated with 1 μM RA (Sigma, St Louis, MO) and/or 1 μM NE (Sigma) for 24 hours. Four incubation wells were used for each condition, and total RNA was extracted in the same manner.

For immunohistochemical analysis, proliferated keratinocytes were washed with phosphate-buffered saline and collected into 4% paraformaldehyde phosphate-buffered solution.

Human skin samples

Human epidermis was obtained from the forearm and abdomen of a healthy male volunteer (age 40 years) using a suction blister. The tissues were immediately washed with phosphate-buffered saline and stored in Isogen for RNA extraction. Tissue specimens (5 mm in diameter) for immunohistochemical analysis were also punched out of the forearm, upper thigh, abdomen, and cheek of four volunteers (ages 60–69 years). These tissue samples were immediately frozen in liquid nitrogen.

The design and performance of skin sampling was in agreement with the Declaration of Helsinki Principles. All subjects gave written and informed consent. The study was approved by the Ethics Committee at Kao Corporation.

RNA extraction and RT–PCR analysis

Total RNA was extracted with guanidine-isothiocyanate using Isogen with proteinase K and DNase I treatment (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The mRNA of each UCP subtype was analyzed with RT–PCR. Briefly, cDNA was synthesized from total RNA (5–50 ng) using TaqMan Reverse Transcription Reagents (Applied Biosystems Japan, Tokyo, Japan) and amplified using a PCR SuperMIX or AccuPrime PCR SuperMIX (Invitrogen, Carlsbad, CA) with individual gene-specific primer sets (Table 1), according to the manufacturer's instructions. The number of cycles for PCR was 25 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard and 35 for UCP1, UCP2,

and UCP3 (UCP3L and UCP3S) in human skin samples. In cell samples, it was 25 for GAPDH, 35 for UCP1, 25 for UCP2, and 30 for UCP3. Human genomic DNA (Clontech, Mountain View, CA), human skeletal muscle DNA (Biochain Institute, Hayward, CA), and human skin cDNA (Biochain Institute) were also analyzed.

RT–PCR products were electrophoresed in agarose gel and analyzed by Molecular Imager FX (Bio-Rad Laboratories, Inc., Tokyo, Japan). Some electrophoresed bands were extracted from agarose gel using silica particles of QIAEX II (Qiagen), purified with glass powder, and sequenced with forward primers and reverse primers using an ABI Prism 377 DNA sequencer (Applied Biosystems Biosystems Japan, Tokyo, Japan).

Furthermore, cDNA samples from keratinocytes were analyzed by real-time PCR with a TaqMan PCR kit (Applied Biosystems Japan) using an ABI Prism 7700 (Applied Biosystems Japan) according to the manufacturer's instructions. Sequences of primer sets and fluorescence-labeled probes for real-time PCR in this investigation are shown in Table 2. Quantitative data were analyzed by analysis of variance, followed by Bonferroni's method for multiple comparisons between pairs.

Immunohistochemical analysis

Tissue specimens obtained from the skin were fixed with 10% formalin, paraffin embedded, sectioned (5 μm thick), and exposed to

Table 1. Primer sets for RT–PCR for UCP1, UCP2, UCP3, and GAPDH

Target gene	GenBank Accession No.	Amplicon size (bp)	Primer	Sequence[1]
Human UCP1	NM021833	134	f[2]	TCTCTCAGGATCGGCCTCTA
			r[3]	GTGGGTTGCCCAATGAATAC
Human UCP2	NM003355	538	f	CATTCTGACCATGGTGCGTACTGA
			r	GTTCATGTATCTCGTCTTGACCAC
Human UCP3L	U84763	263	f	CTCCAGGCCAGTACTTCAGC
			r	GATGCACCGTTTTCTTCC
Human UCP3S	U82818	321	f	AAGGAGAAGCTGCTGGACTA
			r	TGTACTCTTCACCGCTACATCC
Human GAPDH	J04038	226	f	GAAGGTGAAGGTCGGAGTC
			r	GAAGATGGTGATGGGATTTC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcriptase–PCR; UCP, uncoupling protein.
[1]Nucleotide sequences are shown from 5′-end to 3′-end.
[2]Front primer.
[3]Reverse primer.

Table 2. Primer sets and probes for real-time PCR for UCP1, UCP2, UCP3, and GAPDH

Target gene	GenBank Accession No.	Amplicon size (bp)	Primer	Sequence[1]
Human UCP1	NM021833	77	f[2]	GCCTTTGTGAAAAACAACATATTAGC
			r[3]	GCGCAAAATCCAGCGATAA
			p[4]	TGTCGTCCCCTGCCACTTGGTG
Human UCP2	NM003355	71	f	CCTCCTGAAAGCCAACCTCA
			r	AGAAGCCTGCCCCAAAGG
			p	ACAGATGACCTCCCTTGTCACTTCACTTCT
Human UCP3L	U84763	189	f	CACAGCCTTCTACAAGGGATTTACAC
			r	CTTCCATTCTTAACTGGTTTCGGA
			p	ACCTATGAGCAGCTGAAACGGGCCCT
Human GAPDH	J04038	226	f	GAAGGTGAAGGTCGGAGTC
			r	GAAGATGGTGATGGGATTTC
			p	CAAGCTTCCCGTTCTCAGCC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UCP, uncoupling protein.
[1]Nucleotide sequences are shown from 5′-end to 3′-end.
[2]Front primer
[3]Reverse primer.
[4]Probe.

a rabbit anti-human UCP1 antibody (No. ab10983; Abcam, Cambridge, UK), UCP3 antibody (No. UCP32-A; Alpha Diagnostic International, San Antonio, TX), or non-immune rabbit IgG (Chemicon International, Temecula, CA). The UCP1 antibody recognizes epitope mapping near the N-terminal of human UCP1, and the UCP3 antibody recognizes that near the C-terminal of the long and mature form of human UCP3L. Briefly, following treatment with target unmasking fluid (Sanbio BV, Am Uden, Netherlands) for 10 minutes at 90 °C, or with a microwave for 10 minutes at 120 °C, sections were treated with 0.3% hydrogen peroxide solution for 30 minutes at room temperature, dehydrated, blocked with 3% skimmed milk in phosphate-buffered saline for 120 minutes, and then exposed to primary antibodies (2 μg ml^{-1} for UCP1 and 20 μg ml^{-1} for UCP3) or control IgG (same concentration to primary antibody) for 120 minutes at 4 °C. Bound antibody was visualized by a horseradish peroxidase-labeled anti-rabbit IgG kit (Nichirei, Tokyo, Japan) with diaminobenzidine or amino ethyl carbazole, followed by counterstaining with hematoxylin.

Human keratinocytes (NHEK-B) fixed with 4% paraformaldehyde phosphate-buffered solution were paraffin embedded, sectioned (3–4 μm thick), exposed to a rabbit anti-human UCP1 antibody, and visualized similar to the methods for the tissue sections.

To confirm localization of UCP in mitochondria, the same immunohistochemical staining was performed using the antibody for porin (No. ab15895; Abcam).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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