Involvement of Nurr-1/Nur77 in corticotropin-releasing factor/urocortin1-induced tyrosinase-related protein 1 gene transcription in human melanoma HMV-II cells.
（ヒトメラノーマ HMV-II 細胞における CRF 及び urocortin-1 による tyrosinase-related protein 1 遺伝子発現作用）

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Abstract

Recent molecular and biochemical analyses have revealed the presence of corticotropin-releasing factor (CRF) and urocortin (Ucn), together with their corresponding receptors in mammalian skin. The melanosomal enzyme tyrosinase-related protein 1 (TRP1) is involved in modulation of pigment production in response to stressors. Although CRF and Ucn are thought to have potent effects on the skin system, their possible roles and regulation have yet to be fully determined. This study aimed to explore the effects of CRF and Ucn on TRP1 gene expression using human melanoma HMV-II cells. The mRNA of CRF, Ucn1, Ucn2, and CRF receptor type 1 (CRF_1 receptor) was detected in HMV-II cells. CRF and Ucn1 stimulated TRP1 gene transcription via the CRF_1 receptor, and increased both Nurr-1 and Nur77 mRNA expression levels. Both CRF- and Ucn1-induced Nurr-1/Nur77 acted via a NGFI-B response element on the TRP1 promoter. The combination of Nurr-1/Nur77 and microphthalmia-associated transcription factor, a melanocyte-specific transcription factor gene induced by α-melanocyte-stimulating hormone, had additive effects on activation of TRP1 gene transcription. The findings suggest that in human melanoma HMV-II cells both CRF and Ucn1 regulate TRP1 gene expression via Nurr-1/Nur77 production, independent of pro-opiomelanocortin or α-melanocyte-stimulating hormone stimulation.

1. Introduction

The skin is the largest organ of the body, exposed to multiple external chemical, physical and biological stressors. Melanocytes are a type of sensory and regulatory cell with neuroendocrine activity (Tsatmali et al., 2002), producing stress-related neurotransmitters and neuropeptides in response to various endogenous and exogenous factors derived from neighboring keratinocytes (Slominski, 2009; Slominski and Wortsman, 2000). Cutaneous melanin pigmentation occurs in response to external and internal stresses (Slominski et al., 2000a; Slominski et al., 2004b; Ito et al., 2005).

It is well known that ultraviolet radiation (UVR) stimulates α-melanocyte-stimulating hormone (α-MSH) production in human keratinocytes and melanocytes (Chakraborty et al., 1996; Schauer et al., 1994). α-MSH subsequently up-regulates its receptor, melanocortin-1 receptor (MC1R). The binding of α-MSH to MC1R stimulates adenylatecyclase activation, which in turn increases intracellular cAMP levels, resulting in activation of the protein kinase A (PKA) pathway (Lalli and Sassone-Corsi, 1994). The cAMP/PKA-pathway stimulates gene transcription of microphthalmia-associated transcription factor (MITF), a melanocyte-specific transcription factor gene, through a cAMP response element (Bertolotto et al., 1998a). Three enzymes are involved in melanogenesis: tyrosinase, tyrosinase-related protein 1 (TRP1), and DOPA chrome tautomerase (Dct)/tyrosinase related protein (TRP2) (Körner and

Pawelek, 1982; Kobayashi et al., 1994; Yokoyama et al., 1994), with MITF as a key regulator of expression. MITF binds to their binding elements, M box and E box, on the gene promoters of these enzymes, leading to stimulation of melanin synthesis (Yasumoto et al., 1997). MITF-induced TRP1 is mainly involved in the modulation of pigment production in response to stressors.

Corticotropin-releasing factor (CRF) controls the hypothalamic–pituitary–adrenal (HPA) axis during periods of stress (Suda et al., 1985; Vale et al., 1981). CRF is synthesized and secreted in the hypothalamic paraventricular nucleus (PVN) in response to stress, and subsequently stimulates adrenocorticotropic hormone (ACTH), which is encoded by the pro-opiomelanocortin (POMC) gene in pituitary corticotrophs (Gillies et al., 1982; Mouri et l., 1993). ACTH stimulates the release of glucocorticoids from the adrenal glands, and these glucocorticoids play a variety of roles in the target tissues (Whitnall, 1993). Sequentially, glucocorticoids inhibit hypothalamic PVN production of CRF and pituitary production of ACTH (Whitnall, 1993).

Urocortin1 (Ucn1) is a member of the CRF family of peptides, the actions of which are mediated by at least two distinct G protein-coupled receptors: CRF receptor type 1 (CRF_1 receptor) and CRF receptor type 2 (CRF_2 receptor). CRF is highly selective towards the CRF_1 receptor, while Ucn1 binds to both the CRF_1 and CRF_2 receptors. CRF and Ucn1 mediate stress responses, cardiovascular function, and immune functions via these CRF receptors (Kageyama et al., 1999; Suda et al., 2004).

The CRF and POMC system also exists in the skin (Slominski et al., 2007; Skobowait et al., 2011). Indeed, mammalian skin expresses CRF, its related peptide, urocortin (Ucn), their corresponding receptors, POMC, which is further processed to β-endorphin, ACTH, and α-MSH, and glucocorticoids (Slominski et al., 2000b; Slominski et al., 2001; Slominski et al., 2005a; Slominski et al., 2006b). CRF also stimulates POMC gene expression and its subsequent production in human melanocytes (Slominski et al., 2005b). Both CRF and POMC are produced in ultraviolet B-irradiated human melanocytes. CRF activates POMC gene transcription and ACTH release through CRF_1 receptor in these melanocytes (Zbytek et al., 2006). In melanoma cells, both CRF and POMC-related hormones are produced (Kim et al., 2006), and CRF induces POMC mRNA expression through a CRF receptor (Sato et al., 2002). Additionally, CRF regulates cell viability, proliferation, and migration in skin cells (Slominski et al., 2006a; Yang et al., 2007). CRF also stimulates glucocorticoid production through ACTH production in melanocytes and human scalp hair follicles (Slominski et al., 2005b; Ito et al., 2005), while CRF and Ucn1 stimulate expression of tyrosinase-related enzymes in human hair follicle melanocytes (Kauser et al., 2006).

Nur77, also known as nerve growth factor-inducible factor-B (NGFI-B), and Nurr-1 are involved in CRF-induced POMC expression in corticotrophs (Philips et al., 1997: Murphy and Conneely, 1997; Kovalovsky et al., 2002). Both Nurr-1 and Nur77 are transcription factors that constitute the nuclear

receptor 4a (NR4A) subgroup of orphan nuclear receptors. They also have characteristics of immediate early genes and are induced by a variety of extracellular signals. Indeed, it has been reported that interleukin-1β stimulates Nur77 expression in melanoma cells (Rangnekar et al., 1992), while MC1R signaling immediately induces expression in melanocytic cells (Smith et al., 2008). Both Nurr-1 and Nur77 are known to act via a NGFI-B response element (NBRE) on the TRP1 promoter. Indeed, putative NBREs are found on the TRP1 promoter.

Although CRF and Ucn have potent effects on the skin system, their possible roles and regulation have yet to be fully determined. In the present study, we examined the effect of CRF and Ucn1 on TRP1 gene expression using human melanoma HMV-II cells. We also examined the involvement of Nurr-1/Nur77 in CRF/Ucn1-induced and α-MSH/MITF-induced TRP1 promoter activity in HMV-II cells.

2. Materials and Methods

Materials

Human CRF, Ucn1, and α-MSH were purchased from Peptide Institute (Osaka, Japan). Antalarmin was purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Antisauvagine-30 was synthesized by Asahi Techno Glass (Chiba, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. cDNAs were synthesized from total RNA (0.5 μg) with random hexamer primers using the Super Script First-Strand Synthesis System of the RT-PCR Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNAs were used as templates for subsequent PCR reactions using Taq DNA polymerase (Takara Shuzo, Tokyo, Japan). PCR was carried out as previously reported (Kageyama et al., 2010; Yamamori et al., 2007). PCR primer sets specific to each mRNA were designed to span at least one intron to minimize possible contamination of the genomic DNA-derived PCR product. The following primers were used: CRF forward 5'-CTCACCTGCGAAGCGCCTG-3', reverse 5'-GCGGCTGGAAGAAATCCAAG-3'; Ucn1 forward 5'-GTTCCCCAAGGCGTCTTCA-3', reverse

5'-CTTGCCCACCGAGTCGAAT-3'; Ucn2 forward 5'-GTGTCGGCCACTGCTGAGCCTGAGAGA-3', reverse 5'-ATCTGATATGACCTGCATGACAGTGGCT-3'; Ucn3 forward 5'-TGCTGCTCCTGCTGCTGCTC-3', reverse 5'-GTGTCCTGGCGTGGCTTTCCC-3'; CRF_1 receptor forward 5'-CAAACAATGGCTACCGGGAG-3', reverse 5'-ACACCCCAGCCAATGCAGA-3'; CRF receptor type 2a (CRF_{2a} receptor) forward 5'-GACGCGGCACTGCTCCACAG-3', reverse 5'-GCATTCCGGGTCGTGTTGT-3'; CRF receptor type 2b (CRF_{2b} receptor) forward 5'- CCCTCACCAACCTCTCAGGTCC -3', reverse 5'-CAGGTCATACTTCCTCTGCTTGTC-3'; GAPDH-forward 5'-GGTCGGAGTCAACGGATTTG-3', GAPDH-reverse 5'-ATGAGGTCCACCACCCTGTT-3'. The expected sizes of the PCR products of CRF and Ucn1 were 186 bp and 468 bp, respectively, and of Ucn2 and Ucn3 were 195 bp and 310 bp, respectively. The expected sizes of the PCR products of the CRH_1 receptor, CRF_{2a} receptor and CRF_{2b} receptor were 475 bp, 233 bp and 248 bp, respectively. The expected size of the PCR products of GAPDH was 969 bp.

Real-time RT-PCR

Total cellular RNA extraction and cDNA synthesis were performed as described above. First strand cDNAs were then subjected to real-time PCR as follows. Expression levels of human Nurr-1 mRNA and Nur77 were

evaluated using quantitative real-time PCR based on specific sets of primers and probes (TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to standardize the obtained values, because the GAPDH mRNA levels were not changed in any of the treatments. Each reaction consisted of 1 × TaqMan universal PCR Master Mix (Applied Biosystems), 1 × TaqMan Gene Expression Assays Products (Hs01118813_m1 for human Nurr-1, Hs00374230_m1 for human Nur77 and Hs99999905_m1 for human GAPDH) and 2 μl cDNA at a total volume of 50 μl using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as follows: 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min.

Binding motif analyses

TRP1 5' promoter sequences in mice were challenged against E-box finding factors, microphthalmia transcription factor, and NGFI-B binding motifs using MatInspector software (Genomatix) with core similarity 0.75 and matrix similarity 0.85.

Electrophoretic mobility shift assays (EMSA)

HMV-II cells were plated in 10 cm-diameter plates then whole cell extracts obtained using CelLytic-M Mammalian Cell Lysis/Extraction reagent (Sigma). Biotin-labeled probes containing mouse TRP1 promoter

fragments were synthesized as described below and annealed. EMSA was carried out using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA). Binding reactions were loaded onto an 8 × 8 × 0.1-cm 6% polyacrylamide gel in 0.5× TBE buffer and electrophoresed at 100V at 4°C for 2 h. Biotin-labeled double-stranded DNAs were transferred to a positively charged nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Buckinghamshire, UK) using a capillary method. Biotin-labeled DNA was integrated with streptavidin-horseradish peroxidase conjugate. Finally, chemiluminescence was detected using Light Capture imaging apparatus (AE-6961, ATTO, Tokyo, Japan). The sense probes were as follows: consensus sense, 5'-GATCCGTGACCTTTATTCTCAAAGGTCA-3'; NBRE-1 sense, 5'-CAGTGTGTCTGACCTTTTCTTAAGAC-3'; NBRE-2 sense, 5'-CTAGTAGTTAAAGGGCAGGAGAATTC-3'.

Plasmid Construction

The plasmid Nurr-1 promoter-luciferase construct (Nurr-1-Luc) was previously described (Takayasu et al., 2010). The mouse Nur77 promoter-luciferase construct (Nur77-Luc) was obtained by inserting the 5'-promoter region (-1033/+87, +1 designates the transcription start site) of the mouse Nur77 gene into the pA3Luc plasmid with the following primer sets: forward 5'-CCAGGGAATTGACAGAAGAG-3', reverse 5'-GCCGGGACCAGCGACCCAG-3'. The mouse TRP1 promoter-luciferase construct (TRP1-Luc) was obtained as previously described (Bertolotto et al.,

1998b) by inserting the 5'-promoter region (-1310/+60, +1 designates the transcription start site) of the mouse TRP1 gene into unique *Kpn*I-*Xho*I sites of the pGL3Luc plasmid with the following primer sets: forward 5'-TGAAGCCACAGAGAATAAGG-3', reverse 5'-CCAGACAGTAAATCCCAAGC-3'. Deletions in the TRP1-Luc constructs were obtained by inserting various lengths of the 5'-promoter region of the mouse TRP1 gene into unique *Kpn*I-*Xho*I sites of the pGL3Luc plasmid. The reverse primer was the same as that used for TRP1-Luc, and forward primers were selected in accordance with each construct: Δ0 TRP1-Luc 5'-GGCTTACATTTGAATGTTGGC-3', Δ1 TRP1-Luc 5'-GCGTCTCTAATACATCTTCC-3', Δ2 TRP1-Luc 5'-CCATCACAAGGAAACCAGTG-3', Δ3 TRP1-Luc 5'-GGAGAATTCACTGGTGTGAG-3'. A putative NBREs-mutated TRP1-Luc was constructed using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, Palo Alto, CA, USA) according to the manufacturer's instructions with the following oligonucleotides: sense 5'-CCAAATCAGTGTGTCTGA**TT**TTTTCTTAAGACTTTAACC-3', antisense 5'-GGTTAAAGTCTAAGAAAA**AA**TCAGACACACTGATTTGG-3', and those were used for NBRE-2 mutation: sense 5'-GCCTAGTAGTTAAA**AA**GCAGGAGAATTCAC-3', antisense 5'-GTGAATTCTCCTGC**TT**TTTAACTACTAGGC-3' (bold letters denote the substituted nucleotides). The Nurr-1 expression vector was previously described (Takayasu et al., 2010). Nur77 and MITF expression vectors were

constructed by inserting mouse Nur77 and MITF-M cDNA into the pRc/CMV plasmid. Nur77 and MITF-M cDNAs were obtained by PCR from a cDNA library of the mouse corticotroph cell-line AtT-20 and mRNA of the human HMV-II, respectively.

Transfection and cell culture

HMV-II cells were cultured as follows using a cell line provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Cells were maintained in a T_{75} culture flask with DMEM/F-12 HAM (Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin, Invitrogen) under a 5% CO_2-95% air atmosphere at 37°C. Culture media were changed twice a week, and the cells were sub-cultured once a week.

HMV-II cells were plated in 24-well plates with approximately 50% confluence. The following day, the cells were transfected transiently with 1 μg of the test reporter plasmid DNA and/or 0.01-0.55 μg of total expression plasmid DNA using 3 μl of FuGene6 at a final volume of 1.5 ml. The total amount of expression vector was adjusted using the empty plasmid so that levels were the same among groups. When needed, 0.05 μg RSV-β-galactosidase expression vector was co-transfected as an internal control. The next day, the culture medium was changed to growth medium. On the day of each experiment, the culture medium was changed to DMEM/F-12 HAM supplemented with 0.5% FBS, and cells were further

cultured for 12 h. Solutions for all test reagents, at a concentration of 100×, or solvent alone, were added directly to the culture medium in each well, and the cells incubated for the indicated time of each experiment. At the end of incubation, the medium was removed and cells harvested for reporter assay (see below).

Reporter assay

A luciferase assay was performed according to the manufacturer's protocol. At the end of each experiment, cells were washed twice with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, harvested with PicaGene lysis buffer (Tokyo Inki, Tokyo, Japan) and centrifuged at 12,000 rpm for 2 min. For the luciferase assay, 20 μl of each supernatant was used. The reactions were initiated by injection of 100 μl luciferin solution, PicaGene buffer. Light output was measured for 20 s at room temperature using a luminometer (Lumat LB9501, Berthold Technologies, Postfach, Germany). β-galactosidase assay was carried out using a Galacto Light Plus Kit (Tropix, Inc., Bedford, MA) according to the manufacturer's instructions.

Western Blotting

HMV-II cells were cultured in 6 cm-diameter dishes with approximately 70% confluence. On the day of each experiment, the culture medium was changed to DMEM/F-12 HAM supplemented with 0.5% FBS and cells were further cultured for 12 h. Solutions for all test reagents, at a concentration of

100×, or solvent alone, were added directly to the culture medium of each well, and the cells were incubated for the designated period. At the end of incubation, the cells were washed twice with PBS and lyzed with Laemmli sample buffer. Cell debris was pelleted by centrifugation, and the supernatant was recovered. Twenty micrograms of extract was boiled and used for electrophoresis on gradient (4–20%) polyacrylamide gel. Proteins were then transferred to a polyvinylidene fluoride membrane (Daiichi Kagaku, Tokyo, Japan). After blocking with Detector Block® buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), the membrane was incubated for 1 h, detected with anti-TRP1 (sc-25543 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin antibodies (ab8227 Abcam, Cambridge, MA, USA), washed with PBS containing 0.05% Tween 20, and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin G (Daiichi Kagaku). The chemiluminescent substrate SuperSignalWest Pico (Pierce Chemical Co., Rockford, IL, USA) was used for detection and the membrane was exposed to BioMax film (Eastman Kodak Co., Rochester, NY, USA). β-actin expression was assessed as a loading control. Independent experiments were repeated three times and a representative blot shown.

Data Analyses

Most experiments were carried out at least three times. Samples were provided in triplicate or quadruplicate for each group of experiments. All data

are expressed as the mean ± standard error of the mean (SEM). Statistical analyses of data were performed using one-way analysis of variance (ANOVA) followed by the Fisher's PLSD *post hoc* test. The significance level was set at $P < 0.05$.

3. Results

3.1. Expression of CRF, Ucn, and CRF receptor mRNA in HMV-II cells

Expression of CRF, Ucn, and CRF receptor mRNA in HMV-II cells was examined by RT-PCR. HMV-II cells were found to express mainly CRF and Ucn1 mRNA, as well as Ucn2 mRNA at lower levels; however, no Ucn3 mRNA was evident (not shown) (Fig. 1). CRF_1 receptor, but not CRF_2 receptor (not shown), mRNA was also expressed (Fig. 1).

3.2. Effects of CRF/Ucn1 on TRP1 promoter activity and protein expression levels in HMV-II cells

HMV-II cells were incubated with 100 nM CRF or Ucn1 to determine the effects on TRP1 gene transcription. A time course study showed that CRF and Ucn1 significantly increased TRP1 5'-promoter activity, with approximately 1.5-fold increases at 12 h and 24 h, respectively (Fig. 2A). CRF and Ucn1 also significantly increased TRP1 protein levels from 6 h to 48 h (Fig. 2B).

To determine whether a CRF receptor was involved in the CRF/Ucn1-induction of TRP1 gene expression, a CRF receptor antagonist was pre-incubated with CRF/Ucn1 in the HMV-II cells. Pre-treatment with antalarmin, a selective CRF_1 receptor antagonist, completely suppressed CRF/Ucn1-induced TRP1 promoter activity (Fig. 2C). However, pre-treatment with antisauvagin-30, a selective CRF_2 receptor antagonist,

failed to suppressUcn1-induced TRP1 promoter activity (Fig. 2C).

3.3. Effects of CRF/Ucn1 on Nurr-1/Nur77 gene expression levels in HMV-II cells

Next, the involvement of Nurr-1 and Nur77 in CRF/Ucn1-induced TRP1 expression in HMV-II cells was examined. Analyses of a mouse TRP1 promoter of putative transcription factor binding sites revealed the presence of two putative NBREs (Fig. 3A). EMSA was performed to detect direct DNA binding activity in nuclear extracts from HMV-II cells. Both NBRE-1 and NBRE-2 sequences formed DNA-protein complexes in HMV-II cells (Fig. 3B). Subsequently, the effect of CRF/Ucn1 on Nurr-1/Nur77 gene expression levels in HMV-II cells was determined. A time course study showed that CRF and Ucn1 significantly increased Nurr-1 mRNA levels. Maximum effects were observed at 3 h, with approximately 1.9- and 1.6-fold increases compared with the basal level, respectively (Fig. 3C, upper panel). CRF and Ucn1 increased Nur77 mRNA expression levels at 12 h (1.4-fold increase) and at 3 h (1.3-fold increase), respectively (Fig. 3C, lower panel).

To determine whether a CRF receptor was involved in the CRF/Ucn1-induced Nurr1/Nur77 gene transcription cells were pre-treated with antalarmin. Both CRF/Ucn1-induced Nurr-1 and Nur77 promoter activity was blunted as a result (Fig. 3D).

3.4. Effects of Nurr-1/Nur77 on TRP1 promoter activity in HMV-II cells

Next, we examined involvement of Nurr-1/Nur77 in the regulation of TRP1 gene expression. HMV-II cells were transiently transfected with TRP1-Luc with expression plasmids encoding Nurr-1 or Nur77. As shown in Fig. 4A, TRP1 promoter activity was strongly stimulated by both Nurr-1 and Nur77 in a dose-dependent manner.

To determine the involvement of putative NBREs in the trans-activation of TRP1 promoters by Nurr-1/Nur77, reporter plasmids containing deletions and mutations of the TRP1 promoter were constructed. A complete elimination of Nurr-1-induced TRP1 promoter activity was observed in cells transfected with Δ2 TRP1-Luc, which contained a deletion just downstream of NBRE-1 (Fig. 4B). However, Nur77-induced TRP1 promoter activity was observed with this construct, with 1.9-fold increases in promoter activity, although the effect was reduced compared with the wild-type (4.3-fold). Activity was also completely eliminated in cells transfected with Δ3 TRP1-Luc, which contained a deletion just downstream of NBRE-2) (Fig. 4B).

Thus, when NBRE-1 and NBRE-2 in the TRP1 promoter were respectively mutated, Nurr-1/Nur77-induced promoter activity was partially blunted (Fig. 4C). Double mutations of both NBREs led to a complete loss of Nurr-1/Nur77-induced TRP1 promoter activity (Fig. 4C). These results indicate that the two NBREs were involved in the regulation of TRP1 gene transcription induced by Nurr-1/Nur77.

3.5. Effects of CRF/Ucn1 on TRP1 promoter activity induced by NBRE mutations and deletions in HMV-II cells

Next, the role of the NBREs in CRF/Ucn1-induced TRP1 promoter activity was examined. Both CRF- and Ucn1-induced TRP1 promoter activity was completely lost when NBRE-1 was deleted (Fig. 5A). Moreover, activity was greatly reduced by NBRE-1 mutation compared with NBRE-2 mutation (Fig. 5B). Furthermore, activity was completely abolished by both NBRE-1 and NBRE-2 mutations (Fig. 5B).

3.6. Involvement of Nurr-1/Nur77 in α-MSH/MITF-induced TRP1 promoter activity in HMV-II cells.

Nurr-1/Nur77 has been shown to be involved in MC1R signaling in mouse melanoma cells and primary human melanocytes (Smith et al., 2008). We therefore determined whether both Nurr-1 and Nur77 were the target of MC1R signaling in HMV-II cells. A time course study showed that 100 nM α-MSH significantly increased Nurr-1 and Nur77 mRNA levels (Fig. 6A). The maximum effect was observed at 3 h, with approximately 2.5- and 1.5-fold increases, respectively (Fig. 6A). As shown in Fig. 6B, TRP1 promoter activity was strongly stimulated by MITF in a dose-dependent manner.

Both α-MSH and MITF significantly increased TRP1 5'-promoter activity in HMV-II cells (Fig. 6C). Moreover, both α-MSH- and MITF-stimulated TRP1 transcription activity was reduced, but not completely lost, when

NBRE-1 was deleted (Fig. 6C), while a partial reduction was observed with NBRE-1 and NBRE-2 mutations, respectively. However, activity was not completely abolished with both NBRE-1 and NBRE-2 mutations (Fig. 6D). MITF, together with either Nurr-1 or Nur77, had an additive stimulatory effect on TRP1 promoter activity (Fig. 6E).

4. Discussion

HMV-II cells express CRF, Ucn1, Ucn2, and CRF_1 receptor, suggesting that CRF and Ucn play an endogenous role in HMV-II cells via the CRF receptor. The intra-skin regulatory system of the CRF family of peptides relies on a balance between the local concentration of a CRF ligand and the availability of their receptors. Our results are consistent with those of previous studies showing that CRF receptors are expressed in human skin (Slominski et al., 1999; Funasaka et al., 1999). It has been suggested that endogenous CRF and Ucn1 in the skin system act in an autocrine or paracrine manner.

In this study, we found that both CRF and Ucn1 stimulated TRP1 gene transcription and protein expression levels in HMV-II cells. Tyrosinase hydroxylates L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and is thought to be an early obligatory and rate-limiting step in melanogenesis, contributing to both eumelanogenic and pheomelanogenic pathways. TRP1 is involved in the terminal step of eumelanogenesis, with mutations in this gene resulting in reduced melanin in the skin, hair, and eyes (Kenny et al., 2012; Yamada et al., 2011). Thus, TRP1 plays a central role in maintaining cutaneous melanin synthesis.

Our data further suggest that both CRF and Ucn1 stimulate Nurr-1/Nur77 mRNA levels and gene promoter activity in melanoma cells. Both Nurr-1 and Nur77 are common mediators of CRF/Ucn1-induced

activation of the TRP1 gene promoter. In fact, in this study, TRP1 promoter activity was strongly stimulated by both Nurr-1 and Nur77. Although the induction of Nurr-1/Nur77 expression was not so strong, it has been reported that CRF not only induces Nurr-1/Nur77 but also modulates dephosphorylation of the proteins (Maira et al., 2003). Therefore, the effects of Nurr-1/Nur77 on TRP1 gene expression levels may also be affected by the qualitative level through, for example, dephosphorylation of Nurr-1/Nur77. Additionally, elimination of the two NBREs in the TRP1 gene promoter resulted in complete abolishment of Nurr-1/Nur77-induced TRP1 transcriptional activity and CRF/Ucn1-induced transcriptional activity. These results suggest that NBREs are involved in the activation of Nurr-1/Nur77-induced TRP1 gene transcription.

It is possible that CRF/Ucn1 directly or indirectly stimulates TRP1 gene expression levels. Selective antagonists have been used to clarify the role of CRF-related peptides. Here, a selective CRF_1 receptor antagonist, but not a CRF_2 receptor antagonist, had effects on CRF/Ucn1-induced activation of TRP1 gene transcription as well as Nurr-1/Nur77. These results are consistent with those of previous studies showing expression of the CRF_1 receptor mainly in human skin cells such as melanocytes (Slominski et al., 1999; Slominski et al., 2004a). Taken together, the present findings suggest that CRF/Ucn1-induced activation of TRP1 gene transcription, as well as Nurr-1/Nur77, is mediated mainly via the CRF_1 receptor in an autocrine or paracrine manner in melanocytes.

Protective skin pigmentation in response to stresses such as UVR occurs as a result of α-MSH production in keratinocytes and melanocytes. Binding of α-MSH to MC1R mainly activates the cAMP/PKA-pathway, resulting in melanogenesis. MITF is a key transcriptional factor regulating the expression of TRP1 (Wan et al., 2011). Activation of TRP1 gene transcription is regulated via the standard α-MSH-mediated MITF pathway. Our study also found that α-MSH rapidly increased Nurr-1/Nur77 mRNA levels and TRP1 promoter activity in HMV-II cells. It is therefore possible that Nurr-1/Nur77 is also involved in α-MSH-induced activation of TRP1 gene transcription. In fact, MC1R signaling was previously shown to rapidly stimulate transcription of the NR4A subfamily in mouse melanoma cells and primary human melanocytes (Smith et al., 2008). In addition to the standard MITF pathway, α-MSH therefore seems to stimulate TRP1 gene expression at least partially through Nurr-1/Nur77 binding to NBREs.

In fact, MITF was previously shown to act on the TRP1 promoter via binding to the conserved M-box, as well as other sites (Galibert et al., 1999). Moreover, MITF-stimulated TRP1 transcription activity was partially reduced by either NBRE-1 or NBRE-2 elimination. Therefore, MITF seems to act on the TRP1 promoter at least partially via NBREs. An interaction between Nurr-1/Nur77 and MITF may therefore exist during TRP1 gene transcription. It is, however, of interest that the combination of Nurr-1/Nur77 and MITF had additive effects on activation of TRP1 gene transcription. It is therefore possible that Nurr-1/Nur77 stimulates TRP1

gene transcription, independent of MITF.

Although α-MSH stimulates TRP1 through MC1R in melanocytes, POMC, a precursor of α-MSH, was not detected in the HMV-II cells (data not shown). UVR directly or indirectly stimulates α-MSH production in both melanocytes and keratinocytes. It is therefore possible that melanogenesis in melanocytes is regulated by keratinocyte-derived α-MSH in a paracrine manner (Fig. 7). On the other hand, CRF and Ucn1 are produced in melanocytes, and act on melanocytes themselves in an autocrine manner (Fig. 7). These peptides can regulate TRP1 gene expression via Nurr-1/Nur77 production, independent of POMC or α-MSH stimulation.

In conclusion, the present study demonstrated that CRF and Ucn1 stimulate TRP1 gene transcription via the CRF_1 receptor in human melanoma HMV-II cells. Both CRF- and Ucn1-induced Nurr-1/Nur77 act via NBREs on TRP1 promoter activity. Furthermore, the combination of Nurr-1/Nur77 and MITF had additive effects on activation of TRP1 gene transcription. The findings suggest that CRF and Ucn1 regulate TRP1 gene expression via Nurr-1/Nur77 production in human melanoma HMV-II cells, independent of POMC or α-MSH stimulation.

5. Acknowledgments

We thank Drs. Masato Asai and Masanori Yoshida (Nagoya University

Graduate School of Medicine and Hospital) for their helpful discussion. We also thank the Department of Pharmacology, Hirosaki University School of Medicine, Japan, for generously providing us with ABI PRISM 7000. This work was supported in part by Health and Labour Science Research Grants (Research on Measures for Intractable Diseases) from the Ministry of Health, Labour and Welfare of Japan, and by the Young Investigator Award from the Japan Neuroendocrine Society.

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Figure legends

Figure 1. Expression of CRF, Ucn, and CRF_1 receptor mRNA in HMV-II cells

The expected size of the PCR products of CRF was 186 bp, and that of Ucn1 and Ucn2 was 486 bp and 195 bp, respectively. The expected size of the PCR product of the CRF_1 receptor was 475 bp, and that of GAPDH was 969 bp. +, reverse transcriptase-PCR shown in duplicate; -, negative control of reverse transcriptase-PCR.

Figure 2. Effects of CRF/Ucn1 on TRP1 expression levels in HMV-II cells; *, $P < 0.05$ (compared with the basal level)

A. Time-dependent changes in CRF/Ucn1-induced TRP1 promoter activity. Cells were transfected with a TRP1-Luc plasmid then incubated with medium containing 100 nM CRF or Ucn1.

B. Time-dependent changes in CRF/Ucn1-induced TRP1 protein levels, determined by Western blotting. HMV-II cells were incubated in medium containing 100 nM CRF or Ucn1. A representative blot is shown and the average of three independent experiments is given.

C. Involvement of CRF receptors on CRF/Ucn1-induced TRP1 promoter activity in HMV-II cells. TRP1-Luc transfected-cells were pre-incubated with medium containing 100 nM antalarmin (Ant), antisauvagin-30 (AS30) or vehicle for 30 min then incubated for a further 12 h with medium containing

100 nM CRF, Ucn1, or vehicle.

Figure 3. Effects of CRF/Ucn1 on Nurr-1/Nur77 gene expression levels in HMV-II cells; *, $P < 0.05$ (compared with the basal level)

A. Identification of NBREs on the mouse TRP1 promoter. Schematic representation of the TRP1 promoter indicating putative NBREs, the E box, and M box, as identified by *in silico* analysis with MatInspector software (Genomatix).

B. Probes corresponding to NBRE-1 and NBRE-2 were biotin-labeled and incubated with a whole cell lysate extracted from the HMV-II cells, followed by electrophoresis (see *Materials and Methods*). Competition experiments were carried out using a 50-fold molar excess of the unlabeled consensus probe.

C. Time-dependent changes in CRF/Ucn1-induced Nurr-1/Nur77 mRNA levels. Cells were incubated with medium containing 100nM CRF or Ucn1.

D. Involvement of CRF receptors on CRF/Ucn1-induced Nurr-1/Nur77 promoter activity in HMV-II cells. Nurr-1/Nur77-Luc transfected-cells were pre-incubated with medium containing 100 nM antalarmin (Ant), antisauvagin-30 (AS30), or vehicle for 30 min then incubated for a further 3 h with medium containing 100 nM CRF, Ucn1, or vehicle.

Figure 4. Effects of Nurr-1/Nur77 on TRP1 promoter activity in HMV-II cells; *, $P < 0.05$ *vs.* control

A. Dose-dependent effects of Nurr-1 and Nur77 on the regulation of TRP1 promoter activity. TRP1-Luc transfected-cells were co-transfected with increasing doses of expression vectors of each Nur factor (1, 2 and 5% *vs.* TRP1-Luc plasmid) in HMV-II cells.

B. Effects of Nurr-1/Nur77 on activity resulting from TRP1 promoter with deletions. HMV-II cells were transfected with wild-type or TRP1-Luc deletion series, and Nurr-1/Nur77 expression vectors.

C. Effects of Nurr-1/Nur77 on NBREs-mutated TRP1 promoter activity. Basal and Nurr-1/Nur77-induced promoter activities were examined using wild-type or TRP1 5'-promoter constructs in which one or both of the NBREs were mutated.

Figure 5. Effects of CRF/Ucn1 on TRP1 promoter activity induced by NBRE mutations and deletions in HMV-II cells; *, $P < 0.05$ *vs.* basal promoter activity

A. Effects of CRF/Ucn1 on activity resulting from TRP1 promoter with deletions. HMV-II cells were transfected with wild-type or TRP1-Luc deletion series then incubated for 12 h with medium containing 100 nM CRF, Ucn1, or vehicle.

B. Effects of CRF/Ucn on NBREs-mutated TRP1 promoter activity. Basal and CRF/Ucn1-induced promoter activities were examined using wild-type or TRP1 5'-promoter constructs in which one or both of the NBREs were mutated. HMV-II cells were transfected with wild-type or TRP1-Luc

mutation series then incubated for 12 h with medium containing 100 nM CRF, Ucn1, or vehicle.

Figure 6. Involvement of Nurr-1/Nur77 in α-MSH/MITF-induced TRP1 promoter activity in HMV-II cells; *, $P < 0.05$ *vs.* basal promoter activity

A. Time-dependent changes in α-MSH-induced Nurr-1/Nur77 mRNA levels. Cells were incubated with medium containing 100nM α-MSH.

B. Dose-dependent effects of MITF on the regulation of TRP1 promoter activity. TRP1-Luc transfected-cells were co-transfected with increasing doses of MITF expression vectors (5, 10, and 30% *vs.* TRP1-Luc plasmid) in HMV-II cells.

C. Effects of α-MSH/MITF on deleted TRP1 promoter activity. HMV-II cells were transfected with wild-type or TRP1-Luc deletion series then incubated for 12 h with medium containing 100 nM α-MSH, or vehicle. An additional group of cells was co-transfected with MITF expression vectors or a control.

D. Effects of α-MSH/MITF on NBREs-mutated TRP1 promoter activity. Basal and α-MSH/MITF-induced promoter activities were examined using wild-type or TRP1 5'-promoter constructs in which one or both of the NBREs were destroyed. Cells were incubated for 12 h with medium containing 100 nM α-MSH, or vehicle. An additional group of cells was co-transfected with MITF expression vectors or a control.

E. Effects of MITF and/or Nurr-1/Nur77 on TRP1 promoter activity. HMV-II cells were transfected with MITF and/or Nurr-1/Nur77 expression vectors

together with the TRP1-Luc.

Figure 7. Schematic representation of the role of Nurr-1/Nur77 in the regulation of CRF/Ucn1-induced TRP1 gene expression in HMV-II cells The bold line indicates the main signal route of the CRF/Ucn1 pathway.