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Dietary isoflavone increases insulin-like growth factor-I production, thereby promoting hair growth in mice

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Abstract

Sensory neurons release calcitonin gene-related peptide (CGRP) upon activation. We previously demonstrated that CGRP increases insulin-like growth factor-I (IGF-I) production in various tissues of mice including the skin. We demonstrated that isoflavone increases the CGRP synthesis in the dorsal root ganglion (DRG) neurons in rats. Since IGF-I plays a critical role in hair growth, we hypothesized that isoflavones may promote hair growth by increasing the IGF-I production in hair follicles. We examined this hypothesis using wild-type (WT) and CGRP-knockout ($CGRP^{-/-}$) mice. Isoflavone significantly increased the CGRP mRNA levels in DRG neurons isolated from WT mice ($P<.01$). Administration of isoflavone for 3 weeks increased the dermal levels of CGRP, IGF-I and IGF-I mRNA in WT mice, but not in $CGRP^{-/-}$ mice. Isoflavone administration increased the immunohistochemical expression of IGF-I in hair follicle dermal papilla cells in WT mice. Significant enhancements of hair follicle morphogenesis, hair regrowth, and hair pigmentation were also observed in WT mice administered isoflavone. However, none of these effects in WT mice were observed in $CGRP^{-/-}$ mice.

These observations strongly suggest that isoflavone might increase IGF-I production in the hair follicle dermal papilla cells in mice through increasing CGRP production in the sensory neurons, thereby promoting hair growth associated with melanogenesis in mice.
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Keywords: Isoflavone; Calcitonin gene-related peptide; Insulin-like growth factor-I; Hair growth

1. Introduction

The cyclical process of hair growth through three major stages (anagen, the growth phase; catagen, the involution phase; and telogen, the resting phase) is complex and regulated by several factors, including sensory neurons [1]. Sensory neurons contain neuropeptides such as calcitonine gene-related peptide (CGRP) and this CGRP is synthesized in the cell body and transported in vesicles to the peripheral nerve endings [2,3]. Previous reports have demonstrated that sensory neurons play important roles in the promotion of hair growth [4–6].

Insulin-like growth factor-I (IGF-I) is a basic peptide composed of 70 amino acids that is rather ubiquitously distributed in various organs and tissues, including the skin, and promotes growth, survival and differentiation of cells [7]. IGF-I is critically involved in promoting hair growth by regulating cellular proliferation and migration during the development of hair follicles [8,9]. IGF-I receptor null ($Igf\text{-}1r^{-/-}$) mice exhibited hypotrophic skin with a reduced number and size of the hair follicles [10]. Patients with Laron syndrome (primary IGF-I deficiency) show sparse hair growth and decreased epidermal thickness [11,12]. Also overexpression of IGF-I in proliferating and in differentiating keratinocytes resulted in hyperplasia and tumor formation [13,14]. These observations indicate that IGF-I may be a critical component in the promotion of hair growth.

We previously demonstrated that stimulation of sensory neurons with capsaicin increased the IGF-I production by increasing CGRP release in mice [15]. Furthermore, we showed that administration of capsaicin increased the expression of IGF-I in the hair follicle dermal papilla cells, thereby promoting hair growth in mice [16].

Isoflavones have biological activities similar to those of estrogens in vitro and in vivo, and are thus referred as phytoestrogens [17–19]. Estradiol-17β has been shown to increase CGRP synthesis in the DRG neurons through NGF-mediated mechanisms [20]. In this context, we reported that isoflavones, as well as estrogen, increases the transcription of CGRP in the sensory neurons in rats [21].

Taken together, these observations strongly suggest that administration of isoflavones might increase the dermal levels of CGRP and IGF-I in mice, thereby promoting hair growth.

In the present study, we examined this hypothesis using wild-type (WT) mice and CGRP-knockout ($CGRP^{-/-}$) mice.

2. Materials and methods

2.1. Reagents

Fujiflavone P40, contains 43.5% isoflavones which consist of 23.5% daidzin, 13.6% glycitin and 6.3% genistin was kindly supplied by Fujicco (Kobe, Japan) as previously

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Table 1
Composition of experimental diets[a]

Ingredients	Control diet	Isoflavone diet
	g/kg	
Cornstarch	465.692	465.692
Casein	140.000	140.000
Dextrinized cornstarch	155.000	155.000
Sucrose	100.000	100.000
Corn oil	40.000	40.000
Cellulose	50.000	50.000
Mineral mix (AIN-93M-MX)	35.000	35.000
Vitamin mix (AIN-93-VX)	10.000	10.000
L-Cystine	1.800	1.800
Choline bitartrate	2.500	2.500
Tert-butylhydroquinone	0.008	0.008
Isoflavone[b]		5.000

[a] Prepared according to AIN-93M formulation [26].
[b] Fujiflavone P40 (Fujicco, Kobe, Japan).

described [22,23]. In which, the aglycosylated forms (genistein and daidzein) are 2.73% of total isoflavones. All other reagents were of analytical grade.

2.2. Animals

Male C57BL/6 mice (7-8 wks old; Nihon SLC, Hamamatsu, Japan) were used in this study. The care and handing of the animals were in accordance with the National Institute of Heath guidelines. All the experimental procedures described below were approved by Nagoya City University Animal Care Committee.

2.3. Generation of αCGRP-knockout mice

The generation of αCGRP-knockout mice was described previously [24]. The mouse CT/αCGRP genomic DNA was cloned from a BALB/c mouse genomic library in EMBL3 using synthetic oligonucleotide probes derived from the mouse CT/αCGRP cDNA sequence. A 7.0-kb fragment containing exons 3–5 of the mouse CT/αCGRP gene was subcloned into pBluescript (Stratagene). A targeting vector was constructed by replacing the 1.6-kb XbaI-XbaI fragment encompassing exon 5, which is specific for αCGRP, with the neomycin resistance gene and flanking the thymidine kinase gene. This plasmid was linearized with NotI and introduced into 129/Sv-derived SM-1 ES cells by electroporation, after which the cells were selected in medium containing G418 (300 μg/ml) and ganciclovir (2 μmol/L). Homologous recombinants were identified by polymerase chain reaction (PCR) and Southern blot analysis. Targeted ES cell clones were injected into C57BL/6 mouse blastocysts to generate chimeric mice. Male chimeras were then crossbred with C57BL/6 females and germline transmission was achieved. Littermates obtained by breeding heterozygotes with the genetic background of the 129/SvC57BL/6 hybrid were used for phenotypic analysis. Only males were used in this study.

2.4. Genotype determination of CGRP-knockout pups

Genomic DNA was extracted from tails of mice as previously described [24], and was used for PCR analysis. PCR was performed using the external primers of the replaced gene fragment. The wild-type allele and the mutant allele gave different band sizes. Primer sequences and PCR conditions have been described [24,25].

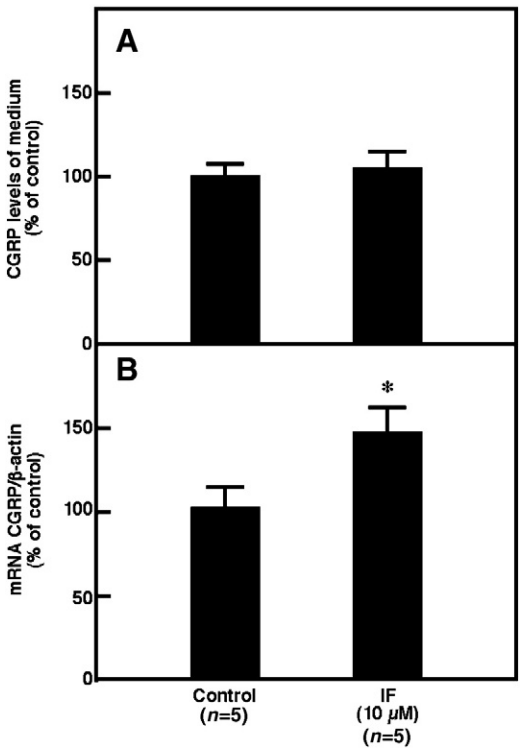

Fig. 1. Effect of IF on the release of CGRP and the CGRP mRNA levels in cultured DRG neurons isolated from WT mice. DRG neurons were incubated with isoflavone (10 μM) for 30 min or with medium only (control). Supernatants were collected, and CGRP levels were measured by enzyme immunoassay. Cellular levels of CGRP mRNA in DRG neurons were determined by quantitative real-time PCR. The value of the control was defined as 100%. Values are expressed as means±S.D. from five experiments. *P<.01 vs. control.

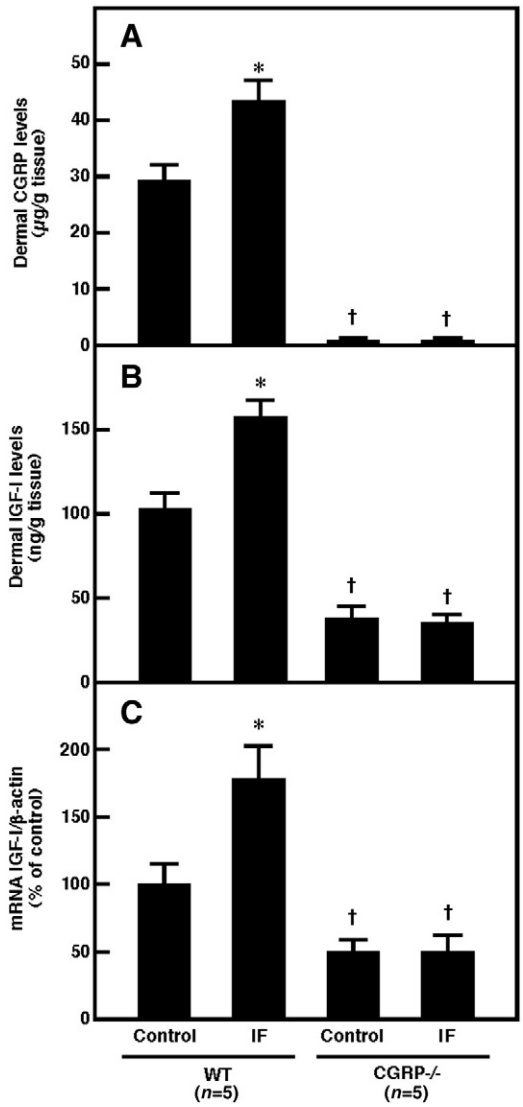

Fig. 2. Effect of isoflavone (IF) administration on dermal levels of CGRP (A), IGF-I (B) and IGF-I mRNA (C) in WT mice and CGRP$^{-/-}$ mice. Skin samples were dissected 3 weeks after administration of isoflavone. Each bar represents mean±S.D. from five animal experiments. *P<.01 vs. control; †P<.01 vs. WT mice.

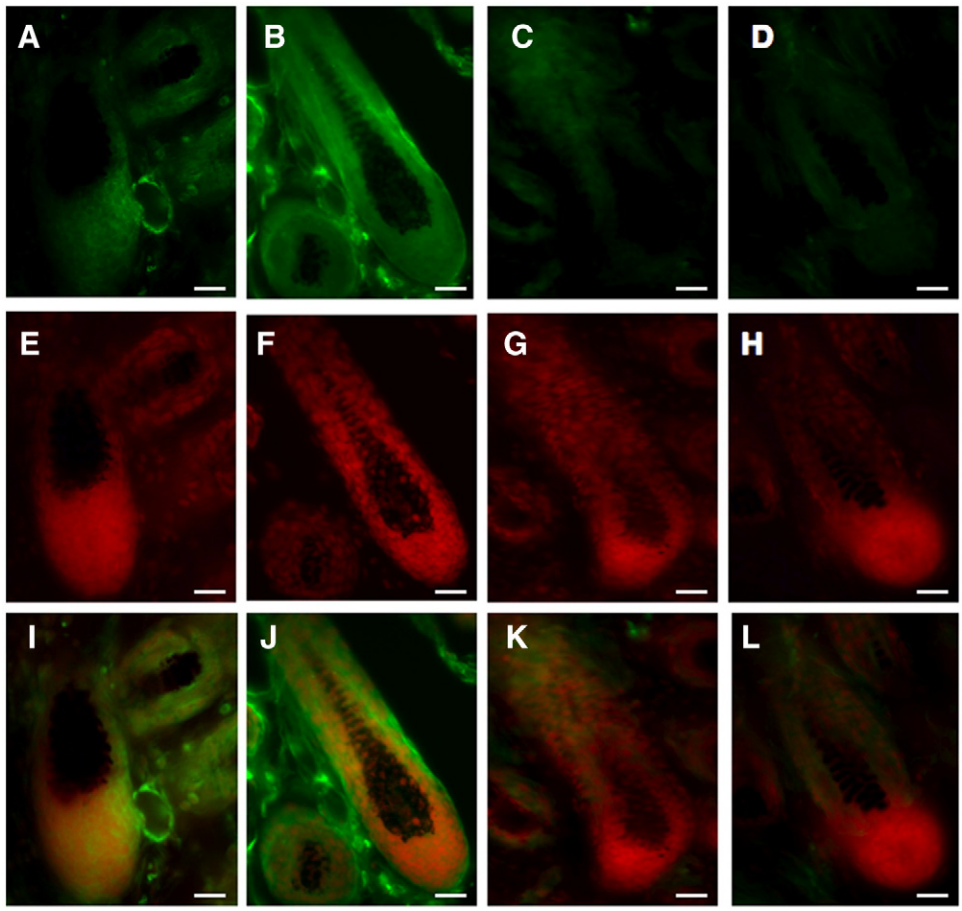

Fig. 3. Effect of isoflavone administration on the expression of IGF-I in the hair follicles of WT and CGRP$^{-/-}$ mice. Immunofluorescent IGF-I staining was determined by anti-mouse IGF-I antibody (green) in hair follicle of WT mice with soy-free diet administration (A, E, I) or with isoflavone administration (B, F, J), and of CGRP$^{-/-}$ mice with soy-free diet administration (C, J, K) or with isoflavone administration (D, H, L). Nuclei (red) were counterstained using propidium iodide (E–H). Typical results are shown in the photographs from five experiments. Scale bar=100 μm.

2.5. Isolation and culture of dorsal root ganglion (DRG) neurons

The lumbar, cervical, and thoracic DRG neurons were dissected from both sides of spinal cord of mice as previously described [25]. After 5 days in culture, the medium was aspirated gently and washed with serum-free Ham's F-12 medium. Cells were incubated with isoflavone (10 μM) for 30 min in Ham's F-12 medium containing 1% supplemented calf serum without nerve growth factor. After incubation, supernatants were sampled and stored at −20°C for CGRP measurements. CGRP levels were determined using a specific enzyme immunoassay kit (SPI-BIO, Massy, France). DRG neurons were sampled and stored at −20°C for CGRP mRNA measurement. CGRP mRNA levels were determined by quantitative mRNA analysis as described below.

2.6. Depilation treatment and administration of isoflavone

Studies started when animals were at 8–9 weeks of age, as their hair was in the telogen phage. Animals were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), the dorsal areas (2 cm in width and 4 cm in length) of mice were shaved with clippers.

Mice were received AIN-93M pellets [26] (isoflavone-free pellets, Clea Japan, Osaka, Japan) for 2 days before shaving the hair. On the day of hair shaving, mice were divided into two groups. Control group were continuously received AIN-93M pellets, and isoflavone (IF) group were received AIN-93M pellets containing 0.5% Fujiflavone P40 [0.22% (wt/wt) isoflavone] for 3 weeks as described previously [16]. Composition of the experimental diets is showed in Table 1. Distilled water was always available to the mice. During the experiment period, the body weight was measured once each week, and the food intake was measured every day. Changes in hair coat were recorded by a photography using digital camera once per weeks for three weeks, until a significant difference was observed from each group. Then the skin from the dorsal areas was removed under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) for determination of dermal CGRP, IGF-I, IGF-I mRNA levels and histological analysis as described below.

2.7. Food consumption and body weight

Food consumption and body weight increase did not differ among the groups. Overall means were 4.6±0.23 g/day food consumption, and body weight increased 2.3±0.14 g 3 weeks after the application.

2.8. Determination of dermal CGRP level

Skin dermal CGRP levels were determined in mice by a modification of the methods described previously [27]. Skins were weighted and homogenized in 2 ml of 2 N acetic acid. Homogenates were bathed in 90°C water for 20 min and then centrifuged at 4500 g for 10 min (4°C). CGRP was extracted from the supernatant using reverse-phage C18 columns (Amersham, Little Chalfont, UK). Columns were prepared by washing with 5 ml methanol onto the column followed by a wash with 20 ml of 0.1% trifluoroacetic acid, and the solvent was evaporated under a stream of nitrogen gas. The concentration of CGRP was assayed by using a specific enzyme immunoassay kit (SPI-BIO, Massy, France). The sensitively of the CGRP assay was 10 pg/ml. The antiserum cross-reacted 100% of rodent α- and β-CGRP according to the manufacturer's data sheet. Results are expressed as micrograms of CGRP per gram of tissue.

2.9. Determination of dermal IGF-I level

Dermal levels of IGF-I were determined in animals by modification of the methods as described previously [28]. The skins were minced and homogenized in 2 ml of 1 N acetic acid according to the manufacturer's instruction. The homogenates were then centrifuged at 3000 g for 10 min. The supernatants were kept at −80°C until assayed for IGF-I concentration by using a specific enzyme immunoassay kit (Diagnostic Systems Laboratories, Webster, TX, USA).

2.10. Quantitative mRNA analysis

Quantitative mRNA analysis was performed as previously described [29]. Total RNAs were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Sample cDNAs were amplified in the Model 7700 Sequence detector (Applied Biosystems, Perkin Elmer Japan, Chiba, Japan) with IGF-I or CGRP primers, dual-labeled fluogenic probes, and a Taqman PCR Kit (Applied Biosystems, Branchburgh, NJ, USA). Thermal cycler conditions were 10 min at 95°C for deactivation preceding 40 cycles for 15 s at 95°C for denaturation and 1 min at 60°C for bath annealing and extension. Known concentrations of serially diluted IGF-I, CGRP and β-actin cDNA generated by PCR were used as standards for quantitation of sample cDNA. Copy numbers of cDNA for IGF-I and CGRP were standardized those for β-actin from same sample.

2.11. Histology and immunohistochemistry

Back skin from mice was fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 10 μm for hematoxylin and eosin staining or immunohistochemistry. For immunohistochemical analyses of IGF-I, skin sections were dewaxed, microwaved in 10 mM sodium citrate (pH 6.0) and then blocked with 10% normal donkey serum (NDS) (Sigma Chemical, St Louis, MO, USA), 0.5% bovine serum albumin and 0.3% TritonX-100 in phosphate-buffered saline (PBS; 0.1 M, pH7.4) for 1 h at room temperature. Sections were then incubated with the primary antibody, a mouse monoclonal anti-IGF-I antibody (Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:200 in PBS containing 2% NDS and 0.3% Triton X-100, overnight at 4°C. After rinsing, sections were performed with Alexa Fluorophore 488 nm donkey anti-mouse at 1:500 (Invitrogen, Mount Waverley, Australia) in PBS containing 2% NDS and 0.3% Triton X-100 for 90 min at room temperature. In control experiments, primary antibodies were omitted to verify the absence of uncontrolled secondary antibody binding. Samples were then mounted and photographed under light Fluorescence microscope (Axio Image A1, Carl Zeiss).

2.12. Quantitative hair follicle number

The number of hair follicles was determined on 10μm paraffin sections under bright-field microscopy as previously described [30]. The calculations were based on an average number of hair follicles from five randomly chosen microscopic fields at 200× magnification.

2.13. Hair melanin assay

The melanin content of hairs was determined following the method described previously [31]. Briefly, full-length hairs were plucked from the dorsal skin of WT and CGRP$^{-/-}$ mice 3 weeks post-depilation with administration of isoflavone, and then 1 mg of these hair shafts were dissolved in 1 ml of a mixture of soluene-350 plus water by heating in a boiling water bath for 45 min [32]. Absorbance of the resulting solutions at 500 and 650 nm that reflects the eumelanin to total melanin ratio was performed, as described previously [33].

2.14. Statistical analysis

Data are expressed as the mean±S.D. The results were compared using an analysis of variance followed by Scheffe's post hoc test. A level of $P<.05$ was considered statistically significant.

3. Results

3.1. Effect of isoflavone on release of CGRP and CGRP mRNA levels in cultured DRG neurons isolated from WT mice

To determine whether isoflavone increases release and synthesis of CGRP in sensory neurons, we examined the effects of isoflavone on the release of CGRP and CGRP mRNA levels in cultured DRG neurons isolated from WT mice. DRG neurons were incubated with isoflavone (10 μM) for 30 min. Isoflavone (10 μM) treatment did not increase the release of CGRP (Fig. 1A), but significantly increased the CGRP mRNA levels in cultured DRG neurons isolated from the WT mice ($P<.01$) (Fig. 1B).

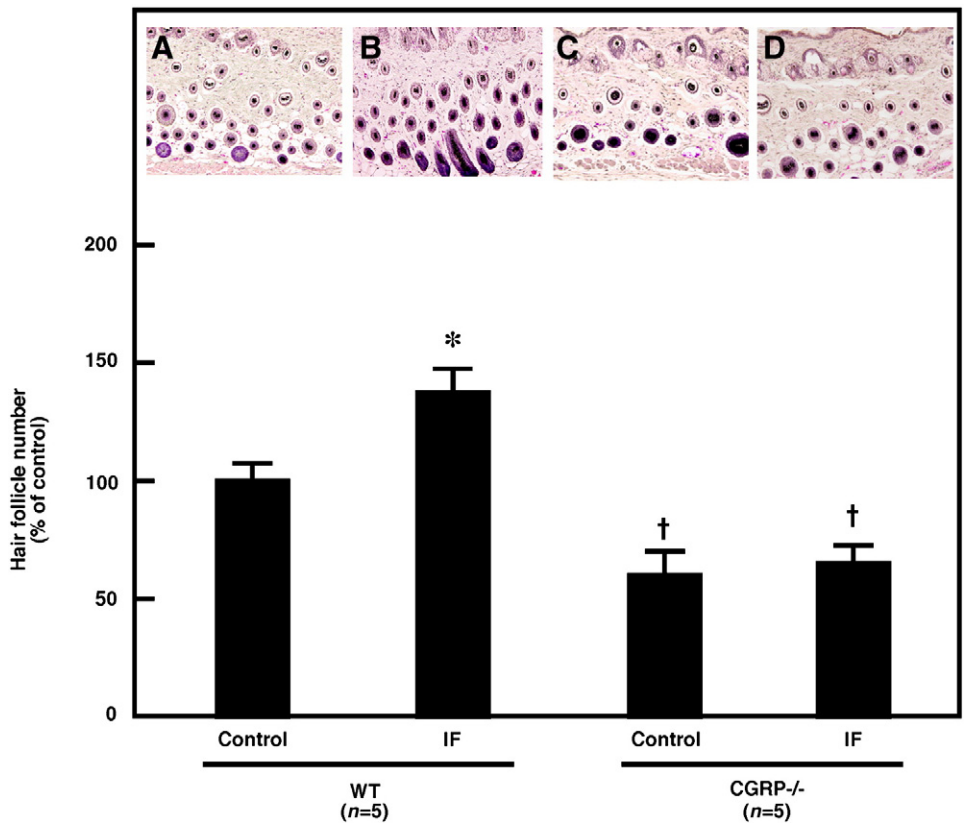

Fig. 4. Effect of isoflavone administration on the number and morphology of hair follicles in WT and CGRP$^{-/-}$ mice. The number of hair follicles was determined on 10μm paraffin sections after hematoxylin and eosin staining at 200× magnification bright-field microscopy. Each bar represents mean±SD from five animal experiments. *$P<.01$ vs. control; †$P<.01$ vs. WT mice. Hematoxylin and eosin staining of skin is shown in insets. (A) WT mice, control. (B) WT mice, isoflavone. (C) CGRP$^{-/-}$, control. (D) CGRP$^{-/-}$, isoflavone.

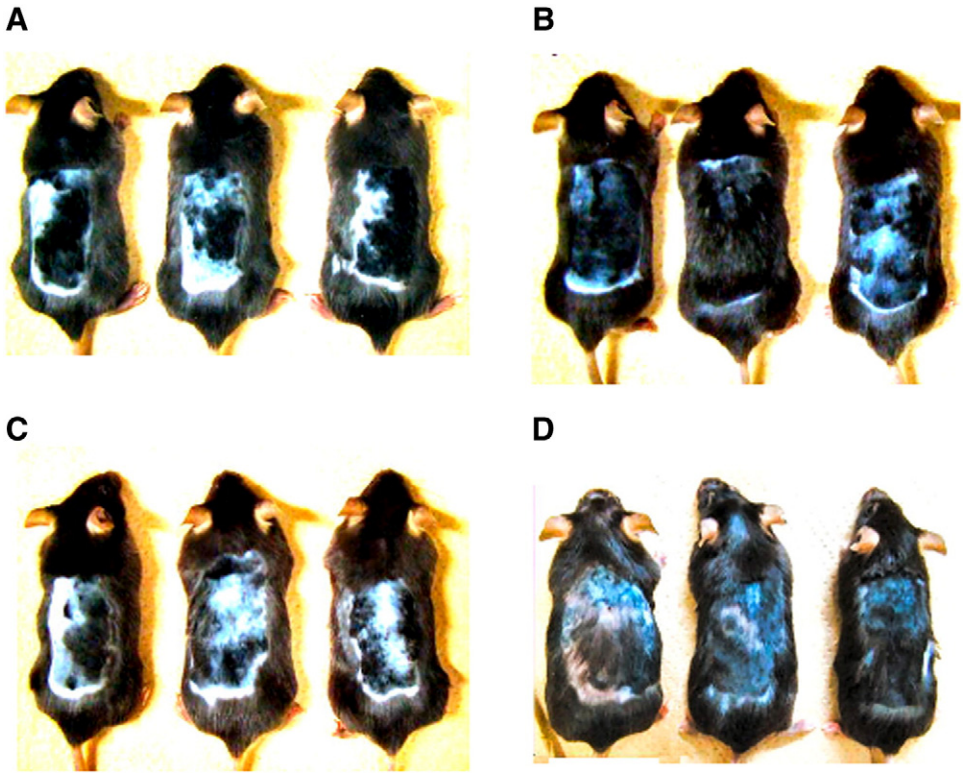

Fig. 5. Effect of isoflavone on hair regrowth in WT mice (A, B) and in CGRP$^{-/-}$ mice (C, D). Hair regrowth was shown at 3 weeks after hair removal in control (A and C) or isoflavone administration (B and D).

3.2. Effect of isoflavone administration on dermal levels of CGRP, IGF-I and IGF-I mRNA in WT and CGRP$^{-/-}$ mice

To examine whether isoflavone increased the dermal levels of CGRP, IGF-I and IGF-I mRNA in mice, we determined the tissue levels of these substances in the skin of WT and CGRP$^{-/-}$ mice following isoflavone administration for 3 weeks. The baseline dermal levels of CGRP, IGF-I and IGF-I mRNA were significantly higher in WT mice than in CGRP$^{-/-}$ mice (P<.01)(Fig. 2). Administration of isoflavone significantly increased the dermal levels of CGRP, IGF-I and IGF-I mRNA in WT mice (P<.01), but not in CGRP$^{-/-}$ mice (Fig. 2).

3.3. Effect of isoflavone administration on immunohistochemical expression of IGF-I in the hair follicles of WT and CGRP$^{-/-}$ mice

Expression of IGF-I in the hair follicles of mice was examined by Immunofluorescent staining. In WT mice, weak immunofluorescence of IGF-I was observed in the follicle bulb and dermal papilla (Fig. 3A, E and I). Administration of isoflavone for 3 weeks clearly increased the IGF-I expression in the outer root sheath and in the dermal papilla in WT mice (Fig. 3B, F and J). However, no such increase in the expression of IGF-I in the hair follicles was found following isoflavone administration in CGRP$^{-/-}$ mice (Fig. 3D, H and L).

3.4. Effect of isoflavone administration on the number and morphology of hair follicles in WT and CGRP$^{-/-}$ mice

To determine whether isoflavone administration affected the number and morphology of hair follicles in WT and CGRP$^{-/-}$ mice, we analyzed the skin sections from these animals, stained with hematoxylin and eosin. Three weeks after hair removal, the follicles penetrated the skin dermis layer and entered the growth phase (Fig. 4, inset A and B).

Administration of isoflavone significantly increased the number of hair follicles in WT mice (P<.01) (Fig. 4), while CGRP$^{-/-}$ mice exhibited hypotrophic skin and had a lower number of the hair follicles than WT mice (Fig. 4, inset C and D) (P<.01) (Fig. 4). Furthermore, isoflavone administration did not affect the number of hair follicles in CGRP$^{-/-}$ mice (Fig. 4).

3.5. Effect of isoflavone administration on hair regrowth in WT and CGRP$^{-/-}$ mice

Hair regrowth was apparently more promoted to a greater degree following isoflavone administration for 3 weeks than following administration of a soy-free diet for 3 weeks in WT mice (Fig. 5A and B). Isoflavone administration had less effect in CGRP$^{-/-}$ mice (Fig. 5C and D).

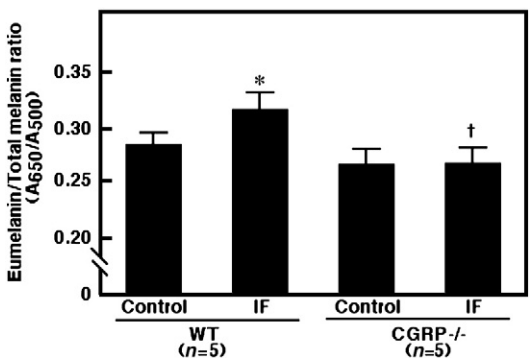

Fig. 6. Spectrophotometric assay of the eumulanin/total melanin ratio in dorsal hairs of WT and CGRP$^{-/-}$ mice administered isoflavone for 3 weeks after depilation. Each bar represents mean±S.D. from five animal experiments. *P<.05 vs. control; †P<.05 vs. IF (WT).

3.6. Effect of isoflavone administration on hair pigmentation in WT and CGRP$^{-/-}$ mice

Darkening was more marked and formation of a subapical yellow band was less in dorsal hairs of WT mice administered isoflavone for 3 weeks than those in dorsal hairs of WT mice administered soy-free diet for 3 weeks after depilation (data not shown). Eumelanin content was significantly increased in dorsal hairs of WT mice administered isoflavone for 3 weeks after depilation compared with that in dorsal hairs of WT mice administered soy-free diet ($P<.05$) (Fig. 6). In contrast, hair plucked from the dorsal skin of CGRP$^{-/-}$ mice showed no significant change of hair pigmentation following isoflavone administration for 3 weeks (Fig. 6).

4. Discussion

In the present study, we demonstrated that while isoflavone increased CGRP production, it did not increase the release of CGRP, in DRG neurons isolated from WT mice. These observations are consistent with our previous report demonstrating that isoflavone increased CGRP production, but did not increase CGRP release, in DRG neurons isolated from rats [21].

We previously reported that capsaicin administration increased IGF-I production by increasing CGRP release in the skin of mice [15], suggesting that administration of isoflavone may also increase the IGF-I production through increasing the CGRP levels in the skin of mice. Consistent with this hypothesis are observations in the present study demonstrating that administration of isoflavone increased the dermal levels of IGF-I and IGF-I mRNA levels in WT mice but not CGRP$^{-/-}$ mice. Since estrogen has been shown to increase the CGRP levels in sensory neurons by enhancing NGF-mediated production of CGRP [20,34,35], isoflavone is referred to as phytoestrogen.

In the present study, although isoflavone did not increase the release of CGRP from sensory neurons in vitro, it increased the dermal levels of CGRP and IGF-I in WT mice. Since numerous endogenous agonists are known to be capable of increasing the release of CGRP from sensory neurons [36], it is possible that the increase of the dermal levels of IGF-I in WT mice following isoflavone administration was induced via enhancement of CGRP release by these endogenous agonists such as anandamide.

IGF-I is synthesized in the hair follicle dermal papilla cells and plays a critical role in the promotion of hair growth by regulating cellular proliferation and migration during the development of hair follicles [8,14]. Consistent with this notion are our previous observations demonstrating that capsaicin promoted the hair growth in mice by increasing the expression of IGF-I in the dermal papilla cells through stimulation of sensory neurons [16]. Since isoflavone increased the dermal levels of IGF-I production by increasing its transcription via enhancing CGRP synthesis in WT mice, it is possible that isoflavone promotes the hair growth by increasing the expression of IGF-I in the hair follicle dermal papilla cells in mice. The results of our present study demonstrating that administration of isoflavone increased the expression of IGF-I in the outer root sheath and in the dermal papilla and increased the number of hair follicles in WT mice, but not in CGRP$^{-/-}$ mice are consistent with this notion. Furthermore, hair regrowth was apparently more promoted to a pronounced degree following administration of isoflavone in WT mice, but scarce effect was noted in CGRP$^{-/-}$ mice in the present study. These observations strongly suggest that isoflavone might promote hair growth by increasing IGF-I production in the dermal papilla through enhancing CGRP synthesis in WT mice.

Hair growth is associated with hair melanogenesis [37,38]. IGF-I has been shown to be expressed at high levels in hair follicles during the growth phase of the hair cycle (anagen) [39], and plays a critical role in regulation of the growth and function of melanocytes [40]. Melanogenesis was significantly increased in dorsal hairs of WT mice administered isoflavone, but not in those of CGRP$^{-/-}$ mice administered isoflavone. These observations strongly suggest that administration of isoflavone may increase melanogenesis in dorsal hairs of WT mice by increasing IGF-I production in hair follicles through enhancing CGRP synthesis in sensory neurons.

We previously reported that administration of isoflavone enhanced capsaicin-induced increase in the expression of IGF-I production in the dermal papilla, thereby enhancing the capsaicin-induced promotion of hair regrowth in WT mice [16]. These observations suggest that simultaneous administration of capsaicin and isoflavone might additively promote the hair growth by increasing the release and production, respectively, of CGRP in sensory neurons, thereby promoting hair growth. Promotion of hair growth was observed in 48 patients with alopecia administered capsaicin and isofalvone for 5 months and this therapeutic effect might be explained, at least in part, by the additive effects of capsaicin and isoflavone on dermal IGF-I production.

Expression of IGF-I in the dermal papilla has been shown to strongly correlate with the therapeutic efficacy of finasteride in patients with androgenetic alopecia (AGA) [41]. These observations strongly suggest that combined use of capsaicin/isoflavone and finasteride may exert enhanced therapeutic efficacy in AGA patients. We are currently investigating this possibility.

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