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ORIGINAL ARTICLE

The gender differences in the inhibitory action of UVB-induced melanocyte activation by the administration of tranexamic acid

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The authors have no conflict of interest.

SUMMARY

Background
Tranexamic acid has an inhibitory action on ultraviolet (UV) B-induced melanocyte activation. This study examined the sex differences in the inhibitory action of tranexamic acid on UVB-induced melanocyte activation.

Methods
We irradiated the eye and ear of male and female mice with UVB at a dose of 1.0 kJ/m^2 using a 20SE sunlamp. We orally administered tranexamic acid (750 mg/kg/day) at 30 min before UVB exposure.

Results
Tranexamic acid inhibited the UVB-induced epidermal melanocyte activation, and the effect was more remarkable under UVB eye irradiation than under UVB ear irradiation. Furthermore, the melanocyte activity suppression effect was stronger in female mice than in male mice. Following the administration of tranexamic acid, the female displayed increased blood levels of β-endorphin and μ-opioid receptor and estradiol receptor β expression in comparison with the male. Furthermore, the effect of melanocyte activity suppression in the female mice was decreased by the administration of tamoxifen (antagonist of estrogen receptor) or naltrexone (antagonist of μ-opioid receptor).

Conclusions
These results suggest that the suppression by tranexamic acid of the UVB-induced melanocyte activation (UVB sensitivity) is stronger in female mice than in male mice and that female hormones and β-endorphin play an important role in this sex difference.

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Tranexamic acid (trans-4-aminomethylcyclohexane-carboxylic acid) is a medical amino acid with an anti-plasmin action that exerts hemostasis, and anti-inflammatory and anti-allergic effects. Furthermore, tranexamic acid inhibits the pigmentation induced by ultraviolet (UV) rays (1, 2). This effect is modulated by α-melanocyte-stimulating hormone (α-MSH) formed from the proopiomelanocortin (POMC) system in the epidermis (3). Moreover, tranexamic acid decreases the expression of tyrosinase which is a melanin synthesis enzyme (4). We previously reported that the expression of prohormone convertase (PC)2, which cleavages from

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POMC to α-MSH, is decreased in the pituitary by the administration of tranexamic acid (5). Although the POMC gene is activated by UVB irradiation, a decrease in the production of α-MSH takes place through the decrease of PC2, and, as a result, a whitening effect is observed.

It is reported that males are more strongly influenced by UV than females. For example, Urbach *et al.* (6) reported that the incidence of basal cell cancer, in males, was higher than that in females. Naruse *et al.* (7) reported that active keratosis was more common in males than in females. On the other hand, the pigmentation in response to UV exposure is stronger in female mice than in males (8). The sex hormones participate in the sex difference. The intervention of estrogen (8) and progesterone (9), which are female hormones, is well known. In addition, many cases of pigmentation caused by a chloasma are seen in women. Female hormones have been mentioned as causes of chloasma pigmentation (10, 11). It is known that tranexamic acid has an effect in the treatment of chloasma (12–14). Therefore, the suppression effect of tranexamic acid on the pigmentation of a chloasma may be stronger in female mice.

This study examined the existence of sex differences in the pigmentation caused by UVB in the ear skin of mice and investigated the mechanism of sex difference in UVB-induced pigmentation.

MATERIALS AND METHODS

Animal experiments

Specific pathogen-free, 8-week-old male and female DBA/2 mice (SLC, Hamamatsu, Aichi, Japan) were subjected to experiments according to the animal care regulations of Suzuka University of Medical Science. The eye or ear was locally exposed to UVB (wavelength 280–320 nm; 20SE sunlamp; Toshiba Co., Tokyo, Japan) for 3 days at a dose of 1.0 kJ/m^2 per day (irradiation time: 60 min/day), with the animals kept under light Nembutal anesthesia. The rest of the body surface was protected from irradiation by aluminum foil (refer to the previous paper for details) (5). In the control experiments, the eye or ear was irradiated with visible light (400–700 nm; FL20SD light source; Toshiba Co.).

Skin samples were obtained from the ear for Dopa staining from six mice per group at 5 days after each session of irradiation and for Western blotting in six mice per group at 3 days after each session of irradiation. Blood samples were obtained 1 day after the final UVB irradiation from an additional six mice per group.

Tranexamic acid treatment

Approximately 750 mg/kg of tranexamic acid (Daiichi Sankyo Healthcare Co., Ltd., Tokyo, Japan) in saline was administered orally to each mouse 30 min before UVB exposure, while saline was administered to the control animals (5).

Naltrexone treatment

Approximately 10 mg/kg of the naltrexone (naltrexone hydrochloride, μ-opioid receptor antagonist; Selleck Chemicals, Houston, TX, USA) in saline was injected intraperitoneally into the treatment mice 30 min before UVB exposure, while saline was injected into the control mice (15).

Tamoxifen treatment

Approximately 10 μg/day of tamoxifen ([2]-1-[p-dimethylamino-ethoxyphenyl]-1,2-diphenyl-1-butene, 17β-estradiol antagonist; Sigma, St Louis, MO, USA) in saline was injected intraperitoneally into the female mice throughout the experimental period. Only saline was injected into the female control animals (16).

The preparation and staining of epidermal sheets

Five days after irradiation, skin samples (0.5 × 0.5 cm) were obtained from the ear and incubated for 2 h at 37°C in 2 mol/l of sodium bromide. The epidermis was then separated from the dermis to obtain epidermal sheets. The stain for Dopa-positive melanocytes used the method of Jimbow & Uesugi (17). All epidermal sheets were examined under coded conditions by one investigator. Briefly, approximately 20 fields of views (magnification: 20×) were chosen for each of the epidermal sheets, and a stack of approximately 30 optical sections was scanned with a *z*-increment of 0–4 μm. From each optical stack, extended focus projections (summation of all optical sections in the stack with each section in focus) were performed, and the areas of cells were calculated using a software program provided with the BioRad MRC500 instrument (BioRad, Hercules, CA, USA). The number of Dopa-positive melanocytes per mm^2 was determined by counting 15–20 fields at 200× magnification.

The Western blot analysis of the ear skin

Fixed ear skin samples were homogenized in Lysis buffer (Kurabo, Osaka, Japan), followed by centrifugation at

8000 × *g* for 10 min. The supernatant from each sample was then isolated and stored at −80°C until analysis. After thawing, equal amounts of protein (20.0 mg/lane) were loaded onto a 4–12% BIS-TRIS Bolt gel (Life Technologies, Carlsbad, CA, USA) and electrophoresed at 200 V for 20 min. Following separation, proteins were transferred to a nitrocellulose membrane using an iBlot Western blotting system (Life Technologies), which was subsequently blocked with 5% skim milk at 4°C overnight. After blocking, the membranes were then incubated at 25°C for 1 h with primary antibodies against μ-opioid receptor (1 : 1000; EN20 Biochem, New York, NY, USA), estrogen receptor β (1 : 1000; Novus Biologicals LLC, Littleton, CO, USA), p-extracellular signal-regulated kinase (pEKR1/2; 1 : 1000; Cell Signaling Technology Inc., Danvers, MA, USA), p-microphthalmia-associated transcription factor (pMITF; 1 : 1000; Cell Signaling Technology Inc.), cAMP response element-binding protein (pCREB; 1 : 1000; Cell Signaling Technology Inc.), p-p38 mitogen-activated protein kinase (p-p38 MAPK; 1 : 1000; Cell Signaling Technology Inc.), Tyrosinase (1 : 500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or β-action (1 : 5000; Sigma). Immunocomplexes on the membranes were then visualized using a horseradish peroxidase-conjugated secondary antibody (Dako Cytomation, Glostrup, Denmark) and immunoStar Zeta (Wako, Osaka, Japan). Images were acquired using the Multi-Gauge software program (Fujifilm, Tokyo, Japan).

The assay of adrenocorticotropic hormone, α-MSH, and β-endorphin in the plasma

Blood samples were collected from the heart at 24 h after the final session of UVB irradiation, and the plasma was fractionated. The plasma levels of adrenocorticotropic hormone (ACTH), α-MSH, and β-endorphin were determined using a commercial ELISA kit (Phoenix Pharmaceuticals Inc., Hayward, CA, USA) according to the manufacturer's instructions. In addition, the % cross-reactivities of this ELISA kit are as follows: ACTH [ACTH (mouse), 100; α-MSH and β-endorphin, 0], α-MSH [α-MSH (mouse), 100; ACTH and β-endorphin, 0], and β-endorphin [β-endorphin (mouse), 100; α-MSH and ACTH, 0].

Statistical analysis

The results obtained from the animal groups were compared using either ANOVA or Student's *t*-test using an ANOVA software program (XHL STAT; Artwork Conversion Software Inc., Santa Cruz, CA, USA). First, we analyzed all data by an ANOVA, and only items with significant differences were further evaluated using the *t*-test. All data are expressed as the means ± standard deviation, and significance was set at $P < 0.05$.

RESULTS

The sex difference in epidermal melanocytes after UVB irradiation following tranexamic acid treatment

Localized UVB irradiation of either the eye or ear significantly increased the number of Dopa-positive melanocytes in the epidermal sheets derived from the ear. The Dopa-positive melanocytes, which increased after UVB irradiation, also decreased in number by the administration of tranexamic acid. The decrease in the number of Dopa-positive melanocytes caused by UVB irradiation was significantly greater in the female mice (Fig. 1).

The sex difference in the plasma levels of ACTH, α-MSH, and β-endorphin after UVB irradiation following tranexamic acid treatment

Localized UVB irradiation of either the ear or eye induced a significant increase in the concentration of ACTH, α-MSH, and β-endorphin in the plasma. No sex difference was observed in the UVB-induced increase in the plasma ACTH, α-MSH, and β-endorphin levels. The level in plasma of ACTH and α-MSH after UVB irradiation decreased in response to the administration of tranexamic acid. However, no sex differences were seen in the levels of ACTH and α-MSH (Fig. 2a, b). On the other hand, the plasma level of β-endorphin was increased after the UVB irradiation by tranexamic acid. Furthermore, a sex difference was observed in the β-endorphin level in plasma after UVB irradiation by tranexamic acid administration: The female mice showed a higher value than the male mice (Fig. 2c).

The sex difference in the expression of the μ-opioid receptor in ear skin

Under UVB eye irradiation, the expression of μ-opioid receptor in the ear skin of female mice increased and also increased by tranexamic acid administration. On the other hand, the altered expression of the μ-opioid receptor of the ear skin was not seen in the male mice (Fig. 3).

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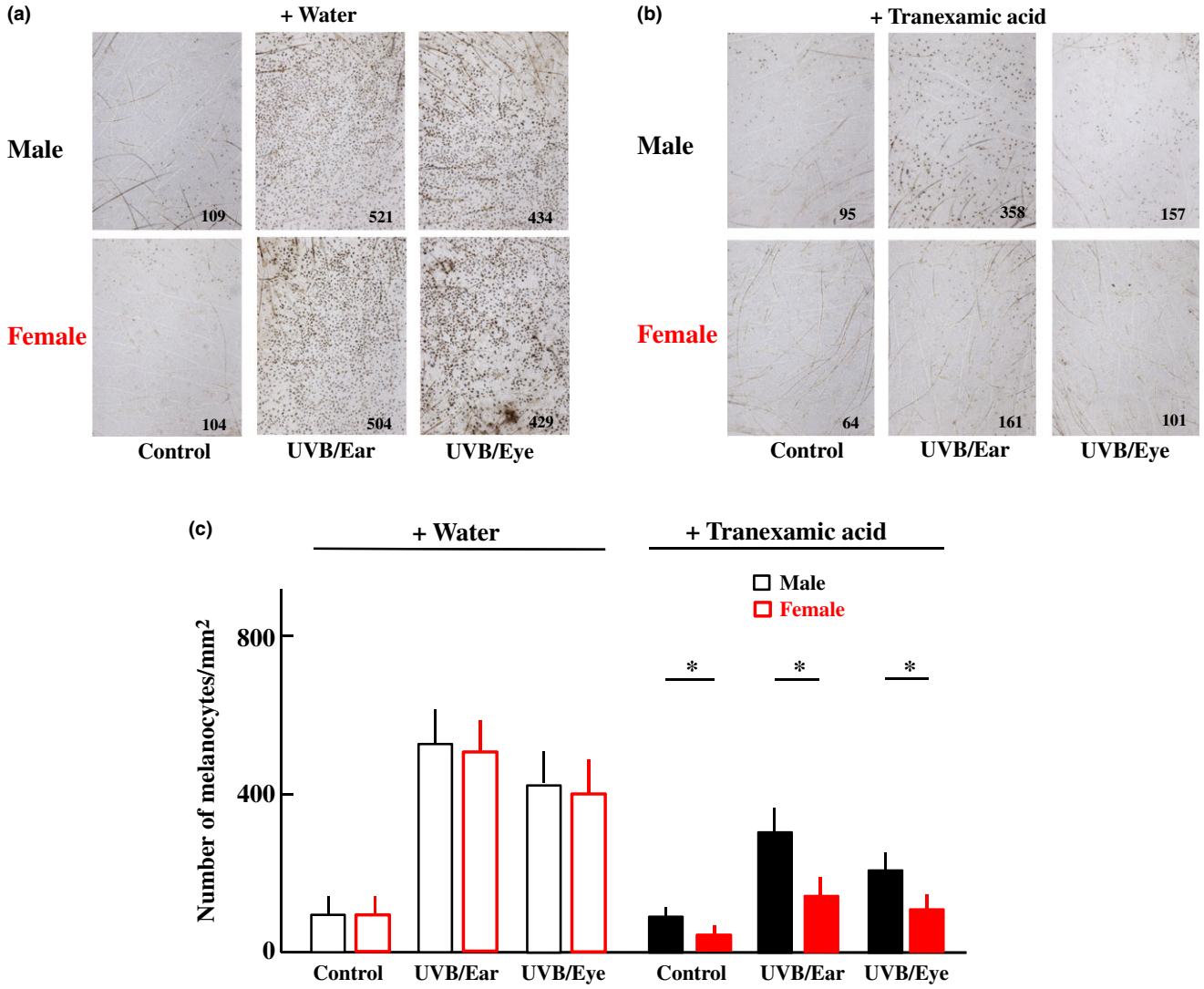

Fig. 1. The sex difference in epidermal melanocytes after UVB eye irradiation following tranexamic acid treatment. Five days after the final exposure of the eye to UVB irradiation, the stained figure (a) and (b) and the number (c) of Dopa-positive melanocytes in epidermal sheets prepared from the ear were examined. The data reflect one typical experiment with six animals. The numbers expressed in (a) and (b) are actual measurements. The values are presented as the means ± SD derived from the six animals (biological replicates; $*P < 0.05$). Scale bar = 100 μm.

The sex difference of tranexamic acid treatment on the epidermal melanocytes after naltrexone injection

The number of melanocytes of the ear in the tranexamic acid treatment group decreased with tranexamic acid in comparison with the nontreatment group following UVB eye irradiation. However, the decreased expression of Dopa-positive melanocytes was suppressed by naltrexone treatment. Furthermore, although the number of melanocytes in the female mice was only 52% than that in the male mice following tranexamic acid treatment, the number of melanocytes in the female mice fell to 13% of the number in males after naltrexone treatment. This difference was significant (Fig. 4).

The effect of tranexamic acid treatment on the plasma levels of 17β-estradiol and on the expression of estrogen receptor β after UVB eye irradiation

To understand the sex differences, we analyzed the properties associated with decreased Dopa-positive melanocytes by examining the plasma levels of 17β-estradiol and examining the estrogen receptor β expression level. In addition, to identify the expression of estrogen receptor

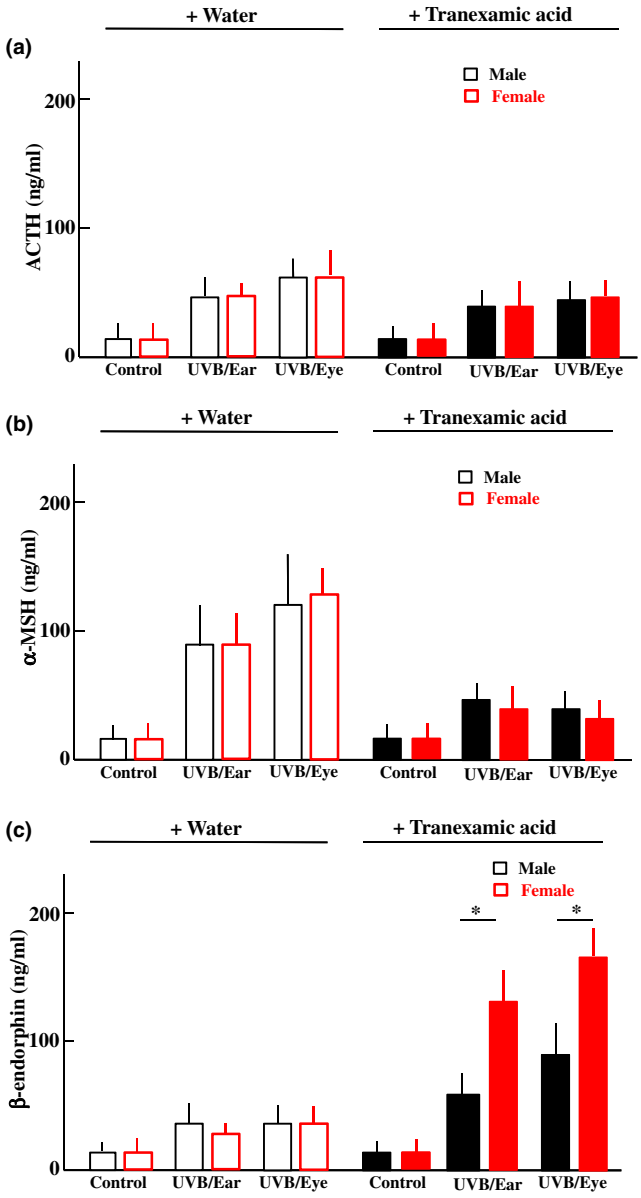

Fig. 2. The sex differences in the plasma levels of adrenocorticotropic hormone (a), α-melanocyte-stimulating hormone (b), and β-endorphin (c) after UVB eye irradiation following tranexamic acid treatment. The values are presented as the means ± SD derived from six animals (biological replicates; $*P < 0.05$).

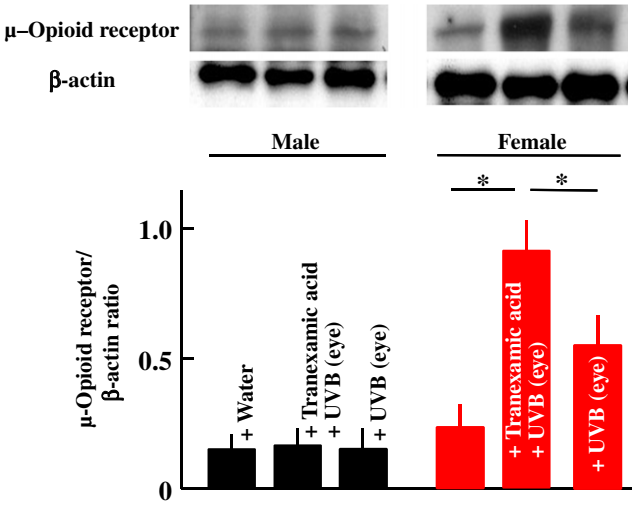

Fig. 3. The sex difference in the expression levels of μ-opioid receptor after UVB eye irradiation following tranexamic acid treatment. The data show the results from one typical experiment involving six animals. The values are presented as the means ± SD derived from six animals (biological replicates; $*P < 0.05$).

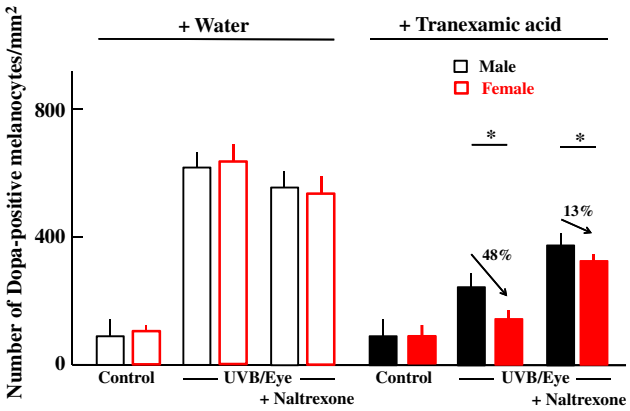

Fig. 4. The sex difference in epidermal melanocytes after tranexamic acid treatment following naltrexone treatment. Naltrexone was injected intraperitoneally into the mice throughout the experimental period. Five days after the final UVB irradiation of the eye, the number of Dopa-positive melanocytes in the epidermal sheets prepared from the ear was determined. The values are presented as the means ± SD derived from six animals (biological replicates; $*P < 0.05$).

in the skin, we also determined the expression of estrogen receptor β.

The 17β-estradiol level in the plasma did not change with UVB eye irradiation. Moreover, the alteration of 17β-estradiol was not seen in tranexamic acid treatment (Fig. 5a). On the other hand, the expression of the estrogen receptor β after UVB eye irradiation was increased by tranexamic acid treatment (Fig. 5b). In addition, in the female mice that received tranexamic acid treatment, the estrogen receptor β was found to be associated with the number of melanocytes (Fig. 5b).

The sex difference of tranexamic acid treatment on epidermal melanocytes after tamoxifen injection

Although the number of melanocytes, which increase in response to UVB eye irradiation, decreases following

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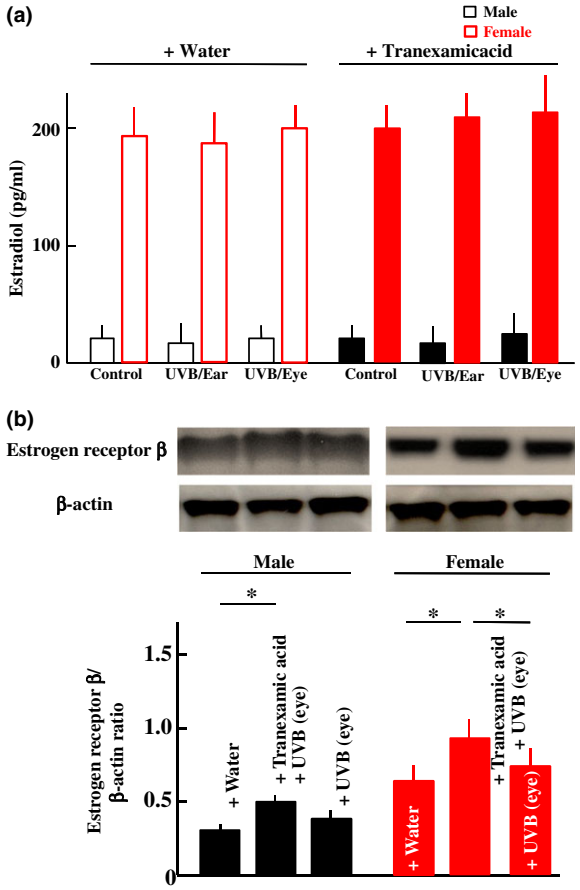

Fig. 5. The sex difference in the plasma levels of 17β-estradiol (a) and the expression levels of estrogen receptor β (b) after UVB eye irradiation after tranexamic acid treatment. The data show the results from one typical experiment involving six animals. The values are presented as the means ± SD derived from six animals (biological replicates; $*P < 0.05$).

tranexamic acid treatment, the number of melanocytes in a tamoxifen-treated male mouse does not change. On the other hand, tamoxifen treatment suppressed the decrement of the number of melanocytes in female mice. Furthermore, female mice showed only 50% of the number of melanocytes after UVB eye irradiation following tranexamic acid treatment. However, in the tamoxifen treatment, the number of melanocytes in the female mice fell to 16% of the number in males. A significant difference was recognized between the male and the female mice in the tamoxifen (Fig. 6).

The sex difference in the expression of the signaling gene in connection with the pigmentation in the melanocytes

During UVB eye irradiation, the gene connected with pigmentation increases in both female and male mice.

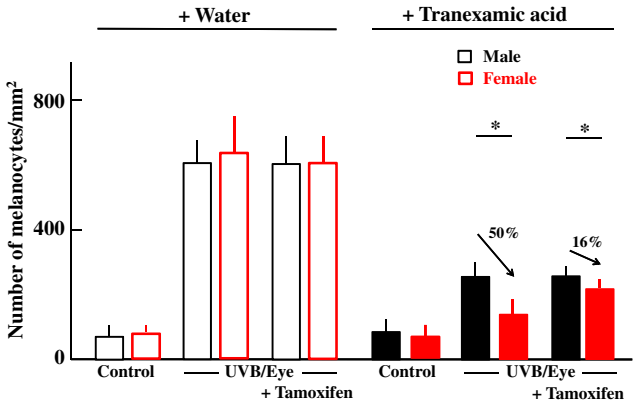

Fig. 6. The sex difference in epidermal melanocytes after tranexamic acid treatment following tamoxifen treatment. Tamoxifen was injected intraperitoneally into the mice throughout the experimental period. Five days after the final UVB irradiation of the eye, the number of Dopa-positive melanocytes in the epidermal sheets prepared from the ear was determined. The values are presented as the means ± SD derived from six animals (biological replicates; $*P < 0.05$).

Furthermore, the expression of p-MITF and tyrosinase after the UVB eye irradiation decreased in both the male and the female mice following the administration of tranexamic acid. On the other hand, p-p38 did not change in the male mice in response to tranexamic acid administration. The decrease was only observed in the female mice (Fig. 7).

DISCUSSION

In the experiment, a sex difference was observed in the suppression of pigmentation after the UVB eye irradiation by tranexamic acid. The suppression effect was observed to be higher in female mice than in male mice. Furthermore, the β-endorphin level in the plasma of female mice increased in comparison with male mice following the administration of tranexamic acid. The expression levels of the estrogen receptor β and μ-opioid receptor were also much higher in female mice than in male mice following the administration of tranexamic acid.

As a result of blocking the combination of plasminogen and plasmin from reaching the melanocytes, tranexamic acid obstructs the disengagement and activation of a melanin generation promotion factor by plasmin, and suppresses the pigmentation, for example, the conversion of the activity of a phospholipase A2 precursor (18), altering the processing from POMC to α-MSH (3), and the disengagement of basic fibroblast growth factor (19). Moreover, in our recent report, it became clear that tranexamic acid obstructed PC2, which performs the processing of α-MSH from the POMC of a pituitary

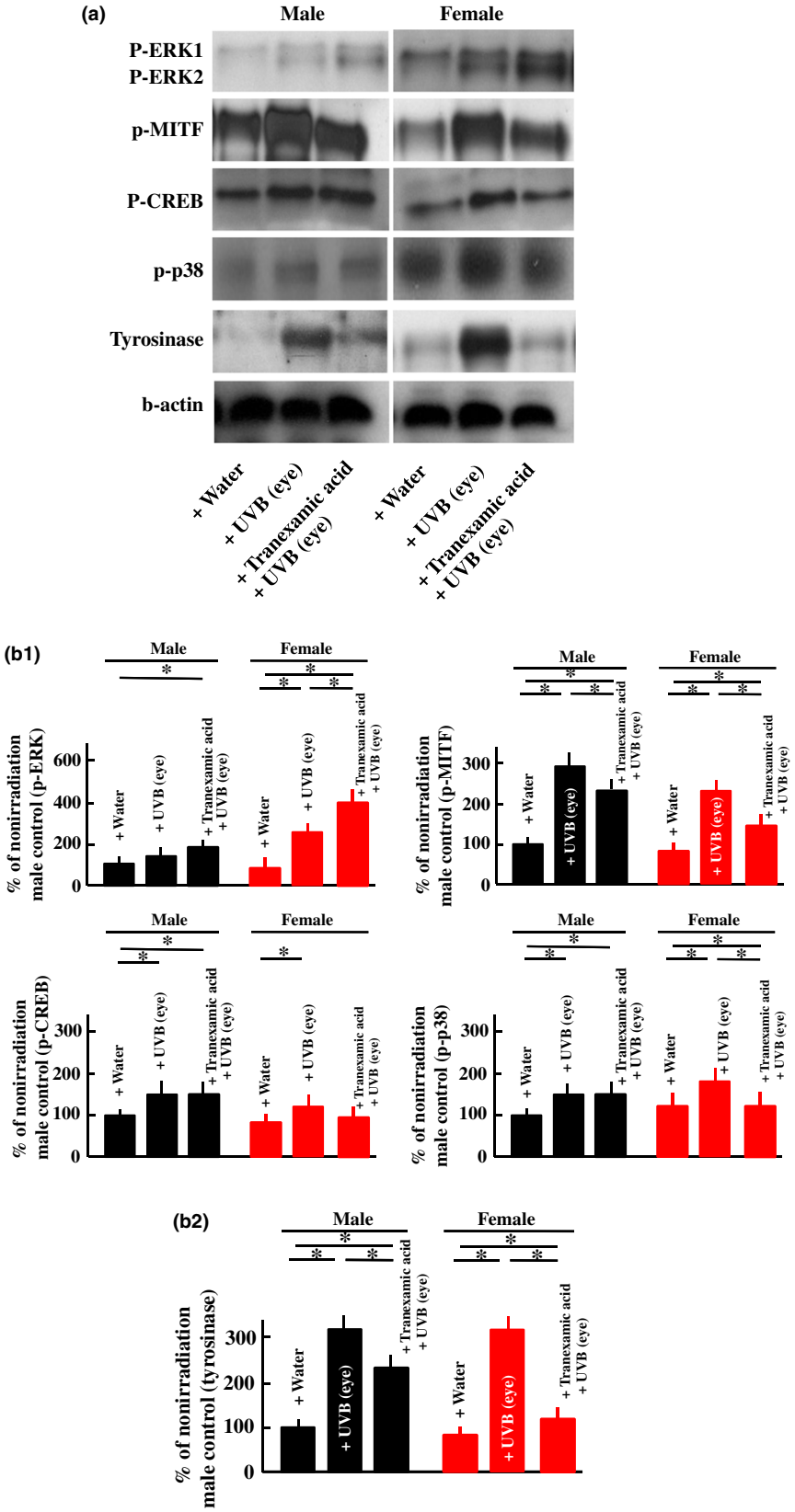

Fig. 7. The sex difference in the expression of p-ERK1/2, p-MITF, p-CREB, p-p38, and tyrosinase in the skin after UVB eye irradiation following tranexamic acid treatment. The data show the results from one typical experiment involving six animals. The values are presented as the means ± SD derived from six animals (biological replicates; $*P < 0.05$).

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gland by UVB eye irradiation (5). However, there are no reports of sex difference.

Although the number of Dopa-positive melanocyte increases in response to UVB irradiation in both male and female mice, the increase was suppressed by tranexamic acid. Moreover, the increased suppression of the Dopa-positive melanocytes was stronger in the female mice than in the males. α-MSH secreted from a pituitary gland falls in response to tranexamic acid; however, we did not observe a sex difference in the level of α-MSH in plasma. On the other hand, there was a sex difference in the level of β-endorphin following the administration of tranexamic acid, which is a hormone of the same POMC system. The expression level of β-endorphin was very high in female mice (Fig. 2c). In this study, both the expression of μ-opioid receptor and the plasma levels of β-endorphin increased in the female mice. On the other hand, in male mice, the expression of μ-opioid receptor did not increase, while the plasma level of β-endorphin, albeit small compared with the female mice, increased. From these findings, we speculate that the increase in the expression of μ-opioid receptor is due to sex differences. Moreover, the sex difference was shortened by naltrexone treatment. In addition, sex differences in exercise (20), cerebral (21), and sickness (22) have been shown to involve β-endorphin. Therefore, we considered that both β-endorphin and μ-opioid receptor were involved in the sex difference. From these results, the β-endorphin/μ-opioid receptor system may be involved with the sex difference in pigmentation after tranexamic acid administration. Although the pigmentation suppression by tranexamic acid administration in the female mice was suppressed by the administration of a μ-opioid receptor inhibitor, we could not completely remove the sex difference in the pigmentation suppression of tranexamic acid. Therefore, it is though that although the β-endorphin/μ-opioid receptor systems participate in a pigmentation suppression of the female mice, it only became clear by this system that the sex differences could not be shown completely. On the other hand, β-endorphin is generally reported to induce pigmentation, which differs from the result of this research (23–25). Therefore, it is necessary to consider the function of tranexamic acid on the β-endorphin/μ-opioid receptor system in greater detail.

It is known that tranexamic acid improves the pigmentation of a chloasma (8). The sex hormone participates in the pathogenic mechanism of a chloasma; however, tranexamic acid did not participate in a secretion of the sex hormone. However, tranexamic acid increased the expression of the estrogen receptor β. When an estrogen receptor inhibitor was administered to mice, the suppression of the pigmentation by tranexamic acid administration was reduced in female mice. Although the suppression rate approached that of male mice, the difference was still statistically significant. Estrogen receptor β is widely distributed over the whole body, and it has important physiological significance. In fact, it may be a key factor in suppressing the incidence of both a climacteric disturbance (26) and a breast cancer (27). As a result, the increased expression of estrogen receptor β after the administration of tranexamic acid may therefore function in many sicknesses in which estrogen and/or estrogen receptor β are concerned, as well as play a role in pigmentation prevention. On the other hand, the increase in estrogen receptor α was evaluated using tranexamic acid in this examination (data not shown). Although estrogen receptor α is known to play a role in the development of a breast cancer, the expression levels observed in this study were very low and not at the level considered to activate breast cancer (28–30). Therefore, tranexamic acid administration was considered to be satisfactory.

In these results, it was thought that the sex difference in the suppression of pigmentation, which was induced by tranexamic acid administration, was produced by the action of both the β-endorphin/μ-opioid receptor and the estrogen/estrogen receptor β. The estrogen receptor is related to the POMC system and controls the upregulation of the POMC system (31–34). A decrement of the μ-opioid system will also decrease the expression of estrogen (35). Therefore, the estrogen/estrogen receptor β and the β-endorphin/μ-opioid receptor may be controlling each activity through an interaction. Further research is needed to elucidate both of these relationships.

In this study, the p38 of the melanin synthesis signal of the melanocytes was suppressed to a greater degree in female mice than in male mice. There are many reports about the relationship between p38 and the estrogen/estrogen receptor. The estrogen/estrogen receptor has been shown to modulate p38 MAPK (36–38). However, the relationship between p38MAPK and the estrogen/estrogen receptor in melanocytes is not known. Moreover, the relationship between p38 MAPK and the β-endorphin/μ-opioid receptor is not known. Therefore, it is necessary to examine the correlation of estrogen/estrogen receptor β-β-endorphin/μ-opioid receptor-p38 MAPK in the melanocytes. In addition, this signal shown here is a part of the gene associated with pigmentation. Therefore, it is necessary to analyze the signal in connection with increased pigmentation. According to the results of these analyses, we demonstrated the mechanism of

comprehensive tranexamic acid and showed a novel effect of tranexamic acid treatment.

CONCLUSIONS

In this experiment, tranexamic acid administration suppressed the pigmentation induced by UVB eye irradiation. Furthermore, it was indicated that there was a sex difference in the suppression. Tranexamic acid is structurally stable, and its safety is high. The pigmentation amelioration effect by the administration of tranexamic acid should therefore be considered in the treatment of women who would like to be healthy and to maintain their appearance.

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