学 位 論 文 概 要

西暦 2022年 01月 23 日

学位申請者
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学位論文題目

New Skin Brightening Mechanisms by Degradation of Tyrosinase in Melanocytes and Digestion of Melanosome in Keratinocytes

学位論文の要旨

This study focused on a new brightening mechanism related to melanosome digestion. We also investigated the brightening effects and mechanisms of propylparaben, *Angelica acutiloba* root extract (touki extract), and *Foeniculum vulgare* Miller fruit extract (fennel extract).

Chapter 2 investigated the potential of pH-dependent tyrosinase degradation, focusing on the low pH of intra-melanosomal environment-induced suppression of melanogenesis. We also investigated whether the mechanism of propylparaben, preservative for cosmetics, is implicated in melanosomal pH regulation at non-cytotoxic concentrations. It has been hypothesized that aspartic and cysteine proteases participate in degradating tyrosinase in acid melanosomes. We found that tyrosinase was degraded via acidification of melanosomes, thereby decreasing its activity; both inhibitors (aspartic protease and cysteine protease) decreased tyrosinase degradation. Na^+/H^+ exchangers and V-ATPase regulate melanosomal pH. We investigated the mechanisms underlying the inhibitory effect of propylparaben on melanin production in B16 melanoma cells. The mRNA expression levels of tyrosinase and related proteins such as tyrosinase-related protein-1 (TRP-1) and dopachrome tautomerase were not affected by propylparaben; however, these proteins levels in melanosomes decreased. We investigated the mechanisms underlying the inhibitory effect of propylparaben on melanin production in B16 melanoma cells. The effects of propylparaben on the mRNA expression of Na^+/H^+ exchangers, Na^+/Ca^{2+} exchangers, and melanosome pH levels were examined. Propylparaben decreased gene expression in both exchangers, and staining with an intracellular pH indicator confirmed the decrease in melanosomal pH. These results suggest that propylparaben downregulates melanin production via acidification of melanosomes.

In Chapter 3 (based on the results of Chapter 2), we focused on improving pigmentation by activating the lysosomal enzyme cathepsin to digest melanosome complexes, and we also

investigated the effects of touki extract. We found that touki extract promoted the activity of total cathepsin, increased the activity and gene expression level of cathepsins K, and significantly digested melanosome complexes in keratinocytes containing melanosome complexes. These results suggest that touki extract disperses melanin and improves pigmentation by promoting cathepsin activity in keratinocytes containing melanosome complexes. Additionally, we found that fennel extract promoted cathepsin more than touki extract.

Chapter 4 investigated how fennel extract affects melanosome complexes degradation in melanosome-containing cultured epidermal keratinocytes. We also investigated the potential of fennel extract to increase intracellular lysosomal digestive enzyme activity via immunostaining and gene expression analysis. Fennel extract reduced the levels of TRP-1, a mature melanosome membrane protein, and increased the expression of cathepsins K and L2. Intracellular cathepsin and TRP-1 colocalization were observed in melanosome-containing keratinocytes. In a three-dimensional human epidermis model, fennel extract increased cathepsin K expression, primarily in the basal and spinous epidermal layers, as well as in the entire stratum granulosum and stratum corneum. Cathepsin L2 expression was increased primarily in the basal and spinous epidermal layers. These results suggest that fennel extract-induced digestive enzymes are involved in melanosome complex membrane protein degradation and keratinization-related melanin dispersion. Moreover, melanosome complexes can be degraded in epidermal keratinocytes by promoting cathepsin K and L2 activities. Thus, fennel may be effective in decreasing senile pigmentation.

These results are expected to be applied to medicated brightening cosmetics in the future, especially to reduce senile lentigo.

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On stimulation of the skin by ultraviolet rays, melanocytes synthesize melanin. Mature melanosomes are then transported to the vicinity of the cell membrane and directly under the cell membrane via long-range bidirectional microtubules and short-range unidirectional actin fibers. Subsequently, melanosomes combine with the cell membrane, are transferred to adjacent keratinocytes, and accumulate in the nucleus of keratinocytes to form a nuclear cap (or melanin cap) that protects keratinocyte DNA from ultraviolet damage. However, during cell proliferation of pigmented cells, there is increased melanin production, increased transfer of melanosomes to keratinocytes, and decreased differentiation potential of keratinocytes. Melanin remains in the epidermis for prolonged periods, leading to pigment spots. Asians are highly concerned about the color of their skin, and most believe that pigmentation affects their appearance. Therefore, skin-brightening agents are highly demanded in Asia. The most influential are Japanese medicinal brightening cosmetics. In Japan, medicated brightening cosmetics (“quasi-drugs”) that inhibit tyrosinase activity, including kojic acid, arbutin. Furthermore, stable vitamin C derivatives such as vitamin C ethyl with antioxidant properties and tranexamic acid with anti-inflammatory properties are being developed. However, agents such as rhododendrol and magnolignan are currently not used because they release hydroxyl radicals from tyrosinase, are highly cytotoxic to melanocytes, and may induce leukoderma. The use of such substances, wherein hydroxyl radicals are generated by tyrosinase to regulate melanin production, poses a risk of vitiligo. Furthermore, they are ineffective in reducing the appearance of senile pigmentation. Therefore, a new brightening mechanism that does not induce leukoplakia needs to be developed. Additionally, there is a growing need for developing skin-brightening ingredients with a mechanism that promotes the degradation of melanosome complexes accumulated in keratinocytes.

This study focused on a new brightening mechanism related to melanosome digestion. First, we investigated the potential of pH-dependent tyrosinase degradation, focusing on the low pH of intra-melanosomal environment-induced suppression of melanogenesis. We then investigated whether the mechanism of anti-melanogenic agent propylparaben is implicated in melanosomal pH regulation at non-cytotoxic concentrations (Chapter 2). Based on the results presented in Chapter 2, we focused on lysosomal cathepsins. We hypothesized that lysosomal functions in highly pigmented keratinocytes could be enhanced because the digestive system promotes the digestion of melanosome complexes and restores decreased cellular proliferation, leading to improved hyperpigmentation. The objective of Chapter 3 was to identify active ingredients from plant extracts for enhancing the proliferation of keratinocytes with melanins, enhancing lysosomal function in the digestion of melanosome complexes, and applying their activity to improve hyperpigmentation (Chapter 3). Chapter 3 found that the *Foeniculum vulgare* Miller fruit extract (fennel extract) had a more substantial effect on cathepsin promotion than the *Angelica acutiloba* root extract (touki extract). Finally, we investigated the degradation of melanosome complexes in cultured human keratinocytes using fennel extract, a natural component with potent antioxidant properties, and we analyzed the mechanism by focusing on the effects of lysosomes (Chapter 4).

Chapter 2 investigated the potential for pH-dependent tyrosinase degradation, focusing on the low pH of intra-melanosomal environment-induced suppression of melanogenesis. We also investigated whether the mechanism of the anti-melanogenic agent propylparaben is implicated in melanosomal pH regulation at non-cytotoxic concentrations. We found that tyrosinase was degraded via acidification of melanosomes, thereby decreasing its activity, and both inhibitors of aspartic protease and cysteine protease decreased tyrosinase degradation. It has been hypothesized that aspartic proteases and cysteine proteases participate in the degradation of tyrosinase in acid melanosomes. Na^+/H^+ exchangers and V-ATPase regulate melanosomal pH. We investigated the mechanisms underlying the inhibitory effect of propylparaben on melanin production in B16 melanoma cells. The mRNA expression levels of tyrosinase and related proteins (TRP-1 and dopachrome tautomerase) were not affected by propylparaben; however, the protein levels in melanosomes decreased. We investigated the mechanisms underlying the inhibitory effect of propylparaben on melanin production in B16 melanoma cells. The effects of propylparaben on the mRNA expression of Na^+/H^+ exchangers, Na^+/Ca^{2+} exchangers, and melanosome pH levels were examined. Propylparaben decreased gene expression in both exchangers, and staining with an intracellular pH indicator confirmed its effect on decreasing melanosomal pH. These results suggest that propylparaben downregulates melanin production via acidification of melanosomes.

Melanosomes are specialized lysosomes. The first protein shared by lysosomes and melanosomes was the lysosomal membrane glycoprotein Lamp-1. Later, Lamp-1 was identified in vesicles implicated in the trafficking of the tyrosinase-related protein family to melanosomes. Melanosomes are the principal repository in melanocytes of lysosomal

hydrolytic enzymes such as β-glucuronidase, β-galactosidase, β-hexosaminidase, cathepsin B, and cathepsin L. In normal keratinocytes, melanosomes incorporated into lysosomes are degraded by lysosomal enzymes when keratinocytes move to the skin surface via turnover, which leads to a lighter skin tone. In contrast, an abnormal accumulation of large melanosome complexes in basal keratinocytes is observed in senile lentigo. Lysosomal cathepsins, enclosed in lysosomes, help maintain homeostasis by participating in the degradation of heterophagic and autophagic materials.

In Chapter 3, based on the results of Chapter 2, we focused on improving pigmentation by activating the lysosomal enzyme cathepsin to digest melanosome complexes, and we also investigated the effects of touki extract. The results confirmed that touki extract promoted the activity of total cathepsin, increased the activity and gene expression level of cathepsin K in cathepsin, and significantly digested melanosomes in keratinocytes containing melanosomes. These results suggest that touki extract is a valuable substance that disperses melanin and improves pigmentation by promoting cathepsin activity in keratinocytes containing melanosomes in spots. We also found that fennel extract had a more substantial effect on the promotion of cathepsin than the touki extract.

Chapter 4 investigated how fennel extract affects melanosome degradation in melanosome-containing cultured epidermal keratinocytes. We also investigated the potential of fennel extract to increase intracellular lysosomal digestive enzyme activity via immunostaining and gene expression analysis. Fennel extract reduced the expression of TRP-1, a mature melanosome membrane protein, and increased cathepsin K and L2 expression. Intracellular cathepsin and TRP-1 colocalization were observed in melanosome-containing keratinocytes. Cathepsin L2 expression levels were lower in the basal layer than in the stratum corneum side of the hyperpigmented region. In addition, melanosome complex degradation was suppressed in cathepsin L2-knockdown cells. In a three-dimensional human epidermis model, fennel extract increased cathepsin K expression, primarily in the basal and spinous epidermal layers and in the entire stratum granulosum and stratum corneum. The expression of cathepsin L2 increased primarily in the basal and spinous epidermal layers. These results suggest that fennel extract-induced digestive enzymes are involved in melanosome complex membrane protein degradation and keratinization-related melanin dispersion. Moreover, melanosome complexes can be degraded in epidermal keratinocytes by promoting cathepsin K and L2 activities. Therefore, fennel may prove effective in fading senile pigmentation.

In summary, we achieved our initial research objectives. We identified a new brightening mechanism in which tyrosinase is degraded by the acidification of melanosomes, thereby decreasing its activity. Based on this result, we investigated the effect of plant extracts by degrading melanosome complexes in keratinocytes to lighten pigment spots and clarified its mechanism. The results indicate that plant extracts such as fennel extract decrease senile pigmentation because they promote the degradation of melanosome complexes from the basal

layer to the stratum corneum of the epidermis by inducing cathepsin K and L2 expression. These results are expected to be applied to medicated brightening cosmetics in the future, especially to reduce senile lentigo.

Summary

Applicant for degree :

(Haifeng Zeng)

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