Effects of Isopropyl Unoprostone on Melanogenesis in Mouse Epidermal Melanocytes

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Purpose: To investigate the effects of isopropyl unoprostone (referred to as unoprostone) on melanin synthesis and the nature of melanin.

Methods: M1 and M2, potential intraocular oxidized metabolites of unoprostone, were added to the culture medium in a cultured immortal line of mouse epidermal melanocytes, melan-a, once a day for 2 weeks at concentrations of 5 nM and 50 nM. Vehicle solution was utilized as a control. The amounts of pyrrole-2,3,5-tricarboxylic acid (PTCA), an oxidized product of eumelanin, and aminohydroxyphenylalanine (AHP), a hydrolyzed product of pheomelanin, were measured by high-performance liquid chromatography.

Results: A 5-nM concentration of M2 significantly increased PTCA production (20.84 ± 3.17 ng/10^6 cells mean ± SD) compared with the control (14.58 ± 4.62 ng/10^6 cells) (P = .04), although other concentrations did not affect the synthesis of PTCA or AHP. A 5-nM concentration of M2 significantly increased the PTCA/AHP (1.86 ± 0.18) ratio compared with the control (1.37 ± 0.41) (P = .04). A 50 nM concentration of M2 slightly increased the PTCA/AHP (1.74 ± 0.58) ratio, but M1 did not affect this ratio.

Conclusions: These results indicate that M2 affects not only melanogenesis but also the nature of melanin, which could result in unoprostone-generated iridial pigmentation.

Key Words: Eumelanin, isopropyl unoprostone, melanocyte, melanogenesis, pheomelanin.

Introduction

The prostaglandin (PG)-related compound, isopropyl unoprostone (referred to as unoprostone), is widely used as an anti-glaucoma ophthalmic solution because of its efficacy in reducing intraocular pressure (IOP) and its few adverse effects.1–3 One of the major adverse effects of unoprostone ophthalmic solution is iridial pigmentation. Yamamoto and Kitazawa4 have reported that unoprostone induces iridial pigmentation, and we sometimes observe patients with iridial pigmentation due to unoprostone use in daily clinics. Bee and associates,5 however, have reported that unoprostone does not induce iridial pigmentation in monkey eyes based on the results of a long-term in vivo trial.

Although latanoprost has been reported to induce iridial pigmentation by increasing the number of melanin granules rather than by the proliferation of iris melanocytes, the mechanism of unoprostone-induced iridial pigmentation is largely unknown.

The melanogenic pathway has been investigated, and it has been revealed that melanin granules are divided into two subtypes, eumelanin and pheomelanin. Eumelanin is a dark-brown to black pigment, and...
pheomelanin is a yellow to reddish-brown pigment. The nature of these two melanins determines the color of the skin, hair, and even the iris. Prota and associates have reported that iris pigments contain pheomelanin and eumelanin, and that the nature of these two melanins is variable depending on iris color.

In the current study, we investigated the effects of unoprostone on the formation of pheomelanin and eumelanin in mouse epidermal melanocytes by high-performance liquid chromatography (HPLC).

Materials and Methods

All procedures were carried out in compliance with the ARVO Statement for the Care and Use of Animals in Ophthalmic and Vision Research.

Preparation of Melanocytes and Chemicals

Melan-a, an immortal line of mouse melanoblasts, was donated by Dr. DC Bennet. Unoprostone, 13, 14-dihydro-15-keto-20-ethyl-PGF2α-isopropyl ester and oxidized metabolites of unoprostone, referred to as M1 and M2, were provided by Ueno Fine Chemical Industry, Ltd (Osaka). M1 and M2 dissolved in ethanol were diluted in culture medium to prepare the final concentrations, and their vehicle solutions ethanol were diluted in culture medium to prepare the basal control. M2, 0.001% ethanol solution, was added to the culture medium once a day for 2 weeks as a basal control.

Culture Conditions

As previously described, melan-a was cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Rockville, MD, USA) with 10% fetal bovine serum (Life Technologies), 4 mM L-glutamine (Sigma-Aldrich Japan, Tokyo), 100 units/mL penicillin (Sigma-Aldrich), and 100 μg/mL streptomycin (Sigma-Aldrich) at 37°C in humidified air with 5% CO2.

PG-exposure to Melanocytes

Confluent melan-a cells were passaged with a split rate of 1 to 4 on 6-well culture plate at a cell density of 5 x 10⁴ cells/well. M1 and M2 were added to the culture medium once a day for 2 weeks at concentrations of 5 nM and 50 nM. The vehicle for the 50 nM of M1 and M2, 0.001% ethanol solution, was added to the culture medium once a day for 2 weeks as a basal control.

Determination of Melanin Content

As described previously, HPLC was employed for the quantitative analysis of melanin content, pyrrole-2,3,5-tricarboxylic acid (PTCA), an oxidized product of eumelanin, and aminohydroxyphenylalanine (AHP), a hydrolyzed product of pheomelanin.

HPLC Settings

A liquid chromatograph was employed with detectors, an ultraviolet detector (269 nm, Intelligent UV/VS 875-UV; JASCO, Tokyo), or an electrochemical detector (400 mV, 840-EC, JASCO), for the determination of PTCA or AHP, respectively. The columns used were a 18°C 7-μm column (Finepak Sil, 25 cm × 4.6 mm, JASCO) for PTCA and a 15°C 10-μm column (Catechol Pak ODS-T, 25 cm × 4 mm) for AHP. Maintaining temperatures for PTCA or AHP measurement were at 40°C or 30°C, respectively. The mobile phases were (a) 0.1 M potassium phosphate buffer, pH 2.1: methanol, 94:6 (v/v) for PTCA; and (b) 0.1 M sodium octanesulfonate and 0.1 mM Na2EDTA: methanol, 93:3 (v/v) for AHP. The flow rate was 0.7 mL/min.

Sample Preparation for PTCA Analysis

A sample was homogenized in 1 mL of 1 M H2SO4 and transferred to a tube. The tube was vigorously mixed, and 3% KMnO4 was added to 20-μL portions while mixing. Each addition was made immediately after the KMnO4 color had disappeared. At 10 minutes after the addition, the residual KMnO4 and MnO2 were decomposed by the addition of 100 μL of 10% Na2SO3. The resulting solution was extracted twice with peroxide-free ether. The combined ether extract was evaporated in a concentrator, and the residue was dissolved in 200 μL of water and then centrifuged. A 10-μL aliquot was injected into the chromatograph for the analysis of PTCA.

Sample Preparation for AHP Analysis

The number of samples for each hydrolysis was the same as that for the permanganate oxidation. A sample was taken in a tube, and 20 μL of 50% H3PO4 and 500 μL of 57% hydriodic acid were added. The mixture was heated at 130°C in an oil bath for 16 hours. After cooling, the hydrolysate was transferred in the concentrator at 60°C. Traps containing NaOH pellets were set between the concentrator and the pump. The residue was dissolved in 990 μL of 0.4 M HClO4 and centrifuged, and a 10-μL aliquot was usually injected into the chromatograph for the analysis of AHP.

Results

Production of Melanin Derivative

Figure 1 shows that 5 nM M2 increased the production of PTCA by 43.0% compared with the control (P = .04). However, no other conditions significantly affected the production of PTCA or AHP.
compared with controls. A 50 nM concentration of M2 tended to reduce the production of PTCA and AHP compared with 5 nM M2.

**Effects on Nature of Melanin**

Both 5 nM and 50 nM concentrations of M2 increased the ratios of PTCA over AHP by 35.8% and 27.7%, respectively (Figure 2). The increase in PTCA over AHP ratio of 5 nM M2 was significantly higher than that of the control \( (P = .04) \). M1 did not affect the ratio compared with the control.

**Discussion**

Although some clinical studies have been reported regarding unoprostone-generated iridal pig-

**Figure 1.** Production of melanin derivative. Effects of unoprostone on pyrrole-2,3,5-tricarboxylic acid (PTCA) (A) and aminohydroxyphenylalanine (AHP) (B) are depicted. Bar = standard error. \( * P = .04 \) vs. control, Mann-Whitney U-test \( (n = 5) \). PG: prostaglandin.

**Figure 2.** Effects on nature of melanin. Y-axis expresses pyrrole-2,3,5-tricarboxylic acid (PTCA)/aminohydroxyphenylalanine (AHP) ratio. Bar = standard error. \( * P = .04 \) vs. control, Mann-Whitney U-test \( (n = 5) \). PG: prostaglandin.
and α-oxidation in the eye; we therefore concluded that the main metabolites involved in actions in the eye are M1 and M2, especially M2, because of their intraocular pharmacokinetics. According to this study, maximum concentrations of M1 and M2 in the iris tissue were approximately 10 nM and 20 nM, respectively. Thus we employed 5 nM and 50 nM as exposed concentrations of M1 and M2 solutions. We have revealed that M2 induces much more endogenous PGE₂ than M1 (in submission), and Nordlund and associates have reported PGE₂-inducing melanogenesis. These results may explain in part why M2 affected the nature of melanin and increased the PTCA production in the current study.

The current results indicate that 5 nM M2 induces a greater production of PTCA and AHP than 50 nM M2. Although we did not obtain any obvious evidence that the effect of these metabolites on melanocyte proliferation are different (unpublished data), a higher concentration of M2 may be toxic to melanin production and/or melanocyte proliferation.

The melanogenic pathway has been investigated and several important steps have been clarified in the last decade. Tyrosinase is a key enzyme that induces melanin in the melanogenic pathway. Iridial pigmentation by latanoprost has been investigated, and latanoprost has been reported to induce iridial pigmentation due to an increase in tyrosinase activity. We revealed that M1, M2, and acid of latanoprost, an active intraocular form of latanoprost, enhanced melanogenesis by increasing tyrosinase activity without altering the proliferation of melan-a and that M1, M2, and acid of latanoprost did not produce a significant change in total melanin synthesis (in submission). In addition, unoprostone metabolites may affect tyrosinase activity. It is not fully understood how the switch from pheomelanogenesis to eumelanogenesis is generated. The eumelanin pathway is favored in the absence of thiol compounds such as cysteine. It has been reported that the switch from pheomelanogenesis to eumelanogenesis might be regulated by the availability of thiol compounds. Unoprostone metabolites may therefore convert pheomelanogenesis to eumelanogenesis by influencing the regulation of thiol compounds.

Although the current study revealed that unoprostone metabolites affect melanogenesis and the nature of melanin, further investigation is required for the following reasons. We employed mouse epidermal melanocytes in the current study to obtain enough cells to generate the HPLC study. Mouse epidermal melanocytes, however, can show different reactions to PG administration than iris melanocytes. Therefore, we cannot simply apply these results to a case of iridial hyperpigmentation. The current study is an in vitro study using purely isolated melanocytes, and it is not unusual for results obtained by in vitro studies to be inconsistent with those obtained by in vivo studies. We therefore must carry out an in vivo study to clarify the mechanisms of iridial pigmentation in response to unoprostone.

It is not clear whether iridial hyperpigmentation in response to unoprostone is critical. This side effect may simply be a cosmetic effect. However, an investigation of its mechanism may lead to new research frontiers and may ultimately result in therapeutic applications.

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References


