Nle^{4}DPhe^{7}α-Melanocyte–Stimulating Hormone Increases the Eumelanin:Phaeomelanin Ratio in Cultured Human Melanocytes

Gillian Hunt, Sylvia Kyne, Kazumasa Wakamatsu,* Shosuke Ito,* and Anthony J. Thody

Department of Dermatology, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom; and *Fujita Health University School of Health Sciences, Toyoake, Aichi, Japan

In mammals, melanin exists in two chemically distinct forms: the red-yellow phaeomelanin and the brown-black eumelanin. Although administration of the pigmentary hormone α-melanocyte–stimulating hormone (αMSH) and its synthetic analogue Nle^{4}DPhe^{7}αMSH induces skin darkening in man, the increases in melanogenesis in cultured human melanocytes in response to these peptides are relatively small. However, it is possible that MSH affects the eumelanin:phaeomelanin ratio rather than total cellular melanin. Thus, this study examined the specific effects of Nle^{4}DPhe^{7}αMSH on the two melanins in cultured human melanocytes, quantifying eumelanin and phaeomelanin by high performance liquid chromatography. Nle^{4}DPhe^{7}αMSH induced significant increases in the eumelanin content of these cells while having lesser and varied effects on the levels of phaeomelanin. As a consequence, the eumelanin: phaeomelanin ratio was increased in every culture. These results demonstrate that Nle^{4}DPhe^{7}αMSH affects melanin type in human melanocytes and suggest a possible mechanism by which this peptide induces skin darkening in man. ***Key words: melanogenesis/melanins/skin pigmentation. J Invest Dermatol 104:83–85, 1995***

Skin pigmentation is the result of melanin synthesis in the epidermal melanocytes and protects against ultraviolet radiation (UVR)-induced damage. There are two chemically distinct types of melanin: the red-yellow phaeomelanin and the brown-black eumelanin [1]. Both types of melanin are present in human epidermis [2]. Tyrosinase (EC 1.14.18.1) catalyzes the conversion of tyrosine to dopaquinone, which then undergoes a series of oxidation reactions resulting in the formation of eumelanin. However, in the presence of sulphydryl compounds, dopaquinone also can act as a precursor for phaeomelanin. The relative amounts of eumelanin and phaeomelanin in the epidermis may determine human skin color.

The darkening effects of the pigmentary hormone α-melanocyte–stimulating hormone (αMSH) and its synthetic analogue Nle^{4}DPhe^{7}αMSH on murine coat color are associated with increased tyrosinase activity and increases in eumelanin relative to phaeomelanin in the hair follicular melanocytes [3,4]. Although administration of αMSH and Nle^{4}DPhe^{7}αMSH to human subjects results in skin darkening [5,6], and cultured human melanocytes have MSH receptors [7,8], there are several reports that human melanocytes are relatively refractory to the effects of MSH peptides *in vitro* [9]. This apparent unresponsiveness may be related to the use of cholera toxin and phorbol esters as melanocyte mitogens; Hunt *et al* [9] demonstrated that αMSH and Nle^{4}DPhe^{7}αMSH increase melanogenesis and stimulate tyrosinase activity in human melanocytes cultured in the absence of these mitogens. These findings have been generally confirmed by Abdel Malek *et al* (personal communication). However, the increases in melanogenesis in cultured human melanocytes in response to MSH peptides are relatively small, and it is possible that the peptides influence the eumelanin:phaeomelanin ratio, as in murine hair follicular melanocytes, rather than increase total cellular melanin. Therefore, this study examined the specific effects of Nle^{4}DPhe^{7}αMSH on eumelanin and phaeomelanin levels in cultured human melanocytes.

MATERIALS AND METHODS

Culture of Human Melanocytes Human melanocytes were isolated from the foreskins of six individuals (age range 8 months to 13 years). Cultures were maintained in MCDB 153 (Sigma, St. Louis, MO) adjusted to 1 mM Ca^{++} and 0.3 mM tyrosine and supplemented with bovine hypothalamic extract, various growth factors, and amino acids but without cholera toxin and phorbol esters, as described previously [9]. Melanocytes from three Caucasians and three non-Caucasians (one individual of mixed Middle Eastern/Caucasian background and two Asians) were used in this study to provide maximal variation in basal eumelanin and phaeomelanin levels.

Effect of Nle^{4}DPhe^{7}αMSH αMSH and Nle^{4}DPhe^{7}αMSH have been shown to have similar dose-related effects on the melanin content of human melanocytes [9] but, because of its increased stability, the latter peptide was used in this study. Human melanocytes that had been maintained *in vitro* for 7–10 d were cultured in the presence of 10^{-8} M Nle^{4}DPhe^{7}αMSH, the concentration that has maximal effect on melanogenesis [9], for a further 7 d. Fresh medium and peptide were added every 2–3 d. Control melanocytes from the same donor were maintained in parallel in the absence of peptide for 7 d.

Manuscript received June 14, 1994; revised August 27, 1994; accepted for publication September 15, 1994.

This work was presented in part at the British Society for Investigative Dermatology Meeting, Bath, September 16–17, 1994. Abstract: *Br J Dermatol* 131 (in press).

Reprint requests to: Dr. Gillian Hunt, University Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, United Kingdom.

Abbreviations: αMSH, α-melanocyte–stimulating hormone; TRP, tyrosinase-related protein.

0022-202X/95/$09.50 • SSDI0022-202X(94)00254-5 •

Measurement of Eumelanin and Phaeomelanin Eumelanin and phaeomelanin were measured in aliquots of greater than 10^6 melanocytes, as described previously [10]. Eumelanin was oxidized to pyrrole 2,3,5-tricarboxylic acid, phaeomelanin was hydrolyzed to aminohydroxyphenylalanine, and both products were quantified by high performance liquid chromatography. One nanogram of pyrrole 2,3,5-tricarboxylic acid and 1 ng of aminohydroxyphenylalanine correspond to 50 ng eumelanin and 5 ng phaeomelanin, respectively.

RESULTS

Eumelanin and phaeomelanin were present in all melanocyte cultures. Although there was considerable variation in the levels of each (eumelanin range 0.04–2 $\mu g/10^6$ melanocytes and phaeomelanin range 0.2–10.3 $\mu g/10^6$ melanocytes), phaeomelanin was more abundant than eumelanin in every culture. The cultures from the non-Caucasians contained the most eumelanin, whereas the melanocytes from the two Asians had the highest levels of phaeomelanin **(Fig 1*A*)**. However, the eumelanin:phaeomelanin ratio was unrelated to the racial origin of the melanocytes **(Fig 1*B*)**.

The effects of Nle^{4}DPhe^7αMSH on subsequent eumelanin levels are shown in **Fig 1*A***. Eumelanin content ranged from 0.2 to 7 $\mu g/10^6$ melanocytes, a statistically significant increase in eumelanin levels; $p < 0.02$ by Wilcoxon signed-ranks test. However, the degree of response was variable **(Table I)**. Culture 4, from the mixed-race individual, showed only a 9% increase in eumelanin and was deemed to be unresponsive. Responsiveness was unrelated to both the basal level of eumelanin and the initial eumelanin: phaeomelanin ratio.

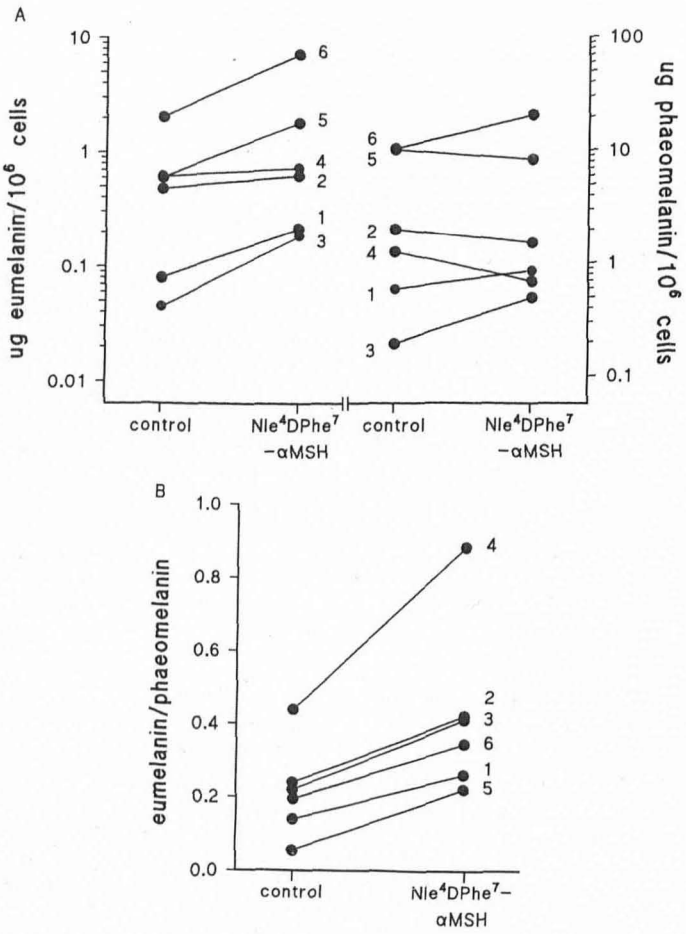

Figure 1. Effect of Nle^{4}DPhe^7αMSH on eumelanin and phaeomelanin content and the eumelanin:phaeomelanin ratio in cultured human melanocytes. Cells were cultured in the presence of 10^{-8} M Nle^{4}DPhe^7αMSH for 7 d; control cells were maintained in parallel in the absence of peptide. Eumelanin and phaeomelanin were quantified by high performance liquid chromatography. Cultures 1, 2, and 3 were from Caucasians, culture 4 from a mixed-race (Middle East/Caucasian) individual, and cultures 5 and 6 from Asians. *A*) Eumelanin and phaeomelanin; *B*) eumelanin:phaeomelanin ratio.

Table I. Changes in Eumelanin and Phaeomelanin Levels and the Eumelanin:Phaeomelanin Ratio in Cultured Human Melanocytes After Exposure to Nle^{4}DPhe^7αMSH[a]

Culture	Race	Eumelanin	Phaeomelanin	Eumelanin: Phaeomelanin
1	Caucasian	+168	+44	+86
2	Caucasian	+31	−24	+75
3	Caucasian	+376	+157	+86
4	Mixed	+9	−45	+100
5	Asian	+227	−19	+340
6	Asian	+257	+98	+77

[a] Human melanocytes were cultured in the presence of 10^{-8} M Nle^{4}DPhe^7αMSH for 7 d, and eumelanin and phaeomelanin were quantified by high-performance liquid chromatography. Changes are expressed in percent relative to untreated (control) cells from each culture.

The effect of Nle^{4}DPhe^7αMSH on phaeomelanin was less clear. Although the levels of this melanin after MSH treatment ranged from 0.5 to 20.5 $\mu g/10^6$ melanocytes, only half of the cultures showed increases; the remaining cultures showed decreases **(Fig 1*A*, Table I)**. Again, there was no relation between basal levels of phaeomelanin or the initial eumelanin:phaeomelanin ratio and the degree of responsiveness. However, those cultures that showed decreases in phaeomelanin had the smallest increases in eumelanin, whereas those cultures in which phaeomelanin was increased also had the largest increases in eumelanin **(Table I)**; $p < 0.05$ by Spearman rank test.

The most consistent effect of Nle^{4}DPhe^7αMSH was on the eumelanin:phaeomelanin ratio, which was increased in every culture **(Fig 1*B*)**; $p < 0.01$ by paired t test. In the majority of cultures, the increase in this ratio was between 75% and 100%. However, culture 5 demonstrated a 340% increase **(Table I)**.

DISCUSSION

The recent cloning and characterization of the human and murine melanocortin receptors [11,12] have renewed interest in the role of pro-opiomelanocortin peptides as pigmentary hormones. Alleles at the extension locus, which encodes the MSH receptor, are associated with distinctive mammalian coat colors [13]; thus the functioning of the MSH receptor-ligand unit appears to be an important determinant of the type of melanin produced [14]. However, the present results demonstrate that melanin type in human epidermal melanocytes is also regulated by MSH.

Eumelanin and phaeomelanin have been measured in cultured human melanocytes on one previous occasion [15]. However, these melanocytes had been cultured in the presence of artificial mitogens, which increase eumelanogenesis in murine melanoma cells [16]. This justifies our use of a culture system free of these additives.

It is widely accepted that increased tyrosinase activity is associated with increased melanogenesis. Nle^{4}DPhe^7αMSH stimulates tyrosinase activity in human melanocytes, with maximal increase at 10^{-8} M [9], the concentration at which eumelanogenesis was increased in the present study. In addition, tyrosinase mRNA levels are increased by the peptide [17].† However, tyrosinase-related proteins (TRPs) also may be involved in regulating melanin type in human melanocytes. Although the exact role of TRP-1 in melanogenesis is unclear, there is evidence for its involvement in eumelanogenesis in cultured human pigment cells, as TRP-1 mRNA is detectable only in those cells producing eumelanin [15]

† Donatien PD, Lunec J, Thody AJ: αMSH increases tyrosinase messenger RNA in normal human melanocytes (abstr.). *Pigment Cell Res* 6:287, 1993.

and Nle^{4}DPhe^{7}αMSH increases TRP-1 mRNA levels in the majority of human melanocyte cultures [17]. Although MSH also increases the expression of TRP-2 (dopachrome tautomerase) in human melanocytes (Abdel Malek *et al*, personal communication), the role of this protein in regulating melanin type in these cells has not been determined.

At present, it is unknown whether the effects of Nle^{4}DPhe^{7}αMSH described in this study result from changes in the synthesis or breakdown of the melanins. The former is perhaps the more probable. However, the effect of the peptide on phaeomelanin levels is less than that on eumelanin content. Similar results are seen in murine melanoma cells, in which αMSH and Nle^{4}DPhe^{7}αMSH induce dose-related increases in eumelanogenesis but have relatively little effect on phaeomelanogenesis [16] (G. Hunt *et al*, unpublished observations). It is not clear why phaeomelanin is increased in some melanocyte cultures and decreased in others, but possible explanations include availability and utilization of sulphydryl compounds, which combine with dopaquinone in phaeomelanogenesis, and the relative activities of the enzymes involved in the synthesis of the two melanins.

In our previous studies on the effects of Nle^{4}DPhe^{7}αMSH on melanogenesis in cultured human melanocytes [9,17], a third of all cultures were unresponsive to the peptide. In the present study, although eumelanogenesis was unchanged in one culture, none of the cultures were totally unresponsive to the peptide. However, the relatively small responses in cultures 2 and 4 may not have been detected if total melanin had been measured spectrophotometrically, leading to the conclusion that these cultures were unresponsive.

It has been suggested that Nle^{4}DPhe^{7}αMSH and other synthetic analogues of MSH may have applications as artificial tanning agents and could protect those individuals who tan poorly against the damaging effects of UVR [18]. In addition to cosmetic effects, changes in the relative proportions of the two melanins may be important in determining susceptibility to UVR-induced skin damage, as phaeomelanin, in contrast to eumelanin, produces free radicals when irradiated [19].

MSH itself has been largely dismissed as having physiologic significance in human skin pigmentation because plasma levels are usually low. However, αMSH-like peptides are present in the skin [20], possibly originating in the keratinocytes [21], and these locally produced pro-opiomelanocortin peptides may have a role in regulating skin physiology [22]. UVR stimulates the production of αMSH by human keratinocytes [21], increases the number of MSH receptors on murine melanoma cells [23], and stimulates the binding of Nle^{4}DPhe^{7}αMSH to human melanocytes [24]. In addition, MSH receptors recently have been identified in cultured human keratinocytes [25]. These receptors are similar to those in murine melanoma cells and are also up-regulated by exposure to UVR and MSH [25]. Thus, it has been proposed that MSH and its receptor are functional in mediating the effects of UVR in the skin and regulate the epidermal-melanin unit [26]. The data presented here indicate a possible role for MSH in regulating melanin type in human melanocytes.

We thank the Medical Research Council (UK) for financial support, Professor M.E. Hadley, University of Arizona, for providing Nle^{4}DPhe^{7}αMSH, and Carole Todd for her excellent technical assistance.

REFERENCES

1. Ito S: High-performance liquid chromatography (HPLC) analysis of eu- and phaeomelanin in melanogenesis control. *J Invest Dermatol* 100:166S–171S, 1993
2. Thody AJ, Higgins EM, Wakamatsu K, Ito S, Burchill SA, Marks JM: Phaeomelanin as well as eumelanin is present in human epidermis. *J Invest Dermatol* 97:340–344, 1991
3. Burchill SA, Thody AJ, Ito S: Melanocyte stimulating hormone, tyrosinase activity and regulation of eumelanogenesis and phaeomelanogenesis in the hair follicular melanocytes of the mouse. *J Endocrinol* 109:15–21, 1986
4. Levine N, Lemus-Wilson A, Wood SH, Abdel Malek ZA, Al-Obeidi F, Hruby VJ, Hadley ME: Stimulation of follicular melanogenesis in the mouse by topical and injected melanotropins. *J Invest Dermatol* 89:269–273, 1987
5. Lerner AB, McGuire JS: Effect of alpha- and beta-melanocyte stimulating hormone on the skin colour of man. *Nature* 189:176–179, 1961
6. Levine N, Sheftel SN, Eytan T, Dorr RT, Hadley ME, Weinrach JC, Ertl GA, Toth K, McGee DL, Hruby VJ: Induction of skin tanning by subcutaneous administration of a potent synthetic analogue. *JAMA* 226:2730–2736, 1991
7. Donatien PD, Hunt G, Pieron C, Lunec J, Taieb A, Thody AJ: The expression of functional MSH receptors on cultured human melanocytes. *Arch Dermatol Res* 284:424–426, 1992
8. De Luca M, Siegrist W, Bondanza S, Mathor M, Cancedda R, Eberle AN: α-melanocyte stimulating hormone (αMSH) stimulates normal melanocyte growth by binding to high affinity receptors. *J Cell Sci* 105:1079–1084, 1993
9. Hunt G, Todd C, Cresswell JE, Thody AJ: αMelanocyte stimulating hormone and its analogue Nle^{4}DPhe^{7}αMSH affect morphology, tyrosinase activity and melanogenesis in cultured human melanocytes. *J Cell Sci* 107:205–211, 1994
10. Ito S, Fujita K: Microanalysis of eumelanin and phaeomelanin in hair and melanomas by chemical degradation and liquid chromatography. *Anal Biochem* 144:527–536, 1985
11. Chhajlani V, Wikberg JES: Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. *FEBS Lett* 309:417–420, 1992
12. Mountjoy KG, Robbins LS, Mortrud M, Cone RD: The cloning of a family of genes that encode the melanocortin receptors. *Science* 257:1248–1251, 1992
13. Cone RD, Mountjoy KG: Molecular genetics of the ACTH and melanocyte stimulating hormone receptors. *Trends Endocrinol Metab* 4:242–247, 1993
14. Jackson IJ: Colour-coded switches. *Nature* 362:587–588, 1993
15. Del Marmol V, Ito S, Jackson I, Vachtenheim J, Berr P, Ghanem G, Morandini R, Wakamatsu K, Henz G: TRP-1 expression correlates with eumelanogenesis in human pigment cells in culture. *FEBS Lett* 327:307–310, 1993
16. Kuzumaki T, Matsuda A, Wakamatsu K, Ito S, Ishikawa K: Eumelanin synthesis is regulated by co-ordinate expression of tyrosinase and tyrosinase-related proteins. *Exp Cell Res* 207:33–40, 1993
17. Hunt G, Donatien PD, Lunec J, Todd C, Kyne S, Thody AJ: Cultured human melanocytes respond to MSH peptides and ACTH. *Pigment Cell Res* 7 (in press)
18. Hadley ME, Sharma SD, Hruby VJ, Levine N, Dorr RT: Melanotropic peptides for therapeutic and cosmetic tanning of the skin. In: Vaudry H, Eberle AN (eds.). *The Melanotropic Pepides. Ann NY Acad Sci* 680:424–439, 1993
19. Persad S, Menon IA, Haberman HF: Comparison of the effects of UV-visible irradiation of melanins and melanin-hemato-porphyria complexes from human black and red hair. *Photochem Photobiol* 37:63–68, 1983
20. Thody AJ, Ridley K, Penny RJ, Chalmers R, Fisher C, Shuster S: MSH peptides are present in mammalian skin. *Peptides* 4:813–816, 1983
21. Schauer E, Trautlinger F, Kock A, Schwarz A, Bhardwaj R, Simon M, Ansel JC, Schwarz T, Luger TA: Proopiomelanocortin-derived peptides are synthesized and released by human keratinocytes. *J Clin Invest* 93:2258–2262, 1994
22. Slominski A, Paus R, Wortsman J: On the potential role of proopiomelanocortin in skin physiology and pathology. *Mol Cell Endocrinol* 93:C1–C6, 1993
23. Bolognia J, Murray M, Pawelek J: UVB-induced melanogenesis may be mediated through the MSH-receptor system. *J Invest Dermatol* 92:651–656, 1989
24. Thody AJ, Hunt G, Donatien PD, Todd C: Human melanocytes express functional melanocyte-stimulating hormone receptors. In: Vaudry H, Eberle AN (eds.). *The Melanotropic Peptides. Ann NY Acad Sci* 680:381–390, 1993
25. Chakraborty A, Pawelek J: MSH receptors in immortalised human epidermal keratinocytes: a potential mechanism for coordinate regulation of the epidermal-melanin unit. *J Cell Physiol* 157:344–350, 1993
26. Pawelek JM, Chakraborty AK, Osber MP, Orlow SJ, Min KK, Rosenzweig KE, Bolognia JL: Molecular cascades in UV-induced melanogenesis: a central role for melanotropins? *Pigment Cell Res* 5:348–356, 1992