

# Ultraviolet B Irradiation of the Eye Activates a Nitric Oxide-dependent Hypothalamopituitary Proopiomelanocortin Pathway and Modulates Functions of $\alpha$ -Melanocyte-stimulating Hormone-responsive Cells

Keiichi Hiramoto, Nobuyo Yanagihara, Eisuke F. Sato, and Masayasu Inoue

Department of Biochemistry and Molecular Pathology, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno, Osaka 545-8585, Japan

**Ultraviolet B radiation increases DOPA-positive melanocytes in the skin specifically at the site of exposure. We found unexpectedly that ultraviolet B irradiation of the eye increased the concentration of  $\alpha$ -melanocyte-stimulating hormone in plasma and systemically stimulated epidermal melanocytes in mice. To test the possible involvement of hypothalamopituitary proopiomelanocortin system in the systemic activation of skin melanocytes, ultraviolet B was also irradiated to the eye after hypophysectomy. Hypophysectomy strongly inhibited the ultraviolet B-induced stimulation of melanocytes. To elucidate the pathway by which ultraviolet B irradiation of the eye activated the hypothalamopituitary system, we examined the effect of bilateral ciliary ganglionectomy and denervation of the optic nerves on the ultraviolet B-induced melanocyte stimulation. Ciliary ganglionectomy, but not optic nerve denervation, strongly inhibited melanocyte stimulation by localized irradiation of the eye. Furthermore, melanocyte stimu-**

**lation by localized ultraviolet B irradiation of the eye was not observed in mice that lack the inducible type of nitric oxide synthase. These results clearly indicate that a signal evoked by ultraviolet B irradiation of the eye is transmitted in a nitric oxide-dependent manner through the ciliary ganglia involving the first branch of the trigeminal nerve to the hypothalamopituitary proopiomelanocortin system, resulting in upregulation of  $\alpha$ -melanocyte-stimulating hormone secretion and consequent stimulation of melanocytes in the skin. The novel network involving the trigeminal nerve and nitric oxide-dependent signaling pathway might play important parts in the activation of proopiomelanocortin-dependent biologic reactions, such as  $\alpha$ -melanocyte-stimulating hormone-induced stimulation of melanocytes in the skin, in ultraviolet B-enriched environments. Key words: melanocytes/nitric oxide/proopiomelanocortin/signaling/ultraviolet B. *J Invest Dermatol* 120:123–127, 2003**

**U**ltraviolet (UV) B irradiation induces oxidative stress and inflammation that increase the mutation rate of DNA and promote the aging of the skin. The reaction of melanocytes to such radiation is therefore of critical importance for the protection of animals from UVB toxicity. It is well known that UVB stimulates epidermal melanocytes to proliferate and promote the synthesis of DOPA-containing pigments at the site of irradiation (Jimbow and Uesugi, 1982; Rorsman and Tegner, 1988; Cario-Andre *et al*, 1999). We unexpectedly observed, however, that localized UVB irradiation of the eye resulted in strong stimulation of epidermal melanocytes, as manifested by increased proliferation and synthesis of DOPA-containing pigments. It has been well documented that  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) derived from proopiomelanocortin is secreted from the pituitary gland to stimulate MSH-receptor containing cells, such as dermal

melanocytes (Eberle, 1988; Catania and Lipton, 1993). To elucidate the mechanism by which UVB irradiation of the eye strongly activates epidermal melanocytes, we investigated the possible involvement of cerebral proopiomelanocortin by examining the effect of UVB in hypophysectomized animals.

We also investigated the signaling pathway responsible for the stimulation of epidermal melanocytes by UVB irradiation of the eye. Inflammatory cytokines have been known to increase the synthesis of  $\alpha$ -MSH by activating the proopiomelanocortin system in the fornix (Lipton, 1989; Lipton and Catania, 1997). Because UVB elicits inflammation at the site of irradiation, we hypothesized that inflammatory mediators, including nitric oxide (NO), may underlie the signaling mechanism responsible for the stimulation of epidermal melanocytes.

We demonstrate here for the first time that localized UVB irradiation of the eye strongly activates the hypothalamopituitary proopiomelanocortin system through an inducible nitric oxide synthase (iNOS)-dependent neuronal network that involves the first branch of the trigeminal, thereby upregulating  $\alpha$ -MSH secretion that stimulates  $\alpha$ -MSH-receptor containing cells, such as melanocytes in the skin. The physiologic significance of the NO-dependent neuronal network that activates hypothalamopituitary proopiomelanocortin system in response to UVB irradiation of the eye is discussed with respect to the

Manuscript received May 6, 2002; revised August 12, 2002; accepted for publication September 21, 2002

Reprint requests to: Masayasu Inoue, Department of Biochemistry and Molecular Pathology, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno, Osaka 545-8585, Japan. Email: inoue@med.osaka-cu.ac.jp

Abbreviations:  $\alpha$ -MSH,  $\alpha$ -melanocyte-stimulating hormone; iNOS, inducible nitric oxide synthase.

biologic defense mechanism of animals living in a UV-enriched environment.

## MATERIALS AND METHODS

**Animal experiments** Specific pathogen-free, 8 wk old male DBA/2 Crj mice (Charles River Japan, Yokohama, Japan), intact or hypophysectomized C57/B6J mice (SLC, Hamamatsu, Japan), and iNOS-knockout (iNOS<sup>-/-</sup>) C57/B6J mice (Jackson Laboratories, Bar Harbor, ME) were subjected to experiments according to the animal care regulations of Osaka City University Medical School. Under light ether anesthesia, the eye, ear, or dorsal skin was locally exposed to UVB (wavelength of 280–320 nm) after covering the remaining body surface with aluminum foil at a dose of 2.5 kJ per m<sup>2</sup> using a 20SE sunlamp (Toshiba Co., Tokyo, Japan). The dose of UVB irradiated was confirmed by an UV radiometer (Topcon Co., Tokyo, Japan). Localized irradiation of the eye without affecting the surrounding tissues was ensured by topical application of 0.1 ml of 10% 2-ethylhexyl-p-methoxycinnamate, a potent UV absorbent, to the periorbital skin 10 min before irradiation; the absorbent completely prevented UVB-induced injury of the periorbital skin as judged on the basis of the failure to detect cells with the typical characteristics of sunburn. In control experiments, the eye or the ear was irradiated with visible light (400–700 nm).

**Ciliary ganglionectomy and optic nerve denervation** In some experiments, ciliary ganglionectomy was performed bilaterally as described previously (Jacob and Berg, 1987). Mice were subjected to pentobarbital anesthesia, and a small incision was made along the caudal portion of the lower lid of the eye. The posterior portion of the eyeball was carefully exposed under a stereomicroscope, and the ciliary ganglia were bilaterally crushed several times with fine forceps. Bilateral denervation of the optic nerves was performed as described previously by clamping the nerves for 3 min with a jeweler's forceps at the exit from the globe (Meyer *et al*, 1994). Histologic examination revealed that the surgical treatment induced a marked atrophy of the retina, indicative of effective denervation. Animals were subjected to experiments 10 d after the surgery.

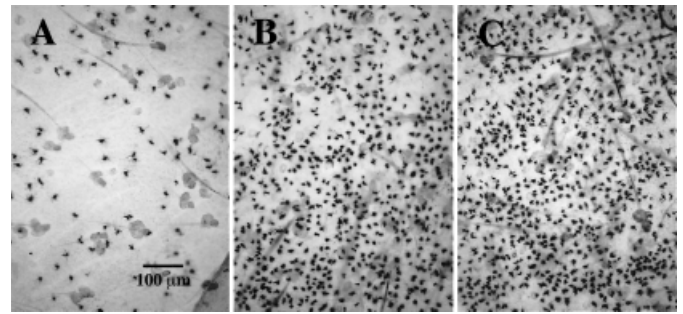
**Preparation and staining of epidermal sheets** Five days after irradiation of mice, skin samples (0.5 × 0.5 cm) were obtained from the ear or dorsal skin and incubated for 2 h at 37°C in 2 M NaBr. The epidermis was then separated from the dermis to obtain epidermal sheets. DOPA-positive melanocytes in the epidermal sheets were stained as described (Jimbow and Uesugi, 1982). After washing of the epidermal sheets with phosphate-buffered saline, they were incubated at 37°C in phosphate-buffered saline containing 0.1% L-DOPA (Sigma, St Louis, MO). The sheets were then washed in phosphate-buffered saline, fixed overnight at 25°C in 10% formalin, dehydrated, and stained for DOPA-positive melanocytes.

**Assay of  $\alpha$ -MSH in plasma** The blood samples were collected from the conjunctiva using capillary phenomenon at 24 h after the final UVB irradiation. The plasma concentration of  $\alpha$ -MSH was determined as described (Surendran *et al*, 1995) with an LC-6A high-performance liquid chromatography system (Shimadzu Co., Kyoto, Japan) equipped with a C18-ODS column (Shiseido Co., Tokyo, Japan). Chromatography was performed with 0.1 M sodium phosphate buffer (pH 2.5) containing 20% acetonitrile and 0.2% triethylamine as the mobile phase at a flow rate of 1 ml per min.

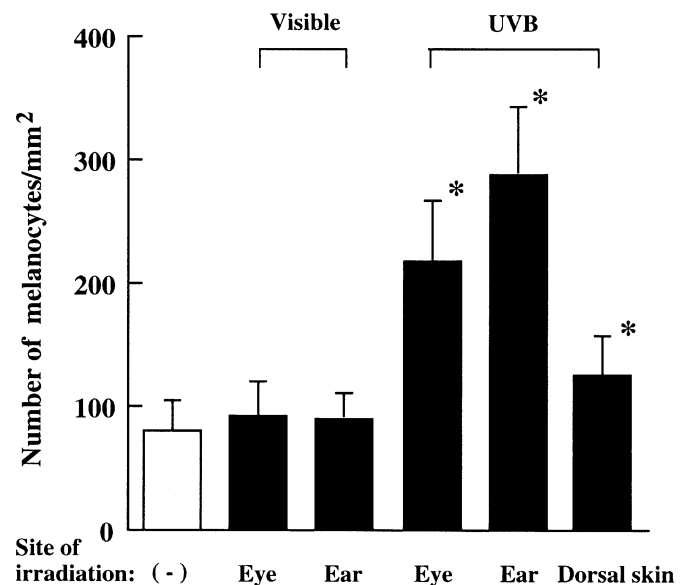
**Statistical analysis** Data are expressed as mean  $\pm$  SD derived from six to nine animals. Data were subjected to analysis of variance with the use of STAT-VIEW 512 software (SAS Campus Drive, Cary, NC).  $p < 0.05$  was considered statistically significant.

## RESULTS

**Effect of localized UVB irradiation on dermal melanocytes** Figures 1 and 2 show that localized UVB irradiation of either the eye or the ear significantly increased the number of DOPA-positive melanocytes in epidermal sheets derived from the ear. The number of such cells in the ear was not affected by exposure of animals to visible light and was increased only slightly in mice whose dorsal skin had been exposed to UVB (Fig 2). Thus, localized UVB irradiation of the



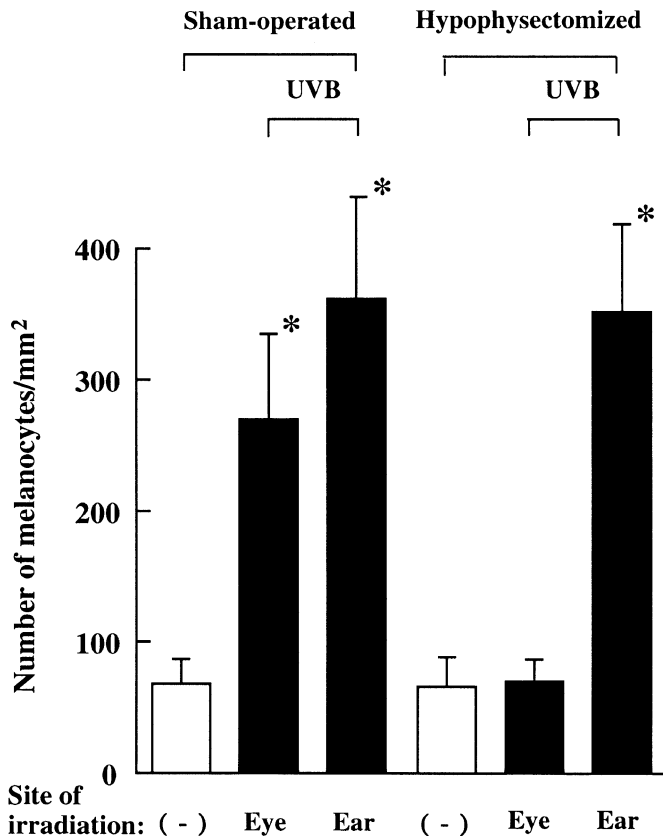
**Figure 1.** Effect of UVB irradiation on epidermal melanocytes in the skin. Five days after bilateral UVB irradiation (2.5 kJ per m<sup>2</sup>) of the eye (B) or the ear (C) of DBA/2Crj mice, epidermal sheets of the ear were prepared and stained by the split-DOPA technique. (A) Nonirradiated control ear. Data show one typical experiment from six animals. Scale bar = 100  $\mu$ m.



**Figure 2.** Effect of irradiation on the proliferation of epidermal melanocytes. Five days after exposure of the eye, ear, or dorsal skin of DBA/2Crj mice to either UVB or visible light, the number of DOPA-positive melanocytes in epidermal sheets prepared from the ear was determined. Values are mean  $\pm$  SD derived from six animals. Other conditions were the same as in Fig 1. Open column (-), nonirradiated control ear. \* $p < 0.05$ .

eye stimulated epidermal melanocytes in the ear to a similar extent as did direct irradiation of the skin.

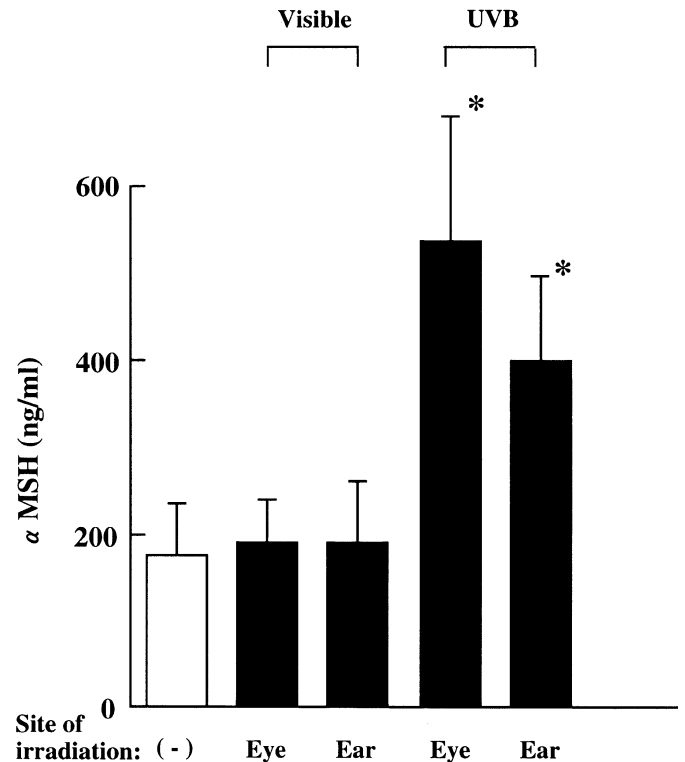
**Effect of hypophysectomy on melanocyte stimulation by UVB**  $\alpha$ -MSH derived from proopiomelanocortin is secreted from the pituitary gland to stimulate epidermal melanocytes (Eberle, 1988; Catania and Lipton, 1993). To investigate the mechanism by which localized UVB irradiation of the eye stimulated epidermal melanocytes in the ear, we examined the effect of such treatment in hypophysectomized animals (Moncada *et al*, 1991). Although epidermal melanocytes in the ear of hypophysectomized mice responded normally to localized UVB irradiation of this organ, irradiation of the eye failed to activate melanocytes in the ear (Fig 3). The epidermal melanocytes in sham-operated animals responded normally to UVB irradiation of the eye and the ear. These observations suggest that, whereas UVB irradiation of the skin results in direct activation of melanocytes *in situ*, the signal evoked by localized irradiation of the eye stimulates epidermal melanocytes through activation of the hypothalamopituitary pathway.



**Figure 3. Effect of hypophysectomy on the activation of melanocytes by UVB.** Ten days after hypophysectomy or sham surgery, C57/B6J mice were subjected to UVB irradiation of the eye or ear. The number of DOPA-positive melanocytes in the ear was determined 5 d after the irradiation. Values are mean  $\pm$  SD derived from six animals. Other conditions were the same as in Fig 1. Open columns (-), nonirradiated control ear. \* $p < 0.05$ .

**Effect of UVB irradiation on plasma levels of  $\alpha$ -MSH** To confirm the possibility that the signal evoked by UVB irradiation of the eye stimulates epidermal melanocytes through activation of the hypothalamopituitary pathway, we measured the concentration of  $\alpha$ -MSH in plasma before and after exposure of the eye and ear to either UVB or visible light (Fig 4). As expected, localized UVB irradiation of the eye induced a significant increase in the concentration of  $\alpha$ -MSH in plasma. The UVB-induced increase in plasma  $\alpha$ -MSH was greater in animals locally UVB irradiated to the eye than to the ear. Thus, the signal evoked by UVB irradiation of the eye is likely transmitted to the hypothalamopituitary proopiomelanocortin system, thereby enhancing the secretion of  $\alpha$ -MSH to stimulate epidermal melanocytes.

**Effect of optic nerve denervation and colliery ganglionectomy** Given that the penetrability of UVB in biologic tissues is relatively low (Wei *et al*, 1995), it likely affects preferentially cells present in the superficial layers of the eye, such as those in the cornea and iris but not in the retina. To delineate the pathway by which UVB irradiation of the eye activates the hypothalamopituitary proopiomelanocortin system, we examined the effect of such treatment in animals that had been subjected to either ciliary ganglionectomy or denervation of the optic nerves (Jacob and Berg, 1987; MacMicking *et al*, 1995). Optic nerve denervation had no appreciable effect on the melanocyte response to UVB irradiation of the eye or the ear (Fig 5). In contrast, ciliary ganglionectomy prevented the stimulation of epidermal melanocytes by UVB irradiation of the eye, without affecting the cellular response to the irradiation of



**Figure 4. Effect of irradiation on the plasma concentration of  $\alpha$ -MSH.** Five days after irradiation of the eye or the ear of DBA/2Crj mice with either UVB or visible light, the plasma concentration of  $\alpha$ -MSH was determined. Values are mean  $\pm$  SD derived from six animals. Other conditions were the same as in Fig 1. Open column (-), nonirradiated control ear. \* $p < 0.05$ .

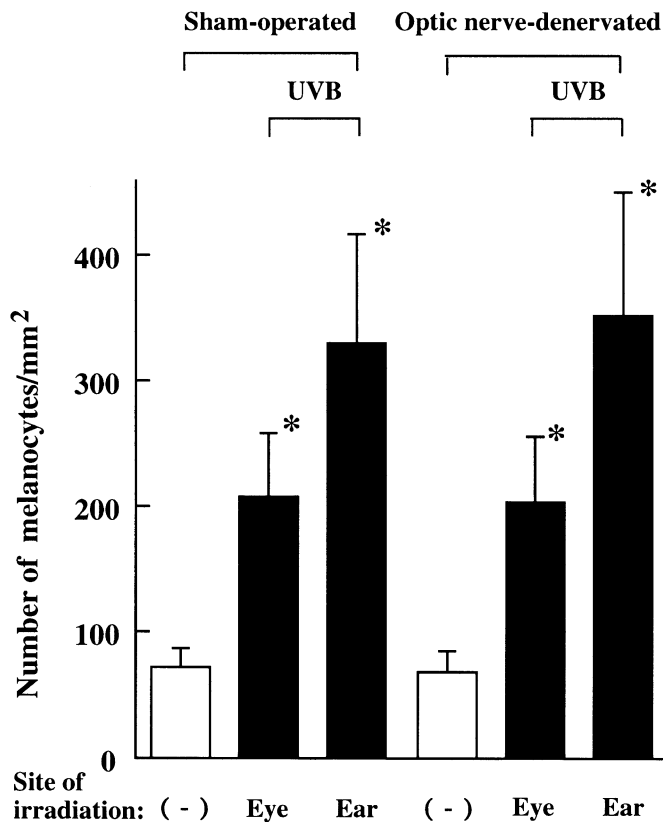
the ear (Fig 6). These observations indicate that the signal evoked by UVB irradiation of the eye is transmitted to the hypothalamopituitary system through a neuronal network that includes the first branch of the trigeminal nerves rather than through the optic nerves.

**Effect of UVB irradiation on epidermal melanocytes in  $iNOS^{-/-}$  mice** Hazardous stimuli, including inflammatory cytokines, upregulate the expression of iNOS, and NO produced by the enzyme modulates inflammatory reactions (Iturriza and Eberle, 1989; Zuclich, 1989; Surendran *et al*, 1995). To investigate whether NO contributes to the signaling responsible for the stimulation of epidermal melanocytes by UVB irradiation of the eye, we examined the effect of such treatment in  $iNOS^{-/-}$  mice. UVB irradiation of the eye of these animals failed to activate epidermal melanocytes, whereas the local response of melanocytes to the irradiation of the ear occurred normally (Fig 7). Thus, NO produced by iNOS appears to be required for the mechanism by which the UVB-induced signal in and around the eye is transmitted to the hypothalamopituitary proopiomelanocortin system.

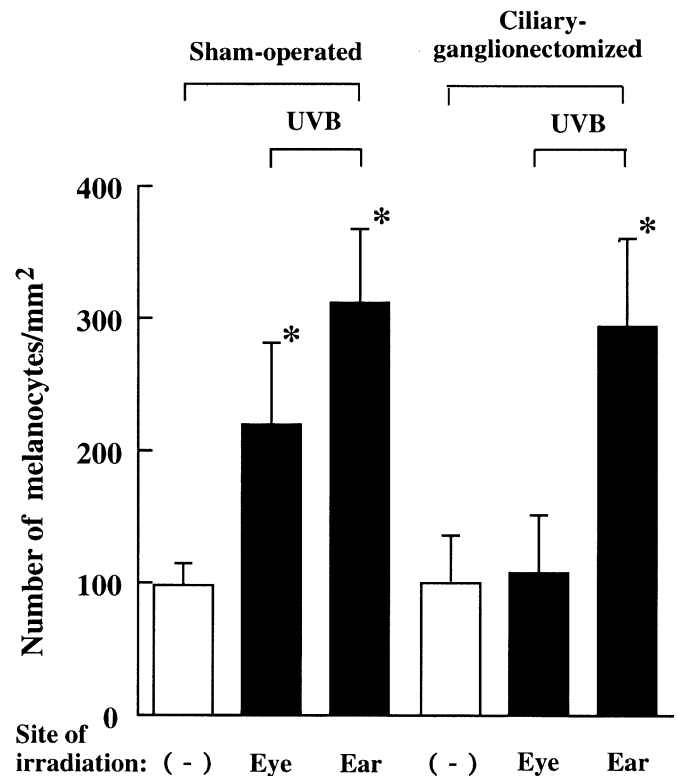
## DISCUSSION

This study demonstrates that UVB irradiation of the eye activates the hypothalamopituitary proopiomelanocortin system in an iNOS-dependent manner via a neuronal network that includes the first branch of the trigeminal nerves passing through the ciliary ganglia, thereby resulting in the stimulation of  $\alpha$ -MSH-sensitive melanocytes in the nonirradiated skin.

Because the penetrability of UVB in biologic tissues is relatively low, only cells present in the superficial layers of the eye,



**Figure 5. Effect of optic nerve denervation on the activation of melanocytes by UVB.** Ten days after denervation of the optic nerves, DBA/2 Crj mice were subjected to UVB irradiation of the eye or the ear. Five days after irradiation, the number of DOPA-positive melanocytes in the ear was determined. Values are mean  $\pm$  SD derived from six animals. Other conditions were the same as in Fig 1. Open columns (-), nonirradiated control ear. \* $p < 0.05$ .



**Figure 6. Effect of ciliary ganglionectomy on the activation of melanocytes by UVB.** Ten days after ciliary ganglionectomy, DBA/2 Crj mice were subjected to UVB irradiation of the eye or the ear. Five days after irradiation, the number of DOPA-positive melanocytes in the ear was determined. Values are mean  $\pm$  SD derived from six animals. Other conditions were the same as in Fig 1. Open columns (-), nonirradiated control ear. \* $p < 0.05$ .

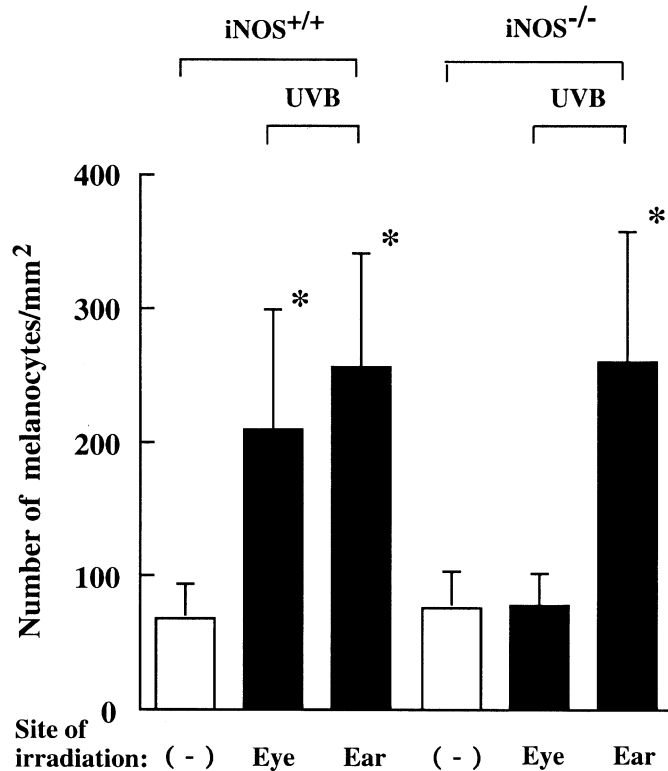
such as those in the cornea and iris but not those in the retina, would have been affected by localized UVB irradiation. In fact, histologic examination and western blotting analysis revealed that UVB irradiation of the eye elicited inflammatory reactions in the cornea and iris, and strongly upregulated the cellular expression of iNOS, heat shock protein (HSP)-70, HSP-90, and substance P, a mediator of a sense of pain, in both tissues (data not shown). Thus, the inflammatory reaction in and around the cornea and/or the iris elicited by UVB irradiation seems to underlie the mechanism for the signaling of cellular reaction to the hypothalamus, a major center for stress response. Consistent with this hypothesis, inflammatory cytokines have been known to increase the synthesis of  $\alpha$ -MSH by activating the proopiomelanocortin system in the fornix (Lipton, 1989; Lipton and Catania, 1997). It has been known, however, that various types of heat shock proteins, such as HSP-70 and HSP-90, are frequently upregulated in inflammatory tissues by nonspecific hazardous stimuli. Thus, critical roles of the proteins upregulated in the cornea and iris in the UVB-induced signaling should be studied further.

It should be noted that NO generated by iNOS is a potent modulator of inflammatory reactions (Iturriza and Eberle, 1989; Zuclich, 1989; Surendran *et al*, 1995). This study also demonstrates that UVB irradiation of the eye, but not of the ear, failed to activate epidermal melanocytes in iNOS<sup>-/-</sup> mice. Preliminary experiments using NOC12, a potent NO donor, revealed that instillation of the agent also stimulated the proliferation of epidermal melanocytes of nonirradiated mice, although the response was fairly small as compared with that induced by UVB. These observations are consistent with the hypothesis that the UVB-induced inflammatory reactions in the cornea and/or the iris underlie the mechanism for the NO-dependent signaling of cellular

response to activate the hypothalamopituitary proopiomelanocortin system, thereby enhancing the secretion of  $\alpha$ -MSH to stimulate  $\alpha$ -MSH-receptor responsive cells, such as melanocytes in the skin. Preliminary experiments revealed that UVB irradiation of the eye increased plasma concentration of  $\alpha$ -MSH in normal mice but not in iNOS<sup>-/-</sup> mice. Because UVB irradiation of the ear stimulated melanocytes both in normal and iNOS<sup>-/-</sup> mice, NO may not play a direct part in the mechanism of cellular activation within the skin. The molecular mechanism for the iNOS-dependent signaling from the UVB-irradiated eye to the hypothalamopituitary proopiomelanocortin system should be studied further.

Apart from the molecular mechanism for the signaling, this study clearly shows that localized UVB irradiation of the eye activates the hypothalamopituitary proopiomelanocortin system via the iNOS-dependent neuronal network involving the first branch of the trigeminal nerves passing through the ciliary ganglia; therefore  $\alpha$ -MSH-receptor containing cells are stimulated, such as DOPA-positive melanocytes in the skin. Given the high mutagenic activity of UVB, stimulation of the proliferation of, and of the synthesis of melanin pigments by epidermal melanocytes might play important parts in the protection of the underlying cells against UVB toxicity (Luger *et al*, 1998). Thus, the signaling pathway activated by UVB irradiation of the eye to increase plasma levels of  $\alpha$ -MSH might function as ultra-sensitive mechanism by which UVB toxicity could be minimized in animals living in UV-enriched environment.

It should be noted, however, that epidermal melanocytes were activated by the localized irradiation of the eye even in the absence of UVB toxicity in the nonirradiated skin. Thus, the stimulation of epidermal melanocytes by UVB irradiation of the eye may reflect a systemic response of various cells to  $\alpha$ -MSH, rather



**Figure 7. Effect of UVB irradiation on epidermal melanocytes in iNOS-knockout mice.** C57/B6J mice deficient in iNOS (iNOS<sup>-/-</sup>) and their wild-type (iNOS<sup>+/+</sup>) littermates were subjected to UVB irradiation of the eye or the ear. Five days after irradiation, the number of DOPA-positive melanocytes in the ear was determined. Values are mean  $\pm$  SD derived from six animals. Other conditions were the same as in Fig 1. Open columns (-), nonirradiated control ear. \* $p < 0.05$ .

than reflecting a protective role of the signaling pathway against DNA damage in the skin. Consistent with this hypothesis, the receptor for  $\alpha$ -MSH has been known to occur in a wide variety of cells, such as leukocytes (i.e., monocytes, macrophages, lymphocytes, and neutrophils), keratinocytes, adipocytes, endothelial cells, and melanocytes (Luger *et al*, 1998). This fact suggests that  $\alpha$ -MSH is a multifunctional hormone that regulates a wide variety of biologic events, including inflammatory reactions. In fact, in addition to stimulate melanogenesis,  $\alpha$ -MSH has been known to exhibit both immunostimulatory (at its low levels) and immuno-

suppressive activities (at its high concentrations) (Luger *et al*, 1998). Therefore, the signaling pathway activated by UVB irradiation of the eye to stimulate the hypothalamopituitary proopiomelanocortin system might play an important part in the modulation of a wide variety of cells expressing receptors for  $\alpha$ -MSH and related hormones. Possible roles of the NO-dependent novel pathway involving the trigeminal nerves and hypothalamopituitary proopiomelanocortin system in the survival of animals in UV-enriched environments, such as those in the Antarctic and surrounding regions, should be studied further.

## REFERENCES

- Cario-Andre M, Bessou S, Gontier E, Maresca V, Picardo M, Taieb A: The reconstructed epidermis with melanocytes. a new tool to study pigmentation and photoprotection. *Cell Mol Biol (Noisy-le-Grand)* 45:931-942, 1999
- Catania A, Lipton JM: Alpha-melanocyte stimulating hormone in the modulation of host reactions. *Endocr Rev* 14:564-576, 1993
- Eberle AN: The melanotropins. In: Eberle AN, (ed). *Chemistry, Physiology and Mechanisms of Action*. Basel: Karger, 1988: pp 173-208
- Iturriza FC, Eberle AN: Secretion of melanocyte-stimulating hormone and adrenocorticotropin from transplanted pituitary pars intermedia in stressed and non-stressed rats. *Neuroendocrinology* 49:610-616, 1989
- Jacob MH, Berg DK: Effects of preganglionic denervation and postganglionic axotomy on acetylcholine receptors in the chick ciliary ganglion. *J Cell Biol* 105:1847-1854, 1987
- Jimbow K, Uesugi T: New melanogenesis and photobiological processes in activation and proliferation of precursor melanocytes after UV-exposure: ultrastructural differentiation of precursor melanocytes from Langerhans cells. *J Invest Dermatol* 78:108-115, 1982
- Lipton JM: Neuroimmune networks. In: Goetzl EJ, Spector NH, (eds). *Physiology and Diseases*. New York: Alan R. Liss, 1989, pp 243-250
- Lipton JM, Catania A: Anti-inflammatory actions of the neuroimmunomodulator  $\alpha$ -MSH. *Immunol Today* 18:140-145, 1997
- Luger TA, Scholzen T, Brzoska T, Becher E, Slominski A, Paus R: Cutaneous immunomodulation and coordination of skin stress responses by  $\alpha$ -melanocyte-stimulating hormone. *Ann NY Acad Sci* 840:381394-381092, 1998
- MacMicking JD, Nathan C, Hom G, *et al*: Altered response to bacterial infection and endotoxin shock in mice lacking inducible nitric oxide synthase. *Cell* 81: 641-650, 1995
- Meyer RL, Miotke JA, Benowitz LI: Injury induced expression of growth-associated protein-43 in adult mouse retinal ganglion cells in vitro. *Neuroscience* 63:591-602, 1994
- Moncada S, Palmer RM, Higgs EA: Nitric oxide physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109-142, 1991
- Rorsman H, Tegner E: Biochemical observations in UV-induced pigmentation. *Photodermatology* 5:30-38, 1988
- Surendran N, Ugwu SO, Sterling EJ, Blanchard J: High-performance liquid chromatographic assay for melanoton-1 ([Nle<sup>4</sup>-Dphe<sup>7</sup>] $\alpha$ -melanocyte-stimulating hormone) in biological matrices. *J Chromatogr B* 670:235-242, 1995
- Wei XQ, Charles IG, Smith A, *et al*: Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 375:408-411, 1995
- Zulich JA: Ultraviolet-induced photochemical damage in ocular tissues. *Health Phys* 56:671-682, 1989