

Ultraviolet B Radiation Acts through the Nitric Oxide and cGMP Signal Transduction Pathway to Stimulate Melanogenesis in Human Melanocytes*

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Ultraviolet B (UVB) radiation is the main physiological stimulus for human skin pigmentation; however, the molecular mechanisms underlying this process are still unclear. Recently, nitric oxide (NO) and cGMP have been involved in mediation of skin erythema induced by UVB. Therefore, we investigated the role of NO and cGMP in UVB-induced melanogenesis. In this study, we demonstrated that UVB stimulation of melanogenesis was mimicked by exogenous NO donors. Additionally, we showed that NO stimulated cGMP synthesis and that cGMP was also a potent stimulator of melanogenesis. Furthermore, the inhibition of the melanogenic effect of NO by guanylate cyclase inhibitor demonstrated that NO mediated its effect through the activation of guanylyl cyclase. Interestingly, 1 min after UVB irradiation, we observed a significant increase in cGMP content in melanocytes. The effects of UVB on cGMP production and on melanogenesis were blocked by both guanylate cyclase and NO synthase inhibitors. Additionally, inhibition of cGMP-dependent kinase also prevented the stimulation of melanogenesis by UVB and NO. Therefore, we concluded that NO and cGMP production is required for UVB-induced melanogenesis and that cGMP mediated its melanogenic effects mainly through the activation of cGMP-dependent kinase.

Epidermal melanin is responsible for skin darkening and is synthesized by melanocytes as the result of a cascade of enzymatic reactions. Tyrosinase, which converts tyrosine to dopaquinone, is the rate-limiting enzyme involved in melanin synthesis and represents the major regulatory step in melanogenesis. Numerous stimuli are able to alter melanogenesis of cultured pigmented cells; vitamin D metabolites (1), retinoids (2, 3), melanocyte-stimulating hormone (4–6), forskolin, cholera toxin, isobutylmethylxanthine (7, 8), diacylglycerol analogs (9, 10), and UV irradiation (11–14). Until now the molecular mechanism involved in the induction of melanogenesis by these agents was not fully elucidated. A role for the cAMP pathway has been proposed on the basis that adenylate cyclase activators such as melanocyte-stimulating hormone, forskolin, and cholera toxin or phosphodiesterase inhibitors (e.g. isobutylmethylxanthine) can stimulate melanogenesis (6). Addition-

ally, the protein kinase C (PKC)¹ pathway was thought to be involved in the regulation of melanogenesis. Indeed, 1-oleyl-2-acetyl-glycerol, a PKC activator, stimulates melanogenesis (9, 10, 15), and a direct correlation between the level of PKC activity in melanocytes and the activity of tyrosinase has been shown (16). However, induction of melanogenesis by UVB or by 1-oleyl-2-acetyl-glycerol is unaffected by RO 31-8220, a PKC inhibitor (17), leading to the conclusion that protein kinase C does not play a pivotal role in the control of melanogenesis. In humans, only UVB radiation represents an established physiological stimulus of melanogenesis, and despite many efforts to identify the molecular events triggered by UVB radiation, the mechanisms underlying UVB-induced melanogenesis remain to be elucidated. Neither cAMP nor PKC pathways appear to be involved in this process.

In addition, UVB radiation is also known to cause erythema by increasing blood flow in the skin microcirculation. Recently, this inflammatory process was shown to be blocked by NO synthase inhibitors, indicating that UVB radiation induces erythema through the release of NO (18). NO is a free radical gas synthesized during the conversion of L-arginine into L-citrulline by NO synthase and is considered a major intracellular and intercellular messenger molecule (19). Generally, NO elicits its effects through the activation of a soluble guanylate cyclase, leading to an increase in intracellular cGMP content. This pleiotropic, bioregulatory agent displays multiple physiological functions. It is, for example, the messenger of macrophage-dependent cell-mediated cytotoxicity, and it functions as an intercellular messenger in neuronal signaling. It is also involved in vasodilation, platelet aggregation, and the inflammatory process (19).

The presence of NO in skin and its role in UVB-induced erythema prompted us to investigate the role of NO in UVB-induced melanogenesis in cultured human melanocytes. In the present study we showed that chemical NO donors mimic the melanogenic effects of UVB by increasing tyrosinase activity and melanin synthesis. A guanylate cyclase inhibitor blocked the effect of NO donors on cGMP production and on melanogenesis, indicating that NO elicits its melanogenic effects through the cGMP pathway. Interestingly, UVB radiation caused a marked increase in melanocyte cGMP content, and this effect was blocked by a NO synthase inhibitor. Moreover,

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¹ The abbreviations used are: PKC, protein kinase C; UVB, ultraviolet radiation B; NO, nitric oxide; PKG, cGMP-activated protein kinase; L-NMA, N-monomethyl-L-arginine; SNP, sodium nitroprusside; SNAP, S-nitroso-N-acetylpenicillamine; NOR-4, 3-[(±)(E)-ethyl-2'-(E)-hydroxyimino]-5-nitro-3-hexenecabonylpyridine; LY 83583, 6-anilino-5,8-quinolinedione; DEA-NO, diethylamine NONOate; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazole,1-oxyl,3-oxide; 8-br-cGMP, 8-bromo-cGMP; DOPA, 3,4-dihydroxyphenylalanine.

TABLE I
Effect of NO donors on melanogenesis of normal human melanocytes

Melanocytes were incubated for 4 days in the presence of 200 μM SNP, 200 μM SNAP, 100 μM NOR-4, and 50 μM DEA-NO. Media and treatments were changed every day. On the third day, [^3H]tyrosine and [^{14}C]DOPA were added, and then tyrosinase activity and [^{14}C]DOPA incorporation were determined. The control condition consisted of 24-h-old solutions of NO donors. At the end of each treatment, melanocyte viability was assessed by the trypan blue exclusion method; for each NO donor, less than a 10% cytotoxic effect was observed.

	SNP	SNAP	NOR-4	DEA-NO
Tyrosinase activity ^a	285 \pm 21.8	260 \pm 15.2	197 \pm 8.5	245 \pm 5.85
[^{14}C]DOPA incorporation into melanins ^b	193 \pm 12.3	180 \pm 4.1	181 \pm 5	180 \pm 4.8

^a Data are expressed as percentages of control performed as described under "Experimental Procedures." Values are averages of triplicates \pm S.E. of one representative experiment. Basal activity of tyrosinase: 23 \pm 0.96 microunits/mg protein.

^b Data are expressed as percentages of control performed as described under "Experimental Procedures." Values are averages of triplicates \pm S.E. of one representative experiment. Basal incorporation of [^{14}C]DOPA: 281 \pm 24.5 nmol/mg protein.

the melanogenic effect of UVB was also prevented by NO synthase, guanylate cyclase, and cGMP-dependent protein kinase (PKG) inhibitors, demonstrating that the NO-cGMP pathway, through the activation of PKG, is involved in UVB-induced melanogenesis.

EXPERIMENTAL PROCEDURES

Chemicals

L-NMA, SNAP, and carboxy-PTIO were from Affiniti (Nottingham, United Kingdom). Ly 83583, KT 5823, and NOR-4 were from Calbiochem. DEA-NO was from Cayman chemicals (Ann Arbor, Mi). Other chemicals were obtained from Sigma. The cGMP enzyme immunoassay system was from Amersham Corp. All other products used for cell culture and biochemical analysis were obtained as previously reported (14).

Cell Culture

Human melanocyte cultures were initiated from neonatal foreskins of Caucasoid individuals and grown according to a modified method of Eisinger and Marko (20) as described previously (14). Two days before experiments, complete melanocyte growth medium was removed and replaced by minimum growth medium consisting of MCDB 153 containing 2% fetal calf serum and 30 $\mu\text{g}/\text{ml}$ bovine pituitary extract.

NO Donor Treatment

Preliminary experiments were undertaken to define the concentrations of NO donors allowing the best melanogenic response with less than 10% of cell toxicity. Thus, melanocytes were treated for 4 days in the presence of 200 μM SNP, 200 μM SNAP, 100 μM NOR-4 or 50 μM DEA-NO. Twenty-four-hour-old solutions of NO donors (which only contain the inactive byproducts) were used as controls. In addition, we evaluated the amount of NO released by these compounds after 24 h of incubation. This measurement was realized using a nitrite/nitrate photometric assay kit (Cayman Chemicals). Hence, 200 μM SNP, 200 μM SNAP, 100 μM NOR-4, and 50 μM DEA-NO produced, respectively, 158 \pm 8, 150 \pm 2, 65 \pm 5, and 38 \pm 2 μM .

Ultraviolet Irradiation

The source of ultraviolet radiation was a Vilber Lourmat (Marne la Vallée, France) stimulator fitted out with a UVB irradiation source composed of Vilber Lourmat T-20.M-312 (no UVA or UVC emission) mercury vapor, low pressure, hot cathode tubes and a Vilber Lourmat RMX-365/312 radiometer with microprocessor-programmable energy (mJ/cm^2) and a basis enabling six irradiation measurements/s for controlling the energy received by the sample. Melanocytes were irradiated daily for 4 days with 100 mJ/cm^2 UVB (14).

Melanogenic Activity Assays

Tyrosinase Activity in Living Cells (Tyrosinase Activity in Situ)—The early rate-limiting step of the biosynthetic pathway of melanin (hydroxylation of tyrosinase) was estimated during the last day of treatment from the amount of $^3\text{H}_2\text{O}$ released into the medium during the conversion of L-[ring-3',5'- ^3H]tyrosine to dihydroxyphenylalanine according to an adaptation (21) of the methods of Pomerantz (22) and Oikawa *et al.* (23) as described previously (14).

Determination of Melanin Neosynthesis—The distal step of the melanin synthesis pathway was measured by the rate of incorporation of L-[3- ^{14}C]3,4-dihydroxyphenylalanine into newly synthesized melanins (24) during the last 24 h of melanocyte treatment as described previously (14). This assay measures the complete reaction sequence of melanin biosynthesis and reflects the melanogenic activity of tyrosinase,

tyrosinase-related proteins I and II, and inhibitory factors involved in this process.

Cyclic GMP Determination

One million melanocytes were used for each determination. Following stimulation, cells were frozen on dry ice, and cGMP was extracted from the melanocytes with ice-cold 65% ethanol. After centrifugation, the extracts were dried, and the samples were kept at -20°C until use. Concentrations of cGMP were determined by enzyme-linked immunosorbent assay after acetylation of the samples according to the manufacturer's instructions (Amersham).

RESULTS

Effects of NO Donors on Human Melanocyte Melanization—To evaluate the effect of NO on melanogenesis, normal human melanocytes were incubated in the presence of different chemical NO donors. Melanocytes were treated for 4 days in the presence of SNP (readily released), DEA-NO ($t_{1/2} = 2$ min), NOR-4 ($t_{1/2} = 1$ h), and SNAP ($t_{1/2} = 4.6$ h), and then tyrosinase activity and melanin neosynthesis were measured (Table I). Twenty-four-hour-old solutions of NO donors (which only contain the inactive byproducts) were used as controls. All these compounds stimulated tyrosinase activity to the same extent, between 2- and 3-fold. They also increased [^{14}C]DOPA incorporation into trichloroacetic acid-precipitable materials. Thus, NO appears to be a potent activator of melanogenesis. In the following experiments, we indifferently used SNP and SNAP as NO donors.

Cyclic GMP Mediates the Effects of NO on Melanogenesis—Then, we wanted to define the second messenger involved in the melanogenic response evoked by NO in melanocytes. In various systems, NO principally acts by stimulating soluble guanylate cyclase, resulting in increased cGMP levels in target cells. Thus, we analyzed whether NO donors could modify the cGMP content in melanocytes (Fig. 1). We observed a dramatic increase (50-fold) in cGMP content after a 10-min exposure to 200 μM SNP. This effect was reversed when melanocytes were pretreated during 30 min with 20 μM PTIO, which is a NO scavenger, or with 10 μM LY 83583, a specific inhibitor of guanylate cyclase. These results indicate that NO activates guanylate cyclase in melanocytes.

Since NO increases cGMP in melanocytes, we investigated the effects of cGMP on melanization. human melanocytes were exposed to 8-br-cGMP, a permeable and nonhydrolyzable analog of cGMP (Fig. 2). Addition of 5 and 10 mM 8-br-cGMP in culture medium of melanocytes for 4 days caused 2- and 5-fold increases, respectively, in tyrosinase activity. Moreover, melanin neosynthesis was also increased by 8-br-cGMP (1.8-fold with 5 mM and 4-fold with 10 mM), indicating that cGMP is able to mimic the melanogenic effects of NO.

To confirm the role of cGMP in the mediation of NO-stimulated melanogenesis, we incubated melanocytes in the presence of 200 μM SNAP together with 10 μM LY 83583 or 500 nM KT 5823, an inhibitor of PKG, and then the melanogenic activity was measured (Fig. 3). NO-induced stimulation of tyrosinase

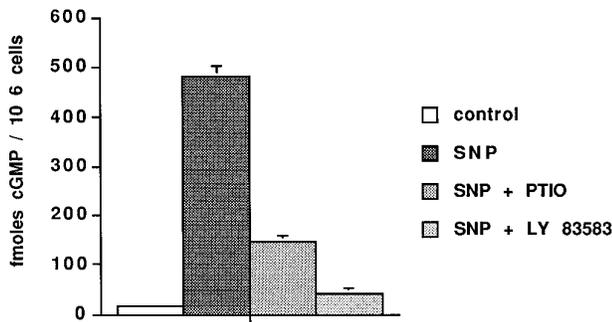


FIG. 1. **Production of cGMP by human melanocytes in response to SNP.** Melanocytes were exposed for 10 min to 200 μ M SNP. When indicated, cells were preincubated for 30 min with 20 μ M PTIO or 10 μ M Ly 83583 before addition of SNP. Each value represents the mean \pm S.E. (bars) of triplicates of one representative experiment.

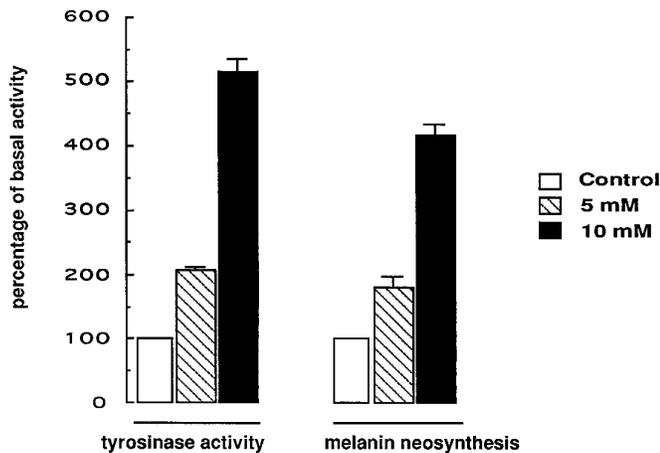


FIG. 2. **Effect of 8-br-cGMP on tyrosinase activity and melanin synthesis of melanocytes.** Melanocytes were exposed to 8-br-cGMP for 4 days, and the tyrosinase activity and melanin synthesis were measured. Results were normalized to cell number and are expressed as percentages of basal activity from nontreated melanocytes. Each value represents the mean \pm S.E. (bars) of triplicates of one representative experiment.

activity (Fig. 3A) and melanin biosynthesis (Fig. 3B) was markedly inhibited by LY 83583 and KT 5823. These results indicate that cGMP, probably through the activation of PKG, is involved in the transmission of the melanogenic effects of NO.

UVB-induced Melanogenesis Is Mediated by NO and cGMP—To assess whether the NO-cGMP pathway is involved in the stimulation of melanogenesis by UVB, we performed cGMP measurement at different times following UVB irradiation (Fig. 4). As soon as 30 s after a 100 mJ/cm² UVB irradiation, we observed a 3-fold increase in intracellular cGMP concentration, which reached about 330 fmol/1 million cells at 3 min after irradiation. This up-regulation was transient, since 10 min after the irradiation, cGMP levels came back to basal (data not shown). As expected, preincubation of melanocytes with Ly 83583 prevented the UVB-induced cGMP accumulation. Furthermore, L-NMA, a structural analog of L-arginine that inhibits NO synthase activity, prevented the accumulation of cGMP observed following UVB irradiation, suggesting that the effect of UVB on cGMP is mediated by a stimulation of NO production.

To demonstrate the role of NO and cGMP in UVB-induced melanogenesis, we measured incorporation of [¹⁴C]DOPA into melanins of melanocytes irradiated for 4 days with daily irradiations of 100 mJ/cm² in the presence or absence of 1 mM L-NMA, 10 μ M Ly 83583, and 500 nM KT 5823 (Fig. 5). Such phototreatment in control conditions resulted in a 2-fold in-

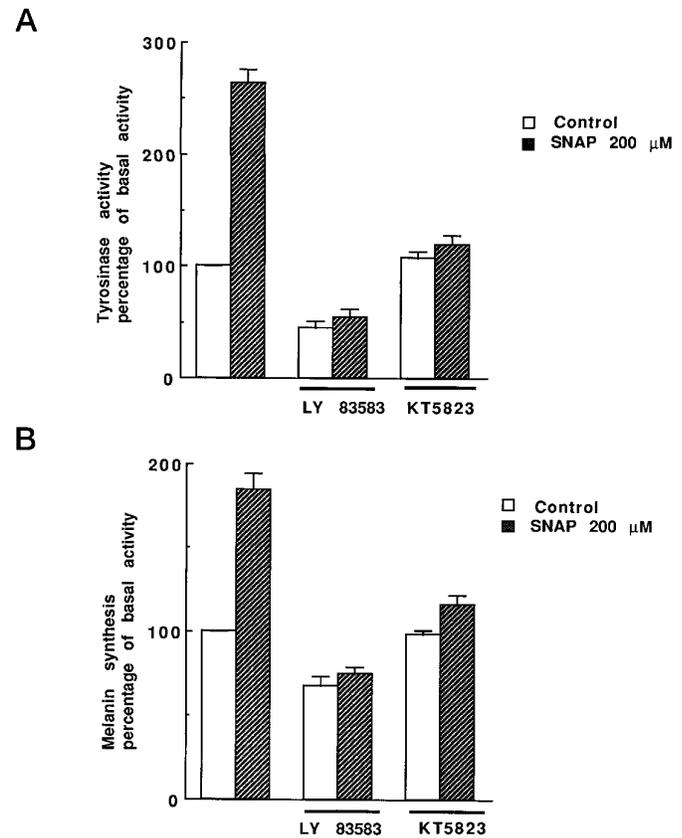


FIG. 3. **Effect of Guanylate Cyclase and PKG inhibitors on NO-stimulated melanogenesis.** Melanocytes were incubated for 4 days in the presence of 200 μ M SNAP. When indicated, cells were preincubated for 30 min with 10 μ M Ly 83583 or 0.5 μ M KT 5823 before the addition of SNAP. Results were normalized to cell number and are expressed as percentages of basal activity from control melanocytes. Each value represents the mean \pm S.E. (bars) of triplicates of one representative experiment.

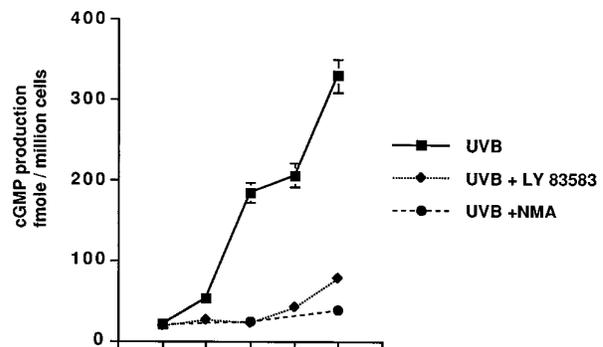


FIG. 4. **Production of cGMP by melanocytes in response to UVB irradiation.** Melanocytes were exposed to 100 mJ/cm² UVB irradiation, and then cGMP was measured after the indicated periods. Cells treated with 10 μ M Ly 83583 or 1 mM L-NMA were preincubated with these compounds for 30 min before irradiation. Each value represents the mean \pm S.E. (bars) of triplicates of one representative experiment.

crease in melanin synthesis. Ly 83583, the specific inhibitor of guanylate cyclase, as well as the PKG inhibitor (KT 5823) markedly impaired the stimulation of melanin synthesis by UVB. Similarly, when the cells were pretreated before each irradiation with L-NMA, we also observed a decrease in UVB-stimulated melanin synthesis. The effects of L-NMA, LY 83583, and KT 5823 on tyrosinase activity were similar to those observed on melanin biosynthesis (data not shown). These results show that NO, through the activation of guanylate cyclase

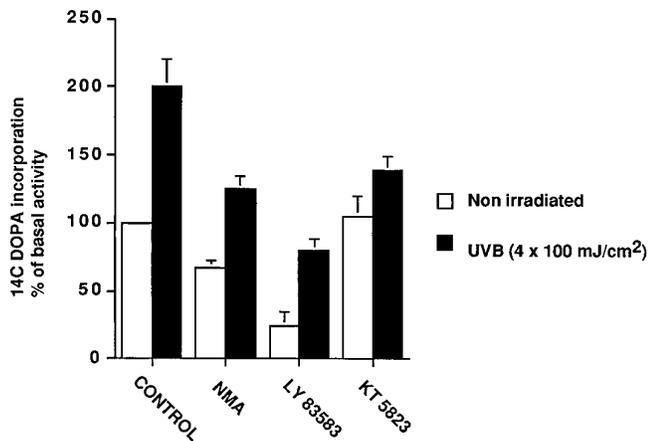


FIG. 5. Effect of NOS, guanylate cyclase, and PKG inhibitors on UVB-stimulated melanin synthesis. Melanocytes were irradiated daily for 4 days with 100 mJ/cm² UVB irradiation. When indicated, cells were preincubated for 30 min with 1 mM L-NMA, 10 μ M Ly 83583, or 0.5 μ M KT 5823 before each irradiation. Results were normalized to cell number and are expressed as percentages of basal activity from control, nonirradiated melanocytes. Each value represents the mean \pm S.E. (bars) of triplicates of one representative experiment.

and protein kinase G, plays a key role in UVB-induced melanogenesis.

DISCUSSION

In humans, melanin pigmentation, which plays a key role in the protection against photocarcinogenesis, is mainly stimulated by UVB. However, the molecular mechanisms underlying melanogenesis regulation had not been identified. In this study we show that stimulation of melanogenesis depends on a linear transduction pathway involving UVB, NO, and cGMP. We clearly demonstrate that NO is a potent stimulator of tyrosinase activity and melanin synthesis. Despite the increasing number of potential NO targets (25–27), the effects of this agent appear to be mediated mainly through the activation of intracellular guanylate cyclase, leading to a cGMP increase (28). Indeed, we show here that short term exposure to NO donors induced a marked increase in cGMP content in human melanocytes, and 8-bromo cGMP, a permeable analog of cGMP, was found to enhance both the tyrosinase activity and melanin neosynthesis in human melanocytes. Furthermore, the effects of NO on melanogenesis are prevented by guanylate cyclase or PKG inhibitors. These inhibitors more efficiently affected NO-induced tyrosinase activity than NO-induced melanin synthesis, suggesting that tyrosinase would be the principal target of PKG. These pharmacological arguments converge to demonstrate that cGMP mediates the melanogenic effects of NO.

We also demonstrate that the UVB effect on melanogenesis is mediated by the NO-cGMP transduction pathway. Indeed, we show that UVB induces a strong and rapid raise of cGMP content in melanocytes. This effect can be blocked by guanylate cyclase and NO synthase inhibitors, suggesting that the effect of UVB on guanylate cyclase is mediated by NO. Similarly, the melanogenic effect of UVB is prevented by both guanylate cyclase and NO synthase inhibitors. These results demonstrate that cGMP is required for UVB-induced melanogenesis and strengthen the hypothesis that UVB radiation activates melanocyte guanylate cyclase through stimulation of a NO synthase and subsequent release of NO. The immediate effect of UVB on cGMP content indicates that UVB radiation activates a constitutive NO synthase rather than increases the expression of an inducible NO synthase. This hypothesis is confirmed by immunofluorescence studies that showed the expression of constitutive NO synthase but not inducible NO synthase in human

melanocytes (not shown).

Cyclic GMP could elicit its effects through different pathways. Indeed, cGMP has been shown to bind and regulate K⁺ ion channel activity (29). In addition, cGMP was reported to inhibit a cAMP phosphodiesterase, leading to an increase in cAMP content (30). Since cAMP strongly stimulates melanogenesis (31), this mechanism could be involved in cGMP-induced melanogenesis. Finally, cGMP activates PKG and phosphorylation of target proteins. UVB- and NO-induced melanogenesis is prevented by a PKG inhibitor, indicating that cGMP acts through the activation of this kinase. It is conceivable that PKG phosphorylates some proteins involved in melanogenesis. For instance, tyrosinase, which controls melanogenesis, contains several consensual phosphorylation sites for PKG (32, 33) and was reported to be phosphorylated (16). Furthermore, PKG was thought to mediate the activation of the transcription factor activator protein 1 by NO and cGMP in rodent fibroblasts and epithelial cell lines (34). Hence, we could hypothesize that the activation of activator protein 1 through the binding to the 12-o-tetradecanoyl-phorbol-13-acetate responsive element-like sequence of the tyrosinase promoter can lead to the stimulation of tyrosinase expression and thereby to the stimulation of melanogenesis (33). This hypothesis agrees with a recent report from our laboratory indicating that activator protein 1 is activated during melanization in mouse melanoma cells (35).

Recently, NO and cGMP have been found to be involved in UVB-induced photorelaxation of vascular smooth muscle (18) and skin erythema (36). In these cases, UVB radiation stimulates NO production by endothelial cells or by keratinocytes. Then, NO freely diffuses to neighboring vascular smooth muscle cells to stimulate guanylate cyclase, leading to cGMP production and vasodilation. Since in skin epidermis, melanocytes and keratinocytes closely interact within the epidermal-melanin unit, NO produced by keratinocytes in response to UVB could act as an intercellular mediator to trigger melanin synthesis by neighboring melanocytes. However, in our study NO appears to be produced by UVB-irradiated melanocytes and could act as an autocrine factor or an intracellular messenger to stimulate melanogenesis. Hence, in skin NO could be involved in both autocrine and paracrine regulation of UVB-induced melanogenesis.

In summary, our data demonstrate that NO and cGMP, through the activation of PKG, mediate the effects of UVB radiation on melanogenesis. The present study provides meaningful clues concerning the mechanism underlying UV-induced pigmentation of human skin and facilitates our fundamental understanding of the melanogenesis mechanisms.

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