Topical Application of a Protein Kinase C Inhibitor Reduces Skin and Hair Pigmentation

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To determine whether inhibition of PKC-β activity decreases pigmentation, paired cultures of primary human melanocytes were first pretreated with bisindolylmaleimide (Bis), a selective PKC inhibitor, or vehicle alone for 30 min, and then treated with TPA for an additional 90 min to activate PKC in the presence of Bis. Bis blocked the expected induction of tyrosinase activity by activation of PKC. Addition of a peptide corresponding to amino acids 501–511 of tyrosinase containing its PKC-β phosphorylation site, a presumptive PKC-β pseudosubstrate, gave similar results. To determine whether Bis reduces pigmentation in vivo, the backs of four shaved and depilated pigmented guinea pigs were UV irradiated with a solar simulator for 2 wk excluding weekends. Compared to vehicle alone, Bis (300 μM), applied twice daily to paired sites for various periods encompassing the irradiation period, decreased tanning. Bis also, although less strikingly, reduced basal epidermal melanin when topically applied twice daily, 5 d per wk, for 3 wk to shaved and depilated unirradiated skin. Moreover, topical application of Bis (100 μM) once daily for 9 d to the freshly depilated backs of 8-wk-old mice markedly lightened the color of regrowing hair. These results demonstrate that inhibiting PKC activity in vivo selectively blocks tanning and reduces basal pigmentation in the epidermis and in anagen hair shafts.

Key words: PKC-beta/skin pigmentation/PKC inhibitor/hair
J Invest Dermatol 122:159–166, 2004

Pigmentation due to synthesis and dispersion of melanin protects the skin from harmful effects of sunlight (Quevedo and Holstein, 1998), but unwanted hyperpigmentation can also produce a significant psychologic stress. Development of effective therapeutics to modulate skin pigmentation has been slow due to the complexity of molecular mechanisms regulating pigmentation. Our laboratory has determined that protein kinase C-β (PKC-β) activates tyrosinase (Park et al, 1993), the key and the rate-limiting enzyme in pigmentation (Pawelek and Chakraborty, 1998), by phosphorylating serine residues at amino acid positions 505 and 509 (Park et al, 1999). Loss of PKC-β prevents melanogenesis in cultured pigment cells (Yamanishi et al, 1991; Powell et al, 1993; Yamanishi and Meyskens, 1994; Park and Gilchrest, 1996; Park et al, 1999), suggesting that inhibition of this isoform specifically or as part of pan-PKC inhibition might lead to skin and hair lightening in vivo.

PKC is a family of serine/threonine kinases with at least 11 isoforms (Dekker and Parker, 1994), categorized as classical PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC). PKC-β belongs to the category of cPKC, which also includes PKC-α and PKC-γ (Nishizuka, 1992). PKC-α is ubiquitously expressed (Nishizuka, 1988) whereas the expression of PKC-γ is restricted to brain (Nishizuka, 1984), and PKC-β is expressed in many tissues, including brain, endocrine tissues, liver, spleen, lung, heart, and testis (Nishizuka, 1988). In skin, PKC-β expression appears to be restricted to melanocytes (Park et al, 1991), whereas PKC-α is expressed in melanocytes, keratinocytes, and fibroblasts (Park et al, 1991; Racchi et al, 1994; Reynolds et al, 1994). In general, PKC resides in the cytoplasm in an inactive form and is activated when diacylglycerol (DAG) is generated from the membrane when cell surface receptors interact with their specific ligands (Nishizuka, 1984, 1992). Activation of cPKC requires the presence of both membrane-generated DAG and elevated levels of intracellular Ca^{2+} (Dekker et al, 1995; Newton, 1995; Nishizuka, 1995; Jaken, 1996), whereas DAG alone is sufficient to activate both nPKC and aPKC (Dekker et al, 1995; Newton, 1995; Nishizuka, 1995; Jaken, 1996). Activated PKC then translocates to the plasma membrane or to another particular site within the cell (Clemens et al, 1992; Leach and Raben, 1993; Lehel, 1995a, 1995b; Dempsey et al, 2000) where it phosphorylates its substrate protein(s).

The role of PKC in pigmentation was first suggested by the observation that addition of DAG increased the melanin level in cultured human melanocytes and the PKC inhibitors H-7 and sphingosine blocked DAG-induced increases in the level of melanin in these cells (Gordon and Gilchrest, 1989). Topical application of DAG induced skin pigmentation in guinea pigs in a reversible fashion, whereas a PKC-inactive analog of DAG failed to induce skin pigmentation (Allan et al, 1995), indicating that the activation of PKC increases pigmentation in vitro and in vivo. Conversely, depletion of

PKC by chronic treatment of cells with phorbol esters markedly reduces the basal melanin level and tyrosinase activity in human melanocytes (Park *et al*, 1993) and murine S91 melanoma cells (Park *et al*, 1996). The PKC inhibitors sphingosine and calphostine also block α-melanocyte stimulating hormone induced pigmentation (Park *et al*, 1996), suggesting that the cAMP-dependent protein kinase A pathway known to mediate α-melanocyte stimulating hormone effects ultimately interacts with the PKC pathway.

Bisindolylmaleimide GF 109203X (Bis), a selective inhibitor for cPKC (Toullec *et al*, 1991), is a synthetic derivative (molecular weight 0.4 kDa) of the microbial product staurosporine that exerts its effects at least partly by competing with adenosine-5′-triphosphate (Tamaoki *et al*, 1986). Bis was shown to have higher selectivity for PKC-β than for PKC-α and PKC-γ, with an IC_{50} of 16 nM *versus* an IC_{50} of 20 nM (Toullec *et al*, 1991; Davis *et al*, 1992), and a 10–100-fold higher IC_{50} for other isoforms of PKC and for other serine/threonine and tyrosinase kinases (Toullec *et al*, 1991; Martiny-Baron *et al*, 1993). In our study, Bis reduced pigmentation in cultured human melanocytes, reduced basal pigmentation, and prevented ultraviolet (UV) induced increased melanogenesis (tanning) via topical application in guinea pigs. In mice, topical application of Bis to depilated skin lightened the color of regrowing hairs. In the context of prior studies, these findings demonstrate that inhibition of PKC-β activity reduces pigmentation *in vivo* and *in vitro*.

Results

Competitive inhibition of PKC-β activity reduces tyrosinase activity *in vitro* To test the hypothesis that inhibition of PKC-β activity would decrease phosphorylation of tyrosinase, and subsequently decrease tyrosinase activity, a tyrosinase mimetic peptide (TMP) was employed. TMP is composed of 11 amino acids whose sequence is identical to amino acids 501–511 of tyrosinase (Kwon *et al*, 1987) (Fig 1). A control peptide had identical sequence except for alanines in place of serines, the known phosphorylation sites for PKC-β (Park *et al*, 1999). Paired cultures of subconfluent human melanocytes were treated with 3 μm of TMP or control peptide for 16 h. To enhance their delivery, TMP and control peptides were first treated with lipofectamine for 30 min. Cells were then treated with 10^{-7} M TPA for 90 min to activate PKC, harvested, and analyzed for tyrosinase activity. Compared to the cells treated with control peptide, TMP-treated cells had significantly lower tyrosinase activity (Fig 2*A*).

To confirm that TMP inhibits phosphorylation of tyrosinase by PKC-β, paired plates were treated with 3 μm control peptide or TMP for 16–18 h and subsequently treated with 10^{-7} M TPA for 90 min in the presence of ^{32}P-orthophosphate to activate PKC. Then tyrosinase was immunoprecipitated using a polyclonal antibody specific for tyrosinase (Jimenez *et al*, 1991). Incorporation of ^{32}P-orthophosphate into tyrosinase was significantly less in TMP-treated melanocytes than in control peptide-treated melanocytes (Fig 2*B*). These results are consistent with previous reports (Park *et al*, 1993, 1999) that tyrosinase
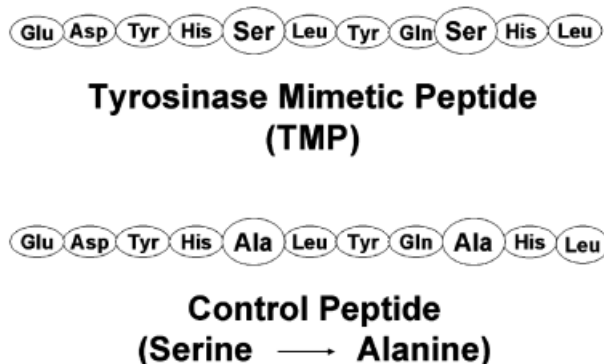

Figure 1
Amino acid sequences of the TMP and control peptide. Serine residues, the site of PKC-β-mediated phosphorylation in TMP, are highlighted. In the control peptide, serine is replaced by alanine, which cannot be phosphorylated.

activity is modulated by PKC-β-mediated phosphorylation and, in combination with the earlier reports, demonstrate that PKC-β inhibition decreases this rate-limiting melanogenic activity.

Inhibition of PKC activity with Bis decreases tyrosinase activity *in vitro* To further demonstrate that inhibition of PKC-β activity would reduce pigmentation, Bis, a commercially available selective inhibitor for cPKC with minimal effect on other serine/threonine kinases including nPKC and aPKC or on tyrosine kinases (Toullec *et al*, 1991), was employed. Paired subconfluent melanocyte cultures were first treated with 50 μM Bis for 30 min, and then treated with 10^{-7} M TPA for an additional 90 min. Preliminary dose–response studies using Bis concentrations of 1–100 μM indicated that 50 μM was the optimal concentration to inhibit TPA-induced increases in tyrosinase activity without any visible effects on cell survival or morphology. Cells were then harvested and tyrosinase activity was measured. The TPA-induced increase in tyrosinase activity was blocked by Bis treatment (Fig 3), further confirming that PKC-β activity is necessary for tyrosinase activity, at least *in vitro*.

Effects of Bis on UV-induced skin pigmentation *in vivo* To test whether inhibition of PKC-β activity *in vivo* reduces skin pigmentation, pigmented guinea pigs were employed as described in *Materials and Methods*. These animals have melanocytes in the interfollicular epidermis and are known to tan in response to UV irradiation (Allan *et al*, 1995). Biopsies of the UV-irradiated sites taken 24 h after the last exposure showed the expected increase in epidermal melanin, as well as epidermal thickening (Fig 4*A*). In all four animals, topical application of Bis decreased skin pigmentation in all application sites (Fig 5). There was no erythema or other evidence of cutaneous irritation during or after the application. Interestingly, the period of Bis application (before, during, and after UV irradiation; during and after only; or during only) did not affect the degree of skin lightening. The effect of Bis on skin lightening was consistently less on Site 1, located closest to the head, however, compared to Sites 2 and 3, which displayed both darker tanning and a more striking reduction in
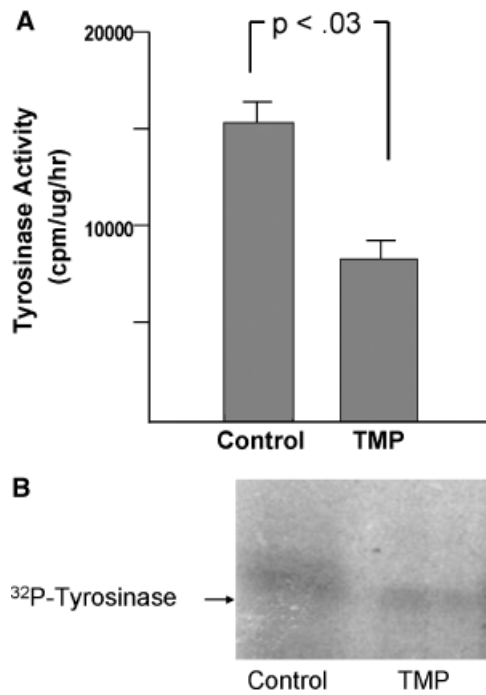
A

B

^{32}P-Tyrosinase →

Control   TMP

Figure 2
TMP inhibits tyrosinase activity by blocking phosphorylation of tyrosinase by PKC-β. (*A*) Paired cultures of subconfluent melanocytes were treated with 3 μm of TMP or control peptide for 16 h, then with 10^{-7} M TPA for 90 min to activate PKC, and then harvested. Tyrosinase activity was measured as previously described (Pomerantz, 1964). Student paired *t* test was employed for statistical analysis. (*B*) Paired cultures of melanocytes were first treated with 3 μm TMP or control peptide for 16 h, and then treated with 10^{-7} M TPA in DMEM without phosphate in the presence of ^{32}P-orthophosphate for 90 min. Cells were harvested and tyrosinase was immunoprecipitated, using a specific polyclonal antibody against tyrosinase. Immunoprecipitated proteins were separated by subjecting them to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gel was dried and exposed to Kodak X-OMAT film to demonstrate ^{32}p-tyrosinase. A representative result from two independent experiments is presented.

pigmentation by Bis. This is consistent with our previous observation that the cephalad part of the guinea pig back does not pigment as darkly in response to UV irradiation or other stimuli (Allan *et al*, 1995), and our reasoning that the degree of observed pigment reduction resulting from PKC-β inhibition depends on the basal or stimulated level of PKC-β activity.

Bis decreased total epidermal melanin and tyrosinase activity To assess the total amount of epidermal melanin in each treatment site, the paraffin-embedded biopsies were sectioned and stained with Fontana–Masson (silver nitrate). There was a consistent decrease in total epidermal melanin in Bis-treated areas compared to vehicle-treated areas (Fig 4*B*). Consistent with these results, split-dopa staining of *en face* sections was far less in Bis-treated sites than vehicle-treated sites (Fig 6).

***In vivo* reflectance confocal microscopy showed decreased melanin in Bis-treated sites** In reflectance-mode confocal imaging of skin, melanin is the best endogenous contrast agent and melanin-containing cells are highly refractile (Rajadhyaksha *et al*, 1995). When imaging pigmented guinea pig skin, melanocytes are easily identifiable
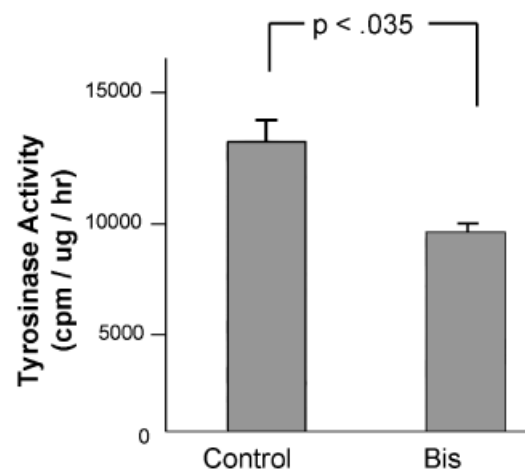

Figure 3
Bis inhibits tyrosinase activity in cultured melanocytes. Paired cultures of subconfluent melanocytes were first pretreated with 50 μm Bis or vehicle for 30 min. Then the cells were treated with 10^{-7} M TPA for an additional 90 min in the presence of Bis. Cells were then harvested and tyrosinase activity was measured. A representative result from three independent experiments is presented and Student paired *t* test was used for statistical analysis.
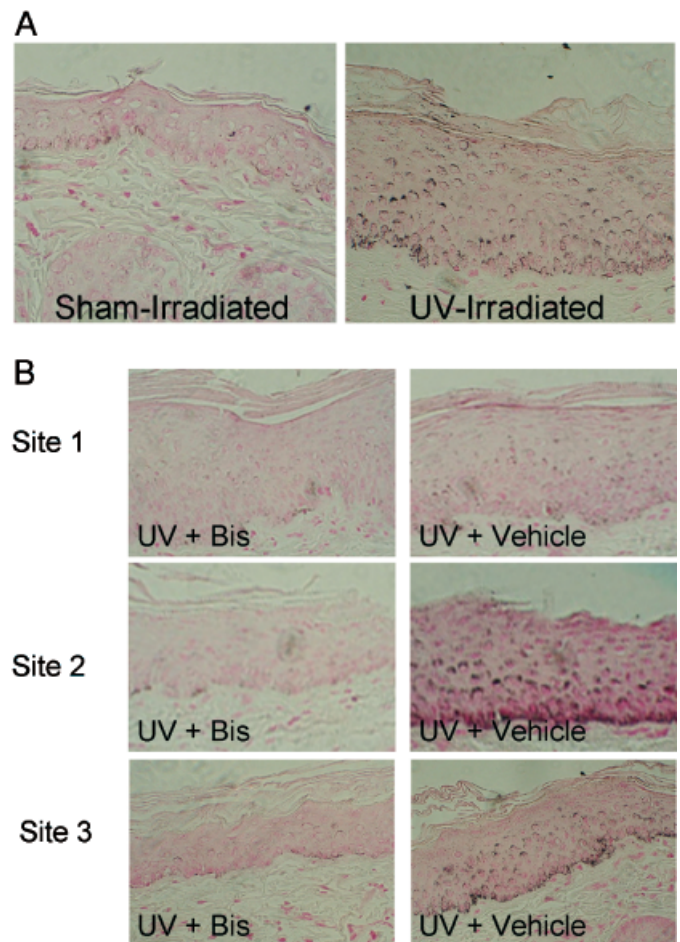

Figure 4
Bis blocks UV- or sham-induced increases in epidermal melanin. Guinea pigs were treated and irradiated over a 2 wk period, as described. Biopsies taken 24 h after the last irradiation were stained with Fontana–Masson to visualize total epidermal melanin. (*A*) UV or sham irradiation only; no topical treatment. (*B*) UV irradiation plus daily treatment with Bis or vehicle alone. Representative sections from each site are shown.
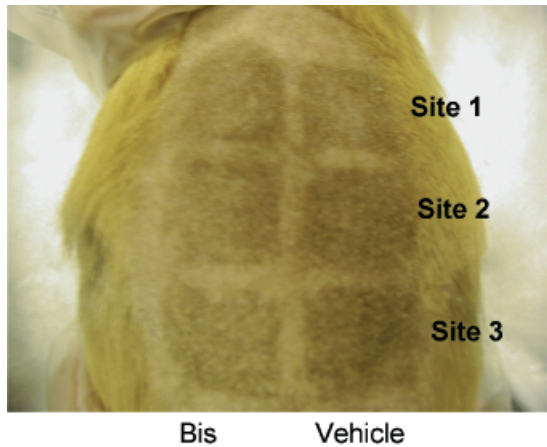
Figure 5
Bis reduces skin pigmentation of guinea pigs. Four pigmented guinea pigs were treated with Bis or vehicle as described in *Materials and Methods*. After the last application, the hair was re-shaved and photographs were taken to demonstrate the effect of Bis on pigmentation.
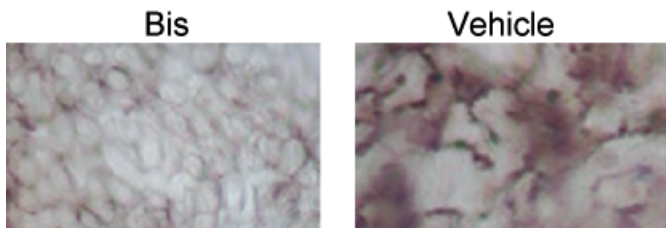

Figure 6
Tyrosinase activity in vehicle- and Bis-treated skin. Guinea pigs were treated and irradiated over a 2 wk period, as described. Biopsies taken 24 h after the last application were processed for *in vivo* split-dopa assay as described in *Materials and Methods*. Brown staining indicates melanin in melanocyte dendrites. Bis-treated and vehicle-treated skin from Site 2 is shown. Sites 1 and 3 showed comparable differences.

Figure 7
Reflectance-mode confocal microscopy of nonirradiated skin and vehicle- and Bis-treated irradiated skin. Images from the suprabasal and basal layer of skin show an overall decrease in brightness (autofluorescence due to melanin present in keratinocytes and melanocytes) of Bis-treated irradiated skin compared to vehicle-treated irradiated skin, corresponding to a decrease in pigmentation. As seen most easily in nonirradiated skin, the suprabasal layer shows the characteristic honey-combed pattern created by keratinocytes with central nuclei and relatively little cytoplasmic melanin, whereas the image obtained at the basal layer is melanocytes that are easily identified by their dendritic morphology. In irradiated vehicle-treated (tanned) skin, both the suprabasal and basal layers of the epidermis contain far more melanin (detected as bright areas) that obscure these patterns. The irradiated Bis-treated skin is intermediate in melanin content but more closely resembles the nonirradiated central images. (*In vivo* confocal images, 30 × water immersion objective lens, image width corresponds to 250 μm.) The cartoon is modified from Daniels *et al* (1968).

by their white color and dendritic morphology (Wang *et al*, 2002). Animals were anesthetized and reflectance confocal microscopy was performed once a week. As shown in Fig 7, the UV-induced increase in melanin was clearly visible after 9 d. In nonirradiated skin, keratinocytes can be visualized in the suprabasal layer as polygonal to round structures with a dark nucleus surrounded by a brighter cytoplasm, whereas in irradiated skin keratinocytes contain far more melanin, seen as bright round spots (Fig 7). After 1 wk and more striking after 2 wk, the overall brightness of keratinocytes was clearly less in Bis-treated skin than in skin treated with vehicle alone, consistent with the reduction of cutaneous pigmentation observed clinically at the end of the irradiation period and with the histologic determinations. Melanocytes could be easily recognized at the basal layer level by their brightness and dendricity, both in nonirradiated and in irradiated skin. As expected, however, melanocytes in irradiated skin were increased in number and size compared to those in nonirradiated skin. Again, this difference was less pronounced in Bis-treated skin than in vehicle-treated skin. These results further confirm that inhibition of PKC-β reduces pigmentation *in vivo* and demonstrate th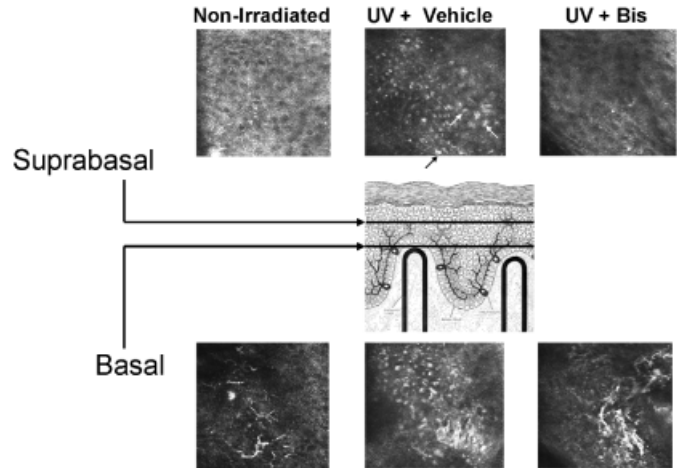e feasibility of performing detailed noninvasive time course studies through serial examination of single animal or human subjects.

Bis decreases basal skin pigmentation *in vivo* In order to determine whether topical application of Bis would also reduce basal pigmentation, the backs of three guinea pigs were shaved and Naired. Bis (300 μM) or vehicle alone was topically applied as described above and biopsies were obtained 24 h after the last applications. Clinically, there was only subtle lightening in the Bis-treated skin but Fontana–Masson-stained sections from the Bis-treated areas showed a significant reduction ($p < 0.03$) in epidermal melanin compared to vehicle-treated areas as assessed by image analysis (Fig 8A,B). Therefore, the effect of Bis is not limited to UV-induced pigmentation. Rather, as anticipated on the basis of the *in vitro* studies, Bis inhibits basal pigmentation as well.

Effect of Bis treatment on murine coat color To determine whether topical application of Bis would also lighten hair color, Bis (100 μM) was applied once daily to freshly depilated mice for 9 d, beginning on day 3 after depilation. As the coat regrew, hair color was strikingly lighter in Bis-treated but not in vehicle-treated areas (Fig 9). The striking loss of coat pigmentation on casual inspection was confirmed by examining individual hairs plucked from vehicle-treated *versus* Bis-treated areas under the dissecting microscope (Fig 9, *inset*).
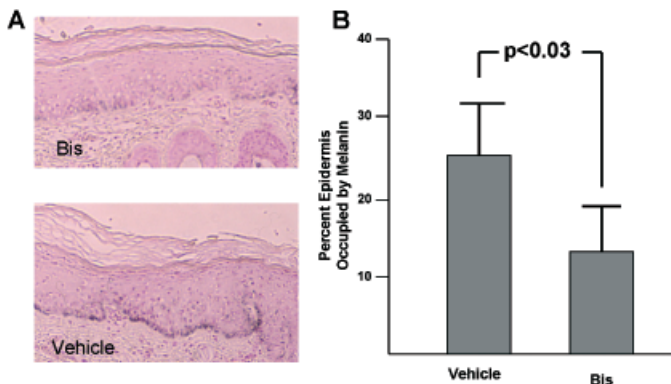
Figure 8
Bis reduces basal pigmentation. Guinea pigs were topically treated with Bis or vehicle alone for 3 wk, as described. Biopsies taken 24 h after the last application were stained with Fontana–Masson to visualize total epidermal melanin. (*A*) Representative sections from Bis- and vehicle- treated areas. (*B*) Percent epidermis occupied by melanin on serial sections from Bis- and vehicle-treated areas determined using computer-image analysis as previously described (Bhawan *et al*, 1991). For statistical analysis, Student *t* test was performed.

Figure 9
Mouse coat color is lightened by Bis treatment. Nine-week-old black mice were depilated to synchronize the hair cycle and initiate a new anagen growth phase. Beginning 3 d later, either 100 μM of Bis (*top*) or vehicle alone (*bottom*) was topically applied once a day for 9 d and mice were photographed on day 13. *Inset*: Hairs from Bis- or vehicle-treated areas were plucked on day 13 and photographed under a dissecting microscope. Representative hairs (midshaft, approximately 0.3 mm in length) from 50 hairs plucked from each area are shown.

Discussion

In combination with previous studies (Park *et al*, 1993, 1999), these results demonstrate that inhibition of PKC-β activity reduces pigmentation both *in vivo* and *in vitro* by preventing phosphorylation of tyrosinase. Although Bis, the inhibitor employed for the *in vivo* studies, is not expected to be completely selective for the β isoform of PKC (Toullec *et al*, 1991), the only detected effects of Bis treatment were those attributable to PKC-β inhibition on the basis of prior *in vitro* studies. Cultured human melanocytes treated with the PKC inhibitor Bis did not display any morphologic changes other than decreased melanin content, and topical application of Bis twice daily for up to 5 wk did not produce any clinically or histologically detectable changes in guinea pig skin other than decreased pigmentation. Skin biopsy cross-sections obtained at the end of the experiment, 24 h after the last application, confirmed that Bis caused no cytologic damage, atrophy, irritation, or inflammation. Visual inspection, histologic analysis, and skin imaging by *in vivo* reflectance confocal microscopy all demonstrated pigmentation reduction by topical application of Bis with no other detectable effect of the treatment. These findings suggest that Bis minimally affects cell behaviors other than melanogenesis, perhaps because of the relative selectivity of Bis for PKC-β (Toullec *et al*, 1991), an isoform that appears to be exclusively expressed in melanocytes in skin (Park *et al*, 1991) and appears to act only by stimulating melanogenesis (Park *et al*, 1993, 1996).

Our results further indicate that restricting Bis application to the 2 wk UV irradiation period is sufficient to block tanning. Additional applications, for 1 wk before irradiation or 2 wk after irradiation, did not further decrease pigmentation. These results are consistent with the expectation that PKC-β is most abundant and active in the period immediately following UV irradiation, given that UV irradiation upregulates the expression of PKC-β mRNA within 1 h (Park and Gilchrest, 1999; Rubeiz *et al*, 2002) and UV
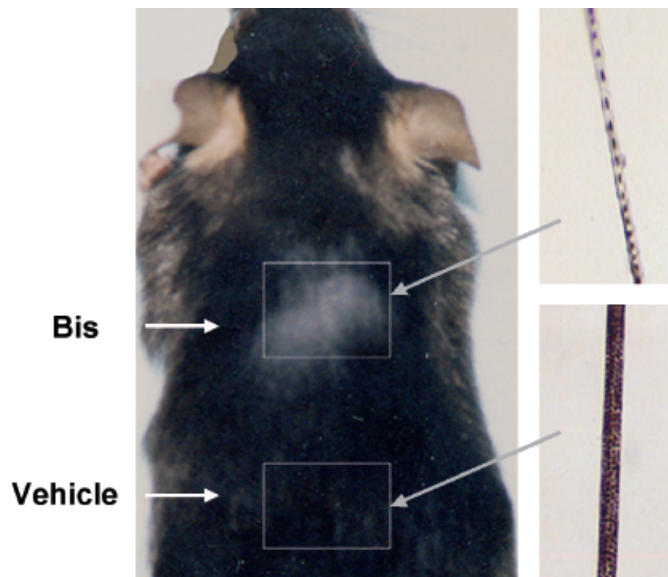
irradiation also rapidly increases the intracellular level of DAG (Punnonen and Yuspa, 1992). Generation of DAG would be expected to activate PKC-β immediately, leading to the activation of tyrosinase. Our findings are also consistent with those of Hallahan *et al* (1991) that UV upregulates the proto-oncogenes c-jun and c-fos, known to be regulated through the PKC-dependent pathway, and that these inductions are inhibited by PKC inhibitors. The lower activity of PKC-β in resting, unirradiated melanocytes should result in Bis having less effect, as observed.

Topical application of Bis also reduced basal pigmentation. Despite a striking difference in melanin content determined by Fontana–Masson staining of the sections obtained from vehicle-treated *versus* Bis-treated areas, however, there was only a subtle difference in pigmentation clinically. This probably reflects the fact that melanin was located primarily in the basal layer (Fig 8*A*), rather than also substantially in the suprabasular layers, as in UV-irradiated skin, given that melanin near the skin surface is perceived as darker than melanin restricted to the basal layer (Quevedo and Holstein, 1998).

Cultured human melanocytes express the α, β, δ, ε, and ζ isoforms of PKC (Oka *et al*, 1995). Among these isoforms, only PKC-β has a well-defined role in the biology of melanocytes. The phorbol ester TPA was found to support long-term growth of melanocytes (Eisinger and Marko, 1982, 1983) via downregulation, not activation of PKC as was later shown (Brooks *et al*, 1991), although the growth rate over a 2 wk period is not accelerated relative to that of melanocytes cultured in an otherwise optimized medium lacking pharmacologic modulators of PKC levels (Gilchrest

and Friedmann, 1987). Downregulation of PKC-α, -δ, -ε, and -ζ has been associated with growth of melanocytic cells, but a causal relationship was not established (Brooks *et al*, 1993; Oka *et al*, 1995).

PKC-α and PKC-β, classical PKCs, have similar activation requirements (Nishizuka, 1992) and both phosphorylate substrate sequences of XRXXSXRX, where X is any amino acid (Kemp and Pearson, 1990). The amino acid sequence of TMP deviates considerably from this sequence, and it remains to be determined whether TMP is a pseudo-substrate only for PKC-β. Unique substrate sequences have not yet been identified for any PKC isoform.

Ideally, these studies would have been performed with an agent known to be a completely specific inhibitor of PKC-β, but no such agent now exists. The TMP peptide has the same sequence as the portion of tyrosinase shown to be phosphorylated exclusively by PKC-β in melanocytes, but it remains unclear whether this specificity is a property of the amino acid sequence or selective localization of PKC-β to the melanosome membrane, adjacent to tyrosinase (Park *et al*, 2000). Hence, TMP introduced into melanocytes with lipofectamine may serve as a pseudo-substrate for other PKC isoforms as well. Bis is described as a selective PKC-β inhibitor (Toullec *et al*, 1991) but at the concentrations employed in our experiments might easily have also inhibited PKC-α, the other cPKC isoforms expressed in both melanocytes and other cell types in skin, notably keratinocytes. Although the exact mechanism of action has not been elucidated, PKC-α has been implicated in the proliferation and differentiation of cultured keratinocytes (Gherzi *et al*, 1992; Stanwell *et al*, 1996; Lee *et al*, 1998). Therefore, topical application of Bis might influence keratinocyte behavior as well as reduce pigmentation through its effects on melanocytes. Despite this ambiguity, however, these data demonstrate that PKC inhibition either in isolated melanocytes or in intact skin reduces pigmentation without having other detectable effects after up to 5 wk of application.

In addition to blocking UV-induced pigmentation and reducing basal pigmentation in the epidermis, inhibition of PKC-β activity also lightened mouse coat color, indicating that Bis penetrates not only into the viable epidermis but also into the follicular root sheath. One application per day almost totally blocks melanogenesis during the anagen phase of the hair cycle.

Although not studied in these experiments, it is likely that the effects of Bis on pigmentation are reversible. The half-life and stability of Bis in tissues have not been determined, but the effects of Bis on PKC activity appear to be transient (Toullec *et al*, 1991), implying that its effect is reversible. Supporting this idea, the activation of PKC by DAG, known to have a short half-life (Nishizuka, 1984), increases pigmentation in guinea pig skin in a reversible fashion (Allan *et al*, 1995).

In summary, we have demonstrated that topical application of a PKC inhibitor reduces skin pigmentation *in vivo*, an effect attributable specifically to PKC-β inhibition. Topical application of such inhibitors may prove beneficial in treating unwanted hyperpigmentation of human skin and/or in lightening hair color.

Materials and methods

Cell culture Primary human melanocytes were cultured from neonatal foreskins as previously described (Park *et al*, 1999). In brief, the epidermis was separated from the dermis after overnight incubation in 0.25% trypsin at 4°C. Primary cultures of melanocytes were established by seeding $0.5–1 \times 10^6$ total epidermal cells per 100 mm dish in Medium 199 supplemented with 10 μg per mL insulin, 10^{-9} M triiodothyronine, 10 μg per mL transferrin, 1.4×10^{-6} hydrocortisone, 80 μM dbcAMP, bovine pituitary extract (35 μg per mL), basic fibroblast growth factor (10 ng per mL), epidermal growth factor (100 ng per mL), and 1%–2% fetal bovine serum. All post primary cultures were maintained in a low calcium (0.03 mM) version of this medium known to selectively support melanocyte growth (Naeyaert *et al*, 1991). Cells at first to third passage were used for all experiments.

UV irradiation The solar simulator consisted of a 1000 W xenon arc lamp (Oriel, Stratford, CT), equipped with a WG 305 cut-on filter (Schott, Germany). The irradiance as measured by an IL 1700 research radiometer (International Light, Newburyport, MA) fitted with a UVB probe (SED 240, serial number 2093, SCS 280 UVB 1#5440, W#3790) was 0.17 mW per cm^2 (or 1.7×10^{-4} W). The animals were anesthetized with 0.3 mL of a mixture of 0.75 mL ketamine (50 mg per mL, Bedford Laboratories, Bedford, OH) and 0.15 mL xylazine (100 mg per mL, Lloyd Laboratories, Shenandoah, IA) and irradiated through an adhesive template exposing three paired 3.2 cm^2 skin sites daily during 7 d for 14 min and 42 s (150 mJ per cm^2), a protocol previously shown to cause dark tanning (Allan *et al*, 1995).

Tyrosinase activity Tyrosinase activity was measured according to Pomerantz (1964). In brief, 5×10^5 cells were briefly sonicated in 80 mM PO$_4$ (pH 6.8) containing 1% Triton-X-100, and tyrosinase was extracted for 60 min at 4°C. 10–50 μg of cellular protein was incubated with 250 nM L-tyrosine, 25 nM L-dihydroxyphenylalanine, 12.5 μg chloramphenicol, and 5 μCi of L-[3,5-^{3}H] tyrosine (40–60 Ci per mmol) for 30–60 min at 37°C. The reaction was stopped by addition of 500 μL of 10% trichloroacetic acid containing 0.2% bovine serum albumin. Trichloroacetic acid soluble material was reacted with Norit A and released ^{3}H$_2$O was measured using a scintillation counter. The activity was expressed as counts per minute of ^{3}H$_2$O released per microgram protein per hour minus the nonspecific incorporation of radioactivity, determined by using lysate boiled for 30 min (background, generally less than 5%–10% of the sample).

Phosphorylation of tyrosinase Phosphorylation of tyrosinase in cultured melanocytes was performed as previously described (Park *et al*, 1999). Cultured melanocytes were preincubated overnight with serum-free Dulbecco's modified Eagle's medium (DMEM) devoid of phosphate, containing 50 μM sodium vanadate and [^{32}P]-orthophosphate (8500–9120 Ci per mmol). Then cells were treated with 12-0-tetradecanoyl-phorbol-13 acetate (TPA) 10^{-7} M for 90 min to activate PKC and lyzed in a buffer containing 20 mM Tris (pH 7.5), 200 mM NaCl, 1% Triton X-100, 2 mM ethylenediamine tetraacetic acid, 2 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 0.5 μg per mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The lysate was clarified by spinning at $800 \times g$ for 15 min, and tyrosinase was immunoprecipitated from the supernatant using polyclonal antibody specific for tyrosinase (Jimenez *et al*, 1991). Immunoprecipitated proteins were separated using 7.5% sodium dodecyl sulfate gel electrophoresis, dried, and exposed to Kodak X-OMAT film.

Animals Outbred pigmented guinea pigs, American Shorthair X Abyssinian (Kuiper Rabbit Ranch, Chicago, IL), aged 12–20 wk at the beginning of the study, were used. The animals had free access to guinea pig chow and chlorinated water and were housed in individual cages. Prior to topical applications, each animal was shaved with an electric clipper (Oster, number 40 blade) to remove

the long hair. The remaining stubble was removed with the commercially available depilatory Nair (Carter-Wallace).

Experimental protocol All protocols were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine. Three separate protocols were employed to study different aspects of pigmentation. To study effects of Bis on UV-induced pigmentation, three pairs of treatment sites (3.2 cm^2) were chosen in areas of comparable baseline pigmentation on the left and right sides of the back, equidistant from the dorsal midline, for subsequent daily applications and UV exposure. The left sites of each pair were treated with vehicle alone (25% dimethylsulfoxide, 75% propylene glycol) and the right sites with 300 μm Bis twice a day. The Site 1 pair was treated for 1 wk before UV irradiation, 2 wk during irradiation, and 2 wk after irradiation. The Site 2 pair was treated twice daily during 2 wk of UV irradiation and 2 wk after irradiation. The Site 3 pair was treated only during 2 wk of irradiation. Weekends were excluded from applications. These three protocols were included to account for the possibilities that some time may be required for Bis to penetrate stratum corneum and reach melanocytes, Bis might be expected to be most effective during the UV irradiation period when PKC is known to be induced (Park and Gilchrest, 1999; Rubeiz et al, 2002), and some time may be required for PKC levels to return to their basal level after irradiation. Photographs were taken and in vivo reflectance confocal laser microscopy was performed during and after the period of application. A higher concentration of Bis (300 μM) was used for in vivo than for studies in vitro in light of the fact that the compound needs first to penetrate the stratum corneum before reaching the target melanocytes.

The possibility that Bis might act as a sunscreen was excluded by metering UV transmittance through the 300 μM Bis preparation versus vehicle alone, applied as an even film to tissue culture plastic. There was no significant difference in transmittance: 0.15 mW vs 0.14 mW in the UVB range and 30 mW vs 28 mW in the UVA range for vehicle versus Bis preparations, respectively.

To study the effects of Bis on basal pigmentation, backs of three guinea pigs were shaved and depilated using Nair as described above. One pair of treatment sites (3.2 cm^2) in the mid-back was treated with either vehicle or Bis (300 μM) twice daily 5 d per wk for 3 wk.

To study the effect of Bis on hair pigmentation, 7–8-wk-old black female mice (C57BL/6) were anesthetized with a ketamine−xylosine mixture (80 mg per kg and 16 mg per kg of body weight, respectively) to depilate the entire back using a wax-rosin mixture as previously described (Stenn et al, 1993). Bis (100 μM) was applied on the cephalad half of the back and vehicle alone was applied to the caudal half of the back beginning 3 d after depilation once daily for 9 d. The 100 μM Bis concentration was employed because skin barrier function is known to be less efficient in mice than in guinea pigs. Photographs were taken to document the color of regrowing hair 24 h after the last application.

In vivo reflectance confocal laser microscopy A commercially available reflectance-mode confocal microscope (Vivascope 1000, Lucid, Henrietta, NY) equipped with an 830 nm diode laser with a maximal power of 25 mW and a 30 × objective lens of numerical aperture 0.9 was used for imaging skin of the anesthetized guinea pigs. Technical details of the microscope and the imaging procedure have been previously described (Rajadhyaksha et al, 1995, 1999). Briefly, the skin is immobilized against the microscope by a ring-template fixture, so the laser beam is directed perpendicular to the skin. Horizontal movement of the beam allows screening of the skin site, whereas axial movement of the beam results in a continuum of en face images from stratum corneum to upper papillary dermis. The images, equivalent to optical sections, have a lateral resolution of 0.5–1 μm and a section thickness (axial resolution) of 3–5 μm, similar to conventional histology. The immersion medium used for imaging was water, as the refractive index of water is closest to that of skin, thereby

reducing artefacts during imaging. Animals were anesthetized during confocal microscopy to minimize motion during imaging, which was performed once a week. All imaging procedures were video-taped and transferred to a computer. Still images were grabbed at the level of the suprabasal layer to visualize spinous keratinocytes and at the level of the basal layer to visualize basal keratinocytes and melanocytes. The VivaScope user interface is driven by a Windows NT based personal computer with menu-driven software for initiating and controlling the scanners, laser illumination power, z-depth focusing, and image capture.

Histologic analysis Punch biopsies (4 mm) were taken from vehicle-treated and Bis-treated skin 24 h after the last application. Specimens were fixed in 10% neutral-buffered formalin for 24 h, dehydrated, and embedded according to standard histologic protocols. Vertical cross-sections, stained with both hematoxylin and eosin and Fontana–Masson silver nitrate were examined under 10 × and 40 × magnification using an Olympus BH-2 microscope as previously described (Allan et al, 1995). The amount of interfollicular epidermis occupied by melanin in serial sections was quantitated by computer-image analysis as previously described (Bhawan et al, 1991).

In vivo split-dopa staining Separate punch biopsies (4 mm) were taken simultaneously from vehicle-treated skin and Bis-treated skin to determine whether tyrosinase activity is reduced by application of Bis in vivo. Each biopsy was placed onto a coverslip, epidermal side down in a thin layer of permount. The biopsy was then covered with 2 M sodium bromide solution for 2 h at 37°C. Dermis was then removed from the epidermis, and the coverslip was rinsed with 0.1 M sodium phosphate buffer (pH 7.4) three times. The entire coverslip with the epidermal sheet was immersed in 0.1% deoxyphenylalanine solution at 37°C for 4–6 h, changing solution after the first 30 min and then every 2 h. The epidermis was checked under the microscope every hour until a dark brown color developed in at least one of the paired sections. The stained epidermis of all paired specimens was then fixed in 10% formalin.

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