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PGE_2 is a UVR-inducible autocrine factor for human melanocytes that stimulates tyrosinase activation

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Abstract

Melanocyte proliferation, dendrite formation, and pigmentation are controlled by paracrine factors, particularly following exposure to ultraviolet radiation (UVR). Little is known about autocrine factors for melanocytes. Prostaglandins activate signaling pathways involved in growth, differentiation and apoptosis. Prostaglandin E_2 (PGE_2) is the most abundant prostaglandin released by keratinocytes following UVR, and stimulates the formation of dendrites in melanocytes. Synthesis of PGE_2 is controlled by $cPLA_2$, which releases arachidonic acid from membranes, and COX-2 and prostaglandin E_2 synthases (PGES), which convert arachidonic acid to PGH_2 and PGH_2 to PGE_2, respectively. In this report we show that multiple irradiations of human melanocytes with UVR stimulates tyrosinase activity, independent of expression of a functional melanocortin 1 receptor, suggesting the presence of a *non-melanocortin* autocrine factor. Irradiation of melanocytes activated $cPLA_2$, the rate-limiting step in eicosanoid synthesis, and stimulated PGE_2 secretion. PGE_2 increased cAMP production, tyrosinase activity and proliferation in melanocytes. PGE_2 binds to four distinct G-protein coupled receptors ($EP_{1–4}$). We show that EP_4 receptor signaling stimulates cAMP production in melanocytes. Conversely, stimulation of the EP_3 receptor lowered basal cAMP levels. *These data suggest that relative levels or activity of these receptors* controls effects of PGE_2 on cAMP in melanocytes. The data are the first to identify PGE_2 as an UVR-inducible autocrine factor for melanocytes that stimulates tyrosinase activity and proliferation, and to show that EP_3 and EP_4 receptor signaling have opposing effects on cAMP production, a critical signaling pathway that regulates proliferation and melanogenesis in melanocytes.

Keywords

PGE_2; melanocyte; UVR; tyrosinase; prostaglandin

Introduction

Melanocytes are long lived cells that reside in the basal epidermal layer and produce melanin, a complex polymer, through a pathway in which tyrosinase activation is rate limiting. Melanin-containing melanosomes are transported along arborizing dendrites in melanocytes, and transferred to keratinocytes, where they form supranuclear caps that protect nuclei from impinging UV rays. Identification of growth factors that stimulate

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dendricity and the production of melanin are of interest because of their potential chemotherapeutic value in preventing UVR-induced skin cancers, including squamous cell carcinoma and melanoma. Prostaglandin E_2 (PGE_2) is a lipid factor produced by keratinocytes in response to UVR and inflammatory conditions such as wound healing (1,2). PGE_2 regulates a broad range of physiological processes in the skin, including immune function, carcinogenesis, cutaneous barrier function and cell growth and differentiation (3–6). Biosynthesis of PGE_2 is controlled by phospholipase A_2 (PLA_2), cyclooxygenase (COX), and prostaglandin E synthase (PGES) enzymes. Type IV phospholipase A_2, also known as cytosolic PLA_2 ($cPLA_2$), is the only PLA_2 that selectively releases arachidonic acid in preference to other fatty acids, and is a rate-limiting step in prostaglandin production (7,8). $cPLA_2$ is tightly regulated both at the transcriptional and post-transcriptional level and is activated by two primary mechanisms: i) phosphorylation at serine residues, and ii) Ca^{2+}-dependent translocation to the nuclear envelope, which is important for coupling with COX enzymes.

Paracrine factors produced by keratinocytes and fibroblasts play an important role in the regulation of melanocyte function. For example, keratinocytes synthesize and secrete endothelin-1 following UVR (9–12) and keratinocyte-derived endothelin-1 stimulates tyrosinase activity (13), melanocyte dendricity (14) and proliferation (15,16). Fibroblasts produce stem cell factor, which induces melanocyte dendrite formation, and pigmentation (17). Autocrine factors for melanocytes are few, compared with paracrine factors. Alpha-melanocyte stimulating hormone (α-MSH) is produced by melanocytes in response to UVR, and controls melanocyte proliferation, melanin production and dendricity through stimulation of the melanocortin 1 receptor (MC1R, 18–21). Our previous work has shown that $PGF_{2\alpha}$, an eicosanoid that binds to the FP receptor, is a UV-inducible autocrine factor for melanocytes that stimulates melanocyte dendrite formation and tyrosinase activation, but not proliferation (22).

PGE_2 binds to 4 different G-protein coupled receptors termed EP_1, EP_2, EP_3 and EP_4. We showed previously that PGE_2 stimulates melanocyte dendrite formation through activation of EP_1 and EP_3, and that melanocytes express the EP_3 receptor in vivo (23). The purpose of the present study was to determine if PGE_2 is released from melanocytes in response to UVR, and its effects on tyrosinase activation and proliferation, and to *begin to identify the* prostanoid receptor(s) that mediates PGE_2 effects on melanocyte pigmentation. Our data show that UVR stimulates PGE_2 synthesis and activates $cPLA_2$ in melanocytes, indicating that PGE_2 is an autocrine factor for melanocytes. Treatment of melanocytes with PGE_2 stimulated the cAMP/PKA pathway, and increased tyrosinase activity, and modestly increased melanocyte proliferation. Through the use of selective agonists and antagonists of EP receptors, we show that EP_4 receptor stimulates, and EP_3 receptor inhibits, cAMP production in melanocytes. These data are the first to demonstrate that PGE_2 is an UV-inducible autocrine factor for human melanocytes that stimulates tyrosinase activation, and that signaling by EP_3 and EP_4 receptors modulate the cAMP/PKA signaling pathway, a critical regulatory pathway of melanocyte function.

Materials and Methods

Reagents

Rabbit polyclonal antibodies to β-actin and to the EP_4 receptor (H-160) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal antibodies against EP_2*and EP* $_3$ *receptor* were purchased from Cayman Chemicals (Ann Arbor, MI); rabbit polyclonal antibodies to $cPLA_2$ phosphorylated on Ser 505 and rabbit polyclonal antibodies to $cPLA_2$ were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies were purchased from Sigma Co

(St. Louis, MO). Full range rainbow molecular weight markers were purchased from Amersham Life Sciences (Arlington Heights, Il). PGE_2, butaprost (EP_2 receptor agonist), sulprostone (EP_3 receptor agonist) and ELISA Assay kits for analysis of PGE_2 were purchased from Cayman Chemicals. *This kit has essentially no cross reactivity with $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGA_2, PGD_2, thromboxane or arachidonic acid (all <1.6%), but does cross react with PGE_1 (70%) and PGE_3 (16.3%).* PAH6809 (EP_2 receptor antagonist) and L 161982 (EP_4 receptor antagonist) were purchased from Tocris Biosciences (Ellisville, MO).

Cells and cell culture

Human melanocyte cultures were derived from *individual* neonatal foreskins as previously described (18). Melanocytes with loss of function mutations in the MC1 receptor were characterized for the response to the MC1R ligand α-MSH as described (24). For analysis of effects of UVR on PGE_2 synthesis and on PGE_2 synthetic enzymes, melanocytes were maintained in of MCDB 153, supplemented with 4% fetal bovine serum (FBS), 14 μg/ml bovine pituitary extract (BPE), 5 μg/ml insulin, 10 ng/ml α-tocopherol acetate, 8 nM 12-*o*-tet-radecanoylphorbol-13-acetate (TPA), and 0.75 ng/ml basic fibroblast growth factor (bFGF). All supplements were purchased from Sigma Co, except FBS, which was purchased from Mediatech (Manassas, VA). For experiments in which effects of exogenous PGE_2 and prostanoid receptor agonists/antagonists were examined (tyrosinase activity assays, BrDu uptake assays, and cAMP assays), *and for experiments in which EP receptor expression was examined by Western blotting,* melanocytes were established in medium as described above, and were changed into medium without TPA and BPE ("−/−" media), 4 days prior to the experiment. Removal of these two factors is important for determining the effects of factors that activate PKC, such as PGE_2, since TPA down-regulates protein kinase C, and cAMP activity, since BPE contains high concentrations of melanocortins that stimulate cAMP formation (24–26). This medium does not support proliferation, however, melanocyte survival is maintained by the presence of bFGF and fetal bovine serum (personal observations).

Irradiation of melanocytes with UVR

Melanocytes were maintained in complete media and irradiation was carried out in phosphate buffered saline (PBS) using a bank of 6 FS20 sun lamps (Westinghouse) that have more than 75% emission in the UVR range (280–320 nm), with a peak emission of 313 nm, and less than 25% UVA rays (>320 nm). Kodacel filter was used to remove UVC rays. For controls, cells were placed in PBS but not irradiated ('sham irradiated").

Western Blotting for EP receptors

Cells were lysed in RIPA buffer (150 mM NaCl, 1%NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl) with protease inhibitors (Boehringer Mannheim, Gmbt, Germany) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set II, Calbiochem). Total cell lysates were resolved on 10% SDS-PAGE and blotted using standard procedures. Visualization of the immunoreactive proteins was accomplished with an enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL).

Tyrosinase activity assay

Melanocytes were *plated at 10^4 cells/cm^2* in triplicate dishes in complete medium, then switched to −/− media 4 days prior to treatment with PGE_2. Melanocytes were treated with PGE_2 (1.5 nM, 3 nM or 30 nM) every other day, for 6 days, in the continuous presence of indomethacin (4 μg/ml) to block endogenous PG production. Twenty-four hours after the final treatment with PGE_2, 0.7μCi ^{3}H-tyrosine (Perkin Elmer, Boston, MA) was added to each dish, and 24 hours later, tyrosinase activity was assayed according to the modified

charcoal absorption method of Pomerantz as previously described (19, 27). Tyrosinase activity was determined using the *in situ* tyrosine hydroxylase activity, based on measuring the ^{3}H-labeled water that is released to the media as melanocytes metabolize ^{3}H-tyrosine to DOPA during the first reaction catalyzed by tyrosinase in the melanin synthetic pathway. Cell number in each dish was counted using a Coulter Particle Counter (Model Z1 Hialeah, FL).

Proliferation Assay

Proliferation was measured using a BrDu cell proliferation assay kit supplied by Calbiochem (San Diego, CA). Cells were plated at 2×10^4 melanocytes/well (triplicate wells) in a 96 well plate in −/− media in the presence of PGE_2, and 8 hours later wells were spiked with BrDu. Twenty-four hours after addition of PGE_2, assay for BrDu uptake was performed using manufacturer's instructions. Cells were pretreated with indomethacin (4 μg/ml for 18 hours prior to the assay) to suppress endogenous prostaglandin production.

Cyclic-AMP assays

Melanocytes (*2.6×10^4 cells/cm^2)* were plated in complete media, then switched to −/− media. Cells were treated with indomethacin (4 μg/ml) overnight to block endogenous prostaglandin production, and indomethacin was maintained in the media for the duration of the experiment. Two hours prior to the addition of PGE_2 or prostanoid receptor agonists and antagonists, isobutylmethylxanthine (IBMX, 2 mM) was added to inhibit phosphodiesterase activity. PGE_2 or agonists/antagonists were added to the medium and cAMP levels were measured 10 minutes later using the Direct Cyclic AMP EIA kit as per manufacturers instructions (Assay Design, Inc., Ann Arbor, MI). *Positive controls consisted of cells treated with 1 μM forskolin, a direct activator of adenylate cyclase, for 10 minutes.* The plate was read on a Finstrument™ microplate Reader (MTX Lab Inc, VA) and the data was analyzed with Deltasoft™ 3 software (BioMetallics, Inc., Princeton, NJ). cAMP was normalized to cell number and expressed as pg/ml/cell.

Statistical Evaluation of Data

Data were expressed as mean ± Standard Error (SE) or Standard Deviation (SD) and were analyzed using Fisher's exact test to determine statistically significant differences in data sets. A p value of <0.05 was considered significant.

Results

UVR stimulates PGE_2 secretion in melanocytes

Melanocytes expressing functional or loss of function MC1R were irradiated with UVR (25 mJ/cm^2), every other day, for a total of 4 irradiations. Two days after the final irradiation, tyrosinase activity was measured and normalized to cell number (Figure 1A). Results are presented as percent of controls, which consisted of sham-irradiated cells. Melanocytes, regardless of their MC1R status, showed an increase in tyrosinase activity in response to UVR, indicating the presence of a UVR-inducible *factor* in melanocytes that is not a melanocortin that binds to the MC1R.

We next determined whether UVR stimulates the synthesis of PGE_2 in melanocytes. Cells were irradiated with UVR (50 mJ/cm^2, five times over the course of 10 days) and 2 days after the final dose of UVR, culture supernatants were collected and PGE_2 levels were determined (Figure 1B). In some dishes, cells were incubated with indomethacin (4 μg/ml) for the duration of the experiment to suppress UVR-dependent activation of COX enzymes. Controls consisted of sham-irradiated cells. Sham-irradiated melanocytes express low but detectable amounts of PGE_2 (7 pg/ml/cell $\times 10^{-5}$) that were reduced by the addition of

indomethacin, indicating low but detectable constitutive activity of COX enzymes. Following irradiation, PGE_2 levels were significantly higher than sham-irradiated cells (16 pg/ml/cell × 10^{-5} vs 7 pg/ml/cell × 10^{-5}, p=0.12). *UVR-dependent up-regulation of PGE_2 synthesis was decreased significantly (p=0.12) by indomethacin, but was not completely eliminated, suggesting that UVR-inducible enzymes other than COX-2 regulate PGE_2 synthesis in melanocytes.* The rate limiting step in eicosanoid synthesis is catalyzed by $cPLA_2$, which is activated by UVR in human keratinocytes (28). To determine if UVR activates $cPLA_2$ in melanocytes, cells were irradiated (10 mJ/cm^2 or 20 mJ/cm^2) and 24 hours later, lysates were blotted for phosphorylated (active) $cPLA_2$ and total $cPLA_2$ (Figure 1C). Irradiation stimulated phosphorylation and expression of $cPLA_2$, similar to effects described in human keratinocytes.

PGE_2 stimulates tyrosinase activity, proliferation, and cAMP production, in melanocytes

Melanocytes were treated with PGE_2 or vehicle every other day for 6 days, and tyrosinase activity was quantified (Figure 2A). Indomethacin (4 μg/ml) was present during the duration of the experiment to block endogenous prostaglandin production. A dose dependent increase in tyrosinase activity in response to PGE_2 was observed. PGE_2 at doses of 1.5 nM and 3 nM induced a 4.5 and 5.8 fold increase in tyrosinase activity compared with cells treated with vehicle ($p<0.05$). At the highest dose of PGE_2 (30 nM) a 10-fold increase in tyrosinase activity, over vehicle-treated cells, was observed ($p<0.05$). To determine the effect of PGE_2 on melanocyte proliferation, cells were pre-treated with indomethacin (4 μg/ml for 18 hours), and plated in triplicate in 96 well plates with PGE_2 or vehicle. Cells were spiked with BrDu 8 hours after plating, and 24 hours after addition of PGE_2, ELISA assay for BrDu uptake was performed (Figure 2B). PGE_2 stimulated a modest (1.2 fold) yet statistically significant ($p<0.001$) increase in BrDu uptake, at doses as low as 1.5 nM.

To determine the effects of PGE_2 on cAMP production, melanocytes were pre-treated with indomethacin for 18 hours (4 μg/ml), and 2 hours prior to the assay, IBMX was added to block phosphodiesterase activity. PGE_2 was added and cAMP was measured 10 minutes later. PGE_2 at nanomolar concentrations stimulated a 2.6 fold and 6.5-fold increase in cAMP at a dose of 1.5 nM and 30 nM respectively, compared with vehicle treated controls (Figure 2C). At higher doses of PGE_2 (1.5–30 μM) cAMP levels reached a maximum of 42 pg/ml/cell × 10^{-6}. While PGE_2 stimulated a cAMP response in melanocytes, the increase in cAMP induced by PGE_2 was much less than that observed following treatment with 1 μM forskolin for 10 minutes (296.6 pg/ml/cell × 10^{-6}, data not shown).

EP_4 receptor signaling stimulates cAMP release

The cAMP/PKA pathway is the main regulator of melanocyte function, through its effects on Micropthalmia transcription factor (Mitf), which stimulates the synthesis of tyrosinase and tyrosinase related protein-1 (TRP-1), and through activation of the MAP kinase pathway, which stimulates proliferation and maintains survival (29–33). The EP_2 and EP_4 receptors couple to G_s to stimulate cAMP production (34). To determine if melanocytes express the EP_2 and EP_4 receptors, melanocytes maintained in −/− media for 4 days were blotted with antibodies against the EP_2 and EP_4 receptors (Figure 3A). Melanocytes expressed both receptors. We next sought to determine the prostanoid receptor that mediates effects of PGE_2 on cAMP production. To determine if EP_2 or EP_4 receptor signaling stimulates cAMP production, melanocytes were treated with PGE_2 alone, or with the EP_2-receptor specific agonist butaprost (BP;100 nM). In some experiments, cells were treated with PGE_2 (1.5 μM) in the presence of the EP_2 or EP_4 receptor antagonists AH6809 (AH; 500 nM, 1 μM, 5 μM) and L 161982 (L; 25 nM, 50 nM, 100 nM) respectively. *The lack of a commercially available EP_4 receptor agonist prevented us from directly assessing the effect of E*P_4 *stimulation on cAMP.* Controls consisted of cells treated with AH6809 alone (5 μM)

or L 161982 alone (100 nM). Ten minutes after treatment with prostanoids or agonists/antagonists, cAMP levels were measured (Figure 3B). As expected, PGE_2 stimulated cAMP production compared with vehicle treated controls (78 pg/ml/cell × 10^{-6} vs. 3.5 pg/ml/cell × 10^{-6} respectively). Butaprost failed to stimulate cAMP production, and pre-treatment of cells with the EP_2 receptor antagonist AH6809 prior to the addition of PGE_2, failed to block PGE_2-dependent increases in cAMP. Higher doses of butaprost (up to 500 nM) also failed to elicit cAMP production (data not shown). In contrast, pre-treatment of melanocytes with the EP_4 receptor antagonist L 161982 resulted in a dose dependent blockade of PGE_2-dependent cAMP production in melanocytes, with complete loss of PGE_2-dependent cAMP response at a dose of inhibitor of 100 nM. These data show that EP_4 receptor signaling stimulates cAMP production in melanocytes.

Human tissues express eight isoforms of the EP_3 receptor that differ in their carboxyl terminal domain, and while most couple to G_i to inhibit cAMP production, some EP_3 isoforms couple to G_s (35–39). Because melanocytes express EP_3 receptor (23, 40), we also tested the effect of sulprostone (1 nM, 50 nM, 100 nM), a selective EP_3 receptor agonist, on cAMP production in melanocytes (Figure 3C). Sulprostone decreased cAMP in melanocytes in a dose dependent manner, completely eliminating detectable cAMP at a dose of 100 nM. These data show that the EP_3 and EP_4 receptor signaling have opposing effects on cAMP production in melanocytes in response to PGE_2.

Discussion

The effects of repetitive UVR on skin are complex, and a recent report indicates that repetitive doses of UVR are protective of subsequent UV-induced DNA damage in vivo (41). Another effect of repetitive UVR is increased pigmentation (tanning response) due to induction of paracrine factors by keratinocytes (42). PGE_2 is a lipid factor that is released by keratinocytes following UVR and in inflammatory conditions, such as wound healing and has multiple functions in the skin including proliferation, differentiation and immune function (43–48). PGE_2 binds to 4 distinct EP receptors with differing affinities for PGE_2, which stimulate multiple signaling pathways including cAMP/PKA (EP_2 and EP_4), Ca^{+2} mobilization (EP_1) and the inositol-triphosphate-diacyl glycerol pathway (EP_3; 49). In the present study we show that normal melanocytes with non-functional MC1R respond to repetitive low dose UVR with increased tyrosinase activity, suggesting the production of a potent, UVR-inducible autocrine factor, distinct from α-MSH or related melanocortins. *Melanocytes produce POMC-derived bioactive peptides that function independent of the MC1R, such as β-endorphin, which binds to the mu-opiate receptor in melanocytes. Therefore, we cannot exclude the possibility that some of the effects of UVR on tyrosinase activity in our MC1R mutants are due to the production of melanocortins that bind to receptors other than the MC1R* (50–53). Our previous studies have shown that $PGF_{2\alpha}$ is produced by melanocytes in response to UV irradiation, and stimulates tyrosinase activity, thus it is likely that some of the effects of tyrosinase activity in MC1 receptor mutants are due to autocrine production of $PGF_{2\alpha}$. However, in this report we show that PGE_2 production is also stimulated by UVR, and PGE_2 can be added to the short list of UV inducible autocrine factors for melanocytes. *Levels of PGE_2 and $PGF_{2\alpha}$ produced by melanocytes under unstimulated conditions are low and are similar (22 and present report). It is difficult to directly compare levels of PGE_2 and $PGF_{2\alpha}$ produced by melanocytes in response to UVR because our previous studies examining effects of UVR on $PGF_{2\alpha}$ production were performed using a solar simulator, which predominantly emits wavelengths in the UVA range. However, both are clearly inducible by UVR, and it is likely that PGE_2 and $PGF_{2\alpha}$ both contribute to melanocyte dendricity and tyrosinase activation. While PGE_2 and $PGF_{2\alpha}$ stimulate melanocyte dendrite formation and tyrosinase activation, PGE_2, in contrast with $PGF_{2\alpha}$, stimulates melanocyte proliferation, suggesting that PGE_2 may*

contribute to UVR-dependent melanocyte proliferation. While the amount of PGE_2 produced by melanocytes is quite small compared with keratinocytes, because prostaglandins are rapidly oxidized, localized synthesis of PGE_2 at the melanocyte cell membrane may result in efficient receptor binding and signaling, *however, it is likely that the* primary effect of PGE_2 on melanocytes occurs through paracrine production of PGE_2 by *keratinocytes. However, it is not known whether the regulation of prostaglandin synthesis in response to UVR differs between melanocytes and keratinocytes. Potential differences in activation of phospholipases, COX-2 enzyme, or PGE synthases in melanocytes and keratinocytes in response to UVR could result in selective production of prostaglandins by melanocytes at certain doses of UVR.* The rate-limiting step in eicosanoid production is the release of arachidonic acid from lipid membranes by $cPLA_2$ (7, 54). UVR stimulates $cPLA_2$ activation and synthesis in keratinocytes (28), and our data indicate that UVR stimulates the activation of $cPLA_2$ in melanocytes. *Two other phospholipases release arachidonic acid from cell membranes; the Ca^{+2} independent phospholipase A2 ($iPLA_2$), which has recently been shown to be expressed by melanocytes* (55), *and secretory phospholipase A2 ($sPLA_2$), which we have shown is expressed by keratinocytes, but not melanocytes* (56). *While $cPLA_2$ is the rate-limiting step in arachidonic acid release, and is inducible by UVR in both melanocytes and keratinocytes, it would be of interest to examine the role of $iPLA_2$ in UV-dependent prostaglandin synthesis in melanocytes.* $cPLA_2$ is activated by the MAP kinase p38, which is involved in the stress responses of keratinocytes and melanocytes (57–61). It will be of interest to determine whether stress-related MAP kinases (p38 and JNK) play a role in UVR-dependent $cPLA_2$ activation in melanocytes.

PGE_2 at nanomolar concentrations stimulated tyrosinase activation in melanocytes. Therefore, conditions in which PGE_2 is released, including exposure to UVR and inflammatory mediators, are expected to increase skin pigmentation in a PGE_2-dependent manner. While several signaling pathways modulate tyrosinase activity, the cAMP/protein kinase A pathway is the best characterized. PGE_2 stimulated cAMP in melanocytes, suggesting a likely mechanism for PGE_2-dependent tyrosinase activation. *We previously examined the effect of PGE_2 on cAMP response in melanocytes and found a small, yet statistically insignificant increase in cAMP in response to PGE_2* (40). *The lack of a significant change in cAMP in the previous report is most likely due to the presence of BPE in the media, which has been shown to suppress cAMP responses to growth factors in melanocytes* (18, 19). The amount of cAMP produced in response to PGE_2 varied from culture to culture; differences in cAMP levels could reflect different levels or activity of prostanoid receptors in individual cultures, or different activities of adenylate cyclase. The EP_2 and EP_4 couple to G_s to stimulate the synthesis of cAMP (34). In addition to G_s, the EP_4 receptor is coupled to the PI3 kinase/AKT pathway (62). Melanocytes express EP_2 and EP_4 receptors by Western blotting, suggesting they are potential candidates for PGE_2-dependent tyrosinase activation through cAMP production. We also examined the effect of EP_3 receptor stimulation on cAMP production, in melanocytes. The selective EP_2 agonist butaprost had no effect on cAMP production even at high doses, and pre-treatment of melanocytes with AH6809, a selective inhibitor of the EP_2 receptor, also failed to block PGE_2-dependent cAMP release, indicating that EP_2 does not mediate effects of PGE_2 on cAMP in melanocytes. *Because the EP_2 receptor is coupled to G_s in virtually all cell types examined, the lack of a response of melanocytes to EP_2 receptor agonists, suggests that the receptor identified by Western blotting is non-functional.* In contrast, the EP_4 receptor functionally couples to cAMP in melanocytes because pre-treatment of melanocytes with L 161982, a selective inhibitor of EP_4 receptor, completely blocked PGE_2-dependent cAMP production in a dose-dependent manner, even at high concentrations of PGE_2. *Unfortunately, because of the lack of a commercially available EP_4 receptor agonist, we are unable to test the effect of stimulation of EP_4 receptor on cAMP production.* Sulprostone, an EP_3 receptor agonist, lowered cAMP levels in melanocytes, showing that EP_3 and EP_4

receptor signaling has opposing effects on cAMP in melanocytes; the net effect of activating these two receptors by PGE_2 is increased cAMP production.

Our previous data shows that PGE_2 stimulates melanocyte dendrite formation through EP_3 receptors(23). Data presented here show that PGE_2 stimulates cAMP release, tyrosinase activity, and melanocyte proliferation. EP_3 and EP_4 receptors are high affinity receptors activated by nanomolar doses of PGE_2. Our data suggest that the relative levels of expression and/or signaling of EP_3 and EP_4 receptors may control tyrosinase activation in melanocytes in response to PGE_2 (Figure 4). EP_3 receptor activation is predicted suppress pigmentation (decreased cAMP) and stimulate dendrite formation (activation of PKCζ), whereas EP_4 receptor activation is predicted to stimulate pigmentation (increased cAMP), in response to PGE_2. However, because EP receptors stimulate multiple pathways, such as PI3-kinase, protein kinase C and MAP kinase (63), which regulate tyrosinase activity (64, 65), the contribution of EP receptor(s) signaling on tyrosinase activity is likely to be complex, and is currently being studied. Regulation of EP receptors by UVR has been demonstrated in murine epidermal keratinocytes in vivo and in vitro (66), and the FP receptor for $PGF_{2\alpha}$ is regulated by UVR in melanocytes in vitro and in human skin in vivo. Therefore, regulation of EP_3 and EP_4 receptors by UVR is a potential mechanism by which effects of PGE_2 on melanocyte pigmentation may be modulated. These data suggest the intriguing possibility that EP_3 and EP_4 receptors could be therapeutic targets in situations in which PGE_2 is released, including post-inflammatory hyper-pigmentation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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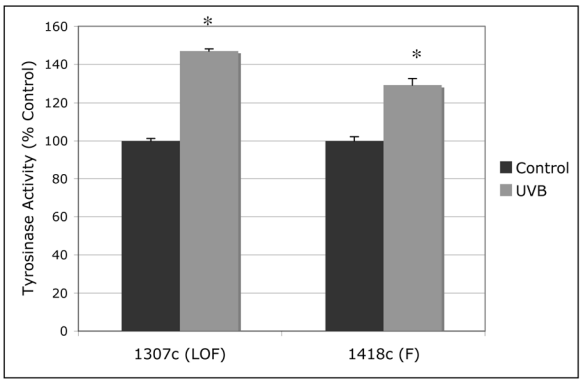

Figure 1A

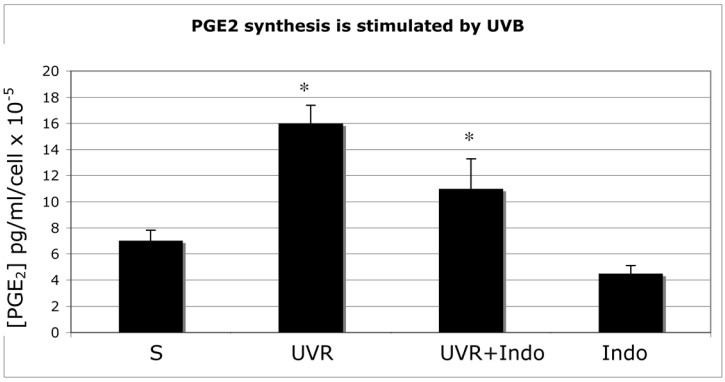

Figure 1B

Effect of UVR on $cPLA_2$ activation

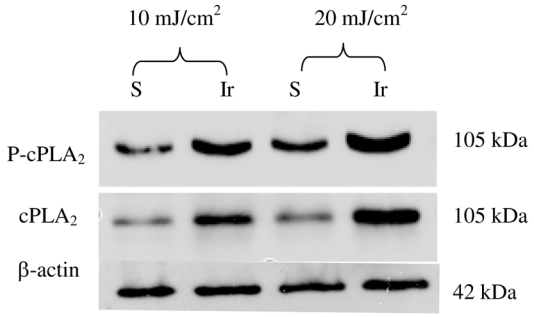

Figure 1C

Figure 1. Evidence that the UVR-induced autocrine factor is not α-MSH, and that UVR stimulates PGE_2 synthesis

A). Tyrosinase activity (represented as percent of controls) is increased in loss-of-function (LOF:1307c) MC1R mutants by UVR *(*p<0.005)*, indicating the presence of an autocrine factor that is not α-MSH stimulates tyrosinase activity in response to UVR. *Results are the average of four separate experiments performed on melanocytes cultured from MC1R mutant melanocytes (1307c) and melanocytes with confirmed functional MC1R (1408c) +/− standard error of the mean (SEM).*

B) Melanocytes showed a 2-fold increase in levels of PGE_2 in response to UVR, which was inhibited by indomethacin, indicating that COX enzymes are activated by UVR in melanocytes. *Differences in PGE_2 were statistically significant between irradiated and sham*

*irradiated cells, and between irradiated cells and cells irradiated in the presence of indomethacin (*p=0.12). Each bar represents the average amount of PGE_2 from 3 independent experiments, +/− SEM, in which each condition was performed in triplicate wells, and in which each experiment was performed using melanocytes cultured from a separate Caucasian donor (n=3).*

C) UVR induced the phosphorylation of $cPLA_2$ and increased levels of $cPLA_2$ protein in melanocytes. Results are representative of 2 experiments. For each experiment, *melanocytes cultured from a separate Caucasian donor were used (n=2).*

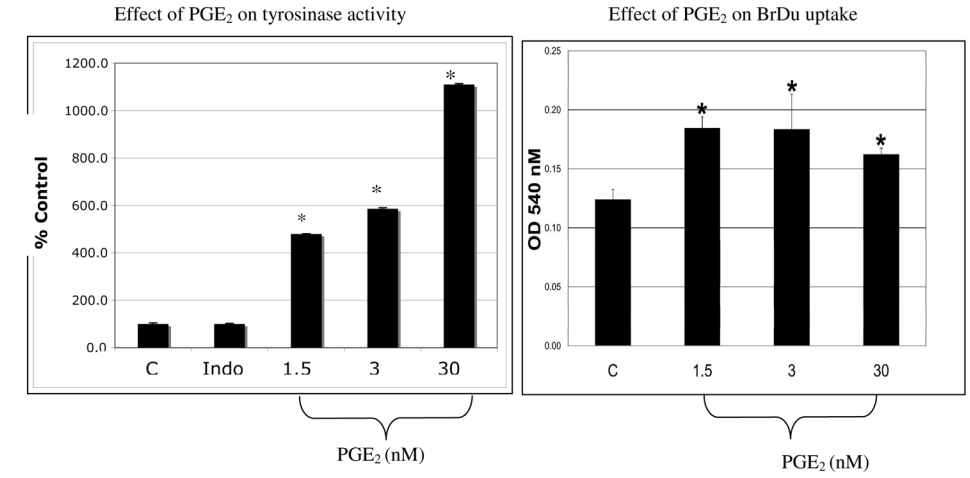

Figure 2A Figure 2B

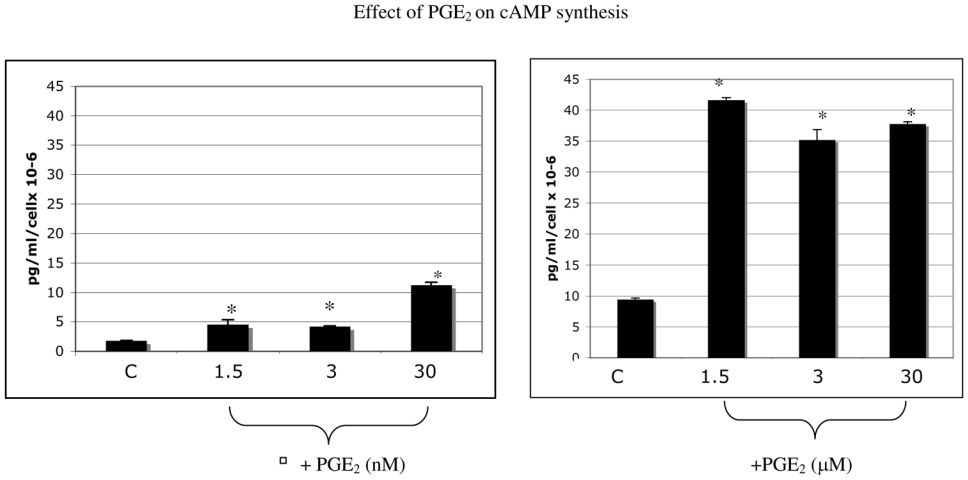

Figure 2C

Figure 2. PGE_2 stimulates tyrosinase activation, proliferation, and cAMP production

A) A dose-dependent increase in tyrosinase activity was observed with PGE_2 treatment. Results represent the average of three separate experiments in which triplicate dishes were analyzed, +/− standard error of the mean (SEM). For each experiment, *melanocytes cultured from a separate Caucasian donor were used (n=3).*

B) Melanocyte proliferation was stimulated by PGE_2 (*$p<0.001$) at each concentration tested. Each bar represents the average of 3 separate experiments +/− standard deviation (SD). *For each experiment, melanocytes cultured from a separate Caucasian donor were used (n=3).*

C) At doses as low as 1.5 nM, PGE_2 stimulated a statistically significant (*$p<0.05$) increase in cAMP compared with vehicle treated controls (4.4 pg/ml/cell × 10^{-6} compared with 1.8 pg/ml/cell × 10^{-6} respectively); a dose of 30 nM PGE_2 resulted in a cAMP response of 11 pg/ml/cell × 10^{-6}. Micromolar doses PGE_2 stimulated a maximum increase in cAMP of 42 pg/ml/cell × 10^{-6}, compared with vehicle treated cells. Each bar represents the averaged cAMP levels of 3 separate experiments +/−SD, in which each experiment was performed in duplicate wells. *For each experiment, melanocytes cultured from a separate Caucasian donor were used (n=3).*

Melanocytes express the EP_2 and EP_4 receptors

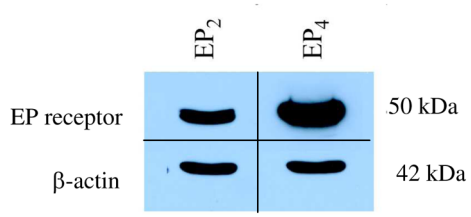

Figure 3A

EP_4 receptor signaling stimulates cAMP production in response to PGE_2

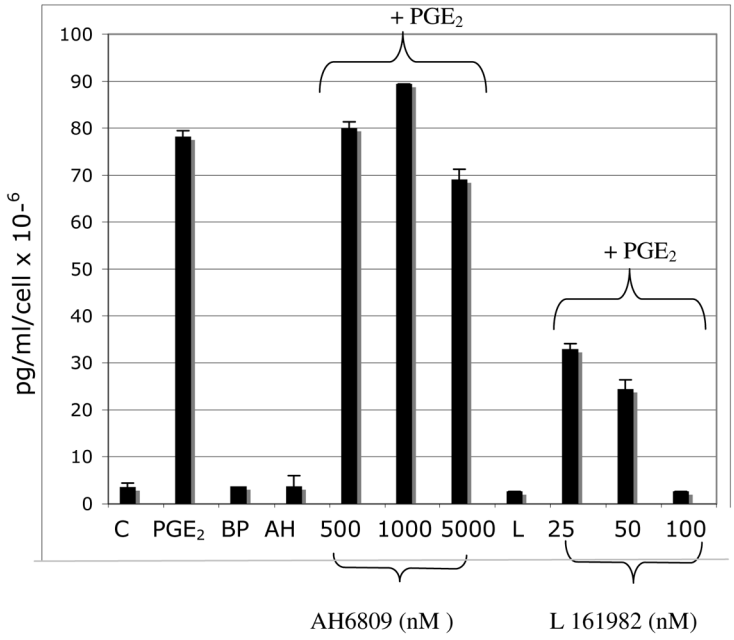

Figure 3B

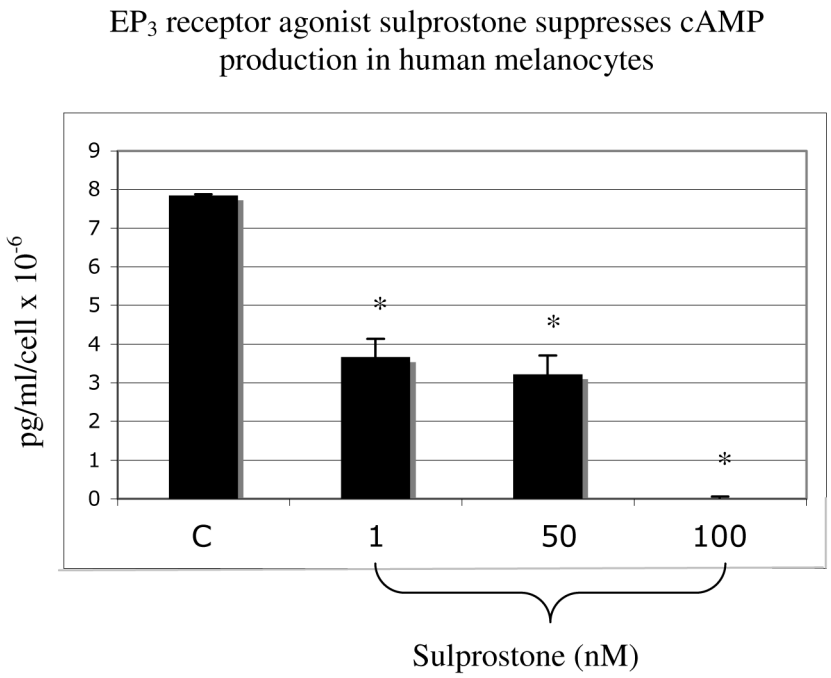

Figure 3C

Figure 3. EP_3 and EP_4 receptor signaling have opposing effects on cAMP production in melanocytes

A) Melanocytes maintained in −/− media for 4 days were lysed and resolved on 10% SDS-PAGE and blotted with antibodies against EP_2 and EP_4. Melanocytes express both EP_2 and EP_4, although steady state levels of EP_4 are higher. *Results are representative of blots on melanocyte cultures from three separate Caucasian donors (n=3).*

B) Treatment of melanocytes with PGE_2 (1.5 μM) stimulates cAMP production (78 pg/ml/cell × 10^{-6}), where as butaprost (“BP”, 100 nM), an EP_2 receptor agonist, had no effect on cAMP. Pre-treatment of melanocytes with the EP_2 receptor antagonist AH6809 (“AH”) failed to block PGE_2-dependent cAMP production, further confirming that EP_2 receptor signaling does not mediate effects of PGE_2 on cAMP. Pre-treatment of melanocytes with the EP_4 receptor antagonist L 161982 (“L”), blocked PGE_2-dependent cAMP production in a dose-dependent manner. Treatment with AH6809 (5 μM) or L 161982 (100 nM) alone had no effect on cAMP levels. *Results are representative of 2 separate experiments performed on melanocytes from a single Caucasian donor (n=2),* done in duplicate wells, +/−SD.

C) Treatment of melanocytes with sulprostone, an EP_3 receptor agonist, lowered baseline cAMP production in a dose dependent manner At a dose of 1 nM, sulprostone lowered cAMP from 7.8 pg/ml/cell × 10^{-6} to 3.6 pg/ml/cell × 10^{-6}; at a dose of 100 nM, sulprostone completely eliminated basal cAMP production. *Results are representative of 2 separate experiments performed on melanocytes from a single Caucasian donor (n=2),* done in duplicate wells, +/−SD.

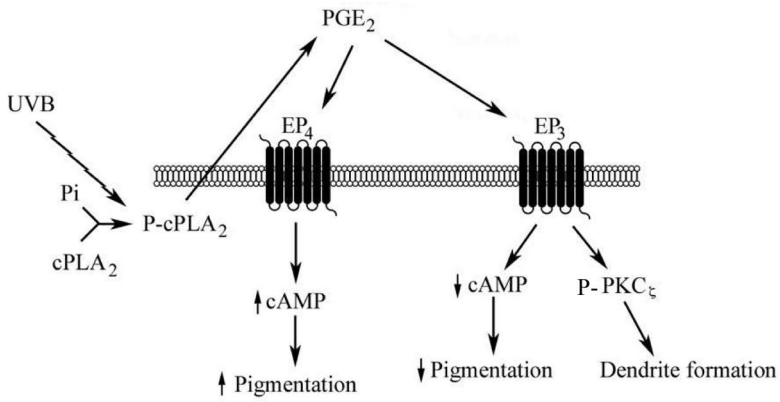

Figure 4. Effects of PGE_2 on melanocyte pigmentation are controlled by EP_3 and EP_4 receptors

UVR stimulates the activation of $cPLA_2$, the rate-limiting step in eicosanoid synthesis. Autocrine production of PGE_2, stimulated by UVR, activates the high affinity receptors EP_3 and EP_4. EP_3 receptor stimulation activates PKCζ, resulting in dendrite formation, and lowers cAMP levels; EP_4 receptor signaling increases cAMP release. The relative levels and or activity of EP_3 and EP_4 receptors is predicted to control the effect of PGE_2 on melanocyte tyrosinase activation and melanin synthesis, in part through modulation of cAMP levels.