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Prostaglandin E₂ regulates melanocyte dendrite formation through activation of PKC ζ

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Abstract

Prostaglandins are lipid signaling intermediates released by keratinocytes in response to ultraviolet irradiation (UVR) in the skin. The main prostaglandin released following UVR is PGE₂, a ligand for 4 related G-protein coupled receptors (EP₁, EP₂, EP₃ and EP₄). Our previous work established that PGE₂ stimulates melanocyte dendrite formation through activation of the EP₁ and EP₃ receptors. The purpose of the present report is to define the signaling intermediates involved in EP₁ and EP₃-dependent dendrite formation in human melanocytes. We recently showed that activation of the atypical PKC ζ isoform stimulates melanocyte dendricity in response to treatment with lysophosphatidylcholine. We therefore examined the potential contribution of PKC ζ activation on EP₁ and EP₃-dependent dendrite formation in melanocytes. Stimulation of the EP₁ and EP₃ receptors by selective agonists activated PKC ζ , and inhibition of PKC ζ activation abrogated EP₁ and EP₃-receptor mediated melanocyte dendricity. Because of the importance of Rho-GTP binding proteins in the regulation of melanocyte dendricity, we also examined the effect of EP₁ and EP₃ receptor activation on Rac and Rho activity. Neither Rac nor Rho was activated upon treatment with EP_{1,3}-receptor agonists. We show that melanocytes express only the EP_{3A1} isoform, but not the EP_{3B} receptor isoform, previously associated with Rho activation, consistent with a lack of Rho stimulation by EP₃ agonists. Our data suggest that PKC ζ activation plays a predominant role in regulation of PGE₂-dependent melanocyte dendricity.

Keywords

melanocyte; dendrite; prostaglandin; protein kinase C ζ

Introduction

Prostaglandin E₂ (PGE₂) is a lipid signaling intermediate produced by cyclooxygenation of arachidonic acid through the action of cyclooxygenase enzymes and PGE₂ synthase enzymes. In the epidermis, PGE₂ is the main prostaglandin produced by keratinocytes in response to ultraviolet irradiation (UVR), and mediates multiple cellular functions including immune regulation, cell growth and differentiation, and carcinogenesis [1–4]. PGE₂ mediates its effects through 4 G-protein coupled receptors (EP₁–EP₄) that are expressed in a cell-type specific fashion and activate diverse signaling intermediates [5,6]. Melanocytes are pigment-producing

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cells that provide photoprotection to the skin through the production and distribution of melanin to keratinocytes. Melanocytes are highly dendritic cells both in culture and in the skin in vivo, and the presence of dendrites is of critical importance for melanosome transfer to keratinocytes. Because melanocytes are a minority cell population within the epidermis, the presence of multiple dendrites facilitates transfer of melanosomes to keratinocytes that surround melanocytes. Previous studies from our laboratory have shown that nanomolar concentrations of PGE₂ stimulate melanocyte dendrite formation, and we identified the expression of 2 of the 4 PGE₂ receptors (EP₁ and EP₃) in melanocytes in vitro [7]. Through the use of EP receptor agonists, we showed that EP₁ and EP₃ receptor signaling stimulates dendrite formation in melanocytes [7]. The EP₁ receptor is a low affinity receptor for PGE₂ that couples to G_q to activate phospholipase C [8,9] and raises intracellular calcium. The EP₃ receptor, unlike the other PGE₂ receptors, has multiple isoforms (8 in human and 3 in mice) and couples to multiple small G-proteins, including G(i) (inhibition of adenylate cyclase), G_{βγ} (increase in intracellular Ca²⁺) and G₁₃ (activation of the small G protein Rho [10]).

Melanocyte dendrite formation is regulated through multiple signaling pathways stimulated by paracrine factors released by keratinocytes in response to UVR [11–13]. In human melanocytes the balance between Rac and Rho activation is a strong determinant of melanocyte dendrite formation [14–17]. Other important determinants of cytoskeletal remodeling in human melanocytes are the protein kinase C (PKC) signaling pathway [18]. Human melanocytes express α, β, δ, ε and ζ PKC isoforms [19,20]. We recently showed that the atypical PKC isoform PKCζ is a signaling intermediate for melanocyte dendrite formation [21]. PKCζ regulates mitogenesis, cell polarity and migration in other cell types and a recent study showed that PKCζ activation stimulates filopodia formation in motile cells [22–25]. Because of the importance of filopodia for melanosome transport and transfer in human melanocytes, PKCζ activation is likely to increase both dendricity and melanosome transfer to keratinocytes [26].

In this report we investigated the role of PKCζ and Rho-GTP binding proteins in PGE₂ dependent melanocyte dendrite formation. We show that both EP₁ and EP₃ receptor signaling activate endogenous PKCζ in human melanocytes but that PGE₂ does not regulate the activity of Rac or Rho in melanocytes. Of the EP₃ receptor has isoforms identified to date [27], only the EP_{3B} isoform has been associated with Rho activation [28]. Using reverse transcription polymerase chain reaction (RT-PCR) we show that melanocytes express only the EP_{3A1} isoform, and do not express EP_{3B}, consistent with the lack of effect of EP₃ receptor agonists on Rho activity. Because inhibition of PKCζ blocked EP₁ and EP₃ dependent dendrite formation, these data suggest that PGE₂, which is released in the skin following UVR, stimulates melanocyte dendrite formation through activation of PKCζ.

Materials and Methods

Reagents

PureCol was purchased from Inamed Biomaterials (Freemont, CA); the BCA Protein Assay kit was purchased from Pierce Chemical (Rockford, IL). Rabbit polyclonal antibodies to β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Sigma Co., (St. Louis, MO). EP receptor agonists (misoprostol and 17-phenyl trinor PGE₂; [17-PT PGE₂]) and the PKCζ pseudosubstrate inhibitor Myr-SIYRRGARRWRKL-OH were purchased from Calbiochem (San Diego, CA). EP₁ and EP₃ polyclonal antibodies were purchased from Cayman Chemicals (Ann Arbor, MI); monoclonal antibodies to phospho-PKC ζ/λ and to PKCζ were purchased from Cell Signaling Technology (Beverly, MA). Our previous work established that melanocytes do not express the λ isoform of PKC, consistent with prior reports [19,20], and therefore the phospho-PKC ζ/λ antibody used in the present study only detects phospho-PKCζ in melanocytes [21]. Streptavidin-horse radish peroxidase, biotin-

conjugated goat anti-rabbit and goat anti-mouse antibodies, amino-ethyl carbizol (AEC) and 3,3'-diaminobenzidine (DAB) were obtained from Vector Laboratories (Burlingame, CA). Silencing RNAs (siRNA) to PKC ζ directed against exon 4 (Cat # 51331) and exons 5 and 6 (Cat # 51331) were used in combination, and were purchased from Ambion Co (Austin TX), as was *Silencer* Negative Control #1 siRNA. Full range rainbow molecular weight markers were purchased from Amersham Life Sciences (Arlington Heights, IL). Chamber slides were obtained from Nalge Nunc International (Naperville, IL).

Cells and cell culture

Neonatal foreskins were obtained according to the University of Rochester Research Subjects Review Board guidelines and were the source of cultured human melanocytes. Human melanocytes were cultured in MCDB 153 supplemented with 1.5% fetal bovine serum (FBS), bovine pituitary extract (15 μ g/ml), phorbol ester (8 nM), basic fibroblast growth factor (1 ng/ml), insulin (5 μ g/ml) and hydrocortisone (500 ng/ml). Melan-a cells (immortalized murine melanocytes), were a gift of Dr. Dorothy Bennett and were cultured in Eagle's minimal essential medium supplemented with 25 mM NaHCO₃, 0.1 mM 2-mercaptoethanol, 5% fetal bovine serum, 1 mM sodium pyruvate, 100 nM tetradecanoyl phorbol acetate as described [31].

Melanocyte dendricity assay

Melanocytes were subcultured onto PureCol coated chamber slides (10⁵ cells) and were treated with misoprostol (an EP₃ receptor agonist; [32]) or 17-PT (an EP₁ receptor agonist; [32]) in the presence or absence of PKC ζ pseudosubstrate inhibitor for 48 hours. In some experiments, PKC ζ was silenced prior to treatment with misoprostol or 17-PT. Slides were fixed in 3.7% formalin/phosphate buffered saline and stained with Mayers hematoxylin (Sigma Co) and cover-slipped with mounting medium containing DAPI (Vector Laboratories). Digital photographs were obtained using a Spot Digital camera (Diagnostics Instruments, Sterling Heights, MI) and the number of dendrites per cell was determined for approximately 300 cells from each experiment. Each experiment was performed on three individual cultures derived from single donors.

Rac and Rho Activity Assays

Activated Rac and Rho were isolated from melanocyte cell lysates using a G-LISA Rac and RhoA Activation Assay Biochem Kits respectively (Cytoskeleton Inc., Denver, CO). Melanocytes (approximately 5 $\times$ 10⁵) were placed in basal media without growth factors for 2 hours and were then treated with 17-PT or misoprostol. Controls consisted of cells treated with diluent. Positive controls were performed for each experiment and consisted of GTP-bound Rho and GTP-bound Rac proteins and were provided by the manufacturer. Lysates (40 μ g) were aliquoted in duplicate onto ELISA plates pre-coated with Rho-GTP binding domain (Rhotekin) or Rac-GTP binding protein (p21 activated kinase binding domain) and activated Rho and Rac were determined according to manufacturer's instructions. Each experiment was performed at least 3 times on pooled cultures from 3 donors each.

Western Blotting

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 gels and blotted using standard procedures. Visualization of the immunoreactive proteins was accomplished with an enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL). Densitometry was performed using NIH 1.62 software and changes in levels of phosphorylated PKC ζ were normalized to actin.

Immunocytochemistry for EP₃ receptor and Melan-a in human skin

Skin sections were fixed in 3.7% formalin/PBS, embedded in paraffin, and 5 μ m sections were cut and deparaffinized using standard procedures. Endogenous peroxidase was quenched by incubation in 0.3% H₂O₂/distilled water. Nonspecific staining was blocked by incubation with Tris Buffered Saline (TBS)/0.1% bovine serum albumen (BSA) overnight at 4°C followed by incubation with rabbit polyclonal antibodies against EP₃ (1:100) in TBS/0.1% BSA for 2.5 hours at room temperature. Negative controls were treated with purified rabbit IgG (1/100; Sigma Co.) instead of the primary antibody. After washes in TBS/0.1% Tween-20 sections were incubated with biotin-conjugated goat anti-rabbit IgG antibodies followed by Streptavidin horseradish-peroxidase for 20 minutes each. The reaction was developed with AEC, and the slides were counterstained in Mayer's hematoxylin. Sections immediately adjacent were stained with mouse monoclonal antibodies against Melan-a (1:50; Dako Corp, Carpinteria, CA) to identify melanocytes using a similar protocol, except that antigen unmasking was performed at 120–123°C, slides were incubated for 45 minutes with the primary antibody and the secondary antibody was biotin conjugated goat anti-mouse IgG followed by Streptavidin horseradish-peroxidase for 20 minutes. The reactions were developed with DAB and the slides were counterstained with Mayer's hematoxylin. Negative controls consisted of slides incubated with mouse IgG instead of the primary antibody.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from melanocytes using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. Total RNA from human kidney was purchased from Stratagene (La Jolla, CA). Reverse transcription was performed using 0.75 μ g of total RNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed using BioRad IQ supermix (Hercules, CA). Primers used for amplification of EP₃ receptor isoforms (A1, A2, B, C, D, E and F) and β -actin are listed in Table 1. Conditions for amplification of the A1, C, E, F isoforms and β -actin were: 95°C, 3 min; 94°C 30 sec, 56°C, 30 sec, 72°C, 1 min (40 cycles) followed by 1 cycle at 72°C. Conditions for amplification of the A2, B and D isoforms were: 95°C, 3 min; 94°C 1 min, 60°C, 1 min, 72°C, 1 min (40 cycles) followed by 1 cycle at 72°C.

Statistical Analysis

Unpaired Student's t-test were performed to determine statistical significance between samples. A difference of <0.05 was considered to be significant.

Results

PGE₂ activates PKC ζ in human melanocytes through EP₁ and EP₃ dependent signaling

Activation of PKC ζ occurs through phosphorylation of its kinase domain that regulates its activity [33]. Therefore, to evaluate the effect of PGE₂ receptor stimulation on PKC ζ activation, melanocytes were treated with PGE₂ (30 nM) and total cell lysates were blotted for P-PKC ζ . Figure 1A shows a representative Western blot of melanocytes treated with PGE₂ and blotted for P-PKC ζ . Phosphorylation of PKC ζ occurred within 5 minutes and persisted at 90 minutes. Denistometry analysis showed an average 1.3 fold change in endogenous levels of P-PKC ζ at 5 and 30 minutes compared with control cells ($p < 0.022$ and $p < 0.05$ respectively; Figure 1B).

Melanocytes express the low affinity EP₁ and high affinity EP₃ receptors for PGE₂ in vitro [7], so we next examined the effect of EP₁ and EP₃ receptor stimulation on PKC ζ activation using agonists that activate these receptors. Melanocytes were treated with the EP₁ agonist 17-PT PGE₂ (25 nM) or the EP₃ agonist misoprostol (500 nM) for time points ranging from 5 to 45 minutes, and total cell lysates were blotted for P-PKC ζ (Figure 2A). The K_i (nM) of 17-PT

for the EP₁ and EP₃ receptors are 7.3 nM and 80 nM respectively [32,34]; the K_i of misoprostol for the EP₃ and EP₁ receptors are 319 nM and 38,000 nM respectively [34]. The levels of P-PKC ζ in untreated melanocytes varied from culture to culture, however, consistent phosphorylation of PKC ζ in response to both 17-PT PGE₂ and misoprostol was detected. PKC ζ phosphorylation occurred 30 minutes following treatment with 17-PT; PKC ζ phosphorylation was detected 15 minutes following treatment with misoprostol. A dose response analysis of 17-PT PGE₂ and misoprostol showed that activation of PKC ζ occurred at doses as low as 1 nM (Figure 2B). To determine if the effects of EP₁ and EP₃ receptor signaling on PKC ζ activation are additive, melanocytes were treated with misoprostol (1 nM) or 17-PT PGE₂ (25 nM) alone or in combination for 30 minutes (Figure 2C). As expected, 17-PT PGE₂ and misoprostol activated PKC ζ as seen by increased levels of P-PKC ζ , however, the combination of both 17-PT PGE₂ and misoprostol was not additive.

The EP₃ receptor activates Rho resulting in neurite retraction [28,35]. To determine whether EP₁ or EP₃ receptor signaling regulate levels of Rho activity in melanocytes, cells were treated with either misoprostol (10 nM) or 17-PT PGE₂ (25 nM) and levels of activated Rho were measured using an ELISA based assay (Figure 2D). EP₁ and EP₃ receptor agonists had no statistically significant effects on levels of activated Rho, although 17-PT PGE₂ did result in a modest (2 fold) but not statistically significant increase in levels of Rho-GTP at 5 minutes (Figure 2D). For each experiment, positive controls using recombinant GTP- Rho protein, provided by the manufacturer, was included. The EP₃ receptor has multiple isoforms (3 in murine cells and 7 in human cells [27]) and Rho activation has been linked to expression of the EP_{3B} isoform in PC12 cells [28]. Analysis of EP₃ receptor isoform expression showed a PCR product of the expected size for the EP_{3A1} isoform (Figure 2E). The EP_{3B} isoform was not expressed in melanocytes, but was detected in kidney RNA, which was used as a positive control (Figure 2E). None of the other EP₃ receptor isoforms examined (A2, B, C, D, E and F) were detected in melanocytes (data not shown).

We next analyzed the effects of 17-PT and misoprostol on Rac activation at doses ranging from 1–500 nM and at time points from 5 minutes to 30 minutes. Changes in Rac-GTP levels were not detected following 17-PT PGE₂ or misoprostol treatment (data not shown). For each experiment, positive controls using recombinant GTP-bound Rac protein, provided by the manufacturer, was included.

Inhibition of PKC ζ activation blocks the effects of EP₁ and EP₃ receptor stimulation of melanocyte dendrite formation

To determine if inhibition of PKC ζ activation blocks the effects of EP₁/EP₃ receptor stimulation on melanocyte dendrite formation, melanocytes were treated with misoprostol (1 nM) or 17-PT PGE₂ (25 nM) in the presence or absence of PKC ζ pseudosubstrate inhibitor for 2 days. We have previously shown that PKC ζ pseudosubstrate inhibitor blocks phosphorylation of PKC ζ in human melanocytes [21]. Indomethacin (3 μ g/ml) was present in the cultures at all times to prevent potential downregulation of prostaglandin receptors by endogenously produced prostaglandins. Controls consisted of cells treated with indomethacin alone, or with pseudosubstrate inhibitor alone. Both misoprostol and 17-PT PGE₂ induced the formation of dendrites in melanocytes with a 2.3 fold and 2.5 fold increase in the percentage of cells with greater than 2 dendrites respectively, compared with cells treated with indomethacin and inhibitor alone (Figure 3A). These results are consistent with our previous report on the effects of EP₁ and EP₃ receptor agonists on melanocyte dendrite formation [36]. PKC ζ pseudosubstrate inhibitor abrogated dendrite formation in response to misoprostol and 17-PT PGE₂, indicating that PKC ζ is a signaling intermediate involved in the effects of EP₁ and EP₃ receptor signaling in melanocyte dendrite formation. Figure 3B shows representative photographs of cells treated with 17-PT or misoprostol in the presence or absence of PKC ζ

pseudosubstrate inhibitor. Cells treated with 17-PT or misoprostol are highly dendritic compared with control cells. The addition of PKC ζ pseudosubstrate inhibitor blocked 17-PT and misoprostol induced dendrite formation.

To verify these results, PKC ζ was silenced and the effects of misoprostol and 17-PT PGE $_2$ on dendrite formation was assessed. Because the immortalized murine melanocyte cell line, melan-a, express about 10-fold more PKC ζ than human melanocytes (unpublished observations) melan-a cells were used for these studies. Melan-a cells share morphologic features with human melanocytes, including a highly dendritic phenotype. Using a combination of siRNA to exon 4 and exons 5 and 6 of PKC ζ , we achieved >75% knockdown of PKC ζ by 24 hours, that persisted at 72 hours (Figure 3C). Twenty-four hours following silencing of PKC ζ , cells were treated with either misoprostol (1 nM) or 17-PT PGE $_2$ (25 nM) in the presence of indomethacin and 2 days later dendricity was analyzed. Controls consisted of melan-a cells treated with scrambled siRNAs. Cells in which PKC ζ was silenced were bi-polar and non-dendritic in the presence of either 17-PT or misoprostol, where as cells treated with scrambled siRNA were highly dendritic in response to 17-PT and misoprostol (Figure 3D). Quantitation of dendricity showed that silencing of PKC ζ resulted in a 2-fold decrease in the effects of 17-PT and misoprostol on dendricity, compared with cells in which PKC ζ was not silenced ($p < 0.05$; Figure 3D).

Melanocytes express the EP $_3$ receptor in skin in vivo

The localization of the EP $_1$ and EP $_3$ receptor expression in the epidermis in vivo has been defined, however the expression of EP $_1$ and EP $_3$ in melanocytes has not been addressed. In murine and human skin EP $_3$ receptor expression is localized to the basal layer of the epidermis within keratinocytes [37,38]. To co-localize EP $_3$ receptor expression in melanocytes, contiguous skin sections were stained with the melanocyte specific antibody Melan-a and with antibodies to EP $_3$. Digital images were then merged to assess the presence of co-localization. EP $_3$ was strongly expressed in basal keratinocytes, and EP $_3$ co-localized with melanocytes (Figure 4). In murine and human skin EP $_1$ receptor expression is localized to the upper differentiated layers of the epidermis [38–40], with little or no localization to the basal epidermal layers where melanocytes reside. Immunocytochemical staining of skin sections for EP $_1$ receptor showed weak positive staining in the upper spinous layer of the epidermis, without expression in melanocytes (data not shown).

Discussion

The effects of prostaglandins and the cross talk between prostaglandin receptors and their signaling intermediates are of intense interest because of the diverse effects of prostaglandins on cell growth, differentiation and carcinogenesis [3,38,41–43]. While the effects of PGE $_2$ on epidermal keratinocytes are reasonably well understood [1,43–45], there has been much less investigation in the effects of PGE $_2$ on human melanocytes. Several years ago we set out to determine the biologic effects of PGE $_2$ on human melanocytes, and showed that nanomolar concentrations of PGE $_2$ stimulated melanocyte dendricity [7]. Melanocyte dendrites are important for prevention of cutaneous cancers because of the requirement for dendrites in the transfer of photoprotective pigment-laden melanosomes to keratinocytes. We identified the expression of two of the four EP receptors for PGE $_2$ on melanocytes in vitro (EP $_1$ and EP $_3$) and showed that signaling by these receptors could independently stimulate the formation of dendrites in melanocytes [7]. Prior reports have shown that the EP $_3$ receptor is expressed in the basal epidermal layer, where melanocytes reside [40] and our data shows that human melanocytes express EP $_3$ in vivo. The biologic relevance of EP $_1$ receptor expression by melanocytes in vitro is uncertain because EP $_1$ expression is restricted to the upper and differentiated layers of the epidermis, and is not present in melanocytes [40]. While it is possible

that UVR could upregulate expression of EP₁ in melanocytes, similar to the EP receptor [13], Tober et al., did not detect upregulation of EP₁ in murine skin following exposure to UVB [38,46]. Therefore, it is possible that EP₁ expression in melanocytes is secondary to cultivation in tissue culture.

We examined the effects of EP₁ and EP₃ receptor signaling on Rac and Rho activity because of the importance of Rho GTP binding proteins in dendrite formation in melanocytes [21,47, 48]. In neuronal cells, EP₃ receptor signaling activates Rho through coupling with G₁₃, resulting in neuron retraction [10]. In PC12 cells, the EP_{3B} isoform of the EP₃ receptor is linked to Rho [28]. There are multiple isoforms of the EP₃ receptor described in human cells [27], all of which regulate cAMP levels, and some of which signal through intracellular Ca²⁺. EP₃ receptor stimulation had no effect on Rho activation in melanocytes, suggesting that the EP₃ receptor does not couple to G₁₃ in melanocytes. A survey of EP₃ receptor isoforms expressed by melanocytes showed that melanocytes do not express detectable levels of the EP_{3B} receptor isoform, consistent with the lack of effect of EP₃ receptor stimulation on Rho activity levels. Of the 7 EP₃ receptor isoforms analyzed, only the EP_{3A1} isoform was detectable in melanocytes. We have shown previously that cAMP activates Rac resulting in dendrite formation in melanocytes. The lack of Rac activation in response to EP₁ and EP₃ receptor agonists is consistent with our prior observation that PGE₂ does not regulate cAMP levels in melanocytes [36].

The PKC ζ isoform of PKC stimulates cytoskeletal rearrangement in several cell types [18, 49,50]. In human melanocytes, lysophosphatidylcholine, which is released by the action of type IIa secretory phospholipase, activates PKC ζ and stimulates melanocyte dendrite formation [21]. In this report we show that PGE₂ activates PKC ζ through EP₁ and EP₃ receptor signaling and that PKC ζ activation directly contributes to EP₁ and EP₃ dependent dendricity. Because PKC ζ pseudosubstrate inhibitor blocked virtually all effects of PKC ζ on EP₁ and EP₃ dependent melanocyte dendrite formation, we conclude that PKC ζ activation is the primary mechanism underlying the effect of PGE₂ on melanocyte dendricity.

PKC ζ activation in response to lysophosphatidylcholine in melanocytes is upstream of Rac, and Rac activation is upstream of PKC ζ activation in other cell types [21,51–53]. Because our data show that PGE₂ has no effect on Rac activity, an alternative mechanism for PGE₂ dependent PKC ζ activation is operative in melanocytes. In keratinocytes, EP₃ receptor signaling stimulates keratinocyte differentiation through the release of ceramide [37]. Ceramide, a sphingolipid-derived second messenger molecule, has been implicated in growth inhibition, regulation of receptor expression, apoptosis, and cellular differentiation [54,55]. Ceramide is a direct activator of PKC ζ [50,56] therefore it is possible that activation of PKC ζ in response to PGE₂ is mediated through EP₃-dependent ceramide release in melanocytes. Other mechanisms by which EP₁ and EP₃ receptor signaling could activate PKC ζ include through stimulation of 3-phosphoinositide-dependent protein kinase (PDK1), or through TC10, that activates PKC ζ through the scaffold proteins Par3 and Par6 [57,58]. Finally, because UVR up-regulates the production and release of lysophosphatidylcholine and prostaglandins by keratinocytes [59–61], activation of PKC ζ in response to these paracrine factors may be additive and appears to be an important signaling intermediate in the dendritic response of human to melanocytes to UVR.

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Figure 1A

Figure 1B

Figure 1. PGE₂ stimulates the activation of PKCζ in human melanocytes

A) Human melanocytes were treated with PGE₂ (30 nM) and total cell lysates were prepared 5', 15', 30' and 90' later and resolved on 10% SDS-PAGE and blotted for P-PKCζ. Membranes were blotted for β-actin as a loading control. PKCζ was phosphorylated at 5 minutes and remained phosphorylated at 90 minutes. The blot shown is representative of 3 separate experiments on pooled cultures from at least 3 donors.

B) Quantitation of levels of P-PKCζ in response to PGE₂ by densitometry of the blots shows a maximum 1.3 fold increase in levels of endogenously phosphorylated PKCζ at 5 minutes following treatment with PGE₂. Each bar represents the averaged densitometry units of P-PKCζ bands (arbitrary units) normalized to β-actin from 3 separate experiments ±SD.

Asterisks indicate a statistically significant difference as determined by unpaired Student's t-test between control and treated groups with a $p < 0.05$ for each time point.

P- PKC ζ in melanocytes treated with 17-Phenyl Trinor PGE $_2$ or Misoprostol

Figure 2A

Figure 2B

Effect of combination of 17-PT PGE $_2$ and Misoprostol on P-PKC ζ

Figure 2C

Effect of 17-PT PGE₂ and misoprostol on Rho activation in human melanocytes

Figure 2D

Figure 2E

Figure 2. EP₁ and EP₃ receptor signaling activate PKCζ, but not Rho-GTP

A) A time course analysis of PKCζ phosphorylation was performed in melanocytes treated with 17-PT PGE₂ (25 nM) or misoprostol (500 nM). Cells were treated for the indicated times and total cell lysates were resolved on 10% SDS-PAGE and blotted for P-PKCζ. P-PKCζ was detected as a 76 kDa band in 17-PT PGE₂ treated cells at 30 minutes (arrow) whereas P-PKCζ was detected at 15 minutes and 30 minutes in cells treated with misoprostol (arrow). β-actin served as a loading control. Results are representative of 2 separate experiments using melanocytes from pooled cultures of 3 separate donors.

B) Cells were treated with the indicated concentrations of 17-PT PGE₂ or misoprostol for 30 minutes and total cell lysates were resolved on 10% SDS-PAGE and blotted for P- PKCζ. β-

actin served as a loading control. Results show that a dose as low as 1 nM is sufficient for phosphorylation of PKC ζ by 17-PT PGE₂ or misoprostol. Results are representative of 2 separate experiments using melanocytes from pooled cultures of 3 separate donors.

C) To determine if the effects of 17-PT PGE₂ and misoprostol on PKC ζ activation are additive, cells were treated with 17-PT PGE₂ (25 nM) or misoprostol (10 nM) alone or in combination for 30 minutes and total cell lysates were resolved on 10% SDS-PAGE and blotted for P-PKC ζ . β -actin served as a loading control. While both agonists increased levels of P-PKC ζ , the combined effect of both agonists was not additive.

D) Melanocytes were treated with either 17-PT PGE₂ (10 nM) or misoprostol (25 nM) for the indicated time points and levels of GTP-bound Rho were determined by ELISA assay. Shown is the averaged fold change in GTP-Rho compared with untreated cells from 3 separate experiments $\pm$ SD. A 2-fold but not statistically significant increase in GTP-Rho was seen at 5 minutes following treatment with 17-PT PGE₂. Levels of activated Rho were slightly decreased in cells treated with misoprostol, but this difference did not achieve statistical significance. For each experiment a positive control consisting of recombinant GTP-bound Rho (supplied by the manufacturer) was included.

E) RT-PCR of human melanocyte total RNA was performed with primers for the EP_{3A1} and EP_{3B} receptor isoforms. PCR products were resolved on a 1% agarose gel. A faint but detectable band of the expected size (526 kD) for the EP_{3A1} isoform was amplified from human melanocytes. For the EP_{3B} isoform, a PCR product of the expected size (900 kb) was detected in human kidney, but not in human melanocytes. NT=no template. Results are representative of 3 separate experiments.

Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 3. Inhibition of PKCζ activation blocks EP₁ and EP₃-dependent melanocyte dendrite formation

A) Melanocytes were treated with either 17-PT PGE₂ (25 nM) or misoprostol (1 nM) in the presence or absence of PKCζ pseudosubstrate inhibitor (Inh; 50 nM) for 48 hours. Indomethacin (Indo) was present in the media at all times to block endogenous prostaglandin production. Controls consisted of cells treated with indomethacin or inhibitor alone. After 48 hours the percentage of cells with more than 2 dendrites was determined in each group. Melanocytes treated with 17-PT PGE₂ or misoprostol showed a 2.5 fold and 2.3 fold increase in the percentage of cells with more than 2 dendrites respectively. The addition of PKCζ pseudosubstrate inhibitor blocked the induction of dendrite formation by both 17-PT PGE₂ and misoprostol. Results shown are the averaged data from three cultures from 3 different donors, $\pm$ SD. Asterisks indicate statistical significance ($p < 0.05$) between Indo and receptor agonist treated groups; # indicate statistically significance differences between agonists and agonist + inhibitor treated groups.

B) Representative photographs of melanocytes treated with 17-PT PGE₂ or misoprostol in the presence or absence of PKC ζ inhibitor (Inh). Cells treated with indomethacin (Indo) alone or inhibitor alone are bi-polar, whereas cells treated with 17-PT PGE₂ or misoprostol demonstrate complex branching that is eliminated in the presence of PKC ζ pseudosubstrate inhibitor. Bar=20 μ m.

C) Melan-a cells were silenced with siRNAs to PKC ζ and 24 and 72 hours total cell lysates were resolved on 10% SDS-PAGE and blotted for PKC ζ and β -actin. Controls consisted of cells incubated with scrambled siRNAs (SC) for 72 hours. siRNA's resulted in almost complete knockdown of PKC ζ at 24 hours that persisted at 72 hours.

D) Melan-a cells treated with either scrambled (SC) siRNAs or siRNAs to PKC ζ were treated with either 17-PT (1 nM) or misoprostol (25 nM) and dendricity was assessed 48 hrs after addition of agonists. Cells treated with SC siRNAs are highly dendritic in the presence of either 17-PT or misoprostol compared with cells treated with siRNAs to PKC ζ . Quantitation of dendricity showed that over 80% of cells exhibited more than 2 dendrites in the presence of either 17-PT or misoprostol. In contrast, cells in which PKC ζ was silenced showed a significantly ($p < 0.05$) reduced response to 17-PT or misoprostol. Results represent the average of three separate experiments $\pm$ SD.

Figure 4. Melanocytes express EP₃ receptor in vivo

Skin sections were stained with antibodies to Melan-a to identify melanocytes (asterisks) and contiguous sections were stained with antibodies to the EP₃ receptor. In EP₃ stained sections, EP₃ receptor expression appears to be concentrated in melanocytes (asterisks). Digital images were then overlaid to co-localize EP₃ receptor expression in melanocytes (arrows). Shown are three representative fields of the basal epidermal layer. The hatched line delineates the basement membrane zone. Bar= 10μm.

Table I

Sequences of primers used in this study

EP ₃ isoform	Forward	Reverse
A1	5'- AGT CAC CTT TTC CTG CAA CC -3'	5'- AGC TTC CAG TGA TGT GAT CC -3'
A2	5'- ATC GTC GTG TAC CTG TCC AAG C -3'	5'- TCA AAA TCC CAT CCA AGA AAC C -3'
B	5'- GAG AGC AAG CGC AAG AAG TCC -3'	5'- TTC CCC AAA ATT CCT CCT GG -3'
C	5'- AGT CAC CTT TTC CTG CAA CC -3'	5'- AGG AGC TAA TTT GCT GTA TTG AGC -3'
D	5'- GTC ATC GTC GTG TAC CTG TCC -3'	5'- TTC CCC AAA ATT CCT CTT GC -3'
E	5'- AGT CAC CTT TTC CTG CAA CC -3'	5'- TCC ATT CAC AAA CCC ATA ACC -3'
F	5'- AGT CAC CTT TTC CTG CAA CC -3'	5'- GAC AAA TCC AAG AAC CAA CG -3'
β-actin	5'- CAC GCACGA TTT CCC GCT CGG-3'	5'- CAG GCT GTG CTA TCC TGT AC-3'