Prostaglandin D2 Uses Components of ROS Signaling to Enhance Testosterone Production in Keratinocytes

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Elevated levels of prostaglandin D2 (PGD2) have been shown to be present in the bald scalp of androgenic alopecia (AGA) patients and to functionally inhibit hair growth. However, its precise mechanism in AGA has yet to be clearly defined. Although testosterone plays a critical role in the initiation and progression of AGA, the existence of a possible link between PGD2 and testosterone in skin has not been investigated. Here we show that human keratinocytes treated with PGD2 show enhanced capacity to convert the weak androgen, androstenedione, to testosterone. At the same time, treatment with PGD2 induced reactive oxygen species as indicated by generation of the lipid peroxidation product, 4-hydroxynonenal. To determine whether these two events are linked, we used the reactive oxygen species scavenger N-acetyl-cysteine, which blocked the enhanced testosterone production from PGD2-treated keratinocytes. Our study suggests the existence of a possible crosstalk between the PGD2-reactive oxygen species axis and testosterone metabolism in keratinocytes. Thus, we propose that AGA patients might benefit from the use of N-acetyl-cysteine or other antioxidants as a supplement to currently available or emerging AGA therapies such as finasteride, minoxidil, and PGD2 receptor blockers.

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INTRODUCTION

Androgenic alopecia (AGA) is a common form of hair loss affecting up to 80% of men and 42% of women (Blumeyer et al., 2011). The critical role of testosterone in the development of AGA has been well established, and current treatments include drugs such as finasteride, which inhibits 5-areductase 2, an enzyme that converts testosterone to dihydrotestosterone. Recent findings by Garza and Cotsarelis have implicated a role for prostaglandin D2 (PGD2), a pro-inflammatory lipid mediator, in the pathogenesis of AGA (Garza et al., 2012). In their study, PGD2 was found to be elevated in the bald scalp of AGA patients and negatively affected the growth of human and mouse hair. In contrast, levels of prostaglandin E2 (PGE2), which has been shown to increase hair growth in some models, were decreased in bald scalp (Geng et al., 1992; Sasaki et al., 2005). The same study also showed that mice overexpressing cyclooxygenase-2, an enzyme upstream of PGD2, in basal keratinocytes had elevated levels of PGD2 in the skin and exhibited many features of human AGA, such as hair follicular miniaturization and sebaceous gland hyperplasia. Based on this model, we decided to focus on the effects of PGD2 on testosterone synthesis in keratinocytes. Our findings suggest that PGD2 but not PGE2 enhanced conversion of androstenedione to its potent androgen derivative, testosterone. Furthermore, PGD2-treated keratinocytes exhibited increased levels of lipid peroxidation products, suggesting formation of reactive oxygen species (ROS). Finally, we show that PGD2-enhanced testosterone formation was mediated by ROS, because this action was blocked by the presence of the antioxidant N-acetyl-cysteine (NAC). Our work supports a possible link between PGD2 and testosterone in AGA and suggests that antioxidant therapies may benefit AGA patients.

RESULTS

PGD2 enhanced testosterone formation in keratinocytes

Because PGD2 has been found to be elevated in bald scalp, we sought to investigate its effects on primary human keratinocytes. Cells were treated with 10 μmol/L of PGD2, PGE2, or equal volume of DMSO for 24 hours. Cells were washed, and media containing the testosterone precursor, androstenedione, was added. The cells were allowed to incubate for 6 and 24 hours, and testosterone levels for each condition were determined as described in the Methods section (Figure 1). Our results from both time points suggest that PGD2- but not PGE2-treated keratinocytes exhibited enhanced production of testosterone compared with untreated cells (2.3-and 1.93-fold, respectively). We next sought to investigate the molecular mechanism by which this response was regulated.

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Abbreviations: 15d-PGJ2, 15-deoxy-delta-12,14-prostaglandin J2; 4HNE, 4-hydroxynonenal; AGA, androgenic alopecia; NAC, N-acetyl-cysteine; PBS, phosphate buffered saline; PGD2, prostaglandin D2; PGE2, prostaglandin E2

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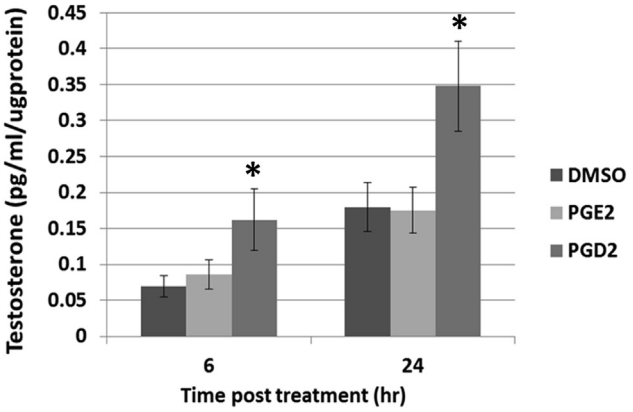

Figure 1. **PGD2 enhanced testosterone formation in keratinocytes.** Human keratinocytes were treated with 10 μmol/L of PGE2, PGD2, or equal volume of DMSO for 24 hours. Cells were washed and incubated with androstenedione (1 nmol/L) for the indicated times. Testosterone levels in supernatants were determined by ELISA, and results were normalized to total protein. Average of three experiments ± standard error of the mean are shown. *$P < 0.05$. hr, hour; PGD2, prostaglandin D2; PGE2, prostaglandin E2.

PGD2 treatment induced ROS in keratinocytes

PGD2 has a relatively short half-life in vivo and has been suggested to mediate some of its biological responses through the formation of its bioactive metabolites, some of which have been shown to induce ROS (Schuligoi et al., 2007; Shibata et al., 2002; Wang and Mak, 2011). Thus, we investigated whether PGD2 treatment induced ROS formation in keratinocytes under our experimental conditions. Cells were treated with 10 μmol/L of PGD2 or PGE2 for 24 hours and formation of 4-hydroxynonenal (4HNE), an indirect marker of ROS and oxidative stress (Liou and Storz, 2015), was assessed by means of immunofluorescence as indicated in the Methods section. Our results showed relatively strong immunoreactivity of the 4HNE antibody in PGD2-treated cells compared with PGE2-treated cells or nontreated controls (Figure 2). These data suggest that PGD2 but not PGE2 induced ROS and oxidative stress in keratinocytes.

PGD2-induced enhanced conversion of androstenedione to testosterone is blocked by NAC

We next sought to investigate a possible role of ROS in PGD2-induced testosterone formation in keratinocytes by scavenging ROS with NAC. Cells were incubated in the presence of 10 μmol/L of PGD2 alone or in combination with 20 mmol of NAC for 24 hours. Cells were washed then subjected to androstenedione for an additional 24 hours, and testosterone formation was assessed as indicated in the Methods section. We found that NAC completely abolished PGD2-induced testosterone enhancement, and its effects on testosterone levels at baseline were not significant (Figure 3). This suggests that enhanced testosterone formation after exposure to PGD2 in keratinocytes is mediated by ROS.

DISCUSSION

The role of prostaglandins in regulating hair growth and their dysregulation in AGA has been documented in the literature. For example, although PGE2 and PGF2α have been shown to stimulate hair lengthening in mice (Sasaki et al., 2005), PGD2 and its receptor CRTH2 have been implicated as negative regulators of hair growth (Garza et al., 2012; Nelson et al., 2013).

Testosterone is a critical factor in AGA development and progression. Our data support a possible existence of cross-talk between PGD2 and testosterone signaling in keratinocytes, which may have relevance to AGA. Here we show that PGD2-treated keratinocytes showed enhanced capacity to convert androstenedione, a weak androgen found to be elevated in AGA patients, to testosterone (Schmidt, 1994). Therefore, high PGD2 levels found in the bald scalp of AGA patients may indirectly lead to increased testosterone (which can be converted to dihydrotestosterone by 5α-reductases), a function that will further drive hair loss. Correlation between testosterone levels and the expression of the PGD2 synthase, LPGDS, has been shown in rats (Zhu et al., 2004). If such action has been preserved in human skin, a positive feedback loop may take place whereby increased testosterone levels drive LPGDS expression and PGD2 synthesis, which in turn further enhance testosterone synthesis from keratinocytes.

Although we didn't define the exact molecular mechanism by which PGD2 induced testosterone synthesis enhancement in keratinocytes, this effect was completely abolished in the presence of NAC, suggesting a ROS-driven function. PGD2 has a relatively short half-life, after which it is further converted to various bioactive derivatives (Schuligoi et al., 2007; Shibata et al., 2002). Induction of ROS by PGD2 treatment may be attributed to 15-deoxy-delta-12,14-prostaglandin J2 (15d-PGJ2), a spontaneous electrophilic metabolite of PGD2 that has been shown to mediate some of its biological actions via ROS (Shibata, 2015; Wang and Mak, 2011). In addition to PGD2,15d-PGJ2 has been also found to be elevated in bald scalp, and its inhibitory effects on hair growth were more profound compared with the parental prostaglandin (Garza et al., 2012). Another recent study implicated 15d-PGJ2 in AGA as treatment with 5 μmol/L of the cyclopentanone-induced apoptosis of cultured dermal papilla, outer root sheath cells, and follicular keratinocytes in cultured human hair follicles (Joo et al., 2016). Nevertheless, although it is tempting to speculate a direct involvement of 15d-PGJ2 in PGD2-induced testosterone synthesis enhancement in keratinocytes, further experiments are required.

Several studies provided evidence for oxidative stress and ROS in alopecia patients, suggesting a pathophysiological function in hair loss. Elevated levels of lipid peroxidation products were detected in the serum of patients of alopecia areata and correlated with disease severity (Abdel Fattah et al., 2011; Bakry et al., 2014). In another study, topical application of lipid peroxides resulted in an early onset of catagen and induced apoptosis of hair follicle cells (Naito et al., 2008).

Although such studies implicate oxidative stress in hair loss, other studies show that antioxidants promote hair growth. For example, the antioxidant epigallocatechin-3-gallate induced growth of human hair follicles and stimulated proliferation of dermal papilla cells (Kwon et al., 2007). Another recent study showed that mice treated with sulphoraphane, an activator of the major cellular antioxidant regulator NRF2, induced hair regeneration and reduced plasma levels of testosterone and dihydrotestosterone

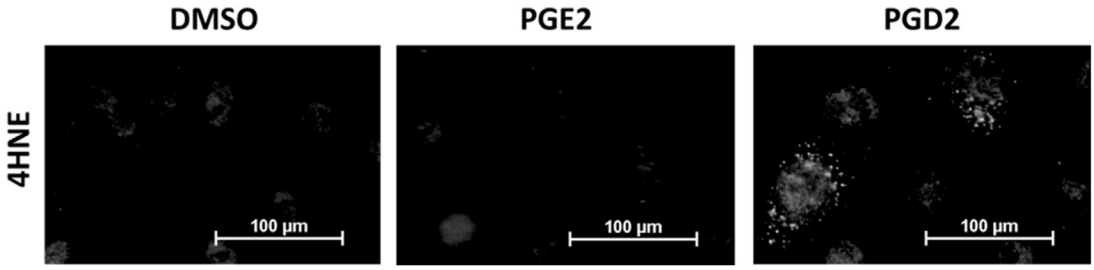

Figure 2. PGD2 induced ROS in keratinocytes indicated by formation of 4HNE. PGD2 induced accumulation of 4HNE in keratinocytes. Cells seeded on glass and treated with PGE2 or PGD2 (10 μmol/L) or equal volume of DMSO for 24 hours. Formation of the final lipid peroxidation product 4HNE was assessed by immunofluorescence. Representative figures are shown, $n = 3$. Scale bar = 100 μm. 4HNE, 4-hydroxynonenal; PGD2, prostaglandin D2; PGE2, prostaglandin E2; ROS, reactive oxygen species.

(Sasaki et al., 2016). Thus, although ROS and oxidative stress appear to negatively regulate hair growth, treatment with exogenous antioxidants or compounds that enhance cellular antioxidant function promote hair growth and may be useful in the treatment of hair loss disorders.

In summary, our study suggests the existence of crosstalk between PGD2 and testosterone, both of which are up-regulated in the bald scalp of AGA patients and inhibit hair growth. High concentrations of PGD2 in bald scalp enhance testosterone production from keratinocytes via a mechanism that includes ROS, a scenario that may overall support AGA progression. We show that this effect of PGD2 on testosterone synthesis in keratinocytes was blocked by the antioxidant NAC. Although additional studies are needed to determine if such interplay applies to the human scalp, our findings support a role for a PGD2-ROS axis in testosterone metabolism and suggest that NAC, or perhaps other antioxidants, may benefit AGA patients.

METHODS

Cell culture

Primary human keratinocytes (GM22251) were purchased from Coriell Institute (Camden, NJ). Cells were seeded on rat type IV collagen (BD Biosciences, San Jose, CA) precoated dishes and maintained in EpiLife keratinocyte growth medium supplemented with human keratinocyte growth supplements and penicillin and streptomycin as recommended (Thermo Fisher Scientific, Waltham, MA).

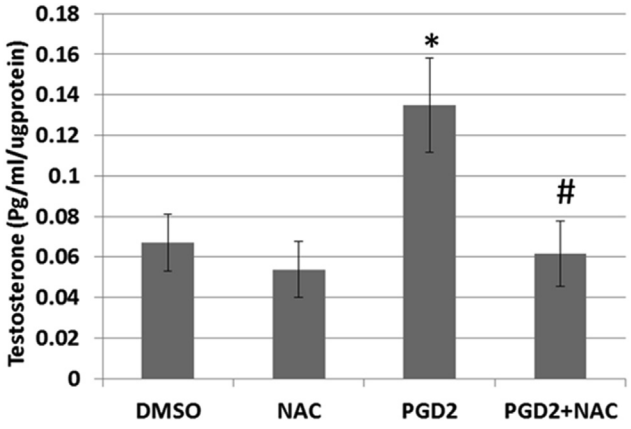

Figure 3. PGD2-induced testosterone formation is mediated by ROS. Keratinocytes were treated with PGD2 (10 μmol/L) alone or in combination with NAC (20 mmol/L) as indicated for 24 hours. Cells were washed and then treated with androstenedione (1 nmol/L) for an additional 24 hours. Testosterone formation was assessed in supernatants by ELISA, and results were normalized to total protein. Averages of three experiments ± standard error of the mean are shown. $^{*}P = 0.018$, $^{\#}P = 0.016$. NAC, N-acetylcysteine; PGD2, prostaglandin D2; ROS, reactive oxygen species.

Reagents

Prostaglandins (Cayman Chemicals, Ann Arbor, MI) were dissolved in DMSO. For all experiments, a final concentration of 10 μmol/L was used. N-acetylcysteine (Sigma-Aldrich, St. Louis, MO) was dissolved in distilled and deionized water as 200-mmol/L stock, pH = 7.4. Stock solution was prepared fresh before each experiment and was further diluted to a working concentration of 20 mmol/L. Androstenedione (gift from Alice Pentland, University of Rochester, Rochester, NY) was dissolved in DMSO and was used at a final concentration of 1 nmol/L.

Testosterone formation

Cells were seeded in type IV collagen-coated 6-well tissue culture plates and allowed to reach 90% confluence. Cells were treated in keratinocyte growth medium without supplements as indicated in each experiment.

Treatment medium was aspirated, and cells were washed three times in phosphate buffered saline (PBS). Cells were then incubated in keratinocyte growth media containing 1 nmol/L androstenedione for various times as indicated (final volume = 1.2 ml/well). Supernatant and protein were extracted and kept at −80 °C until used for analysis. Testosterone formation was assessed by testosterone ELISA (Cayman Chemical, Ann Harbor, MI) according to manufacturer's instructions. All experiments were performed in duplicate, and each duplicate was assessed three times on the ELISA plate. Results were averaged and normalized to total protein as determined by the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA).

Detection of 4HNE by immunofluorescence

Keratinocytes were seeded on type IV collagen-precoated glass coverslips and incubated overnight. Cells were treated with various prostaglandins in keratinocyte growth medium as indicated for 24 hours. Cells were washed three times in cold PBS and fixed in 4% paraformaldehyde in PBS for 15 minutes, followed by 10 minutes incubation in 0.1% triton X-100 at room temperature. Cells were briefly rinsed in PBS and blocked with 5% normal goat serum in PBS for 30 minutes at room temperature. Cells incubated overnight at 4 °C in primary antibody solution containing 1:200 anti-4 hydroxynonenal (#46545, Abcam, Cambridge, MA) in 1% bovine serum albumin in PBS. Cells were washed three times in PBS followed by 1-hour incubation with 488 Alexa Fluor-conjugated anti-rabbit IgG

diluted 1:500 in 1% bovine serum albumin in PBS. Cells were then washed three times in PBS, and coverslips were mounted in DAPI-containing mounting solution (Life Technologies, Grand Island, NY). Three representative images were acquired for each condition using Nikon Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

Institutional approval

Institutional approval was not necessary for this study.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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