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Induction of transforming growth factor-beta 1 by androgen is mediated by reactive oxygen species in hair follicle dermal papilla cells

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The progression of androgenetic alopecia is closely related to androgen-inducible transforming growth factor (TGF)-β1 secretion by hair follicle dermal papilla cells (DPCs) in bald scalp. Physiological levels of androgen exposure were reported to increase reactive oxygen species (ROS) generation. In this study, rat vibrissae dermal papilla cells (DP-6) transfected with androgen receptor showed increased ROS production following androgen treatment. We confirmed that TGF-β1 secretion is increased by androgen treatment in DP-6, whereas androgen-inducible TGF-β1 was significantly suppressed by the ROS-scavenger, N-acetyl cysteine. Therefore, we suggest that induction of TGF-β1 by androgen is mediated by ROS in hair follicle DPCs. [BMB Reports 2013; 46(9): 460-464]

INTRODUCTION

Androgenetic alopecia (AGA) is characterized by androgen-dependent progressive thinning and balding of scalp hair that follows a defined pattern in genetically predisposed subjects (1, 2). Moreover, androgen affects hair growth (3), and the balding scalps exhibit higher levels of 5-alpha reductase and elevated androgen receptor (AR) expression in dermal papilla cells (DPCs) of hair follicles compared to normal hair-bearing

scalps (4-6). In human hair follicles, it is generally accepted that androgen acts via the dermal papilla. Androgen regulates factors derived from DPCs, which are believed to influence the growth of hair follicle components (7-10). The role of androgens in hair growth regulation was previously reported in an in vitro co-culture system of human DPCs from AGA patients and keratinocytes (KC) (11). The study revealed that androgen-inducible transforming growth factor (TGF)-β1 secretion from balding DPCs inhibits epithelial cell growth.

Reactive oxygen species (ROS) are known to be profoundly increased in aged cells. In addition, high ROS levels, induced by oxidative stress, promote damage of cellular DNA, proteins, and lipids. Such damage can lead to cell-cycle arrest, cellular senescence, and even cellular death (12). Interestingly, the ROS hydrogen peroxide has been reported to induce TGF-β in epithelial cells (13). In addition, it was revealed that physiological levels of androgen exposure resulted in increased levels of ROS generation in androgen-sensitive human prostate cancer cells (14).

We postulated that androgen may play a significant role in regulating the ROS balance in DPCs, which, in turn, would influence the secretion of TGF-β1 and inhibit the proliferation of hair matrix epithelial cells. The aim of this study was to clarify the role of ROS in androgen-inducible TGF-β1 expression in DPCs. We investigated the effects of androgen on ROS production and on TGF-β1 secretion in DPCs, and then examined whether an antioxidant treatment could reverse the effects of ROS on androgen-induced TGF-β1 secretion by these cells.

RESULTS

Establishment of AR over-expressing rat DPCs (DP6-AR cells)
We transfected DP6 cells with rat AR cloning retroviral vector (p-Babe-rat AR-HA-puro). Western blot analysis revealed that AR was expressed on the DP6-AR cells, but not the control cells transfected with the puro vector only (data not shown).

Androgen markedly increased ROS generation by the DP6-AR cells

DP6-AR cells were incubated with 10^{-9} and 10^{-7} M R1881, and ROS generation was quantitatively measured by flow cytometry. ROS was generated by R1881 (Fig. 1A). Moreover, exposure to 10^{-9}-10^{-7} M of dihydrotestosterone (DHT) also increased ROS generation (data not shown). The results indicate that the DP6-AR cells generated ROS in response to androgen. We also demonstrated that the androgen-induced ROS generation was suppressed by the ROS scavenger NAC (Fig. 1A). Laser scanning confocal microscopy revealed that exposure to 10^{-7} M R1881 produced a significant increase in ROS in DP6-AR cells; the effect was similar to that of H_2O_2 and was inhibited by NAC (Fig. 1B).
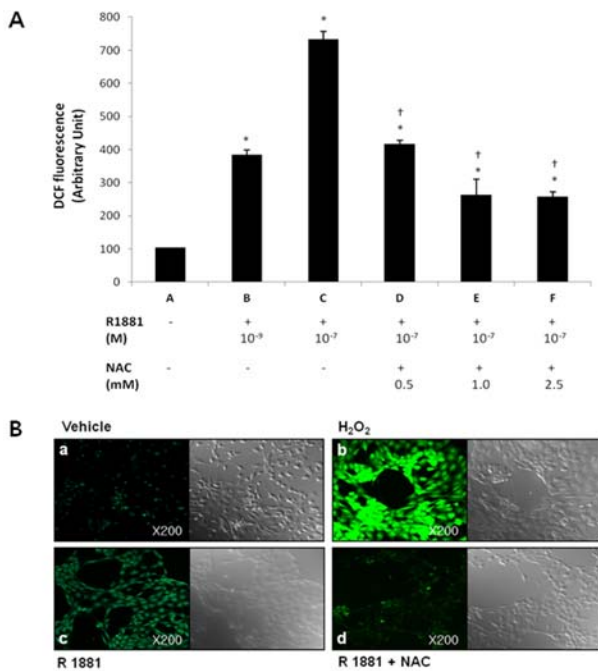
Fig. 1. Intracellular ROS production by DP6-AR cells stimulated with R1881, but then reduced following pre-treatment with NAC. (A) The amounts of ROS induced by R1881 were determined by FACS in DP6-AR cells (lane B, C). Pre-treatment with NAC at doses ranging from 0.5 mM to 2.5 mM for 2 h modulated ROS expression in response to 10^{-7} M R1881 in cultured DP6-AR cells (lane D, E, F). All results are the mean values of experiments performed in triplicate. Error bars indicate standard errors. ANOVA was performed at first and independent t-test was done for post hoc. *P < 0.05 versus control cells transfected with puro (mock) vector only (lane A). †P < 0.05 versus treatment with 10^{-7} M R1881 (lane C). ROS, reactive oxygen species; NAC, N-acetyl cysteine, a ROS scavenger; R1881, methyltrienolone, a synthetic androgen. (B) (a) Vehicle treatment. (negative control) (b) Treatment with 100 μM H_2O_2 (positive control) (c) Exposure of DP6-AR cells to 10^{-7} M R1881. (d) R1881 exposure after pre-treatment with NAC for 2 h.

### Androgen-inducible ROS augmented TGF-b1 secretion from DPCs

ROS has been reported to induce TGF-β in epithelial cells (13) and TGF-β1 is known to inhibit the proliferation of hair epithelial cells (11). To investigate the effects of oxidative stress, DP6-AR cells were treated with 10, 50, or 100 μM H_2O_2. The H_2O_2-treated DP6-AR cells showed 2-3-fold dose-dependent increases in the secretion of active TGF-β1 compared to the untreated cells (Fig. 2).

Androgen-induced TGF-β1 secretion was reversed by a ROS scavenger

Next, we measured the levels of active TGF-β1 in conditioned media after treating the cells with various concentrations of R1881 for 48 h. We found that the levels of active TGF-β1 dose-dependently increased at R1881 concentrations of 10^{-9}-10^{-7} M (Fig. 3). To determine whether ROS scavengers have suppressive effects on androgen-induced TGF-β1 regulation, we analyzed the effects of NAC on androgen-induced TGF-β1 secretion. Pre-treatment of DP6-AR cells with NAC at 0.25-2.5 mM for 2 h reduced the active TGF-β1 secretion when R1881 was added at 10^{-7} M compared to the control that was treated with only 10^{-7} M R1881 (Fig. 3). This result demonstrates that the ROS scavenger reversed the androgen-induced TGF-β1 secretion by the DPCs. These observations indicate that ROS is involved in the increased secretion of TGF-β1 from DPCs induced by androgen.

NAC suppressed the androgen-induced TGF-β1 secretion by the DPCs

We performed promoter assays using the luciferase reporter gene to investigate the suppression of TGF-β1 by a ROS scav-
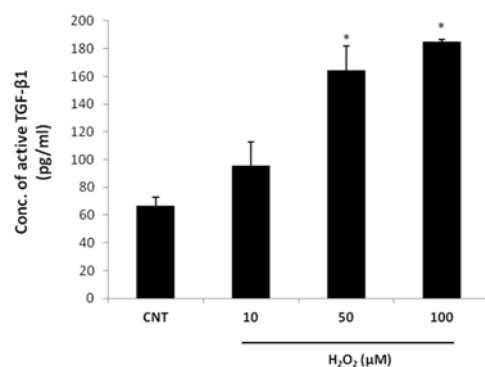
Fig. 2. Analysis of active TGF-β1 levels by ELISA after treatment with H_2O_2. The DP6-AR (5.0×10^5 cells/dish) cells were treated with H_2O_2 at 10, 50, or 100 μM for 24 h and the conditioned media were harvested. Concentrations of the active form of TGF-β1 in the conditioned media were measured using a specific ELISA. All results are the mean values of experiments performed in triplicate. Error bars indicate standard errors. ANOVA was performed at first and independent t-test was done for post hoc. *P < 0.05 versus control. CNT, control; TGF-β1, transforming growth factor-β1.
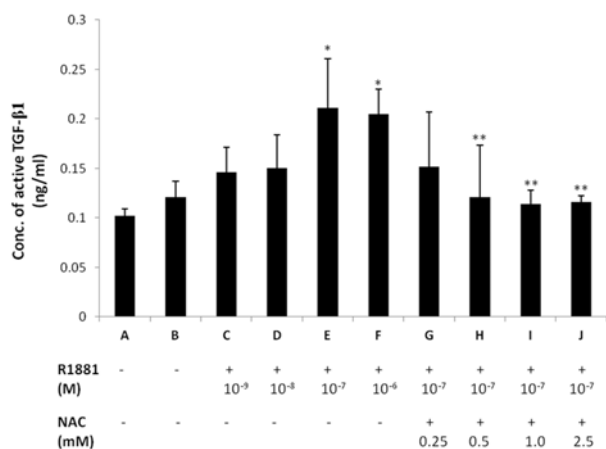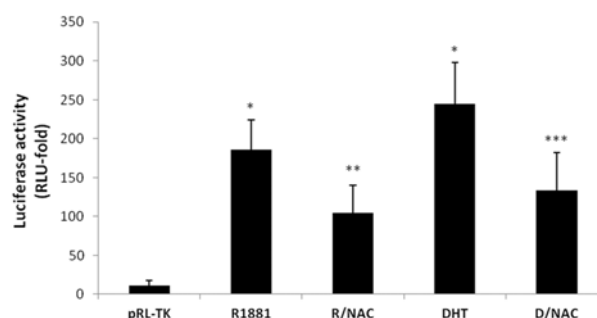
Fig. 3. Androgen-inducible ROS augmented the TGF-β1 secretion by dermal papilla cells. Increased active TGF-β1 secretions were observed after treatment with various concentrations of R1881 for 48 h (lane C, D, E, F). Comparatively, when the cells were initially pre-treated with NAC (0.25-2.5 mM) for 2 h, the active TGF-β1 secretions were reduced (lane G, H, I, J). Lane A represents the control cells transfected with the mock vector only. All results are the mean values of experiments performed in triplicate. Error bars indicate the standard errors. ANOVA was performed at first and independent t-test was done for post hoc. *P < 0.05 versus untreated DP6-AR cells (lane B). **P < 0.05 versus the control which was treated with only R1881 (lane E).

Fig. 4. Luciferase assays analyzing the TGF-β1 promoter levels after treatment with androgen and NAC. The DP6-AR cells were transiently transfected with the pGL3-TGF-β1-Luc reporter plasmid and the pRL-TK vector. First lane, pRL-TK vector only as a negative control; 2nd lane, 10^{-7} M R1881; 3rd lane, 10^{-7} M R1881 + 2.5 mM NAC; 4th lane, 1 nM DHT; 5th lane, 1 nM DHT + 2.5 mM NAC. At 48 h after treatment, the cells were harvested for the luciferase assays. The luciferase activities were measured using a luminometer. All results are the mean values of experiments performed in triplicate. Error bars indicate the standard errors. When the first, the second, and the third lane were compared, ANOVA was performed at first and independent t-test was done for post hoc. In comparison with the 2nd and the 3rd lane, or with the 4th and the 5th lane, independent t-test was done. *P < 0.05 versus a negative control. **P < 0.05 versus the control that was treated with only R1881. ***P < 0.05 versus the control that was treated with only DHT.

enger at the transcriptional level. DP6-AR cells co-cultured in the presence of 10^{-7} M R1881 or 10^{-9} M DHT stimulated the TGF-β1 promoter. Comparatively, luciferase reporter assays demonstrated that TGF-β1 promoter signaling was transcriptionally suppressed after the addition of 2.5 mM NAC in the presence of R1881 or DHT (Fig. 4). This observation suggests that the secretion of androgen-induced TGF-β1 in the DP6-AR cells is suppressed at the transcriptional level by pre-treatment with NAC. These results clearly demonstrate that antioxidants are able to block androgen-inducible TGF-β1 secretion from DPCs.

DISCUSSION

Balding scalp DPCs secret inhibitory factors that affect hair growth (15). In particular, the inhibitory role of androgens in keratinocyte growth was confirmed by Inui *et al.* (11). In addition, it has been reported that androgen-inducible TGF-β1 from balding DPCs is an inhibitory paracrine mediator in AGA (11, 16). Thus, it has been well established that the progression of AGA is associated with androgen and TGF-β1 levels.

Studies by Ripple *et al.* (14) showed that androgens are capable of enhancing oxidative stress in androgen responsive LNCaP prostate carcinoma cells. Thus, it appears that androgens may play a significant role in regulating the cellular redox state, particularly with respect to ROS homeostasis in epithelial cells. In addition, ROS has been reported to induce TGF-β in

epithelial cells (13), and therefore, androgens are involved in ROS generation, and ROS is related to TGF-β secretion. Moreover, these factors are involved in AGA. Consequently, we examined whether androgens are capable of increasing ROS production in dermal papilla cell lines, and if so, whether such mediation plays a role in AGA development.

During sub-cultivation of DPCs, sensitivity to androgens may be low because of the reduced expression level of AR (11). AR has been detected in the DPCs of human skin (5), and the DPCs of bald frontal scalps express higher levels of AR than those of non-balding occipital scalps (4). Therefore, to confirm our hypothesis, we used rat DPCs that over-express AR.

The ELISA and promoter assay showed that ROS induced TGF-β1 secretion in the DPCs, and that NAC inhibits androgen-induced TGF-β1 secretion at the transcriptional level (Fig. 2 and 4). Ascorbic acid, which is a well-known antioxidant, has been shown to stimulate DP cell growth and promote hair shaft elongation *in vitro* and *in vivo* in animal experiments (17). Moreover, green tea epigallocatechin-3-gallate, which is known to have potent antioxidant effects, was reported to promote hair growth (18). These findings, in conjunction with the present study illustrate the beneficial effects of antioxidants on hair growth, and offer a mechanistic basis for these effects.

In conclusion, the present study demonstrates that androgen-induced TGF-β1 regulation is mediated by ROS, and can be prevented by antioxidants in hair follicle DPCs. Moreover, our

findings suggest the potential use of antioxidant therapy for the treatment of androgenetic alopecia.

MATERIALS AND METHODS

ROS generation assay and confocal microscopy

Intracellular H_2O_2 and hydroxyl radical levels were determined by measuring 2',7'-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR) fluorescence. Non-fluorescent DCFDA is hydrolyzed to yield highly fluorescent dichlorofluorescein (DCF) in the presence of intracellular H_2O_2 and related peroxides.

After treatment with 10^{-9} and 10^{-7} M of the synthetic androgen, methyltrienolone (R1881; Sigma, St. Louis, MO), for 24 h, 1.0×10^6 DP6-AR cells were harvested, washed twice with PBS, resuspended in serum-free medium, and incubated with 10 μM DCF-DA in Hanks balanced salt solution (HBSS) at 37°C for 30 min. They were then washed with ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/saline and placed on ice. Cellular fluorescence was measured by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences, Mississanga, ON, Canada). As a positive control, 1.0×10^6 DP6-AR cells were treated with 100 μM H_2O_2 and then processed.

The cells were imaged using a Zeiss LSM 510 META confocal microscope ($200\times$; Carl Zeiss MicroImaging, Inc, Thornwood, NY). The green fluorescence of DCF was excited at 488 nm using an argon laser and detected at 527 nm. Laser attenuation, pinhole diameter, and photomultiplier sensitivity and offset were maintained for all experiments. For imaging, glass coverslips were mounted in a recording chamber filled with HBSS.

TGF-β1 determination by ELISA

The DP6-AR (5.0×10^5 cells/dish) were treated with 10, 50, or 100 μM H_2O_2 for 24 h, and the conditioned media were harvested. After pre-incubation with N-acetyl cysteine (NAC) for 2 h, 1.0×10^5 DP6-AR cells/6-well plates were treated with R1881 (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) for 48 h, and the conditioned media were harvested. The concentration of TGF-β1 in the conditioned media was measured using ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN), as previously described (19).

Others

Chemicals and reagents, as well as the protocols for the plasmid construction, cell culture, transfection, western blot, reporter gene assays, and statistical analyses are described in the supplementary materials and methods.

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