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Short Report

17α-estradiol induces aromatase activity in intact human anagen hair follicles *ex vivo*

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Abstract: For topical treatment of androgenetic alopecia (AGA) in women, solutions containing either estradiol benzoate, estradiol valerate, 17β- or 17α-estradiol are commercially available in Europe and some studies show an increased anagen and decreased telogen rate after treatment as compared with placebo. At present it is not precisely known how estrogens mediate their beneficial effect on AGA-affected hair follicles. We have shown recently that 17α-estradiol is able to diminish the amount of dihydrotestosterone (DHT) formed by human hair follicles after incubation with testosterone, while increasing the concentration of weaker steroids such as estrogens. Because aromatase is involved in the conversion of testosterone to estrogens and because there is some clinical evidence that aromatase activity may be involved in the pathogenesis of AGA, we addressed the question whether aromatase is expressed in human hair follicles and whether 17α-estradiol is able to modify the aromatase activity. Herewith we were able to demonstrate that intact, microdissected hair follicles from female donors express considerably more aromatase activity than hair follicles from male donors. Using immunohistochemistry, we detected the aromatase mainly in the epithelial parts of the hair follicle and not in the dermal papilla. Furthermore, we show that in comparison to the controls, we noticed in 17α-estradiol-incubated (1 nM) female hair follicles a concentration- and time-dependent increase of aromatase activity (at 24 h: 1 nM = +18%, 100 nM = +25%, 1 μM = +57%; 24 h: 1 nM = +18%, 48 h: 1 nM = +25%). In conclusion, our *ex vivo* experiments suggest that under the influence of 17α-estradiol an increased conversion of testosterone to 17β-estradiol and androstendione to estrone takes place, which might explain the beneficial effects of estrogen treatment of AGA.

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Key words: androgenetic alopecia – androgens – enzymes – hair loss – estrogens

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Introduction

Androgenetic alopecia (AGA) affects both sexes but the hair loss patterns are distinct (1). From the early studies by Hamilton we know that androgens play a crucial role in the onset and the progression of AGA (2,3). He showed that none of 20 men with prepuberal gonadal insufficiency developed AGA, whereas all four eunuchoids treated with testosterone developed the phenotype. Later it became clear that among the different androgens dihydrotestosterone (5α-DHT) is the principle pathogen for androgen sensitive hairs because individuals lacking the enzyme type 2 steroid 5 alpha-reductase (5αR), tend not to develop AGA (4–6). AGA can therefore be defined as a DHT-mediated process, characterized by continuous miniaturization of androgen-sensitive hair follicles (7,8). Hence, the aim of AGA treatment is to reverse or to stop the process of hair follicle miniaturization.

Abbreviations: , AGA: androgenetic alopecia; 5αR: 5α-reductase; DHT: dihydrotestosterone.

This can be accomplished by the inhibition of DHT formation or modulating DHT binding to the androgen receptor.

For topical treatment of AGA in women, solutions containing either estradiol benzoate, estradiol valerate, 17β- or 17α-estradiol are commercially available in Europe and some studies show an increased anagen and decreased telogen rate after treatment as compared with placebo (9,10). At present it is not precisely known how estrogens mediate their beneficial effect on AGA-affected hair follicles. In guinea pigs estrogens were shown to prolong anagen (11,12). Thus, estrogens might be able to stop or reverse AGA, but the exact success rates and the underlying mechanisms are unknown or open to question.

Recently, we have shown that 17α-estradiol is able to diminish the amount of DHT formed by human hair follicles after incubation with testosterone (13), while increasing the concentration of weaker steroids. These results suggested an estrogen-triggered induction of those steroidogenic enzymes that convert testosterone to weaker steroids. Because there is some clinical evidence that aromatase activity may be involved in the pathogenesis of AGA, and because aromatase metabolizes testosterone and androstenedione to the weaker steroids 17β-estradiol and estrone, we addressed the question whether 17α-estradiol is able to modify the aromatase activity in human hair follicles.

Materials and methods

Chemicals and media

(1β-^{3}H) Androst-4-ene-3, 17-dione was bought from New England Nuclear, Boston, MA, USA. Williams' E medium was purchased from BioWhittaker/Serva (Heidelberg, Germany) and L-glutamine, insulin, transferrin Triton X-100, NADPH, NADP, NAD, NADH, dextran-coated charcoal, trichloroacetic acid, normal goat serum and sodium selenite from Sigma (Deisenhofen, Germany). The Vectastain Elite ABC kit was purchased from Vector Labs, Burlingame, CA, USA. 17α-estradiol came from Galderma Laboratorium, Freiburg, Germany and the rabbit anti-human aromatase antibody was a generous gift of Dr N. Harada, Jujita Health University, Toyoake, Japan.

Preparation of human anagen hair follicles and volume measurement

Informed consent was obtained from 14 healthy volunteers (seven females/seven males, mean age 28 years) and excisional scalp biopsies were taken from the occiput under local anaesthesia. Two samples were processed on paraffin wax for immunohistochemistry and 12 samples were used for the analysis of aromatase activity. From these 12 intact samples, viable anagen hair follicles were isolated by microdissection as described previously (14). In brief, scalp specimens were placed in Williams' E medium supplemented with L-glutamine (2 mM), insulin (10 μg/ml), transferrin (10 μg/ml) and sodium selenite (10 ng/ml). Under a stereo-dissecting microscope, a scalpel blade was used to remove the epidermis and upper parts of the corium. Intact hair follicles were isolated carefully from the subcutaneous fat with a watchmaker's forceps by gently gripping the outer root sheath of the hair follicles and subsequent slight traction. Groups of three hair follicles were transferred to a 24-well culture dish, placed in 400 μl of Williams' E medium supplemented with L-glutamine (2 mM), insulin (10 μg/ml), transferrin 10 μg/ml) and sodium selenite (10 ng/ml), and digital images were taken at 10-fold magnification using an inverted microscope, a digital camera and the LUCIA M software version 2.995β (Nikon, Duesseldorf, Germany). The software was calibrated with an objective micrometer (Olympus, Tokyo, Japan). The hair follicle volumes were calculated with the 'VolumeEqCylinder' profile. These predefined calculation algorithms were provided by the LUCIA M-software. In this way it was possible to calculate aromatase activity in relation to the volume of hair follicles.

Incubation of human anagen hair follicles

The measured hair follicles were incubated with ^{3}H-1β-androstenedione with or without 17α-estradiol (1 nM, 100 nM, 1 μM) for 24 or 48 h, the culture supernatants were collected, the aqueous phase extracted and radioactive water, as an indicator of aromatase activity, counted. The aromatase activity was expressed in relation to the size (volume) of the examined hair follicle (fmol/mm^3).

Measurement of aromatase activity

Aromatase activity was assessed by measuring the (^{3}H) water produced by stereospecific release of tritium from the C-1-β position of androstenedione (15). Next, 4 μl of an ethanolic solution containing (1β-^{3}H) androst-4-ene-3, 17-dione [1 mCi/ml (37 MBq/ml; 15–30 Ci/mmol)] was added to 400 μl of Willams E medium containing three hair follicles. Incubation was performed in triplicate together with boiled blanks. The aromatase-catalyzed reaction was initiated by addition of 52 μg of a cofactor-mix (NAD, NADH, NADP, NADPH 13 μg each) and hair follicles were incubated for 24 or 48 h at 37°C. At the end of the reaction period, steroids were extracted by adding 1 ml of chloroform/methanol (1:2), mixing and harvesting the aqueous phase. This procedure was repeated three times. The precipitated proteins were sedimented by centrifugation for 10 min at 1500 *g*, and the resulting supernatant was collected. Remaining steroids were removed by adding 400 μl of a suspension containing 4% dextran-coated charcoal. After mixing and storing at 4°C for 15 min the mixture was centrifuged for 20 min at 12000 *g*. Next, 1 ml of the clear supernatant was taken for liquid scintillation counting of (^{3}H) water radioactivity. Correction for blank values was performed and aromatase activity was expressed in terms of fmol estrogen formed/mm^3/h. The results of all probands were grouped for female and male donors and were analyzed by a Wilcoxon/Kruskal–Wallis test for statistical significant differences.

Immunohistochemistry

Immunohistochemistry was performed using a rabbit anti-human aromatase antibody. For this purpose scalp samples from the occiput were fixed in 10% formalin and processed on paraffin wax. Tissue sections were processed by a standard protocol using the Vectastain Elite ABC kit. After deparaffinization and rehydration, tissues were rinsed in phosphate-buffered saline (PBS) containing 0.5% Triton X-100, blocked for 20 min at room temperature with 10% normal goat serum, and incubated

Table 1. Aromatase activity in intact human anagen hair follicles

	Incubation with 17-αestradiol													
	Control		24 h 1 nM		48 h 1 nM		24 h 100 nM		48 h 100 nM		24 h 1 μM		48 h 1 μM	
	female	male	female	male	female	male	female	male	female	male	female	male	female	male
Average (fmol/mm^3)	444.39	320.74	524.59	466.74	558.52	379.12	555.21	541.42	538.31	473.49	700.13	506.80	571.07	338.83
Change from baseline (%)			18.05	45.52	25.68	18.20	24.94	68.80	21.13	47.76	57.55	58.01	28.51	5.64

Direct measurement of aromatase activity was performed with intact human hair follicles as described in *Materials and methods*. Female-drived hair follicles express more aromatase activity as compared with male-derived hair follicles. 1 nM 17α-estradiol induces a concentration- and time-dependent increase of aromatase activity in female-derived hair follicles (e.g. 24 h: 1 nM = +18%, 100 nM = +25%, 1 μM = +57%; 24 h: 1 nM = +18%, 48 h: 1 nM = +25%). By contrast, male-derived hair follicles revealed a transient increase after 24 h of incubation with 17α-estradiol and during the next 24 h a drop of aromatase activity nearly to baseline levels.

with a 1:1000 dilution of anti-aromatase antiserum at 4°C for 18 h. The sections were subsequently rinsed with PBS and incubated with biotinylated goat anti-rabbit linking antibody for 30 min, and thereafter with an avidin–biotin–peroxidase complex (30 min). Immunostaining was visualized using the Vectastain Elite ABC kit. Normal rabbit serum was used as a negative control. All sections were counterstained with Mayer's hematoxylin.

Results and discussion

Women tend to develop AGA later in life and in a milder form than men. With the decline of serum estrogens during menopause many women show an accelerated progression of AGA. Estrogens may play a protective role against the development of AGA, because pregnant women with high levels of estrogens show a prolonged anagen phase, but lose their hair again post-partum (16). In Europe, topically applied estrogens such as 17α-estradiol are used to treat androgenetic alopecia. The female hormone 17β-estradiol can only be used with women, whereas the hormonally almost inactive isomer 17α-estradiol is used in men as well. Although some clinical studies show considerable success with such an approach, the underlying pathways of 17α-estradiol-induced hair regrowth are unknown. Apparently this is not a receptor-mediated hormone effect, as 17β-estradiol is a hormone and 17α-estradiol is not, and because 17α-estradiol cannot bind to estrogen receptors (17). Recently, it has been shown that hair follicles from women with AGA express more aromatase activity as compared with male-derived hair follicles (15), and interestingly those women taking aromatase inhibitors tend to develop AGA rather rapidly (18). These circumstantial lines of evidence indicate a role of aromatase in the pathogenesis of AGA.

In order to unravel the pathways of 17α-estradiol-mediated effects on the hair follicles, we measured aromatase activity in isolated intact human occipital hair follicles by incubating hair follicles with ^{3}H-1β-androstenedione with or without 17α-estradiol (1 nM, 100 nM, 1 μM) for 24 or 48 h. We were able to demonstrate that intact microdissected hair follicles from female donors express significantly more aromatase activity than male-derived hair follicles do (Table 1, Fig. 1). Our results are in line with those of Sawaya and Price who detected more aromatase activity in plucked female-derived hair follicles (15). Using immunohistochemistry (Fig. 2a–c), we also confirmed the results of other groups who detected the aromatase mainly in the epithelial parts (19) of the hair follicle (Fig. 2). However, some cells of the stalk region of the dermal papilla stained also for aromatase. Our results furthermore show that 17α-estradiol has indeed an effect on the measurable aromatase activity. In comparison to the controls,

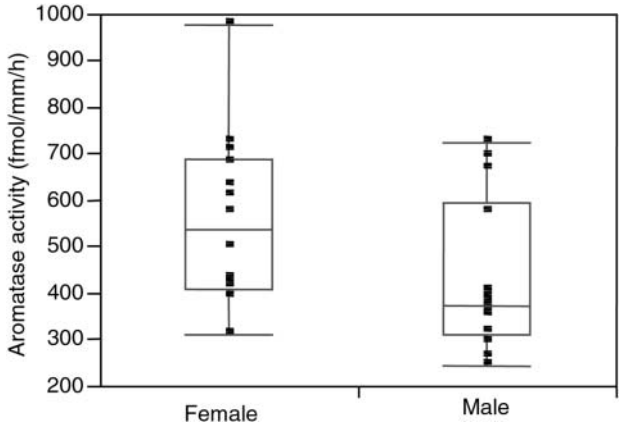

Figure 1. The results of all probands were grouped for female and male donors, were analyzed by a Wilcoxon/Kruskal–Wallis test and a statistical significant difference ($P = 0.032$) was observed. Hair follicles from female donors exhibited a median aromatase activity of 539 fmol/mm^3/h whereas male hair follicles 377 fmol/mm^3/h.

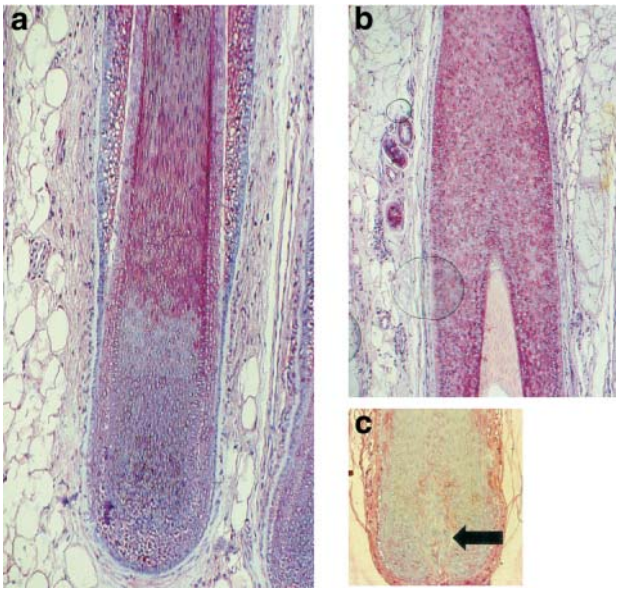

Figure 2.(a–c) Immunolocalization of aromatase within the root sheaths of human anagen hair follicles. Immunohistochemistry was performed as described in *Materials and methods*. The staining results show that aromatase can be detected mainly in the epithelial parts of the hair follicle (a, b), but some cells of the stalk region of the dermal papilla stained also for aromatase (*c*, arrow) (×400).

we noticed in 17α-estradiol-incubated (1 nM) female hair follicles a concentration- and time-dependent increase of aromatase activity (at 24 h: 1 nM = +18%, 100 nM = +25%, 1 μM = +57%; 24 h: 1 nM = +18%, 48 h: 1 nM = +25%) (Table 1). By contrast, male-derived hair follicles revealed a transient increase after 24 h of incubation with 17α-estradiol and during the next 24 h a drop of aromatase activity nearly to baseline levels.

In conclusion, our *ex vivo* experiments suggest that under the influence of 17α-estradiol an increased conversion of testosterone to 17β-estradiol and androstendione to estrone takes place in hair follicles derived from the occiput. In theory this pathway may diminish, at least in female-derived hair follicles, the amount of intrafollicular testosterone available for conversion to DHT. Because DHT is a major mediator of AGA, this pathway may explain the beneficial effect of 17α-estradiol on the development and progression of AGA. Whether such mechanisms take place in AGA-affected hair follicles *in vivo* and whether the detection of aromatase expressing cells in dermal papilla is of physiological significance is not known and should be investigated in the future. It is so far not known whether topically applied 17α-estradiol is able to target the hair follicle *in vivo* at sufficient concentrations. Because aromatase is mainly localized within the root sheaths of the hair follicle (Fig. 2a–c), this enzyme may be more easily accessible by a topical drug, when compared with the deeply situated dermal papilla. Hence, modulation of aromatase activity by topically applied estrogens is a so far unproven but likely possibility.

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