17β-Estradiol and ICI-182780 regulate the hair follicle cycle in mice through an estrogen receptor-α pathway

SANJAY CHANDA,1 C. LEE ROBINETTE,1,2 JOHN F. COUSE,3 AND ROBERT C. SMART1
1Molecular and Cellular Toxicology, Department of Toxicology, and 2Department of Anatomy, Physiological Sciences and Radiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27695; and 3Receptor Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

Estradiol (E2) applied topically twice weekly to mouse skin at doses as low as 1 nmol inhibited hair growth by blocking the transition of the hair follicle from the resting phase (telogen) to the growth phase (anagen). In contrast, application of ≥10 nmol of other steroids produced limited inhibition. Topical treatment with the estrogen receptor (ER) antagonist ICI-182780 reversed the effects of E2, and when applied alone, ICI-182780 caused a telogen-to-anagen transition. Both E2 and ICI-182780 were highly effective at their site of application but not at distant sites, indicating the direct rather than secondary systemic nature of their effects. Western analysis detected a 65-kDa ER-α immunoreactive dermal protein, and Northern analysis revealed the presence of a 6.7-kb ER-α mRNA. A ribonuclease protection assay confirmed the presence of ER-α transcripts but failed to detect ER-β transcripts. These findings implicate a skin-specific ER-α pathway in the regulation of the hair follicle cycle.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Although it appears that the topical application of E2 and ICI-182780 is directly producing their effects locally within the skin, it is also possible that these compounds are absorbed into the general circulation and are acting in a systemic manner to indirectly alter the hair follicle cycle. In support of such a notion are studies demonstrating that multiple subcutaneous injections of high levels of estradiol benzoate retard hair growth in rats (15) and mice (17) and that gonadectomy and adrenalectomy alter hair growth (17). In general, it has been difficult to sort out the action of local vs. systemic factors that regulate the hair follicle cycle. This is further complicated by the fact that numerous steroid-synthesizing enzymes, such as aromatase, 17b-hydroxysteroid dehydrogenase, 3a-hydroxysteroid dehydrogenase, and 5a-reductase, are expressed within the hair follicle (33). In addition, the androgen receptor is also expressed in skin, and androgens are considered to influence the hair follicle cycle (2). Thus the objectives of this study were to examine 1) the specificity and potency of E2 compared with other steroid hormones on the follicle cycle, 2) the potency of ICI-182780 compared with other ER antagonists, 3) whether ER-a and/or ER-b is expressed in mouse skin, and 4) whether the effects of topically applied E2 and ICI-182780 on the regulation of the hair follicle cycle are due to their local effects within the skin or to a secondary systemic effect resulting from their cutaneous absorption.

MATERIALS AND METHODS

Chemicals. All the chemicals, unless otherwise mentioned, were purchased from Sigma Chemical (St. Louis, MO). ICI-182780 was a kind gift from Zeneca Pharmaceuticals (Wilmington, DE).

Animals. Female CD-1 mice (5 wk old) with identical dates of birth were obtained from Charles River Laboratories (Raleigh, NC) and kept in our animal facility for ≈1 wk before use. They were fed rodent chow and water ad libitum, were kept on corncob bedding, and were placed on a 12:12-h light-dark cycle.

Animal treatment. Dorsal or both dorsal and ventral (depending on the experiment) surfaces were clipped once in the beginning of the study and from then on treated with the respective chemicals in 200 µl acetone or acetone alone twice weekly until full hair regrowth occurred. Full hair regrowth is defined as the complete growth of hair over the entire clipped dorsal surface. For the ovarioectomy study, mice were ovarioctomized as previously described (26) by use of halothane anesthesia. The mice were then maintained under halothane anesthesia. After the blood was allowed to clot at room temperature, the samples were centrifuged at 1,550 g for 15 min, and the serum was harvested for storage at −20°C until the assay was performed. A modification of the method previously reported by Cox et al. (9) was used for the RIA. Briefly, after extraction of 25–50 µl of serum once with 4 ml ethyl acetate (carbonyl free grade, Burdick Jackson, Muskegon, MI), the aqueous phase was frozen in a dry ice-methanol bath, and the ethyl acetate was decanted into a test tube for evaporation at 4°C for 20 h.

RIA. Blood for the RIA was collected by cardiac puncture while the mice were maintained under halothane anesthesia. The amount of estradiol in the samples was determined by the method of Lowry et al. (25). The samples were electrophoresed in an 8% polyacrylamide Novex (San Diego, CA) mini-gel, transferred to a polyvinylidene fluoride membrane, blocked with 5% milk, and then incubated overnight with the primary monoclonal antibody to ER-a (either H222 or H226, dilutions 1:2,500; generous gifts from Dr. Geoffrey Greene, University of Chicago). The membranes were washed and incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL; dilution 1:5,000) and finally, signals were detected by Amersham enhanced chemiluminescence detection reagent. H222 binds to an ER-a epitope within the carboxy F domain; H226 binds to an epitope within NH2-terminal transactivation domain of ER-a.

Ribonuclease protection assay. The ribonuclease protection assay (RPA) for ER-a and ER-b mRNA was carried out as previously described (8). Antisense riboprobes were generated from linearized templates by use of the MaxiScrip kit (Ambion, Austin, TX) and the incorporation of [32P]CTP (Amersham) according to the manufacturer’s protocol. The mouse ER-a antisense riboprobe protects a specific fragment of 366 nucleotides (nt), whereas the mouse ER-b antisense riboprobe protects a specific fragment of 262 nt. To equate loading among lanes, all reactions included an antisense riboprobe specific for mouse cyclophilin, generated from the template pT1- Cyclophilin (Ambion) and producing a protected fragment of 103 nt. For all RPA reactions, 5 x 10^4 counts/min (cpm) of each probe, a 1-µg sample RNA, and yeast tRNA (for a final total RNA equal to 25 µg) were mixed and ethanol precipitated at −70°C for 3 h to overnight. The resulting pellets were then processed through the RPA with the Hybspeed kit (Ambion) according to the manufacturer’s protocol. Final analysis of protected fragments was carried out by electrophoresis on a 1.5-mm thick 6% bis-acrylamide-8.3 M urea-1X TBE gel (National Diagnostics, Atlanta, GA), which was then fixed, dried, and exposed to Hyperfilm (Amersham) X-ray film.

Animals. Female CD-1 mice (5 wk old) with identical dates of birth were obtained from Charles River Laboratories (Raleigh, NC) and kept in our animal facility for ≈1 wk before use. They were fed rodent chow and water ad libitum, were kept on corncob bedding, and were placed on a 12:12-h light-dark cycle.

Animal treatment. Dorsal or both dorsal and ventral (depending on the experiment) surfaces were clipped once in the beginning of the study and from then on treated with the respective chemicals in 200 µl acetone or acetone alone twice weekly until full hair regrowth occurred. Full hair regrowth is defined as the complete growth of hair over the entire clipped dorsal surface. For the ovarioectomy study, mice were ovarioctomized as previously described (26) by use of halothane anesthesia. The amount of estradiol in the samples was determined by the method of Lowry et al. (25). The samples were electrophoresed in an 8% polyacrylamide Novex (San Diego, CA) mini-gel, transferred to a polyvinylidene fluoride membrane, blocked with 5% milk, and then incubated overnight with the primary monoclonal antibody to ER-a (either H222 or H226, dilutions 1:2,500; generous gifts from Dr. Geoffrey Greene, University of Chicago). The membranes were washed and incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL; dilution 1:5,000) and finally, signals were detected by Amersham enhanced chemiluminescence detection reagent. H222 binds to an ER-a epitope within the carboxy F domain; H226 binds to an epitope within NH2-terminal transactivation domain of ER-a.

Ribonuclease protection assay. The ribonuclease protection assay (RPA) for ER-a and ER-b mRNA was carried out as previously described (8). Antisense riboprobes were generated from linearized templates by use of the MaxiScrip kit (Ambion, Austin, TX) and the incorporation of [32P]CTP (Amersham) according to the manufacturer’s protocol. The mouse ER-a antisense riboprobe protects a specific fragment of 366 nucleotides (nt), whereas the mouse ER-b antisense riboprobe protects a specific fragment of 262 nt. To equate loading among lanes, all reactions included an antisense riboprobe specific for mouse cyclophilin, generated from the template pT1-Cyclophilin (Ambion) and producing a protected fragment of 103 nt. For all RPA reactions, 5 x 10^4 counts/min (cpm) of each probe, a 1-µg sample RNA, and yeast tRNA (for a final total RNA equal to 25 µg) were mixed and ethanol precipitated at −70°C for 3 h to overnight. The resulting pellets were then processed through the RPA with the Hybspeed kit (Ambion) according to the manufacturer’s protocol. Final analysis of protected fragments was carried out by electrophoresis on a 1.5-mm thick 6% bis-acrylamide-8.3 M urea-1X TBE gel (National Diagnostics, Atlanta, GA), which was then fixed, dried, and exposed to Hyperfilm (Amersham) X-ray film.

RIA. Blood for the RIA was collected by cardiac puncture while the mice were maintained under halothane anesthesia. After the blood was allowed to clot at room temperature, the samples were centrifuged at 1,550 g for 15 min, and the serum was harvested for storage at −20°C until the assay was performed. A modification of the method previously reported by Cox et al. (9) was used for the RIA. Briefly, after extraction of 25–50 µl of serum once with 4 ml ethyl acetate (carbonyl free grade, Burdick Jackson, Muskegon, MI), the aqueous phase was frozen in a dry ice-methanol bath, and the ethyl acetate was decanted into a test tube for evaporation at 4°C for 20 h.

RIA. Blood for the RIA was collected by cardiac puncture while the mice were maintained under halothane anesthesia. After the blood was allowed to clot at room temperature, the samples were centrifuged at 1,550 g for 15 min, and the serum was harvested for storage at −20°C until the assay was performed. A modification of the method previously reported by Cox et al. (9) was used for the RIA. Briefly, after extraction of 25–50 µl of serum once with 4 ml ethyl acetate (carbonyl free grade, Burdick Jackson, Muskegon, MI), the aqueous phase was frozen in a dry ice-methanol bath, and the ethyl acetate was decanted into a test tube for evaporation at 37°C in a dry heat block under a stream of nitrogen. The samples were then reconstituted with 200 µl PBS/gel buffer (0.01 M PBS, 0.1% gelatin, pH 7.0) and incubated overnight at 4°C with 200 µl antibody (diluted 1:1,500,000 with PBS-gel). On the next day, 100 µl of tracer diluted with PBS-gel (−8,000 cpm estradiol-6-(O-carboxymethyl)oximinol-2-[125i]iodohistamine; Amersham) were added and incubated at 4°C for 6 h. Dextran-coated charcoal (500 µl containing 0.05% dextran and 0.05% charcoal in PBS-gel) was added and incubated at 4°C for 45 min to adsorb the unbound hormone. Centrifugation at 1,550 g for 15 min was then used to precipitate the charcoal. The supernatant was decanted, and radioactivity was measured using a gamma counter (1272 Clinigamma, Wallac Instruments, Gaithersburg, MD). The amount of estradiol in the samples was determined from a standard curve that was generated using known amounts of estradiol ranging from 0.098 to 12.5 pg per tube. Extraction efficiency (generally >85%) was monitored by determining the amount of labeled estradiol that was recovered from representative serum samples after ethyl acetate extraction. The values reported have been corrected for these recoveries.
Northern analysis. RNA was extracted by the method of Chomczynski and Sacchi (6). Briefly, fresh tissue was homogenized in 4 M guanidine thiocyanate-25 mM lithium citrate-0.5% sarcosyl-0.12% β-mercaptoethanol-0.1% antifoam A (2 ml/0.1 g tissue). The homogenate was filtered through spectramesh (a macroporous polypropylene filter, 210 µm) into sterile polypropylene tubes to get rid of the hair residuals, and the volume was adjusted back to the original volume with the buffer. After this, 0.1 vol of 2 M sodium acetate (pH 4.0), 1 vol of diethyl pyrocarbonate water-saturated phenol, and 0.2 vol of chloroform:isoamyl alcohol (49:1) were added and vortexed for 15 s. The mixture was allowed to stand on ice for 15 min and then was centrifuged at 10,000 g for 20 min at 4°C (Sorvall RC-5 high-speed centrifuge with rubber adapter). The clear aqueous phase was then carefully taken, an equal volume of cold isopropanol was added, and the mixture was stored at −20°C for 2 h. Samples were then spun at 11,000 g for 20 min at 4°C. The pellet was then resuspended in homogenization buffer and stored overnight at −20°C with an equal volume of cold isopropanol to precipitate RNA. Next day the tubes were centrifuged for 20 min at 4°C. The pellets were then washed with 75% and then 95% cold ethanol and dried in a speed-vacuum centrifuge for 10 min. The RNA concentration was determined by ultraviolet absorption at 260 nm. Samples of total RNA and molecular weight markers (GIBCO-BRL, Gaithersberg, MD) were denatured and subjected to size fractionation by a 1% agarose-formaldehyde gel; the RNA was then transferred to a positively charged nylon membrane (Zeta Probe GT; Bio-Rad, Hercules, CA) by use of a Turbo Blotter System (Schleicher and Schuell, Keene, NH). The filter was baked at 80°C for 1 h. After prehybridization at 65°C for 10 min, the membrane was hybridized at 65°C overnight by use of a 32P-labeled mouse ER-α cDNA probe in a hybridization buffer containing 0.25 M sodium phosphate and 7% SDS. The [32P]cDNA probe was prepared by using a random priming kit from GIBCO-BRL with deoxy-[32P]CTP (New England Nuclear, Boston, MA). The specific activities of the cDNA probe were >106 cpm/µg. The filter was washed several times after hybridization with 0.1X standard sodium citrate (1X SSC: 0.15 mol/l sodium chloride and 0.015 mol/l sodium citrate)-0.1% SDS, and then autoradiographed on Kodak XAR-5 film (Eastman Kodak, Rochester, NY) with an intensifying screen at ~80°C for 36 h.

RESULTS

Effect of topical application of various steroids and ER antagonists on hair growth. The hair follicle cycle of mice is highly synchronized from birth to 12 wk of age, with fixed periods of anagen, telogen, and catagen. The second telogen phase in CD-1 mice begins at 6 wk and lasts until ~9 wk of age, at which time the hair follicles enter the third anagen synchronously. Topically applied E2 (10 nmol/200 µl acetone, twice a week) to mouse skin has been shown to block hair growth by arresting hair follicles in telogen, whereas 17α-E2 was without effect (29). To further determine the specificity and potency of E2, we examined the effect of equimolar doses of a variety of other steroid hormones on hair growth. E2, dihydrotestosterone (DHT), androstenedione, testosterone, or progesterone was applied topically twice weekly at a dose of 10 nmol/200 µl acetone vehicle to the clipped dorsal skin of CD-1 female mice beginning at 6 wk and continuing to the 17th wk of age. As shown in Fig. 1, vehicle control (acetone-treated) mice grew a full coat of hair by 13 wk of age, whereas mice treated with E2 did not demonstrate any hair growth. Compared with the acetone-treated control mice, testosterone treatment produced a slight inhibitory effect on hair growth, whereas DHT delayed full hair growth by 3–4 wk. Interestingly, androstenedione and progesterone stimulated hair growth. These results demonstrate that, of the steroid hormones examined, E2 is the most effective at blocking hair growth.

Twice weekly topical application of the ER antagonist ICI-182780 (10 nmol/200 µl acetone) has been shown to stimulate hair growth by causing telogen hair follicles to enter anagen (29). As shown in Fig. 1B, we compared the effect of equimolar doses of the ER antagonists ICI-182780, nafinoxidene, 4-OH tamoxifen, tamoxifen, and domiphen for their ability to stimulate hair growth. ER antagonists were applied topically twice weekly at a dose of 10 nmol/200 µl acetone vehicle to the clipped dorsal skin of CD-1 female mice beginning at 6 wk and continuing to the 17th wk of age. All of the ER antagonists stimulated hair growth to some extent (Fig. 1B), but none was as efficacious as ICI-182780. Lower doses of nafinoxidene, tamoxifen, and domiphen were not effective, and increasing the doses of these ER antagonists to 100 nmol/treatment abated the stimulatory effect and actually produced a slight inhibitory effect on hair growth (data not shown). Likewise, increasing the dose of 4-OH tamoxifen to 20 nmol also produced a slight inhibitory effect on hair growth. Tamoxifen has previously been shown to produce both estrogen-antagonist and -agonist responses in mice depending on dose and conditions of administration.
Our data are consistent with these earlier findings, in that divergent effects were noted. Appropriately selected low doses of tamoxifen and other antiestrogens that have a similar triphenethylene structure (nafoxidine, domiphen, and 4-OH tamoxifen) appear to be functioning as antagonists in skin similarly to the pure antagonist ICI-182780. However, higher doses of the triphenethylene antiestrogens appear to exhibit agonist activity in the skin. These results demonstrate that, in addition to ICI-182780, other ER antagonists can stimulate hair growth when administered under proper conditions; however, ICI-182780 is the most effective.

Dose-response relationship of E2 and ICI-182780 on hair growth, and effect of coadministration of ICI-182780 with E2. Dose-response studies were conducted to determine whether lower doses of E2 and ICI-182780 were capable of modulating hair growth. Different doses (1, 5, and 10 nmol) of E2 or ICI-182780 were applied topically in 200 µl acetone, twice weekly, to the clipped dorsal surface of 6-wk-old CD-1 female mice. As shown in Fig. 2A, the 1-nmol dose of E2 was found to be inhibitory to hair growth; however, hair growth inhibition was attenuated with time, and by 18 wk of age, the mice had developed a full coat of hair. In contrast, only 20% of the mice treated with 10 nmol E2 developed a full coat of hair by 21 wk of age. For the 5-nmol dose, 100% of the mice had a full coat of hair by 20 wk of age. As shown in Fig. 2B, a dose-dependent stimulation of hair growth by ICI-182780 was apparent at the beginning of the treatment period, but the dose dependency was attenuated at later time points. By 10.5 wk of age, all of the mice treated with all three doses of ICI-182780 developed a full coat of hair. To determine whether ICI-182780 could antagonize the inhibitory action of topically applied E2, mice were treated topically twice weekly with 1 nmol E2 along with 10 nmol ICI-182780 (ICI-182780 was applied first, and then E2 was applied after 1 h). As shown in Fig. 2C, 10 nmol ICI-182780 reversed the inhibitory effect of 1 nmol E2 on hair growth, further supporting the idea that the effects of E2 and ICI-182780 on hair growth are mediated through the ER.

ER-α but not ER-β is expressed in mouse skin. Using an immunohistochemical approach, Oh and Smart (29) demonstrated the presence of an ER immunoreactive protein in the nuclei of the dermal papilla cells of the mouse telogen hair follicle. In the present study, we detected a 6.7-kb ER-α mRNA in RNA isolated from 6-wk-old female mouse skin by using a mouse ER-α cDNA probe (Fig. 3A). In addition, Western analysis, using monoclonal antibody H222 to the carboxy-terminal F region of ER-α, or monoclonal antibody H226 to the NH2-terminal A/B transactivation region of ER-α, revealed a 65-kDa ER-α immunoreactive protein in mouse skin protein extracts from 6-wk-old mice (Fig. 3B). A protein of similar size was detected in uterine extracts with these antibodies (data not shown). These results demonstrate that ER-α is expressed in mouse skin. Next, we wanted to determine whether ICI-182780, E2, or the hair follicle cycle could alter the expression of ER-α protein. Six-wk-old female mice were treated topically twice weekly with acetone vehicle, 10 nmol ICI-182780, or 10 nmol E2 in 200 µl of acetone. At 8 wk of age, mice treated with ICI-182780 had entered anagen (data not shown), and skin protein extracts from these mice demonstrated decreased levels (>50% as demonstrated by laser densitometry) of ER-α H226 immunoreactive protein compared with the vehicle-treated mice, whereas the mice treated with E2 demonstrated 1.5-fold increased levels of the protein (Fig. 3C). At 10 wk of age, the hair follicles of the mice treated with acetone were in anagen (data not shown), and skin protein extracts from these mice had levels of the ER-α immunoreactive protein similar to those of the ICI-treated group, whereas the protein extracts from E2-treated mice demonstrated greater than 3-fold elevated levels of ER-α, and their follicles remained in telogen. These results indicate that the level of ER-α expression is modulated by E2 and ICI-182780 treatment and by the phase of the hair follicle. To determine whether ER-β is expressed in female mouse skin, we conducted an RPA on RNA isolated from mouse skin containing predominantly anagen or telogen hair fol-

![Fig. 2. Dose response of 17β-estradiol (E2) and ICI-182780 and effect of cotreatment on hair growth. Six-week-old mice with exact same date of birth (8 mice/group) were treated topically on the clipped dorsal surface twice weekly with indicated doses of the following substances in 200 µl acetone: A) acetone vehicle alone (○), 1 nmol E2 (+), 5 nmol E2 (○), or 10 nmol E2 (●); B) 10 nmol ICI-182780 (●), 5 nmol ICI-182780 (○), or acetone vehicle alone (○); and C) 10 nmol ICI-182780 and then 1 h later 1 nmol E2 (○), acetone vehicle alone (○), or 1 nmol E2 (●). The same group of control mice is represented in A, B, and C, and the same group of mice treated topically with 1 nmol E2 is represented in A and C.
licles. As shown in Fig. 4, RPA analysis of mouse skin RNA confirmed the presence of ER-α transcripts in RNA isolated from mouse skin containing predominantly telogen or anagen hair follicles; however, ER-β transcripts were not detectable in mouse skin. In addition, ER-β could not be detected using an ER-β-specific antibody and Western analysis (data not shown).

Effect of ventral application of 17β-estradiol and ICI-182780 on dorsal hair growth. To begin to determine whether the effects of E2 on hair growth are mediated within the skin itself or due to a systemic effect of cutaneously absorbed topically applied E2, we injected 1, 5, or 10 nmol E2 in corn oil intraperitoneally (ip) into 6-wk-old female mice twice weekly for 7 wk. Intraperitoneal administration of E2 had no inhibitory effect on hair growth. Previously, we reported that intraperitoneal administration of 10 nmol ICI-182780 at the same dosing frequency was also without effect on hair growth (29). These results suggest that the effects of E2 and ICI-182780 are produced locally within the skin; however, it is possible that these compounds are rapidly metabolized and/or eliminated after intraperitoneal administration. Therefore, in a further effort to determine whether E2 and ICI-182780 are functioning directly within the skin, 6-wk-old mice were treated topically twice weekly with either 1 nmol or 10 nmol E2 or 10 nmol ICI-182780 on the clipped ventral surface (Fig. 5). Hair growth was monitored on both the dorsal and ventral clipped surfaces of the treated mice, with the intention that if the effects were of a systemic nature, modulation of hair growth would be seen irrespective of the site of application. Application of 10 nmol of ICI-182780 promoted hair growth on the ventral surface (site of application) (Fig. 5A) but had no effect on the hair growth on the dorsal surface (Fig. 5B). E2 (1 nmol) inhibited hair growth on the ventral surface (Fig. 5A) but not on the dorsal surface (Fig. 5B). E2 (10 nmol) inhibited hair growth on both the dorsal and the ventral surfaces, but the inhibition of hair growth on the dorsal surface (site away from the site of application) was much attenuated compared with the ventral surface (site of application). Similar results were found.
in experiments in which E2 and ICI-182780 were applied on the clipped dorsal surface and hair growth was monitored on the clipped ventral surface (data not shown). Collectively, these results indicate that the hair growth-modulating effects of topically applied E2 and ICI-182780 are occurring locally rather than systemically.

Effect of ovariectomy on hair growth. It is evident that the topical application of exogenous E2 arrests hair follicles in telogen. Therefore, we wanted to determine whether removal of the ovaries, the major source of endogenous estrogen, would alter the hair follicle cycle. To this end, 6-wk-old female mice whose follicles were in the telogen phase of the hair follicle cycle were subjected to ovariectomy, and hair growth was monitored in this group and compared with the sham-ovariectomized group. As shown in Fig. 6, ovariectomy caused a profound and rapid telogen-to-anagen transition, with accompanying full hair growth. To determine whether the effect of ovariectomy on hair growth could be prevented by topical application of E2, ovariectomized mice were treated twice weekly with 1 nmol E2. As shown in Fig. 6, topical application of E2 prevented the hair growth in ovariectomized mice. Although this dose of E2 was without a systemic effect on hair growth in the dorsal-ventral experiment, we wanted to assure that cutaneous absorption of E2 did not result in high sustained serum E2 levels in ovariectomized mice. RIA analysis revealed that after topical application of 1 nmol E2, serum estradiol increased rapidly within 2 h to levels comparable with those found during proestrous in CD-1 mice but declined to a low physiological range by 12 h (Fig. 7). This rapid decline resulted in an abbreviated exposure to E2 compared with the normal cycling mouse (40). The uterus was capable of responding to these levels of estradiol with less than a half-maximal increase in wet weight (data not shown), whereas under similar topical dosing, hair growth was unaffected in the dorsal-ventral experiment. Therefore, these data further support the notion that the effect of E2 on the hair follicle cycle is due to its direct action in the skin.

DISCUSSION

Estrogen receptors, as detected by Scatchard analysis, have been found in human (14, 31) and mouse skin (38) and, on the basis of [3H]estradiol binding, are present in rat skin (36). Utilizing a monoclonal antibody (H222) to ER, we have previously found that an ER immunoreactive protein was extensively expressed in the nuclei of the dermal papilla of the telogen follicles in mice (29). These results provided direct evidence that the ER is expressed and that its expres-
Estrogen receptor-α protein expression in skin, increases the amount of ER-α expressed in numerous tissues (8, 32). Therefore, it was necessary to determine whether ER-α and/or ER-β is expressed in mouse skin. We found that monoclonal antibodies H226 and H222, directed toward either the NH2-terminal transactivation A/B domain or to the carboxy-terminal F domain of ER-α, respectively, recognize a 65-kDa protein in skin protein extracts. This 65-kDa size corresponds to the known size of the mouse ER-α protein (12, 16). In addition, Northern blot analysis of RNA isolated from mouse skin probed with a mouse ER-α cDNA revealed a 6.7-kb mRNA that corresponds to the size of the mouse ER-α mRNA (41). RPA analysis confirmed that ER-α transcripts are present in mouse skin and also demonstrated that ER-β transcripts are not detectable in mouse skin. These findings, coupled with the previous immunohistochemical localization studies, in which monoclonal antibody H222 detected an ER immunoreactive protein predominantly not exclusively in the nuclei of the dermal papilla cells, indicate that it is ER-α that is expressed in the nuclei of dermal papilla cells of mouse skin.

Earlier studies from our laboratory demonstrated that the topical application of estrogen to mouse skin blocks hair growth by arresting the hair follicle in telogen and that the topical application of the ER antagonist ICI-182780 initiates hair growth by causing the telogen follicle to enter anagen. Although these data are supportive of a skin ER pathway, it is also possible that the effects of topically applied E2 and ICI-182780 were due to a secondary systemic effect subsequent to dermal absorption. Our current results demonstrate that the ventral topical twice weekly application of either 10 nmol ICI-182780 or 1 nmol E2 produces hair growth effects at the site of application but not at remote sites away from the site of application. These results indicate that the effects of E2 and ICI-182780 on hair growth are mediated locally within the skin and are not due to a secondary systemic effect.

Furthermore, the fact that topically applied ICI-182780 can prevent the hair growth-inhibitory action of topically applied E2 provides additional evidence for a skin ER-α pathway that regulates the telogen-anagen hair follicle transition in mice.

We found that topical application of 1 nmol E2 to mouse skin resulted in a rapid increase in serum E2 levels that returned to low physiological levels within 12 h, indicating that E2 is rapidly absorbed through mouse skin and rapidly cleared from the serum. Whereas the serum levels of E2 are not sustained after topical application, a single E2 dose every 3–4 days is sufficient to maintain the hair follicle in telogen. Therefore, it appears that each E2 treatment has a prolonged local inhibitory effect on the hair follicle cycle. Perhaps E2, through ER-α, is inducing the expression of stable proteins that are sufficiently long-lived to block the telogen-anagen transition, or perhaps E2 is sequestered in the skin. We found that topical E2 treatment increases the amount of ER-α protein expression in skin, suggesting that E2 itself may be involved in the regulation of ER-α expression in skin. It is possible that this increase in ER-α protein is sufficient to induce or maintain the putative telogen regulatory signal. In support of this notion are results demonstrating that the levels of ER-α in skin are decreased in ICI-182780-treated mice as well as in the skin of control mice whose follicles have cycled into anagen. In addition, previous studies have demonstrated the absence of the ER immunoreactive protein in the dermal papilla of anagen follicles (29).

The fact that E2 and estrone are produced by the hair follicle itself (35) and that within the skin the ER is predominantly expressed in the dermal papilla (29) suggests that estrogen may function as a paracrine regulator of the hair follicle cycle. However, we found that ovariectomy does induce a rapid telogen-anagen transition with accompanying hair growth and that this effect can be blocked by twice weekly topical application of 1 nmol E2, a dose that has hair growth effects only at the site of treatment. These results suggest that the skin is dependent on serum estrogen or ovarian precursor steroids for the regulation of the telogen-anagen transition. However, it does not appear that the estrous cycle alters or controls the hair follicle cycle in mice. For example, 6-wk-old female mice are reproductively mature and actively cycling, yet the hair follicles of these mice are arrested in telogen from the age of 6 wk to 9 wk. Thus, over this 3-wk period, these mice have undergone ~5 estrous cycles, indicating that the hair follicle cycle is independent of the high and low levels of serum E2 that occur during proestrus and diestrus, respectively. Although it is possible that the effects of ovariectomy are due to events other than or in addition to the decrease in serum estrogen, it is clear that topical treatment with estrogen can prevent the effects of ovariectomy on hair growth. Because ER-α can be activated via an epidermal growth factor receptor pathway in a ligand-independent manner in reproductive tissues (18), additional pathways could be operative in the hair follicle as well. Collectively, our results suggest that estrogen production and uptake in skin are complex and that further investigation will be required to clarify the pathways that regulate the presence of dermal expression and link them with inhibition of hair growth.

Although androgens have been extensively studied as regulators of hair follicle growth and differentiation (34), estrogens have received comparatively little attention. Recently, inhibitors of 5α-reductase have hair growth-promoting properties, indicating an inhibitory role for DHT on hair growth (10, 21). Our current results in mice demonstrate that among the steroids examined, which included E2, testosterone, DHT, progesterone, and androstenedione, E2 is the most effective at blocking hair growth. Based on the hair growth-inhibitory action of 1 nmol E2, it appears that E2 is 10-fold more potent than DHT. ER-binding proteins have been identified in human skin (14, 31) and may represent potential targets for hair growth modulation by E2 or ICI-182780. Among the ER antagonists exam-
ined, ICI-182780 is the most potent at inducing hair growth. All of the ER antagonists examined promoted the telogen-anagen transition and hair growth to some extent; however, higher doses actually inhibited hair growth. The decreased activity of these ER antagonists, as well as their inhibitory action at higher doses, is consistent with the fact that these compounds are partial agonists of ER (20).

In summary, the growth and cyclicity of a hair follicle are controlled through complex and intricate interactions between the epithelial cells of the follicle and mesenchymal cells of the dermal papilla. Although it has been suggested that diffusible factors derived from the dermal papilla cells regulate the follicle cycle, the exact nature of these factors is still unknown. The identification of regulatory molecules such as estrogen and ER antagonists that modulate the telogen-to-anagen transition could allow for the identification of the downstream effectors of ER-α. Identification of the effector pathways may allow for the development of effective therapy to treat hair-related abnormalities like alopecia and hirsutism.

This research was supported by Grant ES-08127 (to R. C. Smart and C. L. Robinette) from the National Institute of Environmental Health Sciences and Grant CA-46637 (to R. C. Smart) from the National Cancer Institute.

Current address for S. Chanda: Cerus Corporation, 2525 Stanwell Drive, Suite 300, Concord, CA 94520.

Address all correspondence and requests for reprints to R. C. Smart, Molecular and Cellular Toxicology, Department of Toxicology, North Carolina State University, Raleigh, NC 27695-7633 (E-mail: rcsmart@unity.ncsu.edu).

Received 14 June 1999; accepted in final form 20 September 1999.

REFERENCES


