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Estrogen formation in stromal cells of adipose tissue of women: Induction by glucocorticosteroids

(aromatase/hormonal regulation/glucocorticosteroid receptors)

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ABSTRACT Stromal cells prepared from adipose tissue of women were maintained in monolayer culture to study the regulation of aromatase activity by hormones. Aromatase activity was stimulated 20- to 100-fold by dexamethasone at a concentration of 250 nM. Half-maximal stimulation of aromatase activity was attained at a dexamethasone concentration of 2.7 nM. The stimulatory effect of dexamethasone was apparent after a preincubation time of 4 hr, and stimulation was maximal after 24 hr of preincubation. The stimulatory effect of dexamethasone was observed only when fetal calf serum also was present in the culture medium. Of the various steroids tested, dexamethasone was the most potent in stimulating aromatase activity. Cortisol was less effective than dexamethasone, whereas corticosterone, at a concentration of 250 nM, caused only a small stimulation of aromatase activity. Progesterone and deoxycorticosterone (250 nM) did not affect aromatase activity. Cytosolic fractions prepared from stromal cells that had been maintained in monolayer culture were found to contain a homogenous population of sites that specifically bound [^{3}H]dexamethasone with relatively high affinity (K_d = 2.9 nM) and low capacity (38 fmol per mg of protein). The stimulatory effect of dexamethasone on aromatase activity was prevented by simultaneous incubation with cortisol 21-mesylate (0.1–10 μM), a compound known to block the binding of glucocorticosteroids to cytoplasmic receptors. The stimulatory effect of dexamethasone also was prevented by incubation of the cells with cycloheximide or actinomycin D. These findings are suggestive that glucocorticosteroids act to increase aromatase activity in stromal cells by inducing the synthesis of new enzyme protein.

In postmenopausal women the principal circulating estrogen is estrone that is formed almost exclusively in extraglandular tissues, primarily in adipose tissue (1). The substrate for aromatization by such adipose tissue is plasma androstenedione (1–5), which originates principally by adrenal secretion. The transfer constant of conversion of plasma androstenedione to estrone is increased greatly in obese postmenopausal women (6), as is the incidence of endometrial carcinoma (6–8). The capacity of human adipose tissue to convert androstenedione to estrone *in vitro* has been demonstrated by using tissue slices and isolated fat cells (9–13). In a recent study, in which human adipose tissue was separated into adipocytes and stromal cells by collagenase digestion followed by differential centrifugation, we found (14) that aromatase activity was present principally in stromal cells, whereas the adipocytes contained only a small fraction of the total activity of adipose tissue. Such stromal cells grow to confluence in monolayer culture and maintain a fibroblast-like appearance.

To define the factor(s) that regulates estrogen formation in adipose tissue, we employed stromal cells prepared from adipose tissue of women as a model system for the evaluation of the effects of various hormones upon the capacity of these cells to convert androstenedione to estrone. We found that dexamethasone caused an increase in the rate of aromatization of androstenedione to estrone by 20- to 100-fold. The results of a detailed study of this phenomenon are presented.

MATERIALS AND METHODS

Source and Preparation of Cells. Tissues were obtained at the time of elective abdominal surgery from women who gave consent in writing. The consent form and protocols were approved by the Human Research Review Committee at the University of Texas Health Science Center at Dallas, Texas. Subcutaneous adipose tissue was immersed in Hanks' balanced salt solution at room temperature and transported immediately to the laboratory. The subcutaneous adipose tissue was separated from skin and was minced finely and incubated for 60 min at 37°C in Krebs bicarbonate buffer that contained bovine serum albumin (Pentex fraction V, Miles Laboratories) (4%), glucose (2 mg/ml), and collagenase (CLS grade, Worthington) (1 mg/ml) (15). After incubation, the digested tissue was filtered through nylon mesh and centrifuged at 400 × *g* for 5 min. The floating layer of fat cells and the infranatant fluid were removed and the stromal cell pellet was washed twice by resuspension in Krebs buffer and recentrifugation.

Culture of Isolated Stromal Cells of Adipose Tissue. Stromal cells were suspended in Waymouth's MB 752/1 medium (GIBCO), containing fetal calf serum (15%) (GIBCO), placed in 35-mm culture dishes (Falcon Plastics) and incubated at 37°C in an atmosphere of air (95%) and CO_2 (5%). The cells were allowed to adhere to the dishes during a period of 3 days, after which time the medium was changed at 2-day intervals. The cells became confluent within 10 days and retained a fibroblast-like appearance. No cells of endothelial appearance were detectable in these cultures. Others (16) have reported that stromal cells derived from human omental adipose tissue accumulate lipid upon reaching confluency. Under the experimental conditions of this study there was no indication of lipid accumulation within the cells.

Radiolabeled Substrates. [1,2-^{3}H]Androstenedione (46.1 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was purchased from New England Nuclear. [1-^{3}H]Androstenedione was prepared from [1,2-^{3}H]androstenedione as described (14, 17).

Incubation Of Cells with [1-^{3}H]Androstenedione and Analysis for [^{3}H]Water. It has been determined previously that the apparent K_m of the aromatase system of stromal cells, with respect to androstenedione, is approximately 25 nM (14). Consequently [1-^{3}H]androstenedione in 30 μl of propylene glycol or 5 μl of ethanol was added to confluent stromal cells in monolayer culture to achieve a final androstenedione concentration in the culture medium of 150 nM. Incubations were con-

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ducted for 6 hr because it had been determined previously that the aromatization reaction rate was linear for more than 6 hr. To establish blank values, incubations were conducted in an identical fashion except in the absence of cells. Aromatase activity was assayed in the medium by the incorporation of tritium from [1-^{3}H]androstenedione into [^{3}H]water as described in detail previously (14). The cells were scraped from the dishes, homogenized, and assayed for protein by the method of Lowry *et al.* (18).

Binding of [^{3}H]Dexamethasone to Cytosol Prepared from Stromal Cells. [6,7-^{3}H]Dexamethasone (50 Ci/mmol) was purchased from New England Nuclear. Stromal cells maintained in culture for 28 days were rinsed five times with phosphate-buffered saline, scraped from the dishes, and homogenized in ice-cold buffer (10 mM Tris·HCl containing 1.5 mM EDTA and 1.0 mM dithiothreitol, pH 7.4) using 20 strokes of a Teflon-glass homogenizer. The homogenization was interrupted for the purpose of cooling (30 sec) after every four strokes. The homogenate was then centrifuged at 100,000 × *g* for 60 min and the supernatant fraction obtained was diluted to 3 mg of protein per ml. Aliquots (0.2 ml) of the cytosol were added to tubes containing 0.03–2.0 pmol of [^{3}H]dexamethasone in the absence or presence of nonradiolabeled cortisol (2 nmol) and incubated for 17 hr at 4°C. Thereafter the tubes were placed in an icebath and 0.5 ml of a suspension of Norit A charcoal (0.25%) and dextran T70 (0.025%, Pharmacia) was added. After Vortex mixing, the tubes were incubated for 20 min and then centrifuged at 1200 × *g* for 15 min at 2°C. The supernatant fluids were decanted into scintillation vials to which 10 ml of Bray's scintillation mixture was added. Specific binding was computed by subtracting the amount of [^{3}H]dexamethasone bound in the presence of nonradiolabeled cortisol from the amount bound in the absence of nonradiolabeled cortisol. The apparent equilibrium dissociation constant (K_d) and binding capacity were computed by Scatchard analysis (19).

RESULTS

Stromal cells of human adipose tissue were preincubated for 48 hr with dexamethasone, in various concentrations, prior to assay of aromatase activity. The results of such an experiment are shown in Fig. 1. Aromatase activity increased as the concentration of dexamethasone in the culture medium was increased up to 25 nM, at which concentration maximal stimulation was achieved. The concentration of dexamethasone required to achieve 50% maximal stimulation was 2.7 nM. When stromal cells were preincubated with dexamethasone (250 nM) for various times prior to assay of aromatase activity, the results presented in Fig. 2 were obtained. Stimulation of aromatase activity by dexamethasone was demonstrable after a preincubation period as short as 4 hr, and maximal stimulation was achieved after a preincubation period of 24 hr. After 24 hr of dexamethasone treatment there was no further increase in aromatase activity. On the basis of these results, a preincubation period of 48 hr with dexamethasone at a concentration of 250 nM was chosen as the standard condition for evaluation of induction of aromatase activity. In a number of experiments in which tissues from different subjects were used, the stimulation of aromatase activity by dexamethasone varied between 20- and 100-fold. This variability was due to differences in the basal rate of aromatase activity, whereas the stimulated rate was reasonably constant from one cell preparation to another [the mean basal rate and SEM in 7 experiments using cells derived from tissues of five subjects was 1.86 ± 0.37 pmol per mg of protein per 6 hr ($n = 17$), whereas the mean stimulated rate was 50.65 ± 2.97 pmol per mg of protein per 6 hr ($n = 28$).].

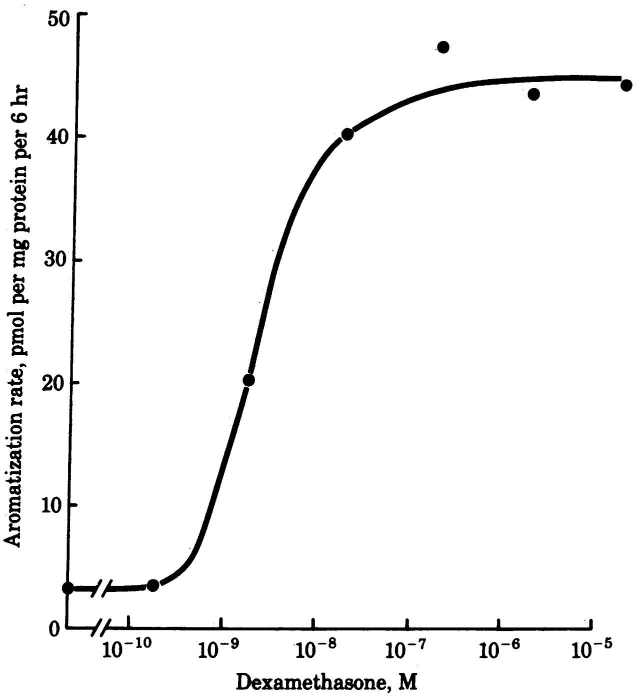

FIG. 1. Aromatase activity of stromal cells prepared from human subcutaneous adipose tissue in response to dexamethasone in various concentrations. Cells maintained in confluent monolayer culture were incubated in Waymouth's MB-752/1 medium containing fetal calf serum (15%) and dexamethasone (2.5×10^{-10} to 2.5×10^{-5} M) for 48 hr. [1-^{3}H]Androstenedione (150 nM) was then added to the dishes. The incubation was continued for 6 hr and aromatase activity was assayed. The data are the means of values obtained from assay of cells of three replicate dishes.

To examine the specificity of the stimulation of aromatase activity by dexamethasone, the stimulation of aromatase activity in stromal cells in monolayer culture by various steroids was examined (Fig. 3). Of the steroids tested, dexamethasone was the most effective in stimulating aromatase activity. Cortisol

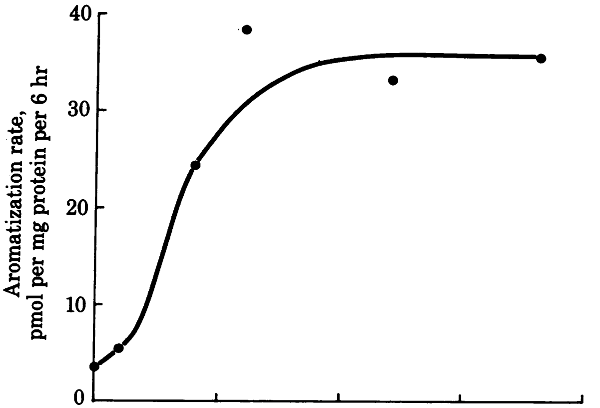

FIG. 2. Aromatase activity in stromal cells prepared from human subcutaneous adipose tissue after various times of preincubation with dexamethasone. Cells in confluent monolayer culture were preincubated in medium containing fetal calf serum alone or in medium containing fetal calf serum and dexamethasone (250 nM) for 4–72 hr. At the end of the preincubation period [1-^{3}H]androstenedione (150 nM) was added to the medium and the incubation was continued for 6 hr. Formation of [^{3}H]water from [1-^{3}H]androstenedione was assayed. The data are the means of values obtained from assay of cells of three replicate dishes.

also stimulated aromatase activity but was less potent than dexamethasone. Cortisol was ineffective at a concentration of 2.5 nM and caused only a 2-fold stimulation of activity at a concentration of 25 nM. Corticosterone, at a concentration of 250 nM, induced only a small stimulation of aromatase activity, whereas deoxycorticosterone and progesterone, each at a concentration of 250 nM, did not stimulate aromatase activity. These results are consistent with the proposition that a receptor specific for glucocorticosteroids is involved in mediating the action of dexamethasone to stimulate aromatase activity.

The presence of a glucocorticosteroid receptor in stromal cells was assessed by analyzing the binding of [^{3}H]dexamethasone to a cytosolic fraction prepared from such cells. By Scatchard analysis of [^{3}H]dexamethasone binding to cytosol prepared from stromal cells, the data presented in Fig. 4 was obtained. The binding capacity was calculated to be 38 fmol per mg of protein. The K_d was computed to be 2.9 nM, a value similar to the concentration of dexamethasone required to achieve a half-maximal stimulation of aromatase activity (Fig. 1). The linear nature of the Scatchard plot obtained was suggestive of a homogenous population of binding sites.

To gain further insight into the role of the glucocorticosteroid receptor in mediating this response in aromatase activity, the effect of cortisol 21-mesylate on the dexamethasone-induced stimulation of aromatase was examined. Cortisol 21-mesylate is believed to be an antagonist for the binding of glucocorticosteroids to cytosolic receptors (20). The effect of cortisol 21-mesylate on the stimulation of aromatase activity by dexamethasone is presented in Fig. 5. Cortisol 21-mesylate was added to the culture medium to achieve various concentrations (10^{-11} M to 10^{-5} M) in the absence or presence of dexamethasone (25 nM). Cortisol 21-mesylate at concentrations of 10^{-7} M to 10^{-5} M prevented the stimulatory effect of dexamethasone on aromatase activity; however, when present in low concentrations (2.5×10^{-11} M to 2.5×10^{-9} M), cortisol 21-mesylate appeared to potentiate the effect of dexamethasone. In the absence of dexamethasone, cortisol 21-mesylate did not affect aromatase activity.

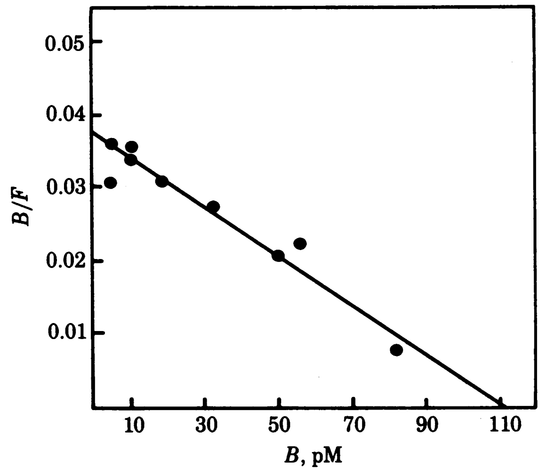

FIG. 4. Scatchard analysis of specific [^{3}H]dexamethasone binding to a cytosolic fraction prepared from stromal cells maintained in confluent monolayer culture. Cytosol (3 mg of protein per ml) was incubated for 17 hr at 4°C with [^{3}H]dexamethasone in increasing concentrations (0.15–10 nM) in the absence or presence of cortisol (10 μM). Bound hormone (*B*) was separated from free (*F*) by charcoal/dextran assay. The amount of [^{3}H]dexamethasone bound in the presence of an excess of nonradiolabeled cortisol was subtracted from the amount bound in the absence of nonradiolabeled cortisol to compute specific binding. K_d = 2.89 nM; binding capacity = 38.0 fmol per mg of protein.

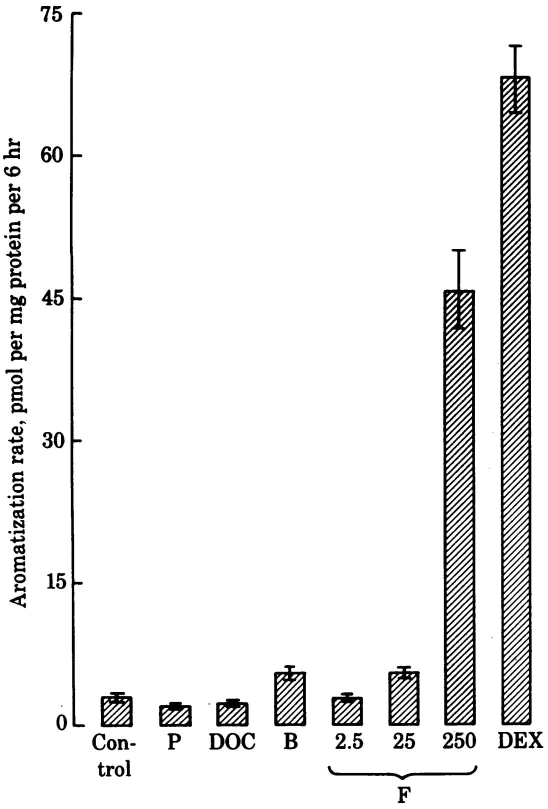

FIG. 3. Effect of various steroids on aromatase activity of stromal cells. Cells maintained in confluent monolayer culture were incubated in medium containing fetal calf serum alone (control) or in medium containing fetal calf serum and 250 nM progesterone (P), deoxycorticosterone (DOC), corticosterone (B), dexamethasone (DEX), or with cortisol (F) at 2.5–250 nM for 48 hr. [1-^{3}H]Androstenedione was then added to the medium and the incubation was continued for 6 hr. Incorporation of ^{3}H into water was then assayed. Each bar represents the mean ± SEM of values obtained from cells of four replicate dishes.

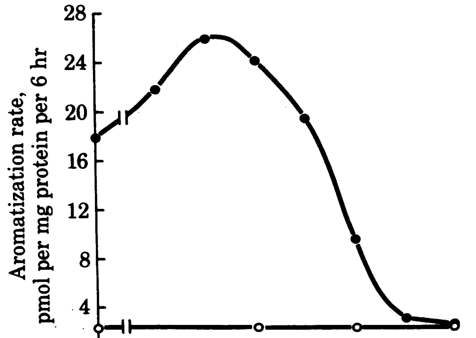

FIG. 5. Aromatase activity of stromal cells in response to dexamethasone in the presence of increasing concentrations of cortisol mesylate. Confluent stromal cells were incubated for 48 hr in medium containing fetal calf serum with cortisol mesylate in increasing concentrations (○). Other cells were incubated for 48 hr in medium containing fetal calf serum and dexamethasone (25 nM) with cortisol mesylate (10^{-11} to 10^{-5}M) (●). [1-^{3}H]Androstenedione (150 nM) was then added and the incorporation of ^{3}H into water was assayed after 6 hr. Each point is the mean of values obtained with cells of three replicate dishes.

To evaluate the role of synthesis of new protein in mediating the action of dexamethasone, the effect of cycloheximide and actinomycin D on the stimulation of aromatization by dexamethasone was investigated. Stromal cells were incubated for 6 hr in medium that did or did not contain dexamethasone (250 nM) in the absence or presence of cycloheximide (50 μg/ml) or actinomycin D (0.5 μg/ml). At the end of this time, the same fresh medium was added containing [1-^{3}H]androstenedione. Aromatase activity was assayed after an additional 6 hr of incubation. In parallel dishes, cells were incubated for 6 hr in medium that did or did not contain dexamethasone with either actinomycin D or cycloheximide. The cells were then washed three times with saline (0.15 M) to remove inhibitors, and fresh control medium or medium containing dexamethasone was added; [1-^{3}H]androstenedione was then added to initiate the assay of aromatase activity. The results of this experiment are presented in Fig. 6. Both cycloheximide and actinomycin D, when present throughout the 12-hr incubation period, inhibited completely the stimulation of aromatase activity by dexamethasone, but did not affect the basal rate of aromatase activity. The aromatase activity in dexamethasone-treated cells that had been washed free of cycloheximide after the 6-hr preincubation period was similar to the activity in dexamethasone-treated cells that had not been incubated with cycloheximide. In contrast, the aromatase activity in cells that had been preincubated with dexamethasone plus actinomycin D and then washed to remove actinomycin D was similar to basal values.

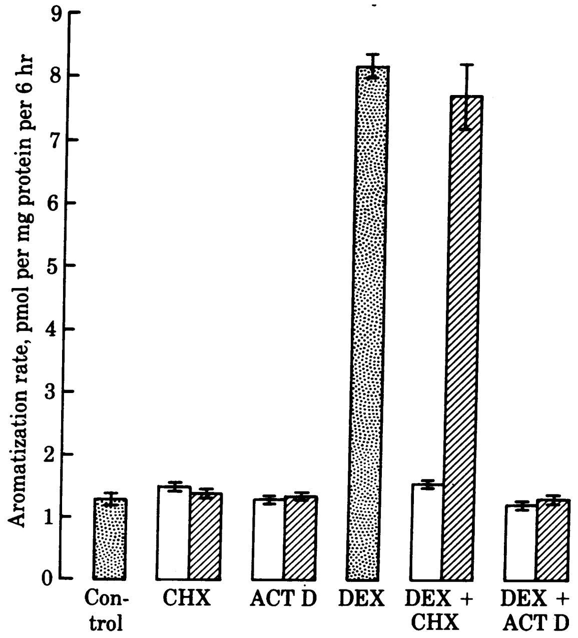

FIG. 6. Effect of cycloheximide (CHX) and actinomycin D (ACT D) on aromatase activity of stromal cells in monolayer culture. Cells were preincubated for 6 hr in medium containing fetal calf serum alone (control) or with medium containing fetal calf serum and dexamethasone (DEX) (250 nM) with or without actinomycin D (0.5 μg/ml) or cycloheximide (50 μg/ml). The corresponding fresh medium containing [1-^{3}H]androstenedione was then added, the incubation was continued for 6 hr, and the formation of [^{3}H]water was assayed (empty bars). Other cells were preincubated for 6 hr with cycloheximide or actinomycin D, either in control medium or with dexamethasone. These cells were then washed with sterile saline to remove cycloheximide or actinomycin D and control medium or medium containing dexamethasone and [1-^{3}H]androstenedione was then added. The incubation was continued for 6 hr and the formation of [^{3}H]water was assayed (hatched bars). Each bar represents the mean ± SEM of values obtained with cells of four replicate dishes.

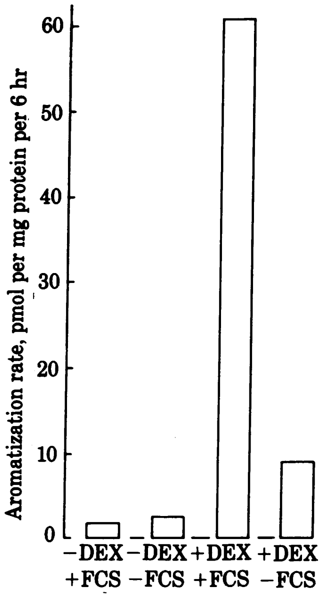

FIG. 7. Effect of dexamethasone (DEX) on aromatase activity of stromal cells incubated in medium with or without fetal calf serum (FCS) (15%). Confluent cells were incubated for 48 hr with dexamethasone (250 nM) in Waymouth's medium with or without fetal calf serum. [1-^{3}H]Androstenedione was then added, the incubation was continued for 6 hr, and the formation of [^{3}H]water was assayed. Each bar represents the mean of values obtained with cells of three replicate dishes.

In all of the experiments described above, cells were maintained in medium containing fetal calf serum (15%). To ascertain whether serum was required to facilitate the action of dexamethasone, cells that were grown to confluence in the presence of serum were maintained for 48 hr in the presence or absence of serum. Dexamethasone (250 nM) was then added to half of the dishes and after 24 hr aromatase activity was assayed. The effect of dexamethasone was reduced greatly when serum was not present in the medium, whereas control rates of aromatase activity were not affected by the absence of serum (Fig. 7).

DISCUSSION

In the present investigation we found that androstenedione was converted to estrogens in stromal cells isolated from human subcutaneous adipose tissue and maintained in monolayer culture. Such cells have been shown to differ from skin fibroblasts in terms of the response of adenylate cyclase to various hormones (21), the presence of lipoprotein lipase (16), and the capacity to accumulate lipid in culture (16). The aromatase activity of these cells was stimulated markedly upon treatment with dexamethasone. The stimulatory effect of dexamethasone was diminished when serum was not present in the culture medium. At present, the nature of the serum requirement is unknown. When cells were incubated with either cycloheximide or actinomycin D, along with dexamethasone, the stimulation of aromatase activity by dexamethasone was blocked completely, whereas control rates of aromatase activity were not affected by these agents. Therefore we suggest that glucocorticosteroids induce the transcription of a mRNA species that directs the synthesis of new aromatase enzyme protein(s). Furthermore, when the cells were washed free of cycloheximide prior to assay of aromatase activity, the stimulation of aromatase activity by dexamethasone was restored within 6 hr, a finding that is suggestive that translation of existing mRNA was rapidly initiated. When the cells were washed to remove actinomycin D prior to measurement of aromatase activity, the stimulation of such activity by dexamethasone was not detectable after 6 hr. This finding is suggestive that a longer time period is required for synthesis of new mRNA and its translation. It also is possible that actinomycin D is not readily removed from cells because it becomes intercalated into the DNA duplex at the base-paired dinucleotide sequence dG-dC and forms hydrogen bonds with guanine residues (22).

These results are suggestive that dexamethasone induces the transcription of a species of mRNA that, in turn, directs the synthesis of new protein. This new protein is likely to be one of the components of the aromatase enzyme complex, perhaps a species of cytochrome *P*-450, or else NADPH-cytochrome *P*-450 reductase. The time course of the stimulation of aromatization activity by dexamethasone is indicative that a preincubation period with dexamethasone of at least 24 hr was required for maximal stimulation, consistent with an induction phenomenon. This action of dexamethasone may be mediated by interaction of the hormone with a cytosolic glucocorticosteroid receptor found to be present in these cells, for the following reasons: (*i*) The concentration of dexamethasone required to elicit half-maximal stimulation of aromatase activity was 2.7 nM, a concentration of dexamethasone similar to that required to cause half-maximal binding to the cytosolic glucocorticosteroid receptors. (*ii*) The order of potency of stimulation of aromatase was as follows: dexamethasone > cortisol > corticosterone. Deoxycorticosterone and progesterone did not stimulate aromatase activity when present in concentrations as high as 250 nM. (*iii*) Stimulation of aromatase activity by dexamethasone was inhibited by cortisol 21-mesylate in a concentration-dependent fashion; the latter compound is believed to be an antagonist for the glucocorticosteroid receptor. It should be noted, however, that cortisol 21-mesylate at low concentrations appeared to facilitate the dexamethasone stimulation of aromatase activity. The reason for this phenomenon is not understood.

Aromatase activity has been demonstrated in a number of tissues, including the granulosa cells of the ovary (23, 24), the Sertoli cells (25) and Leydig cells of the testis (26), the placenta (27), brain (28), and genital skin fibroblasts (29). Aromatase activity of stromal cells prepared from human omental fat is less than that of stromal cells of subcutaneous fat (14). Aromatase activity is not present in stromal cells prepared from epidydimal and perirenal fat of rats, hamsters, and rabbits; nor is it present in mouse 3T3L1 fibroblasts (unpublished observations). In granulosa (24, 30) and Sertoli cells (25) aromatase activity is stimulated by follicle-stimulating hormone. The effect of follicle-stimulating hormone on stimulation of aromatase in the testis of the immature rat appears to be mediated by cyclic AMP; this obtains because follicle-stimulating hormone stimulates cyclic AMP accumulation in these cells and both dibutyryl cyclic AMP and cholera toxin stimulate aromatase activity (31).

Aromatase activity in stromal cells of adipose tissue was not affected by follicle-stimulating hormone or luteinizing hormone but was stimulated by dibutyryl cyclic AMP (unpublished observations). In rat granulosa cells in culture, glucocorticosteroids antagonize the stimulatory effect of follicle-stimulating hormone on aromatase activity (32). On the other hand, dexamethasone causes an increase in aromatase activity of genital skin fibroblasts in culture (29) but has no effect on aromatase activity of stromal cells prepared from human omental fat or from epidydimal or perirenal fat of rats, rabbits, or hamsters (unpublished observations).

The factors that regulate the extraglandular aromatization of androgens *in vivo* are not known. Greater extraglandular aromatization is found in women who are postmenopausal (1), obese (6), or hyperthyroid (33), and in subjects after surgical stress (P. C. MacDonald, personal communication). In both obese subjects and in persons after surgery, increased cortisol production rates have been reported (34). The results of the present investigation are suggestive that glucocorticosteroids may serve a role in the regulation of the rate of synthesis of estrogens in subcutaneous adipose tissue of women.

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