Growth Factor-Mediated Regulation of Aromatase Activity in Human Skin Fibroblasts (43200)

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Abstract. **We investigated the effects of various hormones and growth factors on aromatase activity in cultured human skin fibroblasts. Several potential trophic factors were tested for their ability to modify basal aromatase activity or the response to dibutyryladenosine 3′,5′-cyclic monophosphate and dexamethasone because (i) no endogenous ligand has been identified that is responsible for stimulating aromatase activity in the periphery, and (ii) dexamethasone and cAMP analogs can increase this enzyme's activity in fibroblasts. The effect of insulin and insulin-like growth factors were examined in closer detail because of the clinical association between insulin and hyperandrogenism.**

Pituitary hormones and hypothalamic releasing factors, such as human ACTH (10 n*M*), β-endorphin (10 n*M*), β-lipotropin (10 n*M*), α-MSH (10 n*M*), γ3-MSH (10 n*M*), ovine leteinizing hormone (10 ng/ml), ovine follicle-stimulating hormone (10 ng/ml), ovine thyroid-stimulating hormone (10 ng/ml), rat growth hormone (10 ng/ml), rat prolactin (10 ng/ml), rat corticotropin-releasing factor (10 n*M*), luteinizing hormone-releasing factor (10 n*M*), thyrotropin-releasing factor (10 n*M*), human growth hormone-releasing factor (10 n*M*), and somatostatin (10 n*M*), have no significant effects on aromatase activity. Porcine inhibin A (10 ng/ml) and porcine activin AB (10 ng/ml), two ovarian hormones with structural transforming homology to transforming growth factor-β, also have no effect on aromatase activity. Although basic fibroblast growth factor (1–100 ng/ml), acidic fibroblast growth factor (1 ng/ml), epidermal growth factor (1 ng/ml), platelet-derived growth factor (1 ng/ml), tumor necrosis factor (1 ng/ml), and transforming growth factor-β1 (1 ng/ml) have no effect on basal aromatase activity in human skin fibroblasts, all of these growth factors inhibited the ability of dibutyryladenosine 3′,5′-cyclic monophosphate to stimulate aromatase activity. In contrast, both insulin (100 pg/ml–10 ng/ml) and insulin-like growth factor-1 (1–100 ng/ml) had no effect on cAMP-stimulated aromatase but potentiated the action of dexamethasone (100 n*M*). Thus, there is a clear distinction between the effects of dexamethasone and cAMP on peripheral aromatase. On the basis of the results presented here, it is interesting to speculate that the hyperandrogenism that is often associated with insulin resistance may be due to a combination of growth factor-mediated inhibition of aromatase activity and the failure of peripheral tissues to respond to insulin and metabolize androgens to estrogens. [P.S.E.B.M. 1991, Vol 196]

The aromatization of androgens to estrogens in extraglandular tissues is an important source of C18 steroids in men and postmenopausal women (1, 2). While adipose tissue, muscle, and skin have all been shown to be significant sites of extraglandular aromatization (3, 4), it is only in the ovary that the regulation of this enzyme has been extensively studied (5). In this tissue, aromatase is highly sensitive to pituitary gonadotropins, in particular follicle-stimulating hormone (FSH), and is modulated by several paracrine and endocrine factors. Growth factors are thought to play an important function in the regulation of ovarian aromatase activity. Fibroblast growth factor (FGF), as an example, is a potent ($ED_{50} < 10^{-11}$ *M*) inhibitor of estrogen synthesis in cultured granulosa cells (6), as is transforming growth factor-α (7), tumor necrosis factor (TNF) (8), and epidermal growth factor (EGF) (9). In

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contrast, insulin, insulin-like growth factor-I (IGF-I), transforming growth factor-β (TGFβ), and activin can increase the responsiveness of granulosa cells to FSH.

Human adipose stromal cells (for review, see Ref. 10) and human skin fibroblasts (11–14) have been used in cell culture model systems to study the regulation of extraglandular aromatase activity. As in the ovary, the evidence obtained so far has indicated that there most likely exists a multifactorial regulation of this enzyme, although several pharmacologic agents have been identified that may be second messengers to a putative primary endogenous ligand. Analogs of cAMP and activators of adenylate cyclase (cholera toxin, forskolin) can increase aromatase activity in cultured adipose stromal cells (15). In the presence of serum, however, cAMP and its analogs have little or no effect, possibly due to the inhibitory effects of growth factors in serum (16). We investigated the effects of a wide range of factors in an attempt to identify potential modulators of peripheral aromatase activity. Because dexamethasone (DEX) can enhance aromatase activity in fibroblasts and stromal cells (17, 18), and this synthetic glucocorticoid is known to sensitize many cells to their endogenous trophic stimulus (19), each of the compounds was also tested in the presence of this steroid. The results confirm the potent inhibitory activity of several growth factors and identify IGF-I and insulin as potential regulators of extraglandular aromatase activity. The effects of insulin on the conversion of androgens to estrogens by fibroblasts suggests the possibility that dysfunctions in this process might mediate the hyperandrogenism that is often associated with insulin-resistant diabetes. The results also implicate growth factors in the pathophysiology of this disease.

Materials and Methods

Reagents. Basic fibroblast growth factor (FGF) (20) and acidic FGF (21) were purified from bovine pituitary and brain, respectively. Human ACTH (hACTH), β-endorphin, β-lipotropin, β-MSH, γ3-MSH, CRF, luteinizing hormone-releasing factor, thyrotropin-releasing factor, human GH-releasing factor (hGRF), somatostatin (SRIF), porcine inhibin A (inhibin), and porcine activin AB (activin) were synthesized in these laboratories. Ovine TSH (oTSH; NIADDK-oTSH-12), rat PRL (rPRL; NIADDK-rPRL-I-5), ovine FSH (oFSH; NIADDK-oFSH-17), ovine LH (oLH; NIADDK-oLH-25), and rGH (RP-5) were obtained from the National Hormone and Pituitary Program of the NIADDK. TGFβ1 from human platelets was generously supplied by Dr. M. B. Sporn (National Cancer Institute, Bethesda, MD). EGF was obtained from Dr. G. Gill (University of California, San Diego, CA) and platelet-derived growth factor (PDGF) was a gift from Dr. R. Ross and Dr. E. Raines (University of Washington, Seattle, WA). Human recombinant tumor necrosis factor (TNF) was a generous gift provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). Bovine insulin was purchased from Collaborative Research Inc. (Bedford, MA). Dibutyryladenosine 3′,5′-cyclic monophosphate [$(Bu)_2$cAMP], DEX, and androstenedione were obtained from Sigma Chemical Co. (St. Louis, MO). [1β-^{3}H]Androstenedione (28.0 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The relative distribution of label on the tritiated androstenedione (76% at 1β, 24% at 1α) was determined by New England Nuclear using tritium NMR spectroscopy. Fetal calf serum (FCS) was obtained from Hyclone Laboratories, Inc. (Logan, UT).

Cell Culture. Human skin fibroblasts were generously provided by Dr. R. Ross. The cells were maintained in Hepes-buffered (25 m*M*) Dulbecco's modified Eagle's medium (HDMEM) supplemented with 10% FCS at 37°C in a humidified 93% air-7% CO_2 incubator. All experiments were performed with the 8–20th passage of the same skin fibroblast line.

Aromatase Assay. Aromatase activity was estimated by measuring the stereospecific transfer of ^{3}H from [1β-^{3}H]androstenedione into ^3H_2O (8,22,23). Aliquots of 4.0×10^5 cells were distributed to 16-mm, 24-well culture plates (Flow Laboratories, Inc., McLean, VA) in 1 ml of HDMEM supplemented with 10% calf serum. After a 24-hr incubation, the plates were washed with phosphate-buffered saline, and the cells were incubated for 24 hr in 0.5 ml of HDMEM supplemented with 0.5% FCS and the test compounds. [1β-^{3}H]Androstenedione (0.1 mCi) and unlabeled androstenedione (final concentration, 100 n*M*) were then added to each well and to wells that contained no cells. The dishes were incubated for an additional 3–4 hr with the radiolabeled steroid, after which the plates were removed from the incubator and placed on ice to condense the water vapor. The incubation medium was transferred to 12- × 75-mm tubes, and 0.5 ml of an aqueous suspension of Norit A charcoal (5%) and dextran (0.5%) was added to each tube. The suspension was left at 0–2°C for 30 min with mixing every 10 min, and then was centrifuged to precipitate the charcoal. An aliquot (0.5 ml) of supernatant was added to 4 ml of Ecolite liquid scinitillation counting solution (Westchem, San Diego, CA) and the radioactivity was determined on a Beckman β-counter. Cell protein was determined by the Bradford method using bovine serum albumin as the standard (24). The total radioactivity measured in the supernatant was used to calculate the conversion of substrate to product. Aromatase activity was expressed as a function of the amount of product formed by 1 mg of cellular protein over 1 hr (pmol/mg protein/hr) as described (8, 11, 23).

Results

Human skin fibroblasts were preincubated with various concentrations of FCS for 24 hr in the presence

or absence of $(Bu)_2$cAMP (1 m*M*) or DEX (100 n*M*) to examine the effects of FCS on the aromatase activity (Fig. 1). As described in other cell types (15), FCS attenuates the stimulatory effects of $(Bu)_2$cAMP and increases the effects of DEX on aromatase activity in human skin fibroblasts (Fig. 1). On the basis of these results, a concentration of 0.5% FCS was selected so as to not conceal the inhibitory effects of the putative ligands on $(Bu)_2$cAMP-stimulated aromatase activity and, at the same time, permit the detection of a response in the presence of DEX. This concentration was selected to test the potential regulators of aromatase activity in human skin fibroblasts. Under these conditions of incubations, basal aromatase activity ranged from 0.11 to 0.52 pmol/mg protein/hr (0.30 ± 0.03, mean ± SE, $n = 14$) in the experiments described here.

To determine the effect of growth factors on aromatase activity, we added the growth factors (basic FGF, acidic FGF, TNF, PDGF, EGF, and TGFβ) to the cells at a concentration of 1 ng/ml with or without $(Bu)_2$cAMP (1 m*M*) for 24 hr (Fig. 2). None of the growth factors had a significant effect on the basal aromatase activity. When cells were cultured in the presence of $(Bu)_2$cAMP, however, all of the growth factors tested significantly inhibited the ability of $(Bu)_2$cAMP to stimulate aromatase activity. Among these growth factors, basic FGF was the most active at the dose tested, showing an 80–90% inhibition of activity. These effects are concentration-dependent (Fig. 3) and are observed at concentrations similar to those reported for its mitogenic effects (25).

To examine the possibility that hypothalamic and pituitary hormones may influence aromatase activity in human skin fibroblasts, we also investigated the effects of various hormones on the aromatase activity (Fig. 3). Luteinizing hormone-releasing factor (10 n*M*), thyrotropin-releasing factor (10 n*M*), SRIF (10 n*M*), hGRF (10 n*M*), rCRF (10 n*M*), oFSH (10 ng/ml), oLH (10 ng/ml), rGH (10 ng/ml), rPRL (10 ng/ml), oTSH (10 ng/ml), α-MSH (10 n*M*), γ3-MSH (10 n*M*), β-endorphin (10 n*M*), and hACTH (10 n*M*) had no effect on aromatase activity in human skin fibroblasts in the presence or absence of $(Bu)_2$cAMP (1 m*M*). The gonadal peptides, inhibin (1–10 ng/ml) and activin (1–10 ng/ml), had no effect on aromatase activity in the presence or absence of $(Bu)_2$cAMP (1 m*M*). In view of the subsequent findings outlined below, it is also interesting to note that insulin (10 ng/ml) had no effect on aromatase activity with or without $(Bu)_2$cAMP.

Because DEX can have permissive effects on the cell response to hormones [i.e., GH-releasing factor (26)], we examined the effects of these growth factors and hormones on aromatase activity in combination with DEX. Figure 4 shows the effects of various growth factors on aromatase activity stimulated by DEX. As expected from the results presented in Figure 1, the incubation of cells with DEX (100 n*M*) alone increased aromatase activity in the cultured human skin fibroblasts (2.1- to 5.6-fold over control; 0.39 to 1.93 pmol/

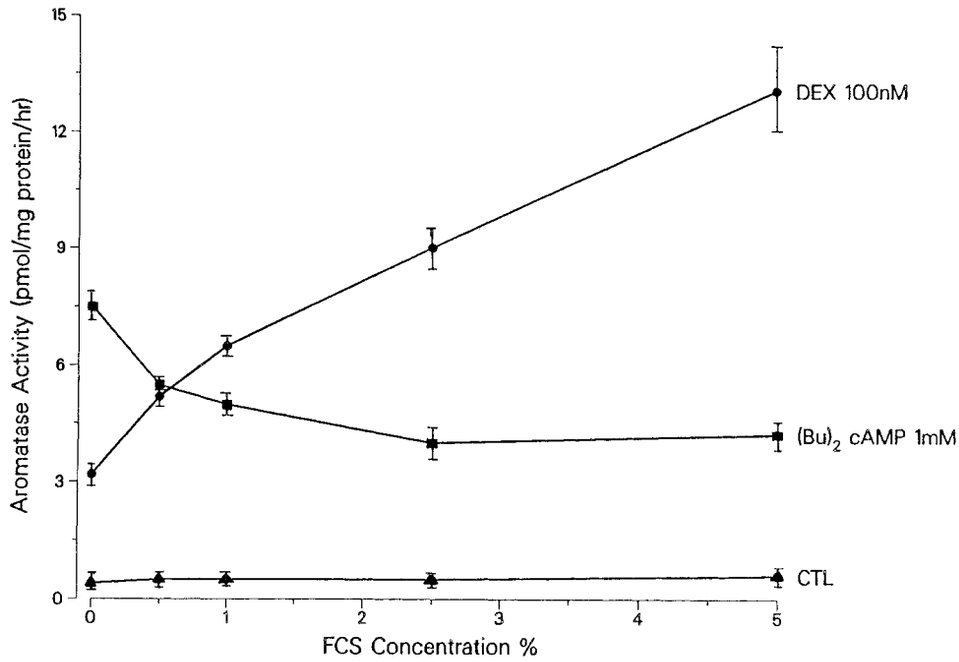

Figure 1. Concentration-dependent effect of FCS on aromatase activity in cultured human skin fibroblasts. Cells were cultured for 24 hr in 1 ml of HDMEM supplemented with 10% calf serum, plates were then washed and incubated for an additional 24 hr in 0.5 ml of HDMEM supplemented with the indicated concentration of FCS with or without (CTL) DEX 100 m*M* or $(Bu)_2$cAMP 1 m*M*, and the changes in aromatase activity were assessed as described in the text. Results are the mean of four determinations ± SE.

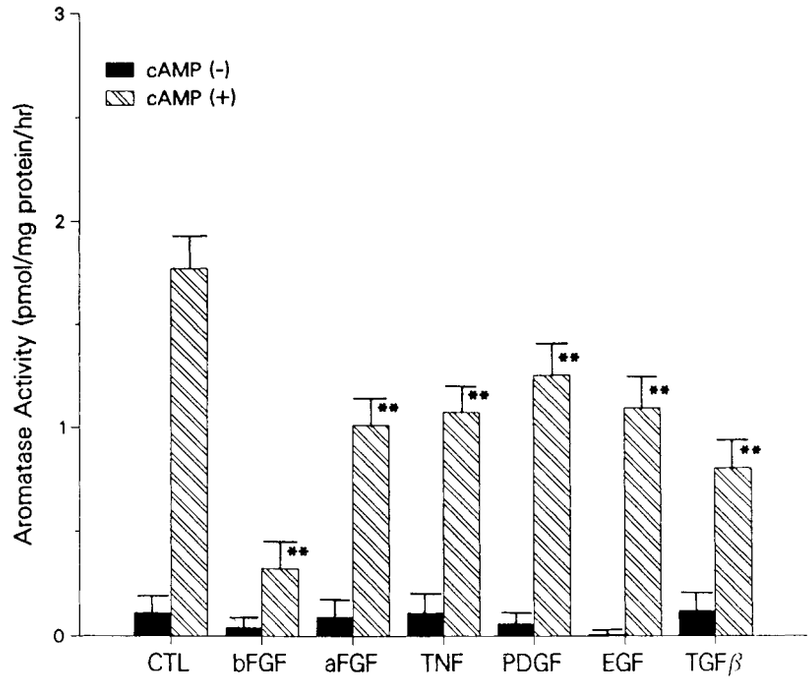

Figure 2. Effects of growth factors on aromatase activity stimulated by $(Bu)_2$cAMP. Cells were treated with various growth factors (1 ng/ml) with or without $(Bu)_2$cAMP (1 m*M*). Control cells (CTL) were cultured in the absence of growth factors. Aromatase activities were determined by measuring ^3H_2O accumulated in medium as described in Materials and Methods. Results are the mean of four determinations ± SE.

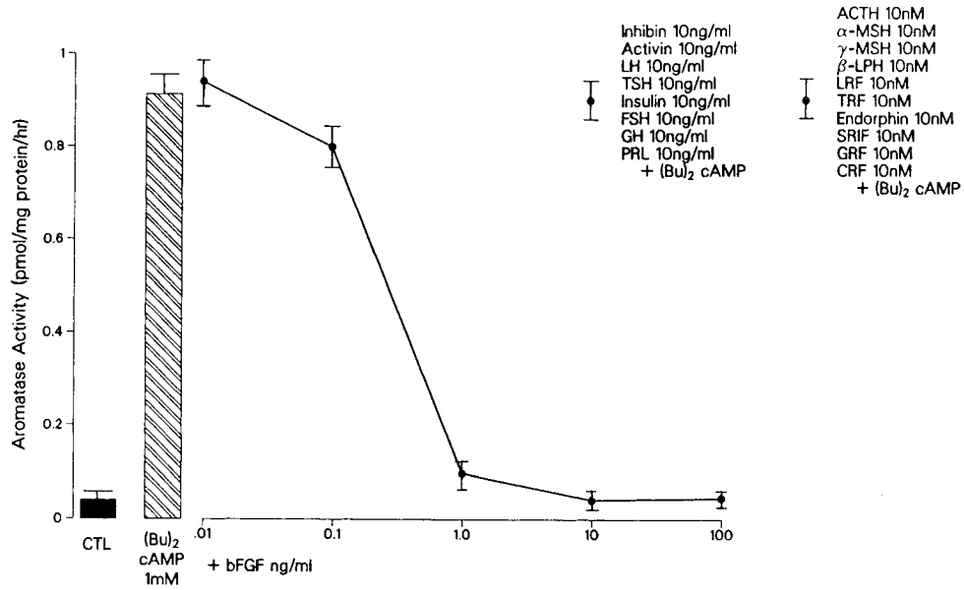

Figure 3. Inhibitory effects of basic FGF (bFGF) on the aromatase activity stimulated by $(Bu)_2$cAMP. Cells were treated with basic FGF or one of several other peptides at the indicated concentrations with $(Bu)_2$cAMP (1 m*M*). Control cells (CTL) were cultured in the absence of any peptides. Aromatase activity was determined by measuring ^3H_2O accumulation in medium. Results are the mean of four determinations ± SE.

mg protein/hr). EGF (10 ng/ml), acidic FGF (10 ng/ml), and TGFβ (10 ng/ml) had no significant effect on DEX-stimulated aromatase activity in cultured skin fibroblasts, thus disassociating cAMP- and DEX-dependent increases in aromatase activity. In contrast, basic FGF could inhibit DEX-dependent increases in aromatase activity. Although the addition of insulin or IGF-I to the cells had no effect on basal aromatase activity (Fig. 5), the co-incubation of insulin or IGF-I with DEX (100 n*M*) resulted in a dose-dependent increase of aromatase activity (100 pg/ml–10 ng/ml), thus establishing a permissive effect of DEX on the cellular response to insulin and IGF-I.

The concentration of insulin (ED_{50} = 0.55 ± 0.11 ng/ml; mean ± SE) capable of increasing DEX-dependent aromatase activity, was 10 times lower than that of

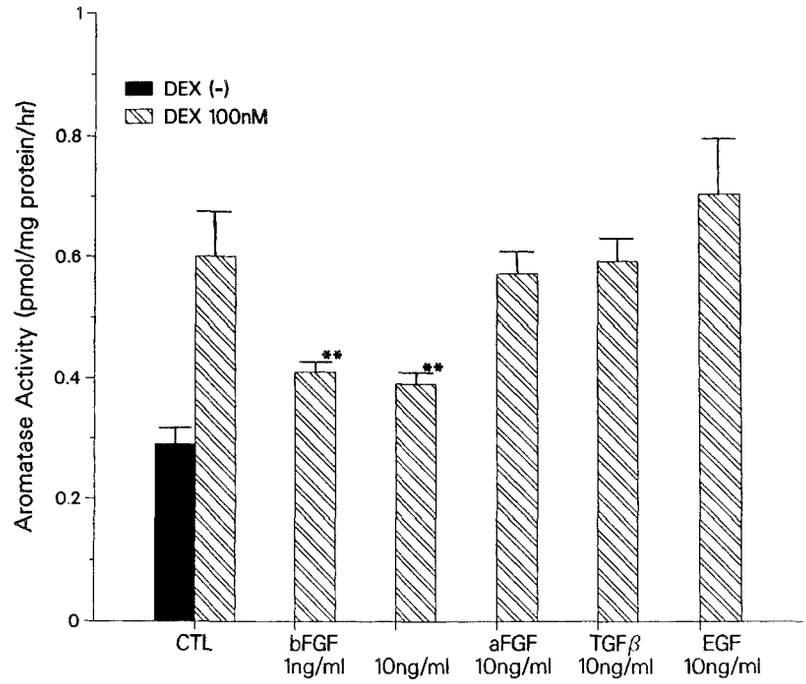

Figure 4. *Effects of growth factors on aromatase activities stimulated by DEX. Cells were co-incubated with the indicated concentrations of* growth factors and 100 n*M* DEX for 24 hr. Control cells (CTL) were cultured in the absence of growth factor. Aromatase activity was determined by measuring ^3H_2O accumulated in medium as described in Materials and Methods. Results are the mean of four determinations ± SE.

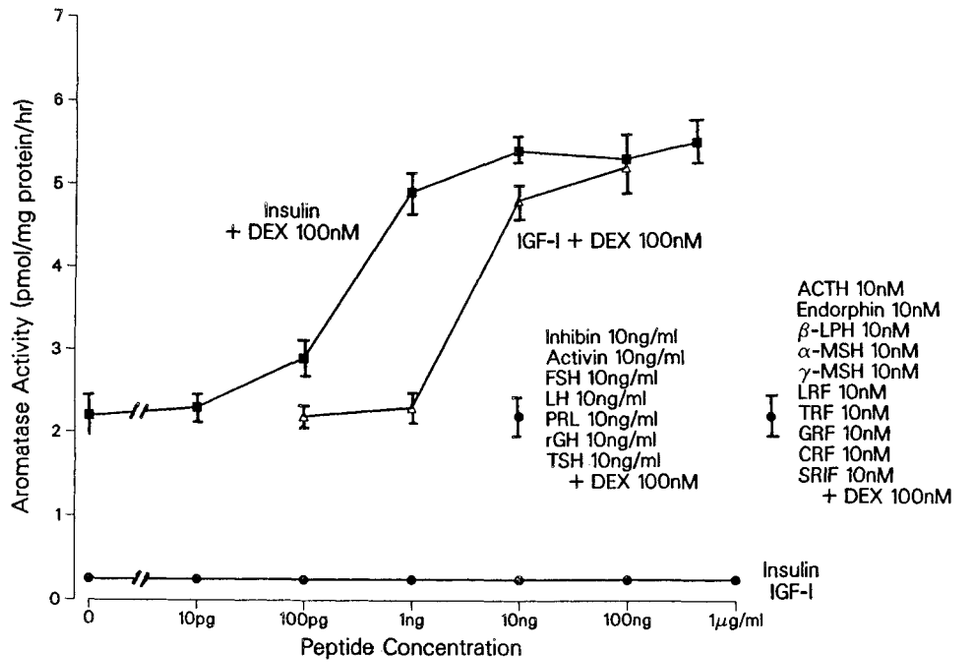

Figure 5. Stimulatory effect of insulin and IGF-I on DEX (100 n*M*) dependent aromatase activity in human skin fibroblasts. Cells were incubated with insulin IGF-I in DEX (100 n*M*). Other peptides indicated were co-incubated with DEX (100 n*M*). Aromatase activity was estimated by measuring ^3H_2O accumulated in medium. Results are the mean of four determinations ± SE.

IGF-I (ED_{50} = 4.38 ± 0.69 ng/ml), indicating that the effects are mediated through the insulin rather than the IGF-I receptor. The maximum stimulatory effect of insulin was obtained at the concentration of 10 ng/ml, and aromatase activity was increased 2.1- to 2.8-fold. Gonadotropin-releasing hormone (10 n*M*), thyrotropin-releasing hormone (10 n*M*), SRIF (10 n*M*), hGRF (10 n*M*), rCRF (10 n*M*), oFSH (10 ng/ml), oLH (10 ng/ml), rGH (10 ng/ml), rPRL (10 ng/ml), oTSH (10 ng/ml), α-MSH (10 n*M*), γ3-MSH (10 n*M*), β-endor-

phin (10 n*M*), hACTH (10 n*M*), inhibin (1–10 ng/ml), and activin (1–10 ng/ml) had no significant effect on aromatase activity in the presence of DEX (100 n*M*) (Fig. 5).

The response to insulin was further investigated to take into account time-dependent changes in activity (Fig. 6). Cells were incuabted with DEX (100 n*M*) with or without insulin (10 ng/ml) for varying periods of time, and the changes in aromatase activity were determined. The co-incubation of insulin (10 ng/ml) with DEX (100 n*M*) increased the aromatase activity throughout the experiment with identical time kinetics that are essentially the same as that of DEX 100 n*M* alone. The only difference detectable was in the changed magnitude of stimulation.

Discussion

In this study, we report the effects of various hormones and growth factors on aromatase activity in cultured skin fibroblasts. None of the pituitary hormones and hypothalamic releasing factors tested had any significant effect on aromatase activity in the presence or absence of $(Bu)_2$cAMP or DEX. Although it has been reported that ACTH can stimulate aromatase activity in adipose tissue (27), we were unable to observe any effect of ACTH in the presence or absence of $(Bu)_2$cAMP or DEX in cultured human skin fibroblasts. This finding is consistent with the observation that the differentiation of 3T3-L1 fibroblasts into adipocytes is accompanied by the appearance of ACTH receptors (28) and suggests that the normal fibroblasts used in this study do not respond to ACTH by virtue of an absence of ACTH receptors.

The effects of acidic and basic FGFs, PDGF, and EGF on aromatase activity stimulated by $(Bu)_2$cAMP in human skin fibroblasts were all inhibitory. This appears to be a common feature in many cell types. Mendelson *et al.* (16) have reported inhibitory effects of growth factors on $(Bu)_2$cAMP-stimulated aromatase activity in adipose stromal cells, and these growth factors have also been shown to inhibit aromatase activity in granulosa cells when stimulated by FSH (6, 9). TNF, which can also inhibit $(Bu)_2$cAMP-stimulated aromatase activity in the fibroblasts, inhibits aromatase activity stimulated by FSH in cultured rat granulosa cells (8). This is not to say that all factors act on peripheral and ovarian cell types with the same effect. As an example, TGFβ1 increases FSH-stimulated aromatase activity in granulosa cells (29), but inhibits the effects of the $(Bu)_2$cAMP-stimulated aromatase activity in the fibroblasts used in this study. Inhibin and activin, two gonadal proteins with structural homology to TGFβ (30–32), have no effect on the aromatase activity of human skin fibroblasts in the presence or absence of $(Bu)_2$cAMP or DEX, but are potent modulators of aromatase activity in granulosa cells (29, 33). While these data support the hypothesis that growth factors may play important roles in the regulation of the activity of aromatase in granulosa cells and extraglandular tissues, they also demonstrate that the responses of the enzyme in various tissues are not the same. Thus, the physiologic function of each growth factor may be significantly different in various tissues. In view of the

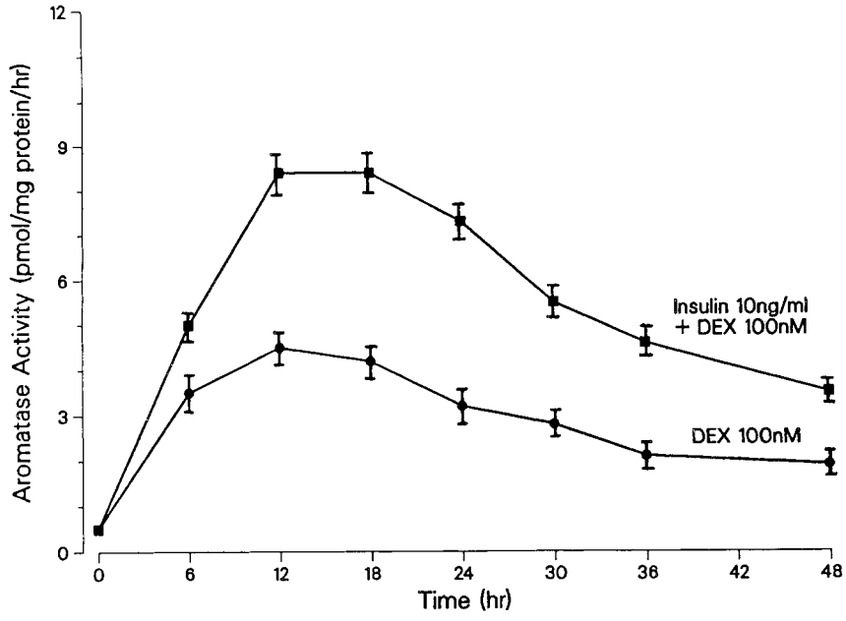

Figure 6. Time course of the effect of insulin on DEX-dependent aromatase activity. Cells were cultured with DEX (100 n*M*) with or without insulin (10 pg/ml) for varying periods of time, whereupon aromatase activity was determined for an additional 3 hr, as described in the text. Results are the mean of four determinations ± SE.

fact that these growth factors are widely distributed in tissues (25, 34) it is most likely that they are playing a paracrine rather than endocrine function *in vivo*.

It has been reported that IGF-I and insulin can potentiate the effects of FSH on aromatase activity in granulosa cells (35, 36). The response of fibroblasts to insulin and IGF-I require the presence of DEX. The observation that in its absence there is no biologic effect, establishes that the physiologic significance of these findings will be highly dependent on *in vivo* studies, the conditions of which may be clearly different in the parameters (serum-free, etc.) set up in *in vitro* models.

Fujimoto *et al.* (18) have demonstrated a time-dependence of aromatase to DEX in human skin fibroblasts when they are cultured in medium containing 15% FCS. Under the cell culture conditions used in the studies reported here (0.5% FCS), aromatase activity stimulated by DEX shows this same type of time-dependent response. Although the addition of insulin to the cells increased the magnitude of this response, it did not change the transient pattern of enzyme induction. Cell culture conditions, however, are now well recognized to modulate aromatase activity (13). Interestingly, their same conditions can have profound effects on the autocrine/paracrine synthesis and release of growth factors by cells in culture.

Growth factors certainly seem to play an important role in the regulation of extraglandular aromatization. On this basis, it is interesting to speculate that the effects of paracrine acting growth factors, insulin, and IGF-I are somehow linked to the mechanisms of hyperandrogenism that exist *in vivo* in patients with hyperinsulinemic, insulin-resistant states (37–40). Although it was believed that hyperandrogenism associated with these syndromes was the underlying mechanism responsible for insulin resistance, it has recently been suggested that the opposite may be true (40). The results presented here support this paradigm. Insulin resistance could effectively prevent efficient androgen metabolism to estrogens. A similar effect could be observed in the ovary where insulin and IGF-I potentiate the effects of FSH (35, 36).

It is noteworthy that hyperandrogenism associated with type A and type B insulin resistance (37), leprechaunism (38), polycystic ovary syndrome (39), and acanthosis nigricans syndrome (40) might also reflect a pathophysiologic function of these factors. To date, very little is known about the *in vivo* physiologic function of these factors (41). In the case of basic FGF, it is widely distributed in tissues (25), synthesized in many cell types (42), and thought to have paracrine, if not autocrine functions (41). It has been associated with some of the complications in diabetes because of its capacity to induce neovascularization (25, 43). By virtue of its capacity to inhibit aromatase activity, the results could be interpreted to suggest that factors like FGF may be involved in the pathophysiology of hyperandrogenism. Thus, growth factor-mediated inhibition of aromatase coupled to insulin resistance could represent an effective mechanism preventing androgen metabolism to estrogens.

The observation that the regulation of peripheral aromatase activity is complex and possibly regulated by a large number of paracrine and endocrine factors rather than one primary ligand emphasizes the importance of examining the physiologic and pathophysiologic effects of these factors *in vivo*. Although we have been unable to find any one primary ligand that might regulate the aromatase activity via cAMP in fibroblasts, the availability of large amounts of growth factors will enable a comprehensive investigation of their role(s) *in vivo* in regulating aromatase activity. At that time, it will be possible to elucidate the physiologic roles of insulin, glucocorticosteroids, and growth factors in the regulation of peripheral aromatase activity.

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