

Progesterone inhibits glucocorticoid-dependent aromatase induction in human adipose fibroblasts

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Abstract

In fibroblasts derived from human adipose tissue, aromatase induction is observed after exposure to 1 μ M cortisol in the presence of serum or platelet-derived growth factor (PDGF). Progesterone suppresses this induction in a dose-dependent manner, 10 μ M resulting in complete inhibition. A reduced cortisol concentration (0.1 μ M) concomitantly reduces the progesterone concentration required for effective inhibition (10–100 nM). This effect of progesterone is specific, as neither the release of cellular enzymes nor aromatase induction by dibutyl-cAMP, which acts independently from cortisol, are affected. However, the inhibitory effect of progesterone requires its presence throughout the induction period. Kinetic studies in intact cells reveal a reduced number of aromatase active sites upon progesterone treatment,

whereas progesterone at near-physiological concentration (100 nM) does not inhibit aromatase activity in isolated microsomes. Semi-quantitative reverse transcriptase PCR analysis shows reduced amounts of aromatase mRNA in progesterone-treated cells, indicating specific inhibition of the glucocorticoid-dependent pathway of aromatase induction. The inhibitory effect of progesterone is not blocked by the anti-progestin ZK114043, excluding action via progesterone receptors and indicating competition for the glucocorticoid receptor. Progesterone must be considered a potential physiological inhibitor of glucocorticoid-dependent aromatase induction in adipose tissue. It is proposed that it is a suppressor of aromatase induction in adipose tissue in premenopausal women.

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Introduction

The enzyme aromatase is responsible for the production of estrogens from androgenic precursors (for review see Simpson *et al.* 1989, 1994). In premenopausal women it is found mainly in the ovaries and during gestation in the placenta (Meyer 1955, Richards 1994). In men and postmenopausal women, estrogens are produced mainly in fibroblasts from adipose tissue (Simpson *et al.* 1994). They contain considerable aromatase activity, but its regulation is only partially understood. Glucocorticoids are powerful inducers of aromatase, but their action depends on the presence of serum (Simpson *et al.* 1981) or serum-derived growth factors like platelet-derived growth factor (PDGF) (Schmidt & Löffler 1994). Dibutyl-cAMP (Bu₂cAMP) is also able to induce aromatase, but this effect is inhibited by serum or serum-derived growth factors (Mendelson *et al.* 1982, 1986). Gonadotropins, which stimulate estrogen production in ovaries, have no influence on estrogen production in adipose fibroblasts (Simpson *et al.* 1989).

Aromatase activity *in vivo* in adipose tissue from healthy donors depends on transcription of the aromatase gene starting from glucocorticoid-dependent promoters (Harada *et al.* 1993, Mahendroo *et al.* 1993). Therefore glucocor-

ticoid action is a prerequisite for estrogen production in adipose tissue. At present, there is little knowledge about physiological mechanisms that may inhibit the glucocorticoid-dependent pathway of aromatase induction in adipose fibroblasts. However, the increased rates of estrogen synthesis in adipose tissue from older (Hemsell *et al.* 1974) or obese (MacDonald *et al.* 1978) patients could be caused by either the enhanced action of inducers or a loss of inhibitors in these patients.

We report here that progesterone inhibits the glucocorticoid-dependent, but not the cAMP-dependent, pathway of aromatase induction in human adipose fibroblasts *in vitro*. We provide evidence that this inhibition is at the transcriptional level and that it results from the interaction of progesterone with the glucocorticoid receptor.

Materials and Methods

Materials

Cell culture media, fetal calf serum (FCS), antibiotics, trypsin solution and collagenase were purchased from Biochrom (Berlin, Germany), BSA from Biomol

(Hamburg, Germany), HEPES, biotin, Norit A, dextran and Serva Blue G-250 from Serva (Heidelberg, Germany), and cortisol, progesterone, transferrin and pantothenate from Sigma (Deisenhofen, Germany). Recombinant human PDGF-BB was obtained from BTS (St Leon-Rot, Germany). [$1\beta,2\beta$ - ^3H]Testosterone was from NEN/DuPont (Dreieich, Germany). RNeasy columns came from Qiagen (Hilden, Germany), RNasin from Promega (Mannheim, Germany), Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) from Amersham (Braunschweig, Germany), *Taq* DNA polymerase from PAN Systems (Nürnberg, Germany) and nylon membranes from Appligene (Heidelberg, Germany). All other molecular biology reagents, including the DIG labeling and detection systems were from Boehringer-Mannheim (Mannheim, Germany). Insulin was a gift from Dr Brocks, Farbwerke Hoechst AG (Frankfurt, Germany). ZK114043 was kindly provided by Dr Parczyk, Schering AG (Berlin, Germany). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Cells and cell culture

Human adipose tissue for the preparation of adipose fibroblasts was obtained during plastic breast surgery from healthy women. The donors gave informed consent according to a protocol, which was approved by the Ethikkommission of the University of Regensburg. Cells were isolated as described (Schmidt & Löffler 1997) and were subcultivated after treatment with 0.25% trypsin in PBS; the detached cells were diluted with medium and placed in 24-well plates or 100-mm dishes (for RNA isolation) and grown as described previously (Schmidt & Löffler 1997). For pretreatment before aromatase induction, the confluent monolayers were washed free of serum constituents and incubated for 48 h with serum-free medium (SF-medium), with replacement of the medium after 24 h. SF-medium consisted of Dulbecco's modified Eagle's medium and Ham's F12 medium in a ratio of 3:1 (both without phenol red) supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), transferrin (2 µg/ml), pantothenate (17 µM), biotin (1 µM) and insulin (1 nM). Culture conditions included a humidified atmosphere with 5% CO₂ at a temperature of 37 °C.

Induction of aromatase and assay of aromatase activity

After the pretreatment phase, aromatase activity in adipose tissue stromal cells was induced by supplementation of SF-medium with cortisol and either 10% FCS or 0.5 nM PDGF-BB. Alternatively, Bu₂cAMP (1 mM) was used as inducer in the absence of cortisol and serum. Progesterone and other antagonists were given immediately before induction was started. The total time of induction was 24 h. The substrate for the aromatase enzyme, [$1\beta,2\beta$ - ^3H]testosterone (38 nM, 1 µCi/well; with exception of

the kinetic experiments), was added 6 h before the incubation was terminated. Incorporation of ^3H into H₂O was measured essentially as described previously (Ackerman *et al.* 1981). Aromatase activity is given as pmol testosterone used/6 h per mg protein calculated as described by Cole & Robinson (1990) and experimentally confirmed for our assay in the laboratory. Whole cell protein was determined by an improved protocol of the method of Bradford (1976), as described by Peterson (1983).

Aromatase assay using placental microsomes

The placental microsomal preparation and the microsomal assay were carried out as described by Kellis & Vickery (1987).

Assay of lactate dehydrogenase and alkaline phosphatase activities

Lactate dehydrogenase activity was determined by the procedure of Bergmeyer & Bernt (1974), and alkaline phosphatase activity as described by Walter & Schütt (1974). After removal of the supernatant for determination of aromatase activity, the cell layers were washed with PBS and then lysed in 1 ml PBS by sonification. The lysates were centrifuged (13 500 g; 5 min) and directly assayed for enzyme activity and cellular protein content.

RT-PCR analysis of aromatase expression

Cells were grown in 100-mm dishes and treated in parallel to the cells used for the aromatase assay. RNA was isolated via RNeasy affinity columns following the manufacturer's instructions. For RT-PCR, 1 µg total RNA was reverse-transcribed (1 h/37 °C) using 200 U MMLV RT and subsequently amplified with 2.5 U *Taq* DNA polymerase for the given number of cycles (1 min/94 °C, 1 min/64 °C, 30 s/74 °C) using the oligonucleotides 5'-ttatgaggagcatgcggtacc (forward) and 5'-tagtgtccagacacctgtc (reverse). After agarose gel electrophoresis and blotting by standard protocols (Sambrook *et al.* 1989), the PCR products were hybridized with 100 ng of a digoxigenin-labeled probe corresponding to the amplified 432 bp cDNA fragment covering parts of exons 9 and 10 of the aromatase gene and detected using the DIG luminescent detection system according to the manufacturer's instructions. The identity of the cloned fragment used for the generation of the probe was confirmed by sequencing. PCR products were quantified by means of a video densitometer.

Statistical analysis

Comparative analyses of aromatase activities between pairs of treatment groups were performed using Student's *t*-test

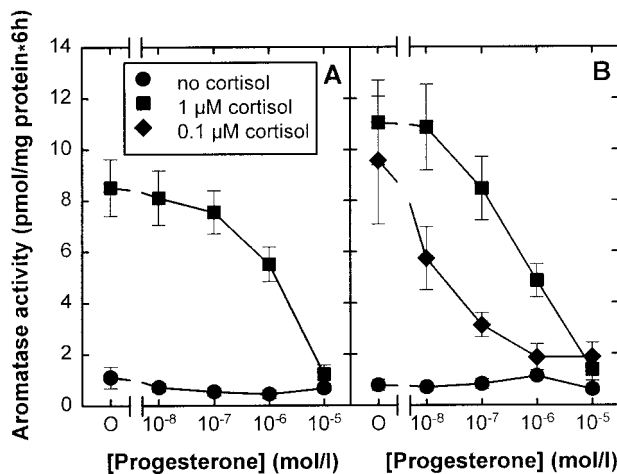


Figure 1 Progesterone inhibits aromatase induction by cortisol. Human breast adipose tissue fibroblasts were incubated for 24 h without or with cortisol in the presence of 10% FCS (A) or 0.5 nM PDGF-BB (B) and the given concentrations of progesterone. The results are means \pm S.E.M. from six (A) or five (B; 3 for 0.1 μ M cortisol) preparations of cells, which were assayed in duplicate. Corresponding values obtained in the presence or absence of cortisol are significantly different (Mann-Whitney U-test) in: (1) the presence of 0–1 μ M progesterone ($P < 0.005$) when 1 μ M cortisol was used for induction; (2) the presence of 0–100 nM progesterone ($P < 0.01$) when 0.1 μ M cortisol was used for induction. In the absence of cortisol, progesterone had no significant effect.

or, when the data for one of the treatment groups to be compared were not distributed normally, the Mann-Whitney U-test. ANOVA was used for comparison of more than two groups.

Results

Aromatase induction in human adipose fibroblasts is observed after treatment with either FCS or PDGF-BB in the presence of cortisol.

To test the influence of progesterone on estrogen biosynthesis in adipose tissue, adipose fibroblasts were incubated with various concentrations of progesterone immediately before aromatase was induced by addition of cortisol together with either FCS or PDGF-BB. With progesterone in concentrations ranging from 100 nM to 10 μ M, aromatase induction was inhibited in a dose-dependent manner. When FCS was used instead of PDGF-BB as a mediator of aromatase induction, the dose-response curve was shifted to the right. About fivefold higher progesterone concentrations are needed to obtain a degree of inhibition comparable to that observed in the presence of PDGF-BB (Fig. 1A and B).

When 0.1 μ M cortisol was used together with 0.5 nM PDGF-BB, the aromatase activities were reduced to about 80% of those seen after treatment of cells with 1 μ M

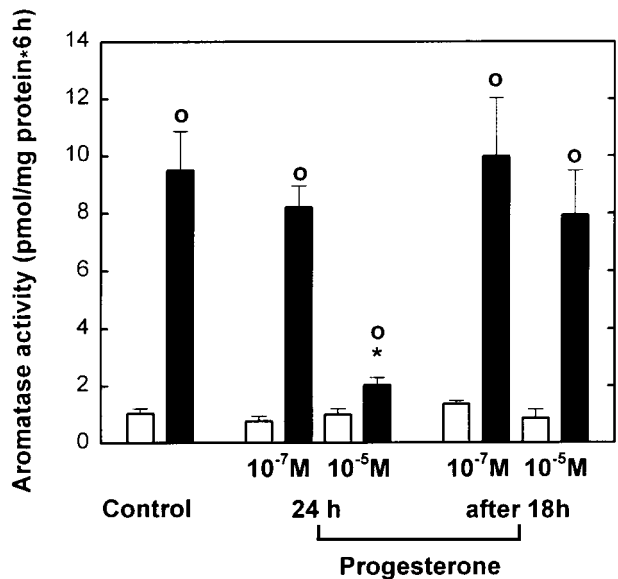


Figure 2 Progesterone action requires its presence throughout the induction period. Adipose fibroblasts were induced with 1 μ M cortisol and FCS for 24 h. Progesterone was added either during the whole induction period (24 h) or after 18 h, i.e. during the last 6 h, concomitantly with the radiolabeled aromatase substrate. Data were obtained in the absence (open bars) or presence (solid bars) of cortisol and represent the means \pm S.E.M. from three preparations of cells assayed in duplicate. Differences from the corresponding values in the absence of cortisol (o) or progesterone (*) are significant ($P < 0.05$; Mann-Whitney U-test).

cortisol. Under these conditions, progesterone was more effective, resulting in almost complete inhibition of aromatase at a concentration as low as 0.1 μ M. In the absence of cortisol, there was no effect of progesterone on aromatase induction with either FCS or PDGF-BB.

Release of lactate dehydrogenase and alkaline phosphatase from the cells does not change during the culture period upon treatment with 10 nM to 10 μ M progesterone (data not shown). Therefore progesterone does not affect overall cell performance, but interacts specifically with the glucocorticoid-dependent pathway of aromatase induction.

When aromatase was induced by treatment of adipose tissue fibroblasts with Bu_2cAMP instead of cortisol together with either PDGF-BB or FCS, aromatase activity reached 34.8 ± 5.7 pmol/6 h per mg protein in the presence of 10 μ M progesterone, compared with 31.4 ± 10.3 pmol/6 h per mg protein in the absence of progesterone (means \pm S.E.M. from three preparations of cells assayed in duplicate).

To exclude the possibility of direct inhibition of aromatase enzyme activity by progesterone, we studied the time-dependence of the progesterone effect (Fig. 2). In the presence of 1 μ M cortisol, 10 μ M progesterone caused almost complete inhibition when present throughout the induction period. However, the addition of even 10 μ M

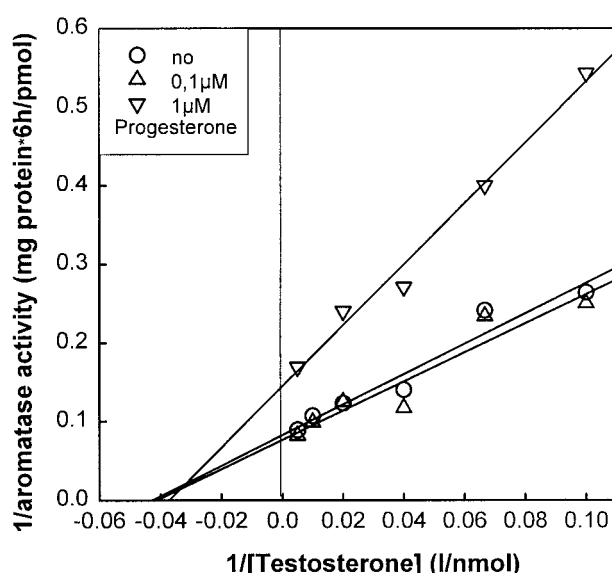


Figure 3 Inhibition of aromatase activity is non-competitive. Lineweaver-Burk plot of aromatase enzyme kinetic measurements on intact adipose fibroblasts induced with 1 μ M cortisol and PDGF-BB. Data are means from duplicate wells from a representative experiment (out of three). Linear regression analysis reveals no differences in the K_m values obtained for the various progesterone concentrations. Progesterone at a concentration of 1 μ M caused a reduction in aromatase activity from 14.3 to 6.5 pmol/6 h per mg protein, whereas at 10 μ M it caused complete inhibition.

progesterone to the cells only for the last 6 h of the 24 h induction period elicits only a slight insignificant reduction in aromatase activity.

Similar results were obtained when human placental microsomes were used instead of adipose fibroblasts. The mean \pm S.E.M. of the relative aromatase activities for three preparations of microsomes treated with vehicle only was $100 \pm 2.7\%$, whereas in the presence of 0.1 μ M progesterone it was $105.8 \pm 3.4\%$. Progesterone at 0.1 μ M has no effect on placental aromatase activity, even though it blocks aromatase induction almost completely in intact adipose fibroblasts in the presence of 0.1 μ M cortisol (Fig. 1B). The slight, but nevertheless significant ($P < 0.05$), inhibitory effect of 10 μ M progesterone to $87.5 \pm 4.2\%$ may indicate a direct inhibitory action of very high doses of progesterone.

The type of inhibition of aromatase activity by progesterone is non-competitive (Fig. 3). At a concentration of 1 μ M cortisol and 0.5 nM PDGF-BB, no change in K_m (25 nM) was observed, whereas the V_{max} decreased (from 14.3 to 6.5 pmol/6 h per mg protein in the presence of 1 μ M progesterone for the experiment shown).

Semi-quantitative RT-PCR analysis revealed a significant reduction in aromatase mRNA in adipose fibroblasts treated with 10 μ M progesterone in the presence of PDGF-BB and cortisol compared with control cells with-

out progesterone treatment (Fig. 4). The remaining aromatase mRNA was in the range from almost undetectable to about 25% and varied between different preparations of cells.

To gain further insight into the mechanism of the progesterone effect, we tested the influence of an anti-progestin without anti-glucocorticoid properties, ZK114043 (Li *et al.* 1995), on aromatase induction (Table 1). It did not block the progesterone effect on aromatase induction and proved to be a weak aromatase inhibitor.

Discussion

Little is known about the physiological regulators of extra-ovarian and extraplacental estrogen production. In fibroblasts from human adipose tissue, induction of the aromatase gene by cortisol has been described (Simpson *et al.* 1994), which depends on the presence of serum or serum-derived growth factors (Schmidt & Löffler 1994). Bu₂cAMP also causes induction of the aromatase gene, but its effect is strongly inhibited by serum or PDGF-BB (Mendelson *et al.* 1982, 1986, Schmidt & Löffler 1994).

Our finding of progesterone inhibition of aromatase gene expression adds a new regulatory mechanism. Kinetic analysis indicates that progesterone has no effect on K_m but reduces the V_{max} of the aromatase enzyme. A plausible explanation for this finding is that the number of active enzyme molecules is reduced by progesterone. Since progesterone is ineffective when added during the final 6 h of the induction period, we conclude that it is not a direct inhibitor of the aromatase enzyme. This conclusion is supported by the finding that progesterone has no inhibitory effect on the aromatase activity of isolated placental microsomes.

Previous work (Evans *et al.* 1987) has shown that aromatase activity is regulated mainly at the transcriptional level. As measured by semi-quantitative RT-PCR analysis, the amount of aromatase mRNA is reduced under the influence of progesterone. This indicates that progesterone inhibits the transcription of the aromatase gene or stimulates the breakdown of its mRNA. Determination of the relative mRNA contents of adipose fibroblasts does not allow us to distinguish between these alternatives. However, taking into account the considerations discussed below, we conclude that progesterone indeed inhibits transcription of the aromatase gene.

There are two different ways by which progesterone could regulate transcription: it could act via progesterone receptors or by interaction with the glucocorticoid receptor. Progesterone receptors have so far been detected in the adipose tissue of animal species such as sheep and rat, but not in human adipose tissue from various locations (Rebuffe-Scrive *et al.* 1990). In undissociated human adipose tissue slices, progesterone had no detectable effect on the expression of the progesterone-regulated

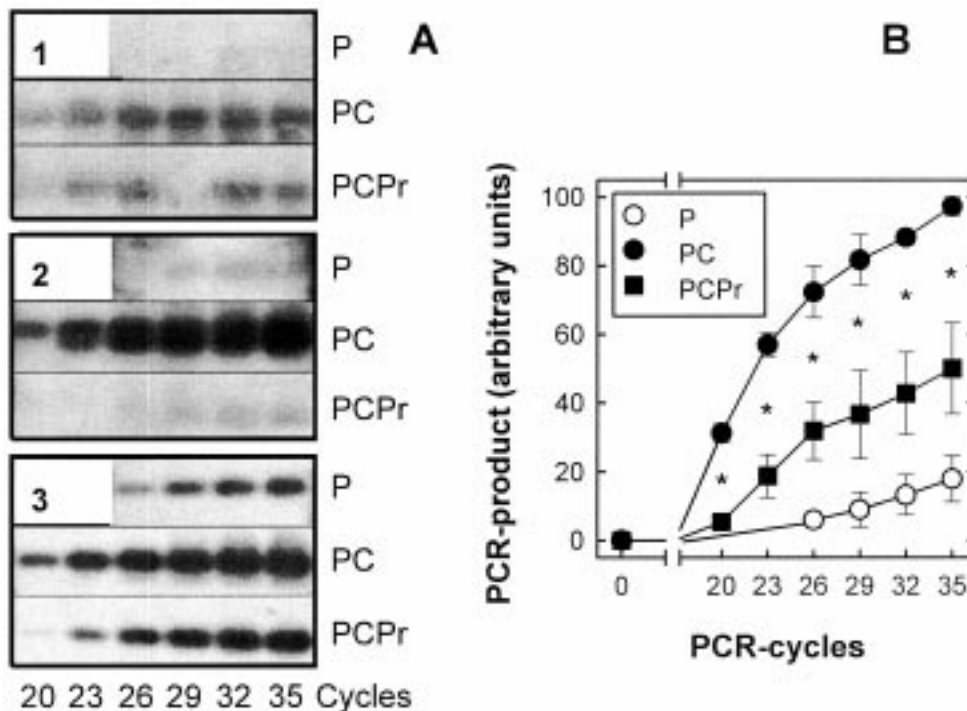


Figure 4 Aromatase mRNA levels are reduced in progesterone-treated cells. Semi-quantitative RT-PCR analysis of aromatase mRNA content of adipose fibroblasts after 24 h treatment with 0.5 nM PDGF (P), 0.5 nM PDGF and 1 µM cortisol (PC) or 0.5 nM PDGF, 1 µM cortisol and 10 µM progesterone (PCPr). Starting from 1 µg total RNA for each condition, RT-PCR was performed as described in Materials and Methods. Equal volumes of PCR mixture were analyzed on the same gel, membrane and film simultaneously. (A) The amount of reduction in aromatase mRNA content of stromal cells after treatment with progesterone is variable and depends on the preparations of cells used (marked 1, 2 and 3). With RNA from cells treated with progesterone, at least three additional cycles are necessary to detect the same amount of PCR product as found in the absence of progesterone (1). In another sample, only traces of PCR product were detected even after 35 cycles (2). For a better comparison, lanes containing PCR products obtained after identical numbers of cycles were placed in vertical rows. The PDGF-only values for 20 and 23 cycles were not determined, because a clear signal was not obtainable under these conditions in initial experiments. (B) The blots shown in (A) and from an additional experiment were quantified densitometrically. Resulting values for signal intensity were normalized and plotted against number of cycles. There is a significant difference between corresponding amounts of PCR products obtained in the absence or presence of progesterone (Student's *t*-test, *P* < 0.05). Note that the exponential product accumulation occurs only in the range below 30 arbitrary units.

metallothionein gene (Brönnegård *et al.* 1994). As adipose fibroblasts represent only a small fraction of cells from adipose tissue (when measured by volume or mass), this does not exclude the possibility that minute amounts of progesterone receptors, which are below the detection limit in whole tissue preparations, are present only in fibroblasts from human adipose tissue. However, recently this issue was directly addressed in adipose fibroblasts by binding studies and RT-PCR analysis, which confirmed that no progesterone receptors and mRNA are present in these cells (Pedersen *et al.* 1996).

Since the progesterone receptor antagonist ZK114043 (Li *et al.* 1995) did not relieve the inhibitory action of progesterone on aromatase induction, an action of progesterone via the progesterone receptor can be excluded.

Our experimental data thus provide independent evidence against the existence of functional progesterone receptors in human adipose tissue, especially in the adipose fibroblasts.

Glucocorticoid receptors are readily detectable in human adipose tissue, and binding of progesterone to the glucocorticoid receptor has been shown previously (Xu *et al.* 1990). The inhibitory potential of progesterone in our experimental system depends on the concentrations of cortisol used, indicating competition for the glucocorticoid receptor between these steroid hormones. Whereas cortisol binding results in a transcriptionally active receptor, progesterone binding appears to be unable to generate an active receptor. A previous study has found that progesterone (about 50 nM) had no effect on aromatase

Table 1 Progesterone action is not blocked by an anti-progestin. Adipose fibroblasts were induced for 24 h with 1 μ M cortisol and 0.5 nM PDGF-BB (PDGF) in the absence or presence of 1 μ M progesterone and/or 1 μ M of the pure anti-progestin ZK114043. The activities observed vary considerably between individual preparations of cells and appear to be due to unknown traits. Therefore data are presented as relative aromatase activities compared with those activities measured in the presence of cortisol and PDGF-BB. Under these conditions, the activities observed were in the range 5–30 pmol/6 h per protein. Results are given as means \pm S.E.M. from three preparations of cells assayed in duplicate.

Treatment of cells	Relative aromatase activity (% of PDGF+cortisol)
PDGF+cortisol	100.0 \pm 4.4
PDGF+cortisol+progesterone	46.4 \pm 4.1
PDGF+cortisol+ZK114043	76.3 \pm 9.2
PDGF+cortisol+progesterone+ZK114043	22.2 \pm 3.3

Differences between individual treatment groups are significant ($P < 0.05$) as determined by ANOVA and Student–Newman–Keuls all pairwise multiple comparison.

induction in adipose fibroblasts in the presence of serum and 250 nM dexamethasone (Lueprasitsakul & Longcope 1990). However, this does not disagree with our results, and allows us to predict that even higher progesterone concentrations will be necessary for competition in the presence of the high-affinity ligand dexamethasone.

Glucocorticoid and progesterone receptors bind to the identical consensus sequence, and it has been shown that induction of gene expression by these hormones in target cells is dependent on the expression of their specific receptor (Strahle *et al.* 1989). Competition for and inactivation of the glucocorticoid receptor by progesterone in adipose fibroblasts would offer an additional possibility for differential regulation of gene expression by steroids in these cells, which could not be the case if progesterone receptors were present. In this view, the absence of progesterone receptors enables progesterone to inhibit glucocorticoid-dependent aromatase induction.

Serum glucocorticoid concentrations exhibit marked circadian alterations, ranging from 0–0.17 μ M during the night to 0.22–0.7 μ M peak concentrations. Therefore it seems reasonable to assume that our data obtained in the presence of 0.1 μ M cortisol are close to physiological levels for most individuals for at least 12 h a day. In the presence of 0.1 μ M cortisol, 100 nM progesterone caused almost complete inhibition of glucocorticoid-dependent aromatase induction, whereas 10 nM produced roughly 50% inhibition (see Fig. 1 B). The former would apply only to late pregnancy, whereas the latter would apply to the luteal phase of premenopausal women, in which 6–60 nM progesterone is found. These considerations suggest that, in premenopausal women, progesterone is able partially to suppress glucocorticoid-dependent aromatase induction. This may explain the increase in adipose tissue aromatase activity in postmenopausal women (Hemsell *et al.* 1974). In addition, an inverse correlation between progesterone concentration and aromatase activity in adipose tissue has been reported (Newton *et al.* 1986), further supporting that notion. Taken together, our results provide evi-

dence that progesterone directly inhibits glucocorticoid-dependent aromatase induction and support the hypothesis that progesterone plays an important role in the regulation of adipose tissue aromatase activity *in vivo*.

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