Peroxisome Proliferator-activated Receptor γ Ligands Inhibit Estrogen Biosynthesis in Human Breast Adipose Tissue: Possible Implications for Breast Cancer Therapy

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ABSTRACT

Estrogen biosynthesis is catalyzed by aromatase cytochrome P-450 (the product of the CYP19 gene). Adipose tissue is the major site of estrogen biosynthesis in postmenopausal women, with the local production of estrogen in breast adipose tissue implicated in the development of breast cancer. In human adipose tissue, aromatase is primarily expressed in the mesenchymal stromal cells and is a marker of the undifferentiated preadipocyte phenotype. Aromatase expression in adipose tissue is regulated via the distal promoter I.4, under the control of glucocorticoids and class I cytokines such as oncostatin M, interleukin 6, and interleukin 11, as well as tumor necrosis factor α. These cytokines, which are expressed in adipose, also inhibit adipocyte differentiation. Therefore, we hypothesized that factors which stimulate adipocyte differentiation should inhibit aromatase expression. These factors include synthetic peroxisome proliferator-activated receptor γ (PPARγ) ligands such as thiazolidinediones, e.g., troglitazone and rosiglitazone (BRL49653) and the endogenous PPARγ ligand 15-deoxy-Δ12,14-prostaglandin J2. We have demonstrated by measurement of aromatase activity and by reverse transcription-PCR/Southern blotting that these PPARγ ligands inhibit aromatase expression in cultured breast adipose stromal cells stimulated with oncostatin M or tumor necrosis factor α plus dexamethasone in a concentration-dependent manner, whereas a metabolite of troglitazone that does not activate PPARγ has no effect. We have also shown that troglitazone inhibits luciferase activity of reporter constructs containing various lengths of the upstream region of promoter I.4 transfected into mouse 3T3-L1 preadipocyte mesenchymal cells, whereas the troglitazone metabolite does not. Because local estrogen production in breast fat is implicated in breast cancer development in postmenopausal women, the actions of PPARγ ligands suggest that they may have potential therapeutic benefit in the treatment and management of breast cancer.

INTRODUCTION

Estrogen biosynthesis is catalyzed by the enzyme aromatase (Refs. 1–4; aromatase cytochrome P-450; the product of the CYP19 gene; Ref. 5). CYP19 is a member of the P-450 superfamily of genes, which currently contains over 600 members in some 40 gene families (6). Aromatase is responsible for catalyzing the aromatization of the A ring of C19 androgens to the phenolic A ring of C18 estrogens, resulting in loss of the C19 angular methyl group as formic acid (7). In humans, aromatase is expressed in a variety of tissues including: the granulosa cells and corpus luteum of the ovary (8, 9); the Leydig cells and germ cells of the testis (10, 11); the syncytiotrophoblast of the placenta (8); various sites in the brain including the hypothalamus and hippocampus (12, 13); adipose tissue of the breast, abdomen, thighs, and buttocks (14, 15); and osteoblasts of bone (16, 17). The human CYP19 gene spans at least 75 kb, with a coding region of ~35 kb containing nine translated exons (II-X; Refs. 18–20; Fig. 1). Aromatase transcripts in the various tissue sites of expression contain different 5′-untranslated first exons because of the use of a number of alternative promoters that regulate aromatase expression in the ovary (promoter II; Ref. 9), placenta (promoter I.1; Refs. 8, 21, and 22), and adipose tissue (promoters I.4 and II; Ref. 23) via alternative splicing mechanisms. Each promoter is differentially regulated with promoter II under the control of follicle-stimulating hormone, the actions of which are mediated by cyclic AMP (24, 25), whereas promoter I.1 is regulated by retinoids (26). Expression via promoter I.4 in adipose tissue requires the synergistic actions of glucocorticoids and class I cytokines or TNFα (27–29). The former uses a Janus-activated kinase 1/STAT3 signaling pathway in which the activated STAT3 binds to a GAS element located upstream of promoter I.4 (28), whereas the latter uses a mitogen-activated protein kinase/AP-1 pathway, resulting in binding of c-fos/c-jun to an AP-1 site upstream of the GAS element (Ref. 29; Fig. 1).

Adipose tissue is the main site of estrogen biosynthesis in postmenopausal women, and aromatase expression in adipose increases with age and body weight (14, 15). Local production of estrogen in breast adipose tissue is implicated in the development of breast cancer in postmenopausal women (15, 30). Estrogen levels in breast tumors are as much as 10 times greater than in the circulation of postmenopausal women (31). This appears to be because aromatase expression within a tumor and the surrounding breast tissue is elevated as a result of the production of factors by the tumor which stimulate aromatase expression (32). Aromatase expression in adipose tissue is primarily located in the mesenchymal stromal cells and appears to be a marker of the undifferentiated preadipocyte phenotype (33–35). Consistent with this, factors known to stimulate aromatase expression in adipose tissue, such as the class I cytokines interleukin 6, interleukin 11, OSM, as well as TNF-α, also inhibit adipocyte differentiation (36, 37). Conversely, factors that stimulate adipocyte differentiation would be anticipated to inhibit aromatase expression. Such factors include ligands of PPARγ such as the TZDs troglitazone and rosiglitazone, as well as natural ligands such as 15d-PGJ2 (38, 39). PPARγ is a member of the superfamily of ligand-activated transcription factors, which includes receptors for steroid, retinoid, and thyroid hormones, and exists in three different isoforms, γ1, γ2, and γ3. It is abundantly expressed in adipose tissue and plays a key role in adipocyte differentiation (38–40). In the present report, we show that TZDs and 15d-PGJ2 do indeed inhibit aromatase expression in human adipose stromal cells of breast origin, whereas a metabolite of troglitazone that does not activate PPARγ has no action on aromatase expression. These findings suggest that troglitazone and other TZDs might have therapeutic utility in the management of breast cancer in postmenopausal women.

MATERIALS AND METHODS

Cell Culture. Adipose tissue was obtained from women undergoing reduction mammoplasty or reduction abdominoplasty, after receiving informed consent. Adipose stromal cells were isolated by collagenase digestion of adipose tissue as described (33) and maintained in primary culture at 50,000 cells/ml in DMEM enriched medium (Trace Biosciences, Sydney, New South Wales, Australia) supplemented with 15% FBS (15% v/v; Trace Biosciences) and allowed to grow until confluent (5–6 days) prior to treatment. After serum

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3 The abbreviations used are: TNF, tumor necrosis factor; STAT, signal transducers and activators of transcription; GAS, IFN-γ activating sequence; OSM, oncostatin M; PPARγ, peroxisome proliferator-activated receptor γ; TZD, thiazolidinedione; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Dex, dexamethasone; Luc, luciferase; AP-1, activating protein-1.
deprivation for 24 h, cells were treated for another 24 h with 250 nM dexamethasone and 5 ng/ml of recombinant human OSM (Sigma, Sydney, New South Wales, Australia) in the presence or absence of the PPARγ ligands troglitazone (Parke-Davis, Ann Arbor, MI), rosiglitazone (BRL49653), or 15d-PGJ2 (Cayman Chemical Co., Ann Arbor, MI) at varying concentrations. Cells were either assayed for aromatase activity or harvested for total RNA.

**Aromatase Assay.** Aromatase activity was determined after incubation of cells with [1,2-3H]estrone (NEN) and expressed as pmol/mg protein/2 h. Concentrations of 15d-PGJ2 >2 μM completely abolished aromatase activity, whereas concentrations <100 nM had little effect. The synthetic PPARγ ligand troglitazone inhibited aromatase activity with an IC50 of <1 μM. Concentrations of 15d-PGJ2 >2 μM completely abolished aromatase activity, whereas concentrations <100 nM had little effect. The synthetic PPARγ ligand troglitazone inhibited aromatase activity with an IC50 of <1 μM. Concentrations of 15d-PGJ2 >2 μM completely abolished aromatase activity, whereas concentrations <100 nM had little effect. The synthetic PPARγ ligand troglitazone inhibited aromatase activity with an IC50 of <1 μM. Concentrations of 15d-PGJ2 >2 μM completely abolished aromatase activity, whereas

**RESULTS**

**PPARγ Ligands Inhibit Aromatase Activity in Human Breast Adipose Stromal Cells.** The effect of PPARγ ligands on aromatase activity in human breast adipose stromal cells was determined. Basal levels of aromatase activity in these cells are low but can be markedly up-regulated in the presence of both glucocorticoids and class I cytokines or TNF-α (28, 29). The PPARγ ligands troglitazone, rosiglitazone (BRL49653), and 15d-PGJ2 inhibited Dex- and OSM-stimulated aromatase activity in human breast adipose stromal cells in a concentration-dependent manner, whereas a troglitazone metabolite that was not a PPARγ ligand had no effect (Fig. 2A). Similar results were obtained when TNF-α was substituted for OSM (Fig. 2B). The endogenous PPARγ ligand 15d-PGJ2 was the most potent inhibitor of aromatase activity, with an IC50 of <1 μM. Concentrations of 15d-PGJ2 >2 μM completely abolished aromatase activity, whereas concentrations <100 nM had little effect. The synthetic PPARγ ligand troglitazone inhibited aromatase activity with an IC50 of <10 μM. Concentrations of troglitazone >10 μM completely abolished aromatase activity.

![Fig. 1. The structure of the human CYP19 gene. The genomic region spans at least 75 kb, with a coding region of ~35 kb containing nine translated exons (III-X), represented by the closed bars. The heme binding region (HBR) is located in exon X, as are two alternative polyadenylation signals that give rise to two different aromatase transcripts of 3.4 and 2.9 kb. The 5′-region of the CYP19 gene contains several untranslated exons, e.g., I.1, I.3, and I.4, represented by the open bars. Expression of aromatase is controlled in a tissue-specific manner via the use of these alternative promoters. Because the various first exons are spliced into a common 3′-splice junction upstream of the start of translation, the coding region and hence the protein product are identical in each tissue site of expression. The identified regulatory elements, AP-1, GAS, and GRE (glucocorticoid response element) upstream of promoter I.4, are indicated. Arom, aromatase.](Image 264x591 to 560x741)

![Fig. 2. PPARγ ligands inhibit aromatase activity in human breast adipose stromal cells.](Image 330x121 to 538x398)
matase activity. To demonstrate that this was not an action of troglitazone to inhibit the catalytic activity of the aromatase enzyme directly, a time course of the inhibitory action of troglitazone was determined (data not shown). Troglitazone only blocked the induction of aromatase activity by Dex and OSM after an incubation of 24 h or greater. Troglitazone had no effect on aromatase activity when it was added simultaneously with the aromatase substrate [1β-3H]androstanedione.

PPARγ Ligands Inhibit Aromatase Expression in Human Breast Adipose Stromal Cells. Troglitazone and 15d-PGJ2 also inhibit aromatase expression in a concentration-dependent manner, as determined by RT-PCR amplification of promoter I.4-specific transcripts (Fig. 3, A and B) or coding region (C and D) for 30 cycles (D, 35 cycles). Integrity of cDNA was checked by amplification of GAPDH. Southern analysis was performed using a 32P-labeled probe to aromatase or GAPDH. Aromatase expression was quantitated by phosphorimaging and normalized against GAPDH.

Fig. 3. PPARγ ligands inhibit aromatase expression in human breast adipose stromal cells. Human breast adipose stromal cells were treated for 24 h with 250 nM Dex and 5 ng/ml OSM in the presence or absence of troglitazone (A and C) or 15d-PGJ2 (B and D). Total RNA was extracted, and 0.25 μg of total RNA was used for RT-PCR amplification of aromatase promoter I.4-specific transcripts (A and B) or coding region (C and D) for 30 cycles (D, 35 cycles). Integrity of cDNA was checked by amplification of GAPDH. Southern analysis was performed using a 32P-labeled probe to aromatase or GAPDH. Aromatase expression was quantitated by phosphorimaging and normalized against GAPDH.
fused to −774 bp of the 5’-regulatory region of promoter I.4 of the CYP19 gene in the pGL3 vector or else the pGL3-basic vector. Fig. 4 demonstrates that in the absence of treatment, basal levels of Luc gene expression were observed in cells transfected with both pGL3-basic vector and −774/+14 P450-I.4/Luc. Treatment with Dex and OSM (which up-regulate aromatase expression through promoter I.4) caused a 5-fold induction in Luc gene expression. Troglitazone caused a concentration-dependent inhibition of Luc gene expression with concentrations >10 μM, reducing expression to basal levels. This result was consistent with the effect of troglitazone on aromatase activity (Fig. 2) and expression (Fig. 3). Treatment with troglitazone had no effect on basal Luc or β-galactosidase expression, and treatment with a 0.1% DMSO vehicle did not affect Luc activity.

Troglitazone Inhibits Aromatase Transcription via Promoter I.4 through Interaction with PPARγ. To determine whether the effect of troglitazone is mediated through its binding to PPARγ, 3T3-L1 cells were transfected with the same Luc reporter gene constructs, −774/+14 P450-I.4/Luc or pGL3-basic vector, and treated with troglitazone and a troglitazone metabolite that is unable to bind to the receptor. Troglitazone inhibited transcription via promoter I.4 in a concentration-dependent manner as before, whereas the troglitazone metabolite had no effect on gene expression at any concentration (Fig. 5), similar to the results obtained in terms of aromatase activity (Fig. 2).

DISCUSSION

PPARγ is a key transcription factor involved in adipocyte differentiation (38, 39). Because of their ability to increase the responsiveness of insulin-sensitive cells to insulin, ligands for PPARγ have been actively studied for their therapeutic utility in the treatment of insulin-resistant diabetes, and currently one of these, namely troglitazone, is in clinical use in the United States for this purpose. More recently it has been found that PPARγ ligands can stimulate differentiation of a number of cell types and not only those of mesenchymal origin, e.g., colon cancer cells (42) as well as breast cancer cell lines (43), suggesting that PPARγ ligands could have therapeutic utility in the treatment of certain forms of cancer. Indeed, in a recent clinical trial of patients with advanced liposarcoma, it was found that troglitazone can stimulate the differentiation of such tumors in vivo (44). The observation that PPARγ ligands can stimulate the differentiation of breast cancer cell lines (43) suggests that these compounds might have utility in breast cancer therapy.

The results presented here provide independent data in support of this contention, i.e., that PPARγ ligands inhibit the expression of aromatase and hence estrogen biosynthesis in adipose tissue, and in particular adipose tissue of the human breast. Aromatase expression is a marker of the undifferentiated preadipocyte mesenchymal phenotype (33–35) and as such is stimulated by factors that inhibit adipocyte differentiation, such as class I cytokines and TNF-α, a number of which are synthesized in adipose tissue itself (36, 37). Consequently, it was anticipated that factors that stimulate adipocyte differentiation and increase the expression of differentiation markers, such as lipoprotein lipase and the insulin receptor, would also inhibit aromatase expression in adipose mesenchymal cells. The results presented here show that this is indeed the case; aromatase expression stimulated by class I cytokines or TNF-α is inhibited by several PPARγ ligands in a concentration-dependent fashion, whereas a troglitazone metabolite, which is not a PPARγ ligand, had no effect on aromatase expression. The results of transfection experiments using chimeric constructs in which the Luc reporter gene is regulated by the aromatase promoter I.4 indicate that this inhibition is a consequence of interaction with the cell signaling pathways involving Janus-activated kinase 1 and STAT3 in the case of the class I cytokines, and AP-1 in the case of TNFα. Interactions of PPARγ with these signaling pathways have previously been described in other systems, for example in a monocytic cell lineage, PPARγ interferes with IFN-γ action via a STAT1- and AP-1-mediated pathway (45). The mechanism whereby PPARγ inhibits cytokine-stimulated aromatase expression via promoter I.4 in adipose stromal cells remains to be elucidated. One possibility is that it competes with the STAT3 and AP-1 pathways for CREB-binding protein, which appears to play a universal role in mediating the transcriptional responses of genes to multiple signaling pathways. This possibility is currently under investigation.

The results presented here suggest that PPARγ ligands could find utility in breast cancer therapy. Presently, most breast cancer hormonal therapies are directed to inhibition of estrogen action or inhibition of aromatase activity. Estrogen receptor antagonists such as tamoxifen display selectivity in the tissue site of action, but generally after
several years of treatment, breakthrough occurs with clonal tumor lines developing that are unresponsive to tamoxifen. Aromatase inhibitors are used as second-line therapy and also have potential as first-line adjuvant therapy but have the disadvantage that they inhibit aromatase indiscriminately in all tissue sites including bone and brain, where they may have adverse effects in terms of bone mineralization on the one hand and possibly cognitive function on the other. Because aromatase expression is regulated differently in different tissue sites through the use of different tissue-specific promoters (35), the possibility is presented that tissue-selective inhibitors of aromatase expression could be developed. Whether PPARγ ligands will demonstrate sufficient tissue selectivity to warrant their development as breast cancer therapeutic agents remains to be determined, but results presented here are sufficiently encouraging to warrant further investigation.

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