

Interactions between Prostaglandin E₂, Liver Receptor Homologue-1, and Aromatase in Breast Cancer

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Abstract

Local synthesis of estrogens within breast adipose tissue by cytochrome P450 aromatase contributes to the growth of postmenopausal breast cancers. One of the major stimulators of aromatase expression in breast is prostaglandin E₂ (PGE₂) derived from tumorous epithelium and/or infiltrating macrophages. Recently, the orphan nuclear receptor, liver receptor homologue-1 (LRH-1), has also been shown to regulate aromatase expression in breast adipose tissue. We therefore examined the expression of, and correlations between, aromatase and LRH-1 mRNA in a panel of breast carcinoma tissues and adjacent adipose tissue. LRH-1 mRNA expression was low in normal breast tissue but markedly elevated in both breast carcinoma tissue and adipose tissue surrounding the tumor invasion (thereby paralleling aromatase expression). Laser capture microdissection localized the site of LRH-1 expression to tumor epithelial cells but not to intratumoral stromal cells. A strong correlation between LRH-1 and aromatase mRNA levels was observed in tumor-containing adipose tissue but not in tumor tissue. Ectopic expression of LRH-1 in primary human adipose stromal cells strongly activated endogenous aromatase mRNA expression and enzyme activity. Finally, treatment of adipose stromal cells with PGE₂ induced expression of both LRH-1 and aromatase. We suggest that PGE₂ derived from breast tumor tissue may increase aromatase expression in the surrounding adipose stroma in part by inducing LRH-1 in these cells. The roles of LRH-1 in breast cancer proliferation merit further study. (Cancer Res 2005; 65(2): 657-63)

Introduction

Numerous recent studies have supported an important role for cyclooxygenase-2 in breast cancer pathology. Cyclooxygenase-2 protein is overexpressed in ~40% of breast tumors (1), and overexpression of cyclooxygenase-2 is sufficient to induce mammary tumorigenesis in transgenic mice (2). Inhibition of cyclooxygenase-2 activity is protective against tumorigenesis in chemical- and oncogene-induced animal models of breast cancer (3–5). Furthermore, epidemiologic studies suggest that nonsteroidal anti-inflammatory drug use protects against breast cancer development, with a 24% reduction in breast cancer risk being associated with nonsteroidal anti-inflammatory drug use in a case-control study of ~6,000 Canadian women (6) and a 28% risk reduction with long-

term regular nonsteroidal anti-inflammatory drug use seen in a recent prospective analysis of the Women's Health Initiative Observational Study of 80,741 postmenopausal women (7).

The mechanisms underlying these protective effects of nonsteroidal anti-inflammatory drugs are unclear. However, it is likely that inhibition of local estrogen synthesis within the breast is a major component of their action. The source of estrogens for postmenopausal estrogen receptor-positive breast tumor growth is via local conversion of circulating androgen precursors due to expression of aromatase within the tumor and surrounding adipose tissue (8). The estradiol concentration within tumor-containing breast tissue is at least 10 times that of the circulation (9, 10), and this arises as a consequence of elevated aromatase expression within the malignant tissue and surrounding stroma in response to tumor-derived stimulatory factors (11, 12). This induction of aromatase expression is associated with a switch in *CYP19* gene promoter usage from the normal adipose-specific promoter I.4 to the gonadal-type promoter II (13–15), and the most potent factor thus far identified that stimulates activity of promoter II is prostaglandin E₂ (PGE₂; ref. 16). Thus, the chemoprotective effects of nonsteroidal anti-inflammatory drugs in postmenopausal women are likely to be mediated at least in part by inhibition of PGE₂-induced aromatase expression in breast adipose tissue.

PGE₂ stimulates aromatase activity and expression in adipose stromal cells by activating prostaglandin receptor subtypes EP₁ and EP₂ linked to protein kinase C (PKC) and protein kinase A (PKA), respectively (16, 17). Activation of both receptor subtypes is necessary for full aromatase activity. The action of PKA is mediated by phosphorylation and activation of members of the cyclic AMP (cAMP)-responsive element (CRE) binding protein/activating transcription factor-1 family of transcription factors that bind to two distinct CRE-like sequences within promoter II (18). Activation of PKC by phorbol esters alone has little effect to stimulate aromatase activity in adipose stromal cells (16), although its action seems to potentiate the effect of PKA. The downstream molecular mechanisms activated by this pathway, and how they interact with the PKA pathway to maximally induce aromatase expression, are unknown.

Whereas PGE₂-induced aromatase expression in breast adipose tissue depends on PKA/PKC/CRE binding protein signaling, basal expression requires the presence of a nuclear receptor half-site (CAAGGTCA) located within the proximal promoter II. Several orphan members of the nuclear receptor superfamily have been shown to bind to this site in different tissues, including SF-1 (ovary, endometriosis; refs. 19–21); COUP-TF, ERR α -1, and EAR-2 (endometriosis, breast tumor fibroblasts; refs. 21–23); and TR α -1 (Sertoli cells; ref. 24). In breast adipose stromal cells, a major binding protein at this site is liver receptor homologue-1 (LRH-1; also known as FTF, hB1F, CPF, or NR5A2; ref. 25). LRH-1 is coexpressed with aromatase in undifferentiated adipose stromal

Note: J. Zhou, T. Suzuki, and A. Kovacic contributed equally to this work.

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cells and binds to promoter II to stimulate transcription. As stromal cells differentiate into mature adipocytes, LRH-1 expression is rapidly lost followed by loss of aromatase (25). Importantly, the ability of PGE₂ to stimulate aromatase promoter II reporter genes expressed in 3T3-L1 preadipocytes is greatly enhanced by LRH-1 cotransfection (25), suggesting that LRH-1 acts as a competence factor for this promoter in adipose stromal cells, sensitizing it to hormonal stimulation by other factors.

This ability of LRH-1 to regulate aromatase expression and estradiol production in breast adipose suggests that it may play a role in breast cancer development. Although this hypothesis has yet to be tested, it is noteworthy that the chromosomal region harboring LRH-1 (1q32.1) has been identified both as a common region of chromosomal gain and as a site of high-level amplification both in primary breast tumors (26) and in a study of 38 breast cancer cell lines (27). 1q32 is also a common amplification in liver (28–30) and ovarian (31) tumors (sites of high LRH-1 expression) but is not commonly amplified in tumors of other tissues that do not normally express LRH-1 (see ref. 32 for a review of comparative genomic hybridization data).

In the present study, we show that LRH-1 expression is increased in human breast tumors and surrounding adipose tissue, that LRH-1 induces aromatase mRNA expression in enzyme activity in primary human adipose stromal cells, and that PGE₂ stimulates LRH-1 expression in these cells. These findings suggest that LRH-1 may play a role in breast cancer pathogenesis in part by increasing aromatase expression in adipose tissue surrounding breast carcinomas.

Materials and Methods

Cell Culture and Stimulation. Human adipose stromal cells were isolated from s.c. adipose tissue obtained with informed consent from women undergoing reduction mammoplasty and cultured as described previously (33). All procedures were approved by the Human Ethics Committee, Monash Medical Centre. Cells were grown until confluent, incubated in serum-free DMEM for 24 hours, and treated with the experimental agents indicated. 3T3-L1 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum.

Aromatase Assay. Aromatase activity was determined after incubation of cells with [³H]androstenedione (NEN Life Science Products, Boston, MA) for 2 hours and measured by the incorporation of tritium into [³H]water as described previously (33).

Adenoviral Constructs and Infection. Recombinant Tet-Off adenovirus containing full-length mouse *Lrh-1* cDNA was constructed according to the manufacturer's instructions (Adeno-X Tet-Off Expression System, Clontech, Palo Alto, CA). Viruses were propagated in HEK 293 packaging cells, and viral titer was determined by plaque assay. Human adipose stromal cells were plated in 80 cm² flasks and allowed to reach 80% confluency before virus infection. Cells were infected with 20 μL of Adeno-X Tet-Off virus [10¹¹ plaque-forming units (pfu)/mL] and 20 μL of Adeno-X TRE-Lrh-1 virus (10¹¹ pfu/mL). Tetracycline (2 μg/mL) was used to suppress exogenous LRH-1 expression.

RNA Isolation and Real-time PCR from Cultured Cells. Total RNA was prepared from primary adipose stromal cell cultures using the QiaAMP RNA Blood Mini kit (Qiagen, Valencia, CA). First-strand cDNA synthesis from 1 μg of total RNA was performed using avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals, Indianapolis, IN) primed by random hexamers. PCR reactions were carried out using the following primer sets (all 5'→3'): *LRH-1* (sense, CTGATACTGGAACCTTTGAA and antisense, CTTTCATTTGGTCAACCTT), *CYP19* coding region (sense, TTGGAATGCTGAACCCGAT and antisense, CAGGAATCTGCCGTGG-

GAGA), *CYP19* promoter II specific (sense, GCAACAGGAGCTATAGAT and antisense, CAGGAATCTGCCGTGGGAGA), *CYP19* promoter I.4 specific (sense, GTGACCAACTGGAGCCTG and antisense, CAGGAATCTGCCGTGGGAGA), *17βHSD1* (sense, AGGGCCGCGTGACGCTGGTGTGTAAC and antisense, CCATCAATCTCCACGCTCCCGG), *EST* (sense, AGAGGAGCTTGTGGACAGGA and antisense, GGGACAATTTCTGGTTCAT), *STS* (sense, ACTGCAACGCCTACTTAAATG and antisense, AGGGTCTGGGTGTGTCTGTC), and *I8S* (sense, CGGCTACCACATCCAAGGAA and antisense, GCTGGAATTACCGCGCT). Real-time PCR amplification was performed on the LightCycler (Roche Molecular Biochemicals) using SYBR Green reaction mix (Roche Molecular Biochemicals) and the primers described above. cDNA samples were diluted 1:10 (*17βHSD1*, *EST*, and *STS*) or 1:20 (*CYP19*, *LRH-1*, and *I8S*) in water immediately before use. Experimental samples were quantified by comparison with standards of known concentration (1–100,000 fg/μL).

RNA Isolation and RT-PCR for Solid and Microdissected Tissues.

For solid tissues, total RNA was extracted with guanidinium thiocyanate followed by ultracentrifugation in cesium chloride. A reverse transcription kit (SuperScript II Preamplification System, Life Technologies, Grand Island, NY) was used in the synthesis of cDNA. To examine the intratumoral localization of LRH-1 mRNA, laser capture microdissection was conducted using the Laser Scissors CRI-337 (Cell Robotics, Inc., Albuquerque, NM). A detailed procedure has been described previously (34, 35). Briefly, ~500 carcinoma or intratumoral stromal cells were collected separately under the microscope from breast carcinoma frozen tissue sections. Total RNA was extracted according to a RNA microisolation protocol described by Niino et al. (35). The synthesized cDNAs were amplified by PCR for 40 cycles. The products were resolved on a 2% agarose ethidium bromide gel, and the images were captured with Polaroid film under UV transillumination. RT-PCR was conducted as described above using the following primer pairs: *LRH-1* (sense, TGAAGCTGCTTCAGAACTGC and antisense, CCGTTCAGGTGCTTGTAGTA), aromatase (sense, GTGAAAAGGGGCAAAACAT and antisense, TGGAATCGTCTCAGAAGTGT), and glyceraldehyde-3-phosphate dehydrogenase (sense, TGAACGGGAAGCTCACTGG and antisense, TCCACCACCCTGTTGCTGTA).

Patients and Tissues. Thirty-eight specimens of invasive ductal carcinoma were obtained from female patients [mean age, 54.4 years (range, 35–74)] who underwent mastectomy from 2001 to 2002 in the Department of Surgery at Tohoku University Hospital (Sendai, Japan). Specimens of nonneoplastic breast tissue and adipose tissue adjacent to the carcinoma were also available in 12 and 27 of these 38 cases, respectively. Specimens for RNA isolation were snap-frozen and stored at –80°C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. The frozen specimens of adipose tissue examined were histologically confirmed not to contain the carcinoma cells using a part of these specimens. Patients examined in this study did not receive irradiation or chemotherapy before surgery. The histologic grade of each specimen was evaluated based on the method of Elston and Ellis (36). Informed consent was obtained from all patients before their surgery, and all research protocols were approved by the Ethics Committee at Tohoku University School of Medicine.

LRH-1 Immunohistochemistry. Goat polyclonal antibody for LRH-1 (sc-5997) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A Histofine kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used for immunohistochemistry. Antigen retrieval for was done by heating the slides in an autoclave at 120°C for 5 minutes in citric acid buffer [2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate (pH 6.0)]. LRH-1 antibody (sc-5997) was used at a dilution of 1:300. The antigen-antibody complex was visualized with 3,3'-diaminobenzidine solution [1 mmol/L 3,3'-diaminobenzidine, 50 mmol/L Tris-HCl buffer (pH 7.6), and 0.006% H₂O₂] and counterstained with hematoxylin. As a positive control, we used formalin-fixed and paraffin-embedded hepatoma cells (HuH7). As a negative control, normal goat, rabbit, or mouse immunoglobulin G was used instead of the primary antibodies, and immunohistochemical preabsorption tests were also done for LRH-1 using the blocking peptide (sc-5997P, Santa Cruz Biotechnology). No specific immunoreactivity was detected in these sections.

Results

Expression of LRH-1 and Aromatase in Breast Tissue.

Aromatase is up-regulated in adipose tissue surrounding breast cancers, and the resulting increase in local estradiol concentration constitutes the major source of estrogens for stimulating the growth of postmenopausal estrogen receptor-positive tumors. We recently described LRH-1 as a novel regulator of aromatase expression in human adipose tissue: LRH-1 mRNA was detected by real-time PCR in human adipose and primary breast cancer tissues (25), although levels were not quantified. Therefore, we quantified LRH-1 mRNA by real-time PCR in a panel of normal breast tissue specimens (predominantly adipose tissue, $n = 12$) as well as in adipose tissue surrounding breast cancers ($n = 27$) and the carcinoma tissue itself ($n = 38$; Fig. 1A). LRH-1 mRNA was ~ 5 times higher in adipose tissue surrounding breast carcinomas than in normal breast adipose tissue and markedly (4-fold) elevated in carcinoma tissue compared with normal tissue. This profile of expression is very similar to that of aromatase, which is expressed ~ 4 - to 5-fold higher in tumor-bearing breast adipose and breast carcinoma tissues than in normal adipose tissue (12, 15, 37).

To examine the mRNA localization of LRH-1 in breast cancer tissues, we performed laser capture microdissection to separate carcinoma cells from intratumoral stromal cells followed by real-time PCR (Fig. 1B). Although some variability in expression of the housekeeping gene *GAPDH* was evident, LRH-1 mRNA was detected only in carcinoma cells but not in intratumoral stromal cells. To confirm these RT-PCR data, we did immunohistochemistry in 38 specimens of invasive ductal carcinoma and related the expression levels to those of the positive control, HuH7 hepatoma cells (set at 100%). LRH-1 immunoreactivity was detected in the nucleus of invasive ductal carcinoma cells (Fig. 1C) in 3 of 38 (8%) cases, and the staining intensity in these cases was 566%, 316%, and 201% of that in HuH7 cells. No staining was observed in intratumoral stromal cells. LRH-1 immunoreactivity was also detected in the nucleus of adipose stromal cells and was markedly expressed in areas adjacent to the cancerous invasion (Fig. 1D) in 4 of 38 (11%) cases. The LRH-1 mRNA level in adipose tissue in these 4 cases was 576% and 300% in 2 cases and no specimens were available in 2 cases. LRH-1 immunoreactivity was negative in nonneoplastic mammary epithelium (data not shown). Note that the failure to detect LRH-1 immunoreactivity in the majority of breast cancer specimens likely reflects antibody sensitivity issues, because (a) the three cases in which LRH-1 immunoreactivity was detected expressed the highest levels of LRH-1 mRNA by real-time PCR and (b) this commercially available antibody has been noted previously to have relatively low affinity for native LRH-1, producing only a weak partial supershift of recombinant LRH-1 in gel shift analysis (25). Nevertheless, the current study clearly localizes the site of LRH-1 expression to tumor epithelium and surrounding adipose tissue.

Because aromatase expression has been reported in both intratumoral stroma and adipose tissue adjacent to the cancerous invasion (11, 12, 38–40), we quantified aromatase mRNA levels by real-time PCR in both carcinoma and adjacent adipose tissues and correlated these findings with the LRH-1 mRNA levels. There was a strong positive correlation between LRH-1 and aromatase mRNA levels in adipose tissue ($r = 0.828$; $P < 0.0001$; Fig. 1E), whereas no significant correlation was detected between these factors in carcinoma tissue ($r = 0.062$; $P = 0.710$; Fig. 1F).

LRH-1 Stimulates Endogenous Aromatase Expression in Adipose Stromal Cells. The correlation between LRH-1 and

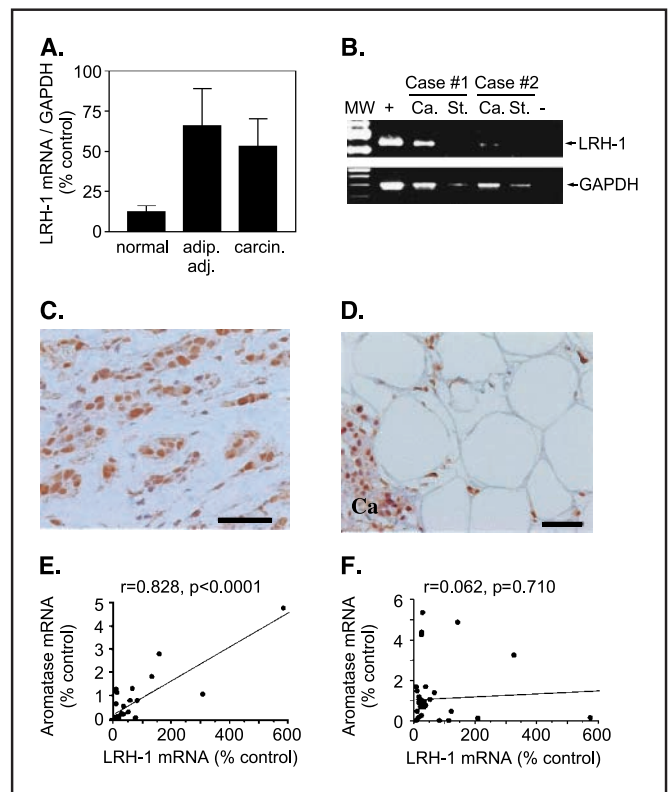


Figure 1. LRH-1 is expressed in breast cancer. **A**, RT-PCR quantification of LRH-1 mRNA in normal breast adipose tissue (*normal*), adipose tissue adjacent to breast carcinoma (*adip. adj.*), and breast cancer tissue (*carcin.*). LRH-1 mRNA was markedly detected in adipose tissue adjacent to the carcinoma ($66.1 \pm 23.0\%$, $n = 27$) and breast carcinoma tissue ($53.2 \pm 17.2\%$, $n = 38$), whereas it was very low in nonneoplastic breast tissue ($12.3 \pm 3.8\%$, $n = 12$). *Columns*, mean; *bars*, SE. mRNA level of LRH-1 in each specimen was evaluated as a ratio (%) of the positive control (HuH7 human hepatocellular carcinoma cells). Mean LRH-1 mRNA levels in the three groups were not statistically different by one-way ANOVA and Bonferroni test ($P = 0.32$). **B**, laser capture microdissection/RT-PCR analyses for LRH-1 in breast carcinoma tissues. mRNA expression for LRH-1 was detected as a specific single band (448 bp) in carcinoma cells but was not detected in intratumoral stromal cells. PCR was done for 40 cycles. +, positive control (HuH7 cells); -, negative control (no cDNA substrate). **C**, immunohistochemistry for LRH-1 in breast carcinoma. LRH-1 immunoreactivity was detected in the nucleus of invasive ductal carcinoma cells but was not observed in the intratumoral stromal cells. Bar, 50 μ m. **D**, immunoreactivity for LRH-1 was detected in the nucleus of adipocytes, especially marked adjacent to the carcinoma invasion. *Ca*, carcinoma cells. Bar, 50 μ m. **E**, association between mRNA levels of LRH-1 and aromatase in adipose tissue adjacent to the carcinoma. A strong positive correlation (linear regression $r = 0.828$, $P < 0.0001$) was detected. mRNA level in each specimen was evaluated as a ratio (%) compared with the positive control (HuH7 cells for LRH-1 and placental tissue for aromatase). **F**, correlation between mRNA levels of LRH-1 and aromatase in carcinoma tissue. No significant correlation (linear regression $r = 0.062$, $P = 0.710$) was detected.

aromatase expression in adipose tissue surrounding breast carcinomas (but not in carcinoma tissue) suggests that aromatase might be a target of LRH-1 action in adipose stromal cells. Although we have shown previously that LRH-1 can stimulate activity of aromatase promoter-luciferase constructs expressed in 3T3-L1 mouse preadipocytes (25), the relevance of this *in vitro* data to expression of the endogenous aromatase gene in human adipose tissue is not clear. A significant difficulty in studying aromatase expression in human adipose stromal cells is that these primary cells are markedly resistant to traditional methods of transfection. Therefore, to manipulate LRH-1 protein levels in these cells, we constructed a recombinant adenovirus expressing LRH-1 from a tetracycline-repressible promoter. In the presence of tetracycline, infection of

primary adipose stromal cells with up to 100 viral pfu had little or no effect on LRH-1 protein levels as measured by Western analysis (Fig. 2A, lanes 1-4). In the absence of tetracycline, however, infection of increasing viral pfu resulted in a dose-dependent increase in LRH-1 protein expression (Fig. 2A, lanes 5-8), indicating that LRH-1 protein levels are increased by viral infection and suppressed in the presence of tetracycline. Infection of LRH-1 into primary adipose stromal cells under basal conditions resulted in a 28-fold increase in aromatase mRNA as measured by real-time PCR (Fig. 2B, lanes 1 and 2). This induction was almost completely inhibited in the presence of tetracycline (Fig. 2B, lanes 3 and 4), indicating a specific response to the exogenous LRH-1 rather than a nonspecific effect of viral infection. When cells were incubated in the presence of the adenylyl cyclase activator forskolin and the PKC activator phorbol 12-myristate 13-acetate (PMA; to mimic the effects of PGE₂), aromatase mRNA levels increased ~20-fold (Fig. 2B, lane 5). LRH-1 expression produced a further 10-fold increase in aromatase mRNA (lane 6), and again, this effect of viral infection was inhibited in the presence of tetracycline (lanes 7 and 8), down to the same level as seen in the absence of infection, indicating that the tetracycline itself had no inhibitory action on aromatase expression. Because LRH-1 is thought to activate aromatase transcription by binding to a response element in promoter II (25), we next quantified aromatase transcripts derived from this promoter. As shown in Fig. 2C, promoter II-specific aromatase transcripts were induced by LRH-1 in almost exactly the same profile as observed for total aromatase transcripts, suggesting that the increase in aromatase mRNA in response to LRH-1 occurs through activation of promoter II.

To assess the effects of LRH-1 on endogenous aromatase protein, a further series of viral-infected cells were assayed for aromatase activity by measuring the release of [³H]water from [1β-³H]

androstenedione (33). LRH-1 produced a modest (~3-fold) increase in aromatase activity in the absence of hormonal stimulation (Fig. 2D, lanes 1 and 2)). Treatment with forskolin and PMA together induced aromatase activity by ~200-fold (lane 5). LRH-1 increased this hormone-induced aromatase activity by a further 2- to 3-fold (lane 6), and again, this effect was abolished in the presence of tetracycline (lane 7). In the presence of LRH-1 and forskolin/PMA, tetracycline had an additional inhibitory effect on aromatase activity (lane 8) that was not seen at the mRNA level (Fig. 2B and C), possibly reflecting nonspecific post-translational effects of viral infection on the aromatase protein. Nevertheless, these data clearly indicate that exogenous LRH-1 is capable of stimulating transcription of the aromatase gene in its native chromatin environment.

To assess the specificity of these effects on aromatase expression, we measured mRNA levels for several other genes involved in estrogen production and metabolism (Fig. 2E). No effect of LRH-1 was seen on aromatase transcripts derived specifically from promoter I.4 or on expression of *STS*, *EST*, or *17βHSD1*. Therefore, increasing the level of LRH-1 in primary adipose stromal cells specifically induces aromatase mRNA expression via promoter II, increases aromatase enzyme activity, and markedly potentiates the stimulatory effect of forskolin/PMA (i.e., of PGE₂) on aromatase expression and activity.

PGE₂ Induces LRH-1 Expression in Adipose Stromal Cells.

Tumor-derived PGE₂ is a well-characterized stimulator of aromatase expression in adipose tissue surrounding breast cancers (16, 17). PGE₂ binds to EP₁ and EP₂ receptors on adipose stromal cells, which are linked to PKC and PKA pathways, respectively. Activation of both pathways maximally stimulates aromatase expression (16). Because LRH-1 expression is increased in adipose tissue surrounding breast

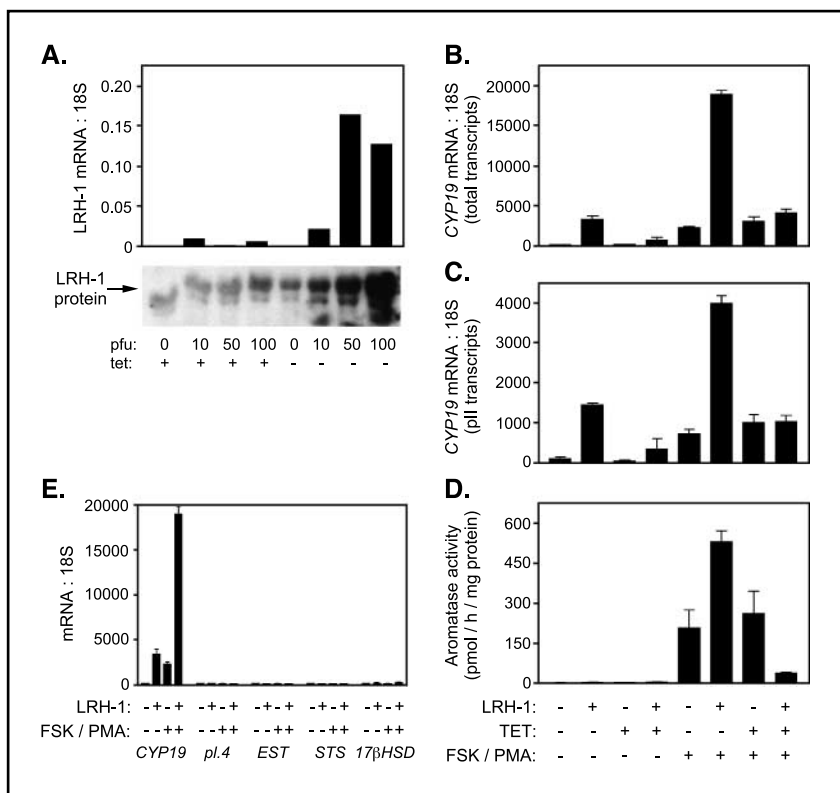
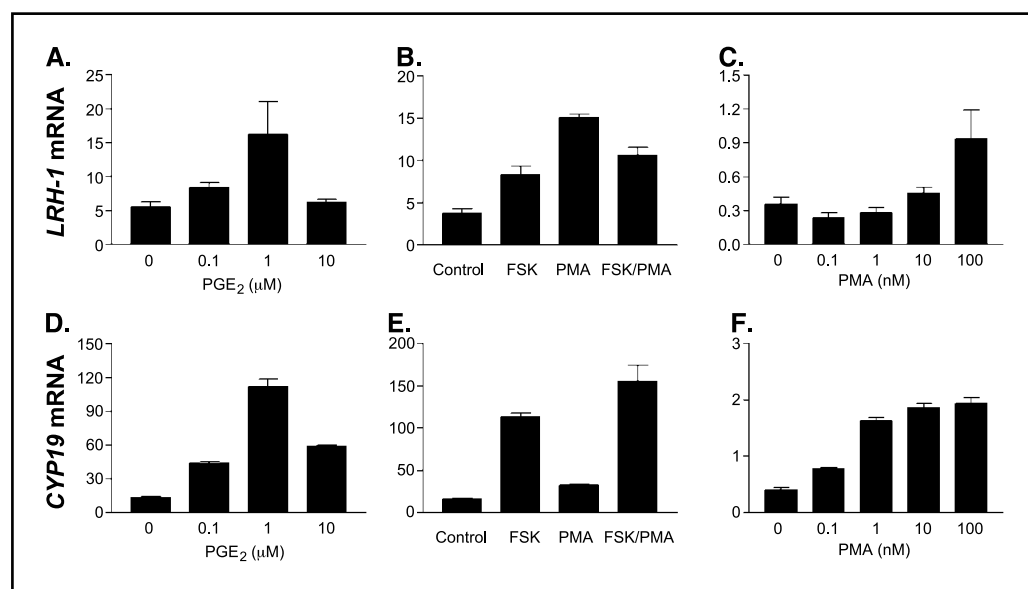


Figure 2. Exogenous LRH-1 stimulates endogenous aromatase expression in adipose stromal cells. A, primary cultures of human adipose stromal cells were infected with tetracycline-repressible adenovirus expressing LRH-1 (0-100 pfu) in the presence or absence of tetracycline. RT-PCR for LRH-1 mRNA (top) and Western analysis for LRH-1 protein (bottom) were done. Adenoviral infection produced a robust increase in LRH-1 mRNA and protein levels that was almost completely inhibited in the presence of tetracycline. B, adenoviral expression of LRH-1 in human adipose stromal cells stimulates endogenous CYP19 mRNA expression. Cells were infected with 10 pfu of virus in the presence or absence of forskolin (FSK) + PMA and/or tetracycline. C, adenoviral expression of LRH-1 in human adipose stromal cells stimulates endogenous CYP19 mRNA expression via promoter II. Cells were infected with 10 pfu of virus in the presence or absence of forskolin + PMA and/or tetracycline. D, adenoviral expression of LRH-1 in human adipose stromal cells stimulates endogenous aromatase activity. Cells were infected with 10 pfu of virus in the presence or absence of forskolin + PMA and/or tetracycline, and aromatase activity was determined by the incorporation of tritium derived from [1β-³H]androstenedione into [³H]water. E, LRH-1 specifically stimulates aromatase expression in adipose stromal cells. Cells were infected with 10 pfu of virus in the presence or absence of forskolin + PMA, and levels of total aromatase mRNA transcripts, transcripts derived from aromatase promoter I.4 (*pl.4*), and *EST*, *STS*, and *17βHSD1* measured by RT-PCR.

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Figure 3. PGE₂ induces LRH-1 mRNA expression in human adipose stromal cells. Human adipose stromal cells were treated with the indicated concentrations of PGE₂ for 24 hours, and (A) aromatase and (B) LRH-1 mRNA levels were quantified by RT-PCR. Similarly, cells were treated with forskolin (25 μmol/L) or PMA (4 nmol/L) alone or in combination for 24 hours, and (C) aromatase and (D) LRH-1 mRNA levels were quantified by RT-PCR. E, adipose stromal cells were treated with increasing concentrations of PMA and aromatase, and (F) LRH-1 mRNA levels were quantified by RT-PCR. Columns, mean CYP19 or LRH-1 levels (fg/μL cDNA) relative to total RNA concentration (triplicate determinations); bars, SD. Similar results were obtained in at least two independent experiments.



carcinomas (Fig. 1), we hypothesized that PGE₂ might stimulate aromatase expression in part by increasing LRH-1 expression. To test this hypothesis, adipose stromal cells were treated with PGE₂, and real-time PCR was performed to quantify LRH-1 and aromatase mRNA (Fig. 3). PGE₂ treatment dose-dependently increased LRH-1 expression, with maximal effect at 1 μmol/L PGE₂ (2.5-fold; Fig. 3A). Aromatase mRNA expression was increased in parallel (Fig. 3D). Higher concentrations of PGE₂ (10 μmol/L) produced a submaximal response for both LRH-1 and aromatase. To determine whether the PKA or PKC pathways mediate PGE₂-induced LRH-1 expression, cells were treated with forskolin and PMA alone or in combination (Fig. 3B). Treatment with forskolin and PMA stimulated LRH-1 expression 2- and 4-fold, respectively. In combination, forskolin and PMA induced LRH-1 expression ~2.5-fold. Aromatase expression was increased 7- and 2-fold by treatment with forskolin and PMA, respectively (Fig. 3E), and 10-fold in the presence of both agents together. Whereas the stimulation of aromatase expression by forskolin is mediated via activation of CRE binding protein to two distinct CRE-like sequences within promoter II (18), the pathway by which PMA potentiates this effect is unknown. Therefore, to confirm that activation of PKC by PMA induces LRH-1 expression in adipose stromal cells, cells were treated with increasing concentrations of PMA, and LRH-1/aromatase expression was quantified. PMA induced a dose-dependent increase in both LRH-1 (Fig. 3C) and aromatase (Fig. 3F) mRNA expression, reaching 5-fold over basal for aromatase and 2.5-fold for LRH-1. These data suggest that increased expression of LRH-1 in adipose stromal cells is one mechanism contributing to the induction of aromatase expression by PGE₂.

Discussion

In the current study, we show that (a) LRH-1 is markedly expressed in adipose tissue adjacent to breast carcinoma, (b) levels of LRH-1 are strongly correlated with that of aromatase in this tissue, (c) ectopic expression of LRH-1 markedly induces endogenous aromatase expression and enzyme activity in adipose stromal cells, and (d) PGE₂ induces LRH-1 mRNA expression in these cells. The findings suggest that one mechanism by which PGE₂ synthesized in

breast carcinomas, stimulates aromatase activity in surrounding adipose tissue is through induction of LRH-1 expression.

Increased aromatase expression in breast cancer adipose tissue is associated with a switch in promoter usage from the normal adipose-specific promoter I.4 to the PGE₂-responsive promoter II (13–15). PGE₂ activates transcription from promoter II in part by activating PKA, which in turn phosphorylates CRE binding protein causing it to bind to two distinct CRE-like sequences within the promoter (18). PGE₂ also activates PKC via EP₁ receptors leading to a maximal induction of promoter II activity (16, 17). The exact mechanisms by which PKC stimulates promoter II are unclear; however, the present results suggest that increased expression of LRH-1 might contribute to this effect. The importance of this lies in the fact that LRH-1 acts as a competence factor for promoter II, sensitizing it to stimulation by PKA (25). Therefore, LRH-1 and PKA signaling act in synergy to stimulate promoter II. Thus, relatively small increases in LRH-1 expression may have major effects on aromatase activity in the context of increased cAMP signaling. This is particularly relevant given that expression of CRE binding protein is also increased in adipose tissue surrounding breast carcinomas (18).

Immunohistochemistry localized the sites of LRH-1 expression to tumor epithelium and surrounding adipose tissue. Expression in adipose tissue correlated significantly with that of aromatase, consistent with a role for LRH-1 in regulating aromatase expression in this tissue, as discussed above. The lack of LRH-1 expression in intratumoral stromal cells is perhaps surprising, however, because these cells also express aromatase (11, 38–40). Previous *in vitro* studies have shown the regulation of aromatase expression in breast fibroblasts by various other transcription factors, including CCAAT/enhancer binding protein (41), EAR-2/3, retinoic acid receptor (23), and ERR (22). Interestingly, although ERR has been reported to activate aromatase in breast tumor fibroblasts, it does not significantly increase aromatase transcription in normal breast preadipocytes (25), and ERR expression in breast cancer tissue is limited to the tumor epithelium rather than to the surrounding adipose stroma (42). Although ERR immunoreactivity is associated with poor outcome, this does not

seem to be related to induction of aromatase because ERR and aromatase do not colocalize in breast tissue, and no correlation between ERR and aromatase expression levels exists in breast cancer tissue (42).

In contrast to tumor stromal cells, tumor epithelium expressed relatively high levels of LRH-1 (Fig. 1C), although no correlation between LRH-1 and aromatase mRNA expression in tumor epithelium was evident. This therefore raises questions as to the possible roles of LRH-1 in breast cancer. Although this is the first study to show LRH-1 expression in cancer tissue, the roles of LRH-1 in tumorigenesis are unknown. LRH-1 is predominantly expressed in tissues of endodermal origin such as liver, pancreas, and intestine (43–46). One of the primary targets of LRH-1 is the α -fetoprotein gene (43), and α -fetoprotein is a well-characterized marker of cancers of these tissues (47). α -Fetoprotein is also expressed in human breast cancer (48) and MCF-7 cells (49), consistent with the expression of LRH-1 described here, and proliferative effects of α -fetoprotein on breast cancer cells

in vitro have been described (50). Our preliminary studies indicate that ectopic expression of LRH-1 in MCF-7 breast cancer cells has pronounced proliferative effects (data not shown). Thus, LRH-1 may play dual roles in breast cancer development—a direct role to stimulate proliferation of tumorous epithelium and an indirect role via stimulation of aromatase expression in the adipose mesenchymal cells surrounding the tumor. For these reasons, the pathophysiologic effects of LRH-1 expression in breast cancer and its role in cell proliferation warrant further investigation.

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