Liver Receptor Homologue-1 (LRH-1) Regulates Expression of Aromatase in Preadipocytes

Estrogen biosynthesis from C_{19} steroids is catalyzed by aromatase cytochrome P450. Aromatase is expressed in breast adipose tissue through the use of a distal, cytokine-responsive promoter (promoter L4). Breast tumors, however, secrete soluble factors that stimulate aromatase expression through an alternative proximal promoter, promoter II. In other estrogenic tissues such as ovaries, transcription from promoter II requires the presence of the Ftz-F1 homologue steroidogenic factor-1 (SF-1); adipose tissue, however, does not express SF-1. We have explored the hypothesis that in adipose tissue, an alternative Ftz-F1 family member, liver receptor homologue-1 (LRH-1), substitutes for SF-1 in driving transcription from promoter II. In transient transfection assays using 3T3-L1 preadipocytes, promoter II reporter constructs were modestly (2–3-fold) stimulated by either activators of PKA or PKC. Quantitative real-time PCR revealed that LRH-1 (but not SF-1) is expressed in the preadipocyte fraction of human adipose tissue at levels comparable with that of liver. Differentiation of cultured human preadipocytes into mature adipocytes was associated with a time-dependent induction of peroxisome proliferator-activated receptor-γ (PPARγ), and rapid loss of LRH-1 and aromatase expression. We conclude that LRH-1 is a preadipocyte-specific nuclear receptor that regulates expression of aromatase in adipose tissue. Alterations in LRH-1 expression and/or activity in adipose tissue could therefore have considerable effects on local estrogen production and breast cancer development.

Estrogen biosynthesis from C_{19} steroids is catalyzed by the enzyme aromatase cytochrome P450 (1). In humans, aromatase is expressed in both the granulosa and luteal cells of the ovary and also in various extra-glandular sites, including the placenta, brain, bone, testis, and adipose tissue (2). Aromatase is encoded by the CYP19 gene, which maps to chromosome 15q21.2 in humans (3, 4). The structure and hormonal regulation of CYP19 are complex: the gene spans 123 kb, with a coding region of 30 kb comprising nine translated exons (4–7). A number of untranslated exons I, each driven by a unique promoter, exist upstream of exon II (8–10). These are spliced to a common site in the 5’-untranslated region. Tissue-specific regulation of CYP19 expression is achieved through the use of these distinct promoters, each of which is regulated by distinct hormonal factors. Thus in the ovary, CYP19 expression is regulated by FSH, which acts (through cAMP) via promoter II (11, 12), whereas in placenta, promoter I.I regulates CYP19 expression in response to retinoids (13). In bone and adipose tissue, by contrast, a distal promoter (promoter L4) drives CYP19 expression under the control of glucocorticoids, class 1 cytokines, or TNFα (14–17).

In postmenopausal women, aromatase activity in adipose tissue is the major source of circulating estrogens (18, 19). In normal breast adipose tissue aromatase activity and CYP19 expression are low. However, in adipose tissue of breast cancer patients, estrogen levels, aromatase activity, and CYP19 expression are elevated (20–23). This occurs in response to tumor-derived factors (such as prostaglandin E2) produced by breast tumor fibroblasts and epithelium as well as infiltrating macrophages (24). It is this local source of estrogen that provides the drive for growth of estrogen receptor-positive tumors and which is the target of anti-estrogen adjuvant therapies in postmenopausal women. However, current strategies of anti-estrogen therapy such as pure estrogen receptor antagonists or aromatase enzyme inhibitors act in a global fashion and inhibit estrogen action or synthesis in all sites of production. This has the potential to result in bone loss and other sequelae of estrogen insufficiency such as cognitive dysfunction and hepatic steatosis with prolonged treatment (25–27). Thus there is a clear need for more specific, tissue-selective anti-estrogens.

The increased CYP19 expression in response to breast tumor-derived factors is associated with a switch in promoter usage from the normal adipose-specific promoter L4 to the cAMP-responsive promoter II (28–30). Since these two promoters are regulated by different cohorts of transcription factors and coactivators, it follows that the differential regulation of CYP19 expression via alternative promoters in disease-free and cancerous breast adipose tissue may permit the development of selective aromatase modulators, which target the aberrant overexpression in cancerous breast, while sparing estrogen action in other sites of synthesis such as normal adipose tissue, bone, and brain (31). A more complete understanding of the mechanisms regulating CYP19 transcription from promoter II in breast adipose tissue is a prerequisite for the development of such selective aromatase modulators.

In classic steroidogenic tissue such as ovary and testis, pro-
The abbreviations used are: SF-1, steroidogenic factor-1; LRH-1, liver receptor homologue-1; ER, estrogen receptor; ERRα, estrogen receptor-related receptor-α; PPARy, peroxisome proliferator-activated receptor-γ; NRE, nuclear receptor half-site; DBD, DNA binding domain; PKA, protein kinase A; PKC, protein kinase C; FSK, forskolin; PMA, phorbol 12-myristate 13-acetate; Ftz-F1, fushi tarazu F1.

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2 C. D. Clyne, C. J. Speed, J. Zhou, and E. R. Simpson, unpublished observations.
the role of LRH-1 in CYP19 transcription has not previously been investigated.

**LRH-1 Is Expressed in Breast Adipose and Cancer Tissue**—SF-1 and LRH-1 are expressed at high levels in steroidogenic tissues and liver, respectively. To address the possible roles of these receptors in the regulation of CYP19 expression in adipose tissue, we determined the expression profiles of each receptor in various cell lines and adipose tissue specimens (Fig. 2A). LRH-1 mRNA expression was detected by RT-PCR in mouse adrenal and liver, human adrenal and liver cell lines (H295R and HepG2), the 3T3L1 mouse preadipocyte cell line, and the MCF-7 human breast cancer cell line. LRH-1 was also expressed in four out of four human adipose tissue specimens, and in four of four primary cultures of adipose stromal cells derived from different individuals. In contrast, expression of SF-1 mRNA was restricted to mouse adrenal and human adrenocortical H295R cells. We also investigated SF-1 and LRH-1 expression in human primary breast cancer tissue (Fig. 2B). All seven breast cancer specimens examined expressed LRH-1, whereas SF-1 was undetectable. Thus LRH-1, but not SF-1, is expressed in breast adipose tissue and cancer tissues.

To quantify LRH-1 mRNA expression in these tissues, we performed real-time PCR (Fig. 3A). Expressed as the ratio of LRH-1 molecules:18S molecules per μg of total RNA; LRH-1 expression in adipose tissue was ~20% that of liver. However, the relative expression in primary cultured adipose stromal cells was much higher, ~11-fold higher than in whole adipose tissue and 2.5-fold higher than in liver. LRH-1 protein was also readily detectable by Western blotting in isolated preadipocytes, but not in whole adipose tissue (Fig. 3B). Thus although LRH-1 mRNA levels in adipose tissue are relatively low compared with liver, LRH-1 expression is enriched in the adipose stromal cell compartment. This suggests that LRH-1, like CYP19, may be a marker of the undifferentiated preadipocyte phenotype.

To test this hypothesis, primary cultured human preadipocytes were induced to differentiate into adipocytes by a 12-day incubation in adipogenic medium (Fig. 3C). Under such conditions lipid droplets became visible after 6 days, and by day 12 ~50% of the cells exhibited abundant lipid accumulation (Fig. 3C, lower panel). The mature adipocyte phenotype was confirmed by a rapid and sustained expression of PPARγ (Fig. 3D, upper panel). LRH-1 mRNA was readily detectable in untreated preadipocytes but was dramatically reduced following 3 days of culture in adipogenic medium and undetectable at days 9 and 12. CYP19 mRNA expression also displayed a time-dependent decrease with progression of differentiation. Therefore, LRH-1 is expressed at high levels in human preadipocytes, but not mature adipocytes. Since this expression profile mirrors that of CYP19, LRH-1 is a potential physiological regulator of CYP19 expression in breast adipose stromal cells.

**Regulation of CYP19 Promoter II by LRH-1—Aromatase activity and CYP19 mRNA expression are strongly induced by prostaglandin E2 derived from breast cancer cells and/or macrophages infiltrating the tumor site (24). PGE2 binds to EP1 and EP2 receptors linked to PKC and PKA signaling pathways, activation of which together maximally stimulates CYP19 expression via promoter II (24). To assess the effect of these pathways on LRH-1-induced CYP19 transcription, 3T3L1 cells were cotransfected with the CYP19 promoter II reporter construct and increasing concentrations of LRH-1 expression construct. Cells were then incubated in the presence or absence of the adenylyl cyclase activator forskolin and the PKC activator PMA for 8 h (Fig. 4). In the absence of stimulation, LRH-1 dose-dependently increased promoter II activity reaching a maximum of 3-fold over basal at 1.0 μg of LRH-1 plasmid. Treatment with forskolin and PMA increased basal promoter II activity 4-fold; however, in the presence of these agents LRH-1 strongly induced promoter II activity reaching a maximum of 30-fold at 1.0 μg of LRH-1.

The synergistic effects of LRH-1 and FSK + PMA raised the possibility that LRH-1 contributes to PKA and/or PKC induction of promoter II. The primary amino acid sequence of LRH-1 contains several potential consensus PKA and PKC phosphorylation sites (PKA: Ser-32, Thr-142, Ser-362; PKC: Ser-32, Ser-126, Thr-154, Ser-350, Thr-512). To determine whether transactivation by LRH-1 can be directly modified by phosphorylation, we constructed a fusion construct in which the DNA binding domain of LRH-1 is replaced by the DBD of the yeast transcription factor GAL4. This fusion construct was transfected into 3T3-L1 cells along with a GAL4-responsive luciferase reporter gene, and cells treated with FSK and PMA, alone or in combination, for 16 h. As a control, we also treated 3T3-L1...
cells transfected with pII-516 with these agents (Fig. 5A). Treatment with FSK increased activity of pII-516 5-fold. PMA, while ineffective on its own, increased FSK-induced activity to 8.5-fold. These changes in activity of pII-516 mirror the effects of FSK and PMA on endogenous aromatase activity in adipose stromal cells (24). 3T3-L1 cells transfected with the GAL4 DBD and a GAL4-responsive luciferase reporter had low levels of luciferase activity that were not altered following treatment with FSK or PMA (Fig. 5B, upper panel). Luciferase activity in cells transfected with the GAL4 DBD/LRH-1 fusion construct were 15-fold higher; however, treatment with FSK or PMA, alone or in combination, did not further affect luciferase activity. Therefore, activity of LRH-1 is not regulated by PKA or PKC signaling pathways. Induction of expression from promoter II by PKA and PKC likely occurs through use of other hormone-sensitive cis-elements, for example the CRE-like element upstream from the NRE (45) in the case of PKA, with LRH-1 functioning as a basal transcription or competence factor. This would be consistent with, and analogous to, the role of SF-1 in cAMP stimulation of promoter II activity in the ovary (12, 48).

We next explored the contribution of the promoter II NRE to transcriptional regulation by FSK, PMA, and LRH-1 (Fig. 6). 3T3-L1 cells were transfected with LRH-1 and either a wild-type promoter II reporter construct (pII-516) or a promoter construct harboring a mutation in the NRE (AGGTCA → AaaTCA (pII-516mNRE)). Under control conditions (Fig. 6, upper panel), LRH-1 increased promoter II activity 2-fold. This modest stimulation was abolished when the NRE was mutated. In the presence of FSK + PMA (Fig. 6, lower panel) activity of pII-516 increased ~4-fold. LRH-1 cotransfection increased luciferase activity by a further 6-fold. Mutation of the NRE did not affect the ability of the promoter to respond to FSK + PMA; however, LRH-1 did not increase activity of this construct. These data suggest that the NRE is required for induction of promoter II by LRH-1, but not by FSK + PMA, and further support the hypothesis that LRH-1 acts as a basal transcription factor, whereas hormone-induced transcription occurs through other, non-LRH-1 mechanisms.

Binding of LRH-1 to the Promoter II NRE—To ascertain whether LRH-1 derived from adipose stromal cells is capable of binding to the promoter II NRE, a synthetic oligonucleotide probe encompassing this sequence was prepared and used in electrophoretic mobility shift assay. In the presence of adipose stromal cell nuclear extracts, two specific protein-DNA complexes were formed (Fig. 7A, lane 2). Formation of each of these complexes was abolished by the addition of a 200-fold molar
excess of non-radiolabeled wild-type probe (lane 3), but not by an excess of probe containing a mutation in the NRE GG dinucleotide (AGGTCA, lane 4). No change in the pattern of protein-DNA complexes was observed when nuclear extracts were preincubated with an antibody directed against either LRH-1 or against an unrelated antigen (the p65 subunit of NFκB, lanes 5 and 6). Fig. 7 also shows the protein-DNA complexes formed using in vitro translated mouse LRH-1 as the source of protein (lanes 8–12). LRH-1 formed at least three specific protein-DNA complexes with the promoter II NRE probe (lane 8), consistent with its known use of multiple inter- 

FIG. 7. LRH-1 binds to the −130 bp AGGTCA motif. A, human adipose stromal cell nuclear extracts (Nuc ext. 5 μg, lanes 2–6) or in vitro transcribed/translated mouse LRH-1 (LRH-1, lanes 8–12) were incubated with radiolabeled probe encompassing the −130 AGGTCA motif (20,000 cpm) in the presence or absence of wild-type (200X self) or mutated (200X mut) non-radiolabeled competitor probe or antibodies directed against either LRH-1 (LRH Ab, 3 μl) or the p65 subunit of NFκB (p65 Ab, 3 μl). Protein-DNA complexes were separated from free probe by gel electrophoresis and visualized by phosphorimaging. B, human adipose stromal cell nuclear extracts or in vitro transcribed/translated LRH-1 or SF-1 were incubated at 22 °C or 37 °C for 3 min either before (22b, 37b) or after (22a, 37a) addition of radiolabeled probe. Protein-DNA protein complexes were visualized as above. exess of non-radiolabeled wild-type probe (lane 3), but not by an excess of probe containing a mutation in the NRE GG dinucleotide (AGGTCA → AaaTCA, lane 4). No change in the pattern of protein-DNA complexes was observed when nuclear extracts were preincubated with an antibody directed against either LRH-1 or against an unrelated antigen (the p65 subunit of NFκB, lanes 5 and 6). Fig. 7 also shows the protein-DNA complexes formed using in vitro translated mouse LRH-1 as the source of protein (lanes 8–12). LRH-1 formed at least three specific protein-DNA complexes with the promoter II NRE probe (lane 8), consistent with its known use of multiple inter- 

FIG. 6. The −130-bp AGGTCA motif is required for LRH-1-induced promoter II activity. 3T3-L1 cells were transfected with pII-516 or pII-516mNRE (both 1.0 μg) in the presence or absence of an expression vector encoding LRH-1 (1.0 μg). Cells were serum-starved (24 h) and then incubated in the presence or absence of forskolin (F, 25 μM) or phorbol ester (P, 4 nM) for 16 h. Cells were then lysed and assayed for luciferase activity. Similar results were obtained in two additional independent experiments.

LRH-1:

- luciferase +

- X luciferase +

- X luciferase +

- F-P luciferase +

- F-P luciferase +

LRH-1

CTRL

FIG. 5. LRH-1 activity is not regulated by PKA or PKC. 3T3-L1 cells were transfected with 1.0 μg of pII-516 (A) or 1.0 μg of pG5Luc (B), in the presence of either pBIND or pBIND-LRH (both 1.0 μg). Cells were serum-starved (24 h) and then incubated in the presence or absence of forskolin (F, 25 μM) or phorbol ester (P, 4 nM) for 16 h. Cells were then lysed and assayed for luciferase activity. Similar results were obtained in three additional independent experiments.

FIG. 4. LRH-1 and PKA/PKC synergize in regulating promoter II. 3T3-L1 preadipocytes were cotransfected with a CYP19 promoter II-luciferase reporter construct (pII-516, 1.0 μg) and various amounts of LRH-1 expression vector in the presence or absence of forskolin (FSK, 25 μM) and phorbol ester (PMA, 4 nM). Luciferase activity is expressed as a ratio of β-galactosidase activity. Similar results were obtained in two additional independent experiments.

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3 D. Mangelsdorf, personal communication.
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noted that mouse and human LRH-1 cDNAs are of different size, and each gives rise to multiple distinct translation products (35, 44, 49).

Unlike other nuclear receptors, the DNA binding activity of human and rat LRH-1 is rapidly and irreversibly disrupted by incubation at 37 °C. Once bound to DNA, however, LRH-1 is resistant to heat treatment (35, 49). We therefore explored the thermal sensitivity of adipose stromal cell protein binding and compared it with that of LRH-1 or SF-1 (Fig. 7B). Adipose stromal cell nuclear extracts were incubated at 22 °C or 37 °C for 3 min either before or after addition of the DNA binding probe and subjected to electrophoretic mobility shift assay. No alteration in the pattern of protein-DNA complexes was observed on incubation at 22 °C before or after addition of probe (lanes 1 and 2, 5 and 6, and 9 and 10). Adipose stromal cell nuclear extracts failed to form complexes if heated to 37 °C before (lane 3), but not after (lane 4), probe addition. The ability of in vitro translated mouse LRH-1 to form complexes was also diminished on heating to 37 °C before, but not after, probe addition (lanes 7 and 8). In contrast, SF-1 binding was unaffected by heat treatment (lanes 11 and 12). The thermal sensitivity of adipose stromal cell nuclear protein binding to the promoter II NRE therefore resembles that of human LRH-1 (49). Note that the relative insensitivity of in vitro translated mouse LRH-1 to heat treatment likely arises from divergence in the amino acid sequences of mouse and human LRH-1 between the DBD and AF2 domain, the region known to confer thermal instability to human LRH-1 (35, 49). Together, these data provide strong evidence that LRH-1 is a component of the protein-DNA complexes formed between adipose stromal cell nuclear extracts and the promoter II NRE.

DISCUSSION

Hormonal treatment is generally the first line adjuvant therapy for patients with metastatic ER-positive breast cancers. Because recent trials have demonstrated the superiority of aromatase inhibitors such as anastrozole over traditional estrogen receptor antagonists in this setting (50), there is much interest in the biochemistry of this enzyme. In particular, the development of tissue-specific aromatase inhibitors that act at the level of transcription of the CYP19 gene is an attractive possibility (31). Since the overexpression of aromatase that occurs in adipose tissue of breast cancer patients arises through aberrant transcription from promoter II, we and others have sought to understand the mechanisms controlling the tissue-specific regulation of promoter II (12, 28, 33, 45, 47, 51).

In the current study we show that LRH-1 is expressed in adipose tissue and can bind and activate promoter II. There are several important implications of these findings. First, that LRH-1 and aromatase are coexpressed in preadipocytes provides a mechanism whereby aromatase expression in adipose tissue can be maintained in the absence of SF-1. Second, the regulation of promoter II by LRH-1 imparts a new level of control of aromatase expression in breast adipose through changes in LRH-1 expression or activity, which could in turn be modified by hormonal regulation, ligand binding, or coregulator recruitment. Finally, the tight confinement of LRH-1 expression to the preadipocyte fraction of human adipose tissue raises the possibility that LRH-1 may participate in more general control of preadipocyte function and/or adipose differentiation. These points are discussed below.

We hypothesized that since breast preadipocytes do not express SF-1, other nuclear receptors may bind the SF-1 site within promoter II to contribute to basal or hormone-induced transcription. Indeed, a previous study implicated the orphan receptor ERRα in regulation through this site in breast cancer cell lines (33). We think it unlikely, however, that ERRα contributes to transcription from promoter II in preadipocytes for the following reasons: ERRα was a very weak transactivator in transient transfection assays in SK-BR3 breast cancer cells (~2-fold stimulation of promoter II (33)). We were also unable to detect ERRα expression in human preadipocytes (not shown), and in our hands ERRα did not activate promoter II in transfection assays (Fig. 1). Finally, ERRα expression is positively correlated with adipocyte differentiation (52), whereas aromatase expression is down-regulated on adipocyte differentiation. In contrast, as shown here, LRH-1 is expressed at a high level in preadipocytes, strongly transactivates promoter II, and, like aromatase, is rapidly down-regulated upon adipocyte differentiation. For these reasons we propose that LRH-1 is a physiological regulator of aromatase expression in preadipocytes.

Little is known about the regulation of LRH-1 expression or activity. The LRH-1 promoter contains several conserved elements that are thought to confer liver-specific expression. These are bound by the hepatocyte nuclear factors HNF1, HNF3β, and HNF4α, which activate LRH-1 transcription in cooperation with other proteins including GATA factors (53, 54). We have not yet addressed the mechanisms by which preadipocyte-specific expression of LRH-1 might occur, but several interesting possibilities can be considered. It has been proposed that GATA factors, in particular GATA-2 and GATA-3, play a major role in controlling adipocyte differentiation (55). GATA-2/3 are preadipocyte-specific factors that maintain the undifferentiated phenotype by inhibiting expression of PPARγ. Adipocyte differentiation is associated with a rapid loss of GATA expression followed by induction of PPARγ and commencement of the differentiation program. Overexpression of GATA-2 or -3 is sufficient to inhibit differentiation (55). Since the LRH-1 promoter contains multiple canonical GATA motifs, and is positively regulated by GATA factors (54), the preadipocyte-specific expression of GATA-2 and -3 may contribute to LRH-1 expression in undifferentiated adipose stromal cells. It is also noteworthy that the LRH-1 promoter contains at least two consensus binding sites for the basic helix-loop-helix leucine zipper protein SREBP (53), which itself is closely associated with the process of adipocyte differentiation (56, 57).

The rapid loss of LRH-1 expression in differentiating adipocytes might suggest an inhibitory effect of PPARγ or other adipogenic agents on LRH-1 expression. Preliminary experiments in our laboratory using ligands for PPARγ and/or retinoid X receptor have not, however, supported this notion (data not shown). An alternative hypothesis would be that LRH-1 inhibits the expression and/or action of PPARγ. In this role, LRH-1 would then play a critical role in adipocyte differentiation. Consistent with this, LRH-1 activity is inhibited by the small heterodimer partner (39, 40), an atypical orphan receptor that lacks a DNA binding domain (58). A small heterodimer partner is expressed in adipose tissue and markedly potentiates the activity of PPARγ (59). Although this occurred through direct interactions between small heterodimer partner and PPARγ, involvement of LRH-1 in this process and in adipocyte differentiation in general warrants further investigation.

In conclusion we have shown that LRH-1 is a preadipocyte-specific nuclear receptor that can stimulate CYP19 transcription. Alterations in LRH-1 expression and/or activity have great potential to influence aromatase expression and estrogen production in adipose tissue. In particular, elucidation of the mechanisms regulating LRH-1 expression in normal and tumor-bearing breast adipose tissue will be an important area of future research.
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