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14. ABSTRACT

This project has focused on the potential role of Estrogen-Related Receptor alpha (ERRalpha) in regulating steroidogenesis. ERRalpha is an orphan nuclear receptor closely related to the Estrogen Receptors (ERs), and while its expression correlates with unfavorable biomarkers and poor prognosis for breast cancer, its function in breast cancer biology is not known. This project was initiated because we observed that a primary coactivator of ERRalpha, PGC-1alpha, induces hepatic gene expression of the initial enzymes in steroidogenesis, including steroidogenic acute regulatory protein (StAR), cytochrome p45011A1 (CYP11A1), cytochrome p45017A1 (CYP17A1), and aromatase (CYP19). We endeavored to characterize ERRalpha's role in regulating the expression of these enzymes in the breast, since increasing evidence points to local production of steroids as a significant source of estrogens in breast cancer, particularly in postmenopausal women (Simpson, 2003). We proposed to examine whether these steroidogenic enzymes are regulated by the ERRalpha/PGC-1alpha pathway in breast cancer cell lines, to determine which ERRalpha cofactors are present in breast, and to examine regulation of steroidogenic enzymes through ERRalpha by other cofactors.

15. SUBJECT TERMS
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INTRODUCTION:

This project has focused on the potential role of Estrogen-Related Receptor alpha (ERRalpha) in regulating steroidogenesis. ERRalpha is an orphan nuclear receptor closely related to the Estrogen Receptors (ERs), and while its expression correlates with unfavorable biomarkers and poor prognosis for breast cancer, its function in breast cancer biology is not known. This project was initiated because we observed that a primary coactivator of ERRalpha, PGC-1alpha, induces hepatic gene expression of the initial enzymes in steroidogenesis, including steroidogenic acute regulatory protein (StAR), cytochrome p45011A1 (CYP11A1), cytochrome p45017A1 (CYP17A1), and aromatase (CYP19). We endeavored to characterize ERRalpha's role in regulating the expression of these enzymes in the breast, since increasing evidence points to local production of steroids as a significant source of estrogens in breast cancer, particularly in postmenopausal women (Simpson, 2003). We proposed to examine whether these steroidogenic enzymes are regulated by the ERRalpha/PGC-1alpha pathway in breast cancer cell lines, to determine which ERRalpha cofactors are present in breast, and to examine regulation of steroidogenic enzymes through ERRalpha by other cofactors.

BODY:

PGC-1alpha induces expression of steroidogenic enzymes CYP11A1 and CYP17A1 in selected cell models (Task 1A).

Over the first two years, we measured gene induction of steroidogenic enzymes by the PGC-1alpha/ERRalpha pathway in many different cellular models. Initially this response was identified in HepG2 cells (see initial proposal), and we also found the response in some, but not all hepatic models. Both CYP11A1 and CYP17A1 were induced in Hep3B cells and primary human hepatocytes (Figure 1), however, this pathway was not active in Huh or H4IIE hepatocytes (data not shown). The inconsistency of this response across cell lines suggests that this response is sensitive to culturing conditions. We examined the response to the PGC-1alpha/ERRalpha pathway in MCF7 and BT474 cells, but did not see induction of either CYP11A1 or CYP17A1. We did notice a slight induction of CYP19 (armoatase) by PGC-1alpha

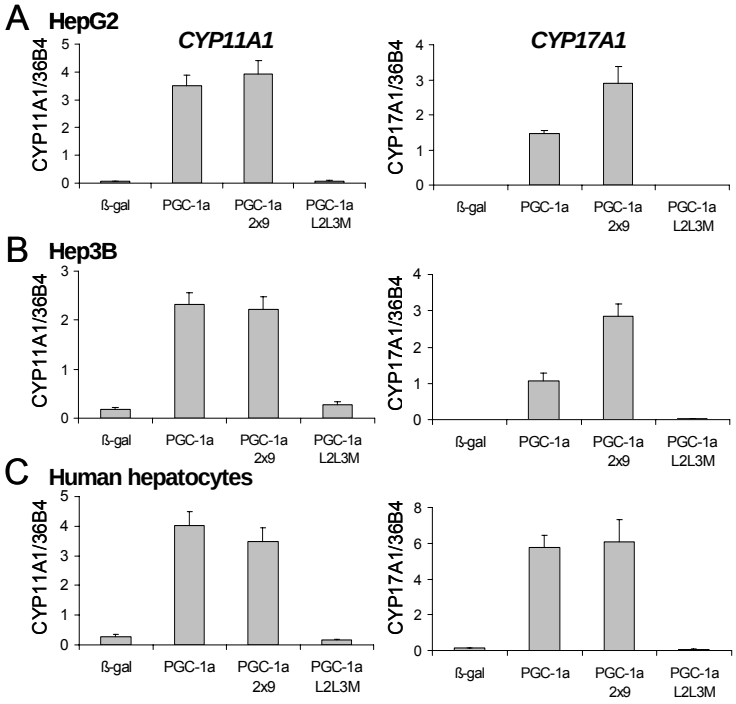

Figure 1. PGC-1α and PGC-1α 2x9 induce gene expression of *CYP11A1* and *CYP17A1* in hepatic cells. Gene expression of *CYP11A1* and *CYP17A1* was measured by quantitative PCR in (A) HepG2 cells, (B) Hep3B cells, or (C) primary human hepatocytes infected with adenoviruses expressing β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M. Gene expression was normalized to 36B4 expression. Error bars represent SEM of three replicates, and each graph is representative of at least three independent experiments.

expression (see 2007 report), suggesting that PGC-1alpha and ERRalpha could induce steroid synthesis from a sex steroid precursor, but *de novo* steroidogenesis does not appear to be induced by PGC-1alpha/ERRalpha. Since primary rat and human hepatocytes exhibit robust activation of these transcripts by ERRalpha/PGC-1alpha while some other hepatic cell lines do not recapitulate this activity (Huh cells, H4IIE cells), cultured models might be subject to changes in transcriptional activity and might not adequately or uniformly represent *in vivo* events. Therefore, additional examination of additional cell lines or more primary breast cells, such as human mammary epithelial cells (HMECs) might be required to continue exploring this aspect in breast cancer cells.

Examination of steroids synthesized by the PGC-1alpha pathway (Task 1B)

We focused our efforts on measuring the steroidogenic potential of the ERRalpha/PGC-1alpha pathway in cell lines with the most robust transcriptional induction of the steroidogenic enzymes, HepG2 cells. We reported the results of these measurements in our first annual report (2007), showing that the ERRalpha/PGC-1alpha pathway induces functional enzymes (CYP11A1 and CYP17A1) that can synthesize DHEA from steroidogenic precursors. High-Performance Liquid chromatography was used to identify the steroids synthesized by HepG2 cells expressing PGC-1alpha. The percentage of pregnenolone that was converted to DHEA or its intermediate, 17alpha-hydroxypregnenolone, is shown in Figure 2A. Additionally, a radioimmunoassay corroborated synthesis of DHEA from both pregnenolone and 22(R)-OH-cholesterol (Figure 2B and 2C). These results indicate that PGC-1alpha is capable of inducing functional enzymes which lead to the synthesis of DHEA. The function of this steroid in the liver is under continued investigation.

Regulation of steroidogenesis through ERRalpha (Task 1A)

Since these enzymes had not previously been shown to be transcriptionally regulated by ERRalpha, we characterized the molecular regulation of CYP17A1 by ERRalpha. We reported in the first annual report (2007) that PGC-1alpha-activated ERRalpha can drive expression of a CYP17A1 reporter. However, we were unable to identify ERRalpha binding by chromatin immunoprecipitation (ChIP) to the promoter region

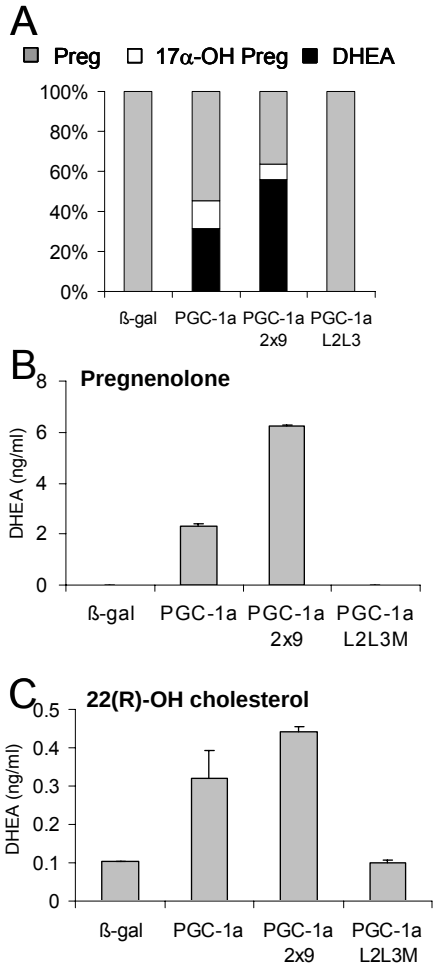

Figure 2. PGC-1α and PGC-1α 2x9 increase the functional activity of CYP11A1 and CYP17A1. A. HepG2 cells were infected with adenoviruses expressing β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M, then were incubated with 200nM [^{3}H]pregnenolone for 6 hrs. Steroids were extracted and separated by HPLC. Bar graph represents area under the curve. B-C. HepG2 cells were infected as above and incubated with 200nM pregnenolone (B) or 10uM 22(R)-OH cholesterol (C), and DHEA was measured using a radioimmunoassay. Error bars represent SEM of three replicates, and each RIA is representative of three independent experiments.

indicated by the transcriptional assay. Additionally, extensive mutagenesis of putative nuclear receptor binding sites (singular and in combination) failed to abrogate the observed induction of CYP17A1 by ERRalpha/PGC-1alpha (data not shown). Therefore, we searched for additional potential ERRalpha binding by performing a scanning ChIP analysis of all the potential ERREs 20kb upstream and downstream of CYP17A1, as well as within all introns of the gene and found a site 15KB downstream of CYP17A1 that ERRalpha appears capable of binding in these cells when PGC-1alpha is present (See figure 3 from Grasfeder et al, 2009).

CYP11A1 is also a novel target of ERRalpha, so we additionally searched for potential ERRalpha binding sites within and 20kb around the gene. We found a site within the first intron that ERRalpha is capable of binding in these cells when PGC-1alpha is present (see figure 4 from Grasfeder et al, 2009).

Identification of cofactors that bind ERRα in breast tissues using T7 phage display technology (Task2A)

Since we had not found a breast cancer cell line in which the PGC-1alpha/ERRalpha pathway could recapitulate steroidogenesis, we searched for novel ERRalpha binding partners in T7 libraries derived from HepG2 cells as well as the proposed MCF7 cells. Screens of the breast T7 cDNA libraries yielded only 2 clones, neither of which were known cofactors. However, analysis of the HepG2 libraries yielded several known cofactors, including PGC-1alpha, Rip140, Asc2, and SRC2, and several additional uncharacterized and hypothetical proteins. Interaction of each of these cofactors with ERRalpha was confirmed by mammalian 2-hybrid assays (See 2008 report). Since the screen of the HepG2 library was successful, it suggests that there might be something wrong with the T7 library derived from MCF7 cells. This library should probably be reconstructed. Additionally, other cell lines should be considered as well, preferably a line which exhibits PGC-1alpha/ERRalpha-induction of steroidogenesis.

Validation of ERRalpha-binding proteins (Task2B)

To characterize the activity of these cofactors with ERRalpha, we examined the ability of these cofactors to activate known ERRalpha target genes. However, most NR cofactors interact with a number of different NRs, many of which might share promoter elements on target genes. Therefore, to minimize potential interference, we customized several of these coactivators by a method previously described (Gaillard et al., 2006), replacing their NR-interacting domains with peptides that selectively bind ERRalpha. We measured gene induction of a number of known ERRalpha targets with these wild-type and customized cofactors in the breast cancer cell line BT474 to determine which cofactors might be responsible for coactivating ERRalpha in breast cancer cells (Figure 3). These results were striking in that only the PGC-1 family members PGC-1alpha or PGC-1beta appeared capable of inducing ERRalpha target genes in this cell line. This result needs to be confirmed in additional cell lines, but it suggests that the PGC-1 family of coactivators contribute to most of ERRalpha's activity in breast cancer cells. Additionally, a customized coactivator for PGC-1beta is needed, and is being constructed as discussed later.

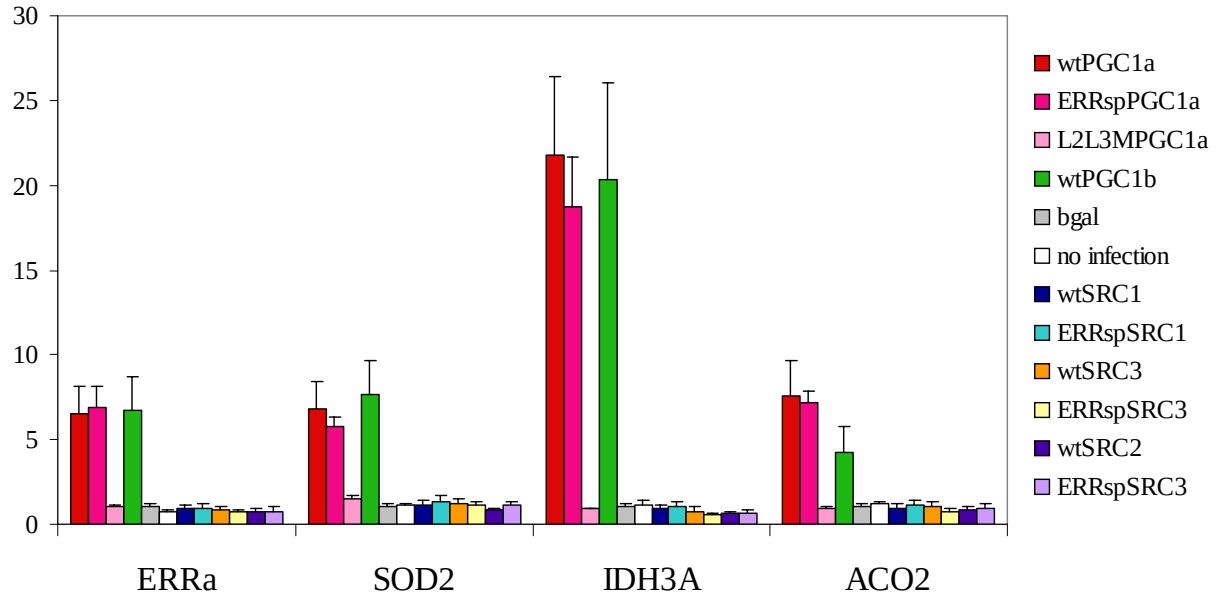

Figure 3. Induction of ERRa target genes by wild-type and customized ERRa-specific coactivators. BT474 breast cancer cells were infected with adenoviruses expressing the indicated coactivators for 48hrs, then RNA was harvested and analyzed by real-time PCR for expression of the indicated ERRalpha target genes. SOD2: superoxide dismutase 2, IDH3A: isocitrate dehydrogenase 3A, ACO2, aconitase 2.

Characterization of ERRalpha-binding proteins (Task2C)

An additional method used to characterize the importance of these coactivators in ERRalpha activity in the breast involved knocking down expression of the cofactors with siRNA. To this end, several siRNA constructs to each cofactor were transfected into SKBR3 cells, and expression of ERRalpha target genes, ERRalpha and oxoglutarate dehydrogenase, were measured by real-time PCR (Figure 4). These results clearly demonstrated that knocking down PGC-1beta decreased ERRalpha target gene expression the most, suggesting that PGC-1beta might be a primary coactivator of ERRalpha in these cells. These results also need to be repeated in additional cell lines.

Finally, we have explored the role of the corepressor, RIP140, binding to ERRalpha. RIP140 has been demonstrated to bind and regulate ERRalpha by us and others (Castet et al., 2006; Debevec et al., 2007). We have additionally found that knocking down RIP140 in SKBR3 cells results in a small increase of transcription of known ERRalpha target genes (Figure 5). Additionally, we found that RIP140 can antagonize PGC-1alpha-mediated coactivation of ERRalpha reporter genes. However, this result is not consistent, suggesting that other factors might be influencing the activity of any or all of these proteins. This interaction with RIP140 could prove consequential to ERRalpha activity, further linking ERRalpha to cancer progression through changes in cellular metabolism.

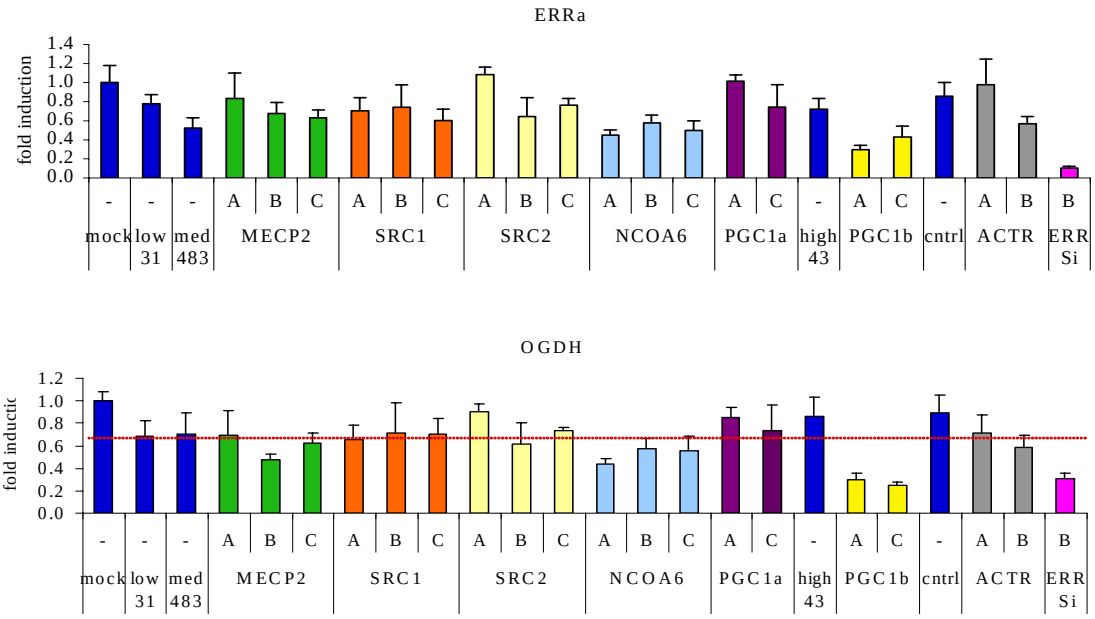

Figure 4. ERRalpha target gene activity with siRNA knockdown of coactivators.
SKBR3 cells were transfected with 2-3 different siRNA constructs for each indicated coactivator for 72hrs, and RNA was harvested for analysis by real-time PCR. All SiRNA showed >80% KD, except NCoA6 ~60-70%. OGDH: oxoglutarate dehydrogenase. MECP2:

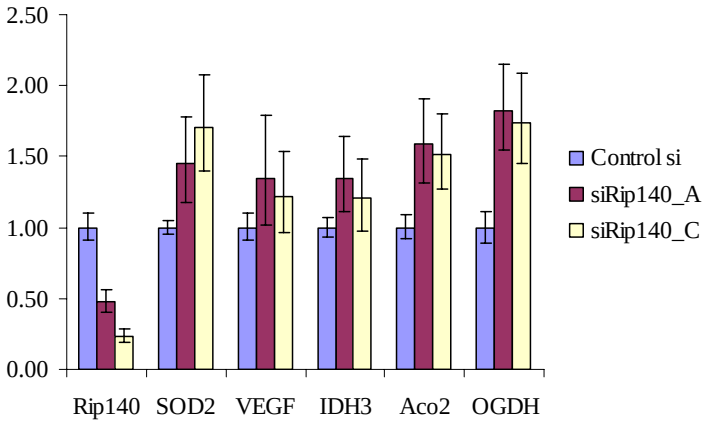

Figure 5. siRIP140 activates ERRa target genes
SKBR3 cells were transfected with control si or siRNA to 2 different regions of RIP140, and RNA was harvested for analysis of ERRalpha target gene expression by real-time PCR. ERRalpha target genes evaluated are SOD2: superoxide dismutase, VEGF: vascular endothelial growth factor, IDH3: isocitrate dehydrogenase 3A, Aco2: aconitase, OGDH: oxoglutarate dehydrogenase.

Identification of cofactors involved in steroidogenesis in the breast (Task 3)

Initial quantitative analysis of ERRalpha cofactors in the breast (Task 3A) was performed using the oncomine database to search *in silico* for studies reporting differential expression of the candidate cofactors. PGC-1alpha has increased expression in hepatocellular carcinoma, clear cell renal cell carcinoma (not shown), and prostate carcinoma (figure 6) when compared to expression in respective normal tissue.

PGC-1beta displays increased expression in cervical carcinoma compared to normal cervix (figure 7). However, as PGC-1beta was only discovered in 2001 (Lin et al., 2002), it has only recently been added to expression arrays, so data is still coming in on this factor. At the time of my departure from the lab, we were in the process of generating an ERRalpha-specific PGC-1beta adenovirus. This product is partially generated, and will be useful within the laboratory for this and other projects within the lab. Having this construct will allow us to ask some of the remaining unanswered questions of this proposal—whether PGC-1beta has a role in binding ERRalpha in the breast and coactivating it to drive expression of steroidogenic target genes (Task3B), or whether knockdown of PGC-1beta can abrogate induction of the genes (Task3D). Additionally, we can examine whether PGC-1beta can induce synthesis of steroids in breast cancer cells (Task 3C).

RIP140 displays increased expression in ovarian, prostate, and lung cancers compared to their normal tissues (data not shown). While RIP140 does not appear differentially regulated in studies comparing breast tumors to normal breast, it does show decreased expression in ER-negative breast cancer compared to ER+ breast cancer in 13 different studies (Figure 8). Since RIP140 generally has a repressive function, this could result in increased ERRalpha activity in ER-negative breast tumors. The potential implications of this are discussed in the conclusion.

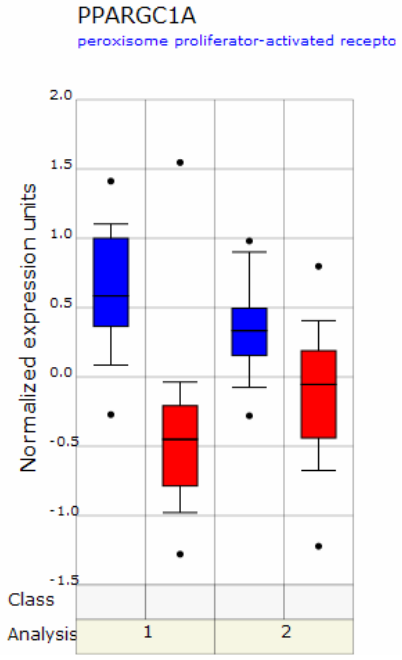

Figure 6. Increased PGC-1alpha expression in prostate cancer
Two studies (1: Lapointe, 2: Dhanasekaran) show significant differential expression of PGC-1alpha in prostate cancer (P-value of 5e-20 and 4.5e-5 respectively. Normal prostate (red) vs. prostate carcinoma (blue).

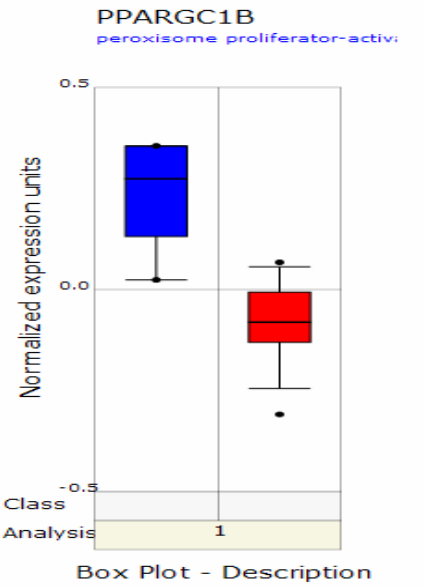

Figure 7. Increased PGC-1beta expression in cervical cancer
PGC-1beta has higher expression in cervical cancer (blue) compared to normal cervix (red).

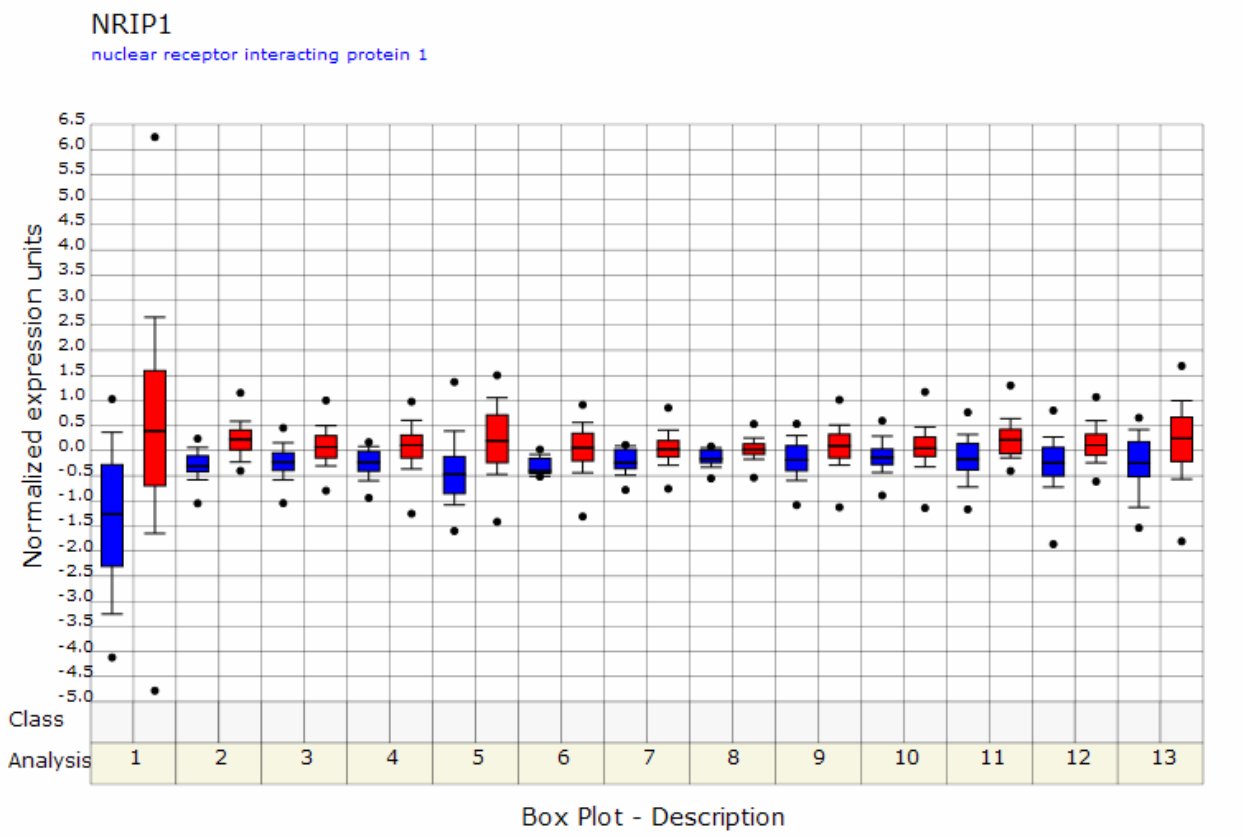

Figure 8. RIP140 has reduced expression in ER-negative breast cancer cells
Thirteen independent studies show that ER-negative breast cancer (blue) has lower expression of RIP140 (NRIP) when compared to ER+ breast cancer (red).

KEY RESEARCH ACOMPLISHMENTS:

- The PGC-1a/ERRa pathway induces StAR, CYP17A1, CYP11A1, and CYP19 in select cell models
- CYP17A1 and CYP11A1 enzymes induced by the PGC-1α/ERRα pathway are functional.
- Steroids produced by the PGC-1α/ERRα pathway include 17αOH-pregnenolone and DHEA
- *De novo* synthesis of DHEA can be initiated from both pregnenolone and 22(R)-OH-cholesterol.
- CYP17A1 gene expression is responsive to cAMP
- Identification of ERRalpha binding sites surrounding the CYP17A1 gene.
- Identification of ERRalpha binding sites in CYP11A1 gene.
- PGC-1alpha expression appears to be required for ERRalpha binding to either site around CYP17A1 or CYP11A1.
- Identification of PGC-1alpha and PGC-1beta as coactivators that can drive expression of ERRalpha target genes in breast tissues.

- Knockdown of ERRalpha corepressor RIP140 drives expression of ERRalpha target genes.
- RIP140 expression is decreased in ER-negative breast cancer cells

REPORTABLE OUTCOMES:

Manuscripts:

Gaillard S, Grasfeder LL, Haeffele CL, Lobenhofer EK, Chu TM, Wolfinger R, Kazmin D, Koves TR, Muoio DM, Chang CY, McDonnell DP. Receptor-selective coactivators as tools to define the biology of specific receptor-coactivator pairs.
Mol Cell. 2006 Dec 8;24(5):797-803.

Grasfeder, LL and McDonnell, DP. Nuclear Receptor Cofactor Interactions as Targets for New Drug Discovery. *NR Coregulators and Human Diseases*. Chapter 19; 559-585. World Scientific Publishing Co. 2008.

Grasfeder LL, Gaillard S, Hammes SR, Ilkayeva O, Newgard CB, Hochberg RB, Dwyer MA, Chang CY, McDonnell DP. Fasting-induced hepatic production of DHEA is regulated by PGC-1{alpha}, ERR{alpha} and HNF4{alpha}. Mol Endocrinol. 2009 Apr 23.

Degrees obtained:

PhD Thesis Defense: Regulation of hepatic metabolism through PGC-1α-mediated pathways. Department of Pharmacology and Cancer Biology, Duke University, December 12, 2007.

Employment/Research opportunities obtained:

Post-doctoral researcher in laboratory of Jason Lieb, Department of Biology and Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill.

CONCLUSIONS:

Summary:

We have characterized the PGC-1alpha/ERRalpha pathway as a novel pathway for initiating steroidogenesis. We have shown that this pathway is capable of inducing the synthesis of functional p450 enzymes Cyp17A1 and Cyp11A1 from either pregnenolone or 22(R)-OH cholesterol. Potential ERRalpha binding sites have been identified around both genes by scanning chromatin immunoprecipiation assays. DHEA is at least one of the resulting steroids produced by this pathway, but it is unclear what its function is.

This pathway appears to be very active in cells of hepatic origin, but we have been unable to find evidence for activity of this pathway in breast cancer cells. Technical issues may be a confounding factor, but evidence for PGC-1alpha activity in breast cancer cells has been sparse as well. Our attempts to identify alternative ERRalpha cofactors have been promising, with PGC-1beta and RIP140 being our top candidates. PGC-1beta is capable of driving expression of known ERRalpha target genes, while knockdown reduces expression of ERRalpha target genes. Generation of the ERRalpha-specific-PGC-1beta will be very useful for further characterizing the specificity of these effects. RIP140 knockdown increases expression of ERRalpha target genes in breast cancer lines, suggesting that it regulates ERRalpha activity. Delivering this siRNA by adenovirus or lentivirus might improve the efficiency of this response and could be

used to examine whether removing this repressor is sufficient to activate steroidogenic genes in breast cancer cells. Since RIP140 binds so many nuclear receptors through its 10 NR-interacting domains, it would be challenging, but useful to customize this cofactor to ERRalpha to determine its effects specifically through ERRalpha and eliminate indirect effects from interactions with other nuclear receptors. Another logical next step is to examine RIP140 expression in ER-positive and ER-negative tumors to see if its expression is reduced in ER-negative tumors.

Implications:

Other studies in the lab have also revealed a role for ERRalpha specifically in ER-negative breast cancer, where knockdown of ERRalpha reduces the migratory potential of MDA-MB231 cells and reduces tumor growth of xenographs in mice (Stein et al., 2008). Combining that work with the overwhelming number of studies showing reduced RIP140 expression in ER-negative tumors suggests a mechanism by which ERRalpha activity could be modulated/activated in ER-negative breast cancer cells. Since ERRalpha is subject to autoregulation, even small changes could be magnified and could contribute to increased ERRalpha activity in the cells, potentially contributing to or resulting in the observed poor clinical outcome of patients with elevated ERRalpha levels in the tumors.

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APPENDICES

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Receptor-Selective Coactivators as Tools to Define the Biology of Specific Receptor-Coactivator Pairs

Technique

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Summary

In the absence of specific high-affinity agonists and antagonists, it has been difficult to define the target genes and biological responses attributable to many of the orphan nuclear receptors (ONRs). Indeed, it appears that many members of this receptor superfamily are not regulated by classical small molecules but rather their activity is controlled by interacting cofactors. Motivated by this finding, we have developed an approach to genetically isolate specific receptor-cofactor pairs in cells, allowing us to define the biological responses attributable to each complex. This is accomplished by using combinatorial peptide phage display to engineer the receptor interacting domain of each cofactor such that it interacts selectively with one nuclear receptor. In this study, we describe the customization of PGC-1α and its use to study the biology of the estrogen-related receptor α (ERRα) in cultured liver cells.

Introduction

The ONRs are a subset of the larger nuclear receptor superfamily of transcription factors, for which no physiologically relevant ligands have yet been identified. For some ONRs, it has been possible to develop useful synthetic agonists and antagonists enabling the elucidation of their function in vivo. For others, it has been extremely difficult to develop small molecule regulators, and consequently, progress in defining their functional roles has been impeded. Some of these problems have been mitigated with the advent of siRNA technology, wherein knockdown of receptor expression can be used to substitute for antagonists. However, the constitutive activity of many ONRs is so low that even quantitative knockdown does not provide the dynamic range needed to identify target genes. Thus, there is a clear unmet need for approaches with which to positively and negatively regulate the activity of ONRs in target cells.

We are interested in defining the physiological roles for ERRα, an ONR in the NR3B subfamily. Structurally, the ERRs are most closely related to the estrogen receptors (ERs), and not surprisingly, significant crosstalk between the signaling pathways regulated by these receptors has been observed. However, ERRα also plays ER-independent roles as a regulator of oxidative phosphorylation, fatty acid oxidation, and lipid handling (Huss et al., 2004; Mootha et al., 2004; Sladek et al., 1997). Absent a ligand, however, it has been very difficult to evaluate the relative physiological and pathological importance of the ERRs in estrogen action and in the regulation of metabolism.

Crystallographic analysis has revealed that ERRα is capable of adopting a transcriptionally active conformation in the absence of any obvious electron density in what would be expected to be its ligand binding pocket (Kallen et al., 2004). However, its overexpression in cells results in only modest activation of transcription. Robust activation is observed when ERRα is coexpressed in cells with the coactivator PGC-1α (Huss et al., 2002), suggesting that in place of a small molecule ligand, the transcriptional activity of this and related ONRs may be regulated by cofactor availability. Attempts to develop small molecule regulators of ERRα have yielded only weak antagonists and inverse agonists whose toxicity and receptor crossreactivity limit their use (Willy et al., 2004).

One approach to selectively increase the transcriptional activity of a weakly active receptor is to fuse it to the strong transcriptional activator VP16. However, this approach does not recapitulate the physiological activity of the native receptor, as negatively regulated genes will likely be turned on by this modified protein. Another approach is to activate the receptor with one of its known cofactors. However, because most of the coactivators that interact with ERRα also interact with multiple receptors and unrelated transcription factors, it has proven difficult, even when complemented with siRNA knockdown technology, to study the biology of this receptor by simply manipulating coactivator levels in cells. Thus, we have developed a methodology that has enabled us to selectively and effectively regulate ERRα transcriptional activity. Specifically, we have used combinatorial peptide phage display to engineer the receptor interaction domain of the coactivator PGC-1α such that it interacts with and activates ERRα in a highly selective manner. We validated the use of this customized coactivator by using it to identify ERRα-regulated genes in HepG2 cells, a study that both confirmed the key role of this receptor in oxidative metabolism and uncovered additional pathways in which it is engaged. This technology, we believe, will have broad application, allowing for the development of customized coactivators for other NRs and transcription factors.

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	ERRα	ERRβ	ERRγ	ERα	ERβ	PR-A	GR	AR	RAR	RXR	ROR	TRβ	VDR	LXR	FXR	LRH	PPARγ
SRC1	+	+	+	+++	+	+	++	-	++	++	-	++	++	++	+	nd	nd
L3-02	+++	+++	+++	-	-	+	++	-	+	-	-	-	-	+	-	nd	nd
L3-07	+++	+++	++	-	-	-	-	-	-	-	-	-	-	+	-	nd	nd
L3-09	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L3-12	+++	+++	+++	-	-	+++	++	-	-	++	-	-	-	+	-	nd	nd
L3-28	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L3-37	++++	+++	+++	-	-	++	-	-	++	+++	-	-	-	-	-	nd	nd
L3-49	+++	+++	+++	-	-	++	-	-	++	+++++	-	-	-	-	-	nd	nd
L3-62	+++	+++	++	-	-	-	-/+	-	-	-	-	-	-	+	-	nd	nd
L3-80	+++	++++	+++	-	-	-	-	-	-	-	-	-	-	-/+	-	nd	nd

Figure 1. Evaluation of the Receptor Selectivity of the ERRα-Selective Peptides Identified by Phage Display

The interaction of some ERRα-selective peptides with other nuclear receptors (NRs) was tested in a mammalian-two-hybrid assay by using a VP16-receptor construct and a peptide-Gal4DBD fusion on a 5xGal4-luciferase reporter. ERR peptide activity is defined as a percent of highest ERR activity: −, <10%; +, 10%–25%; ++, 25%–50%; +++, 50%–75%; ++++, 75%–100%; +++++, >100%; and nd, not determined. ERRα-selective peptides with minimal interactions with other NRs are indicated by light or dark shading (light, one to two other NRs; dark, no other NR). Abbreviations: ERR, estrogen receptor-related receptor; ER, estrogen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; AR, androgen receptor; RAR, retinoic acid receptor; ROR, RAR-related orphan receptor ligand binding domain; TR, thyroid receptor; VDR, vitamin D receptor; LXR, liver X receptor; FXR, farnesoid X receptor; LRH, liver receptor homolog 1; and PPARγ, peroxisome proliferator-activated receptor γ.

Results

Generation of an ERR-Selective Coactivator

Given that ERRα tethers PGC-1α to target gene promoters through either of two LXXLL motifs within the coactivator's (CoA) NR-interaction domain (Huss et al., 2002), we hypothesized that it may be possible to alter PGC-1α's NR specificity by reengineering its NR-interaction surface such that it interacts with ERRα at the exclusion of other receptors. In this manner, we believed that it would be possible to functionally isolate this receptor-coactivator pair in target cells and define the biological consequences attributable to this complex.

Previously, we demonstrated that it was possible to use combinatorial phage display of peptide libraries to identify LXXLL-containing peptides that interact in a highly specific manner with the coactivator binding pocket of individual NRs (Chang et al., 1999; Hall et al., 2000). Thus, using purified recombinant ERRα as bait, we screened M13 phage display libraries containing 10^8 different random or semirandom peptides 12–19 amino acids in length. The full details of this procedure and the characterization of the peptides identified are described elsewhere (Chang et al., 1999; Gaillard et al., 2006). In brief, we first identified LXXLL peptides that interacted selectively with ERRα but did not interact with either ERα or β under the same conditions. Peptides that showed a preference for ERR were expressed in cells as a fusion with the GAL4DBD, and their ability to tether different VP16-NR fusions was tested in a mammalian two-hybrid assay. This secondary screen enabled the elimination of those peptides that were (1) capable of interacting with NRs commonly expressed with ERRα in target cells and (2) receptors known to interact with PGC-1α. The results of this analysis, summarized in Figure 1, indicate that some peptides (L3-09, L3-28, and L3-80) are highly selective for the ERRs under the conditions tested.

There are three LXXLL motifs within PGC-1α (Figure 2A, L1–L3); however, only L2 and L3 appear to interact with NRs. Although L2 is the dominant site of interaction for most NRs, we and others have shown that either L2 or L3 is sufficient to allow full ERRα transcriptional activity (Gaillard et al., 2006; Huss et al., 2002; Schreiber et al., 2003). Therefore, we replaced both of the 19 amino acid regions corresponding to each of the L2 and L3 motifs within PGC-1α with sequences corresponding to the L3-09 peptide (PGC-1α 2x9, Figure 2A). When assayed on a 3xERE-TATA-luciferase reporter (Figure 2B), PGC-1α 2x9 stimulates ERRα transcriptional activity to the same degree as PGC-1α. Equivalence of PGC-1α and PGC-1α 2x9 was also demonstrated in experiments using ERRβ and ERRγ (data not shown). ERRα transcriptional activity was unaffected by the expression of PGC-1α L2L3M, a mutant in which the leucines within the L2 and L3 motifs were changed to alanines. Thus, within the resolution of these reconstituted transcription assays, we conclude that PGC-1α 2x9 is indistinguishable from PGC-1α as an ERRα coactivator.

We next tested whether PGC-1α 2x9 could activate other NRs, in particular those known to be coexpressed with ERRα and/or subject to coactivation by PGC-1α. To this end, we compared the coactivator activity of PGC-1α or the mutants on individual receptors and cognate reporters in the presence or absence of a ligand as required. Of the receptors tested that are known to interact with PGC-1α, only HNF4α activity was enhanced to any significant degree by PGC-1α 2x9, resulting from crossreactivity of the L9 peptide, whereas the transcriptional activity of other receptors such as ERα, PPARγ, GR, LRH-1, or RXRα was unaffected (Figure 2B). Thus, by engineering the LXXLL motifs within PGC-1α, we have been able to develop a highly selective coactivator for ERRα.

Identification of ERRα Target Genes in HepG2 Cells by Using an ERR-Selective PGC-1α

A role for the ERRα/PGC-1α complex has been shown in the regulation of mitochondrial biogenesis, oxidative phosphorylation, and fatty acid oxidation in cardiac muscle and bone precursor cells (Huss et al., 2004; Schreiber et al., 2004). PGC-1α, HNF4α, and other receptors have a well-established role in the regulation of gluconeogenesis in liver cells (Puigserver and Spiegelman,

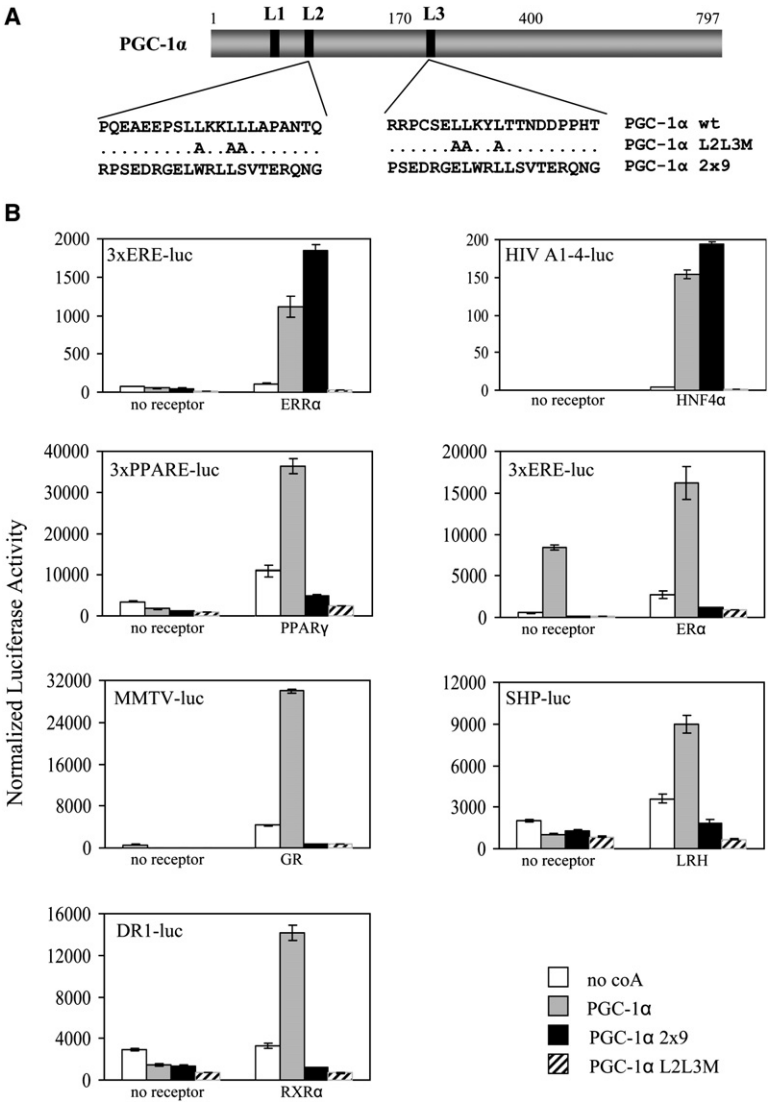


Figure 2. ERR-Selective PGC-1α Preferentially Coactivates ERR

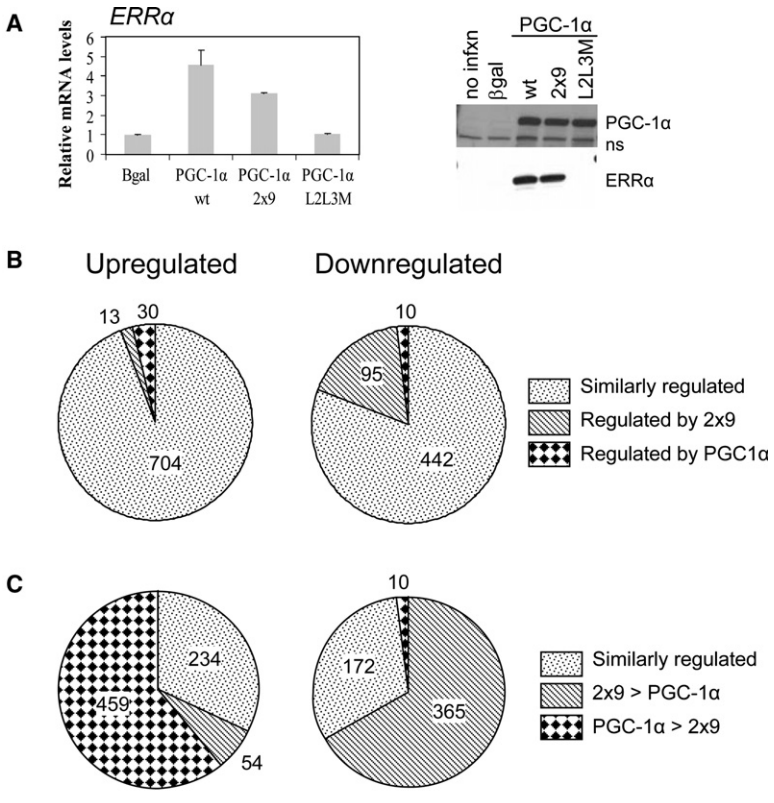
(A) Schematic of PGC-1α LXXLL region mutants.

(B) HeLa cells were transfected with the indicated receptor, cofactor, and luciferase reporter containing the indicated response element. One-hundred nanomolar 17β-estradiol (E2), 100 nM dexamethasone, and 100 nM 9-*cis*-retinoic acid were used to activate ERα, GR, and RXRα, respectively; no hormone indicates an equivalent volume of ethanol. Results are expressed as normalized luciferase activity ± standard error of the mean (SEM) per triplicate sample of cells.

2003), but the role of ERRα in mediating energy metabolism in these cells has not been defined. As an initial step toward addressing this issue, we elected to define the target genes expressed in the hepatocellular carcinoma cell line (HepG2) that were subject to regulation by the ERRα/PGC-1α complex.

We first determined whether PGC-1α 2x9 could activate physiologic targets when transduced into target cells. PGC-1α facilitates transcriptional auto upregulation of the mRNA encoding ERRα (Laganiere et al., 2004). Thus, we measured the expression of ERRα in HepG2 cells overexpressing either PGC-1α or the 2x9 variant. As expected, ERRα mRNA and protein levels were strongly induced by both the PGC-1α and PGC-1α 2x9, but not by either of the control viruses as measured by quantitative PCR (qPCR) and immunoblot, respectively (Figure 3A). We observed no induction of endogenous PGC-1α by either the PGC-1α- or PGC-1α 2x9-expressing adenoviruses (Figure S1 in the Supplemental Data available with this article online) and proceeded to use the targeted PGC-1α to define the ERRα transcriptome in HepG2 cells.

To identify target genes induced by PGC-1α through ERRα, PGC-1α, the 2x9 variant, and the controls were independently transduced in HepG2 cells and the corresponding gene expression profiles were analyzed by using the GeneChip technology (Affymetrix, Santa Clara, CA). The resulting hierarchical clustering diagram (Figure S2) illustrates that the vast majority of genes are similarly regulated by PGC-1α and PGC-1α 2x9, differing only by degree of induction or repression. Of the genes significantly induced, 94% were induced by both PGC-1α and PGC-1α 2x9, whereas 76% of the downregulated genes were repressed by both (Figure 3B). This high percentage of similarity suggests that PGC-1α 2x9 recapitulates the activating capacities of PGC-1α and that a significant number of PGC-1α target genes in HepG2 cells are regulated by ERRα and/or HNF4α. However, quantitative differences exist between the magnitude of induction or repression by PGC-1α 2x9 compared to PGC-1α. PGC-1α induces 61% of the upregulated genes more effectively than PGC-1α 2x9, suggesting that optimal expression of these genes relies on the additional interactions by PGC-1α, in which PGC-1α 2x9 is unable



D

GO category	*Number of genes regulated by both PGCs*	*Bonferroni-adjusted p value*
Tricarboxylic acid cycle	8	0
Oxidative phosphorylation	2	0.00934
Electron transport	22	0
Fatty acid metabolism	6	0.00799
Lipid metabolism	12	0.00384
Lipid transport	6	0.00319
Mitochondrial transport	2	0.81079
Transcription	15	1
Aminoacid metabolism	4	0.3696
Carbohydrate metabolism	10	0.07003

Figure 3. Regulation of Transcription by WT PGC-1α and PGC-1α 2x9 in HepG2 Cells

(A) Induction of ERRα mRNA and protein by PGC-1α constructs. Error bars indicate SEM of three biological replicates.

(B) Qualitative summary of genes significantly regulated by PGC-1α or 2x9 or both.

(C) Quantitative differences in gene regulation by PGC-1α or 2x9 based on hierearchical clustering. Expression levels from clusters compared between treatment groups using unpaired Student's t test ($p < 0.002$, where degree of induction or repression was different, $p > 0.196$ for similarly regulated clusters).

(D) Gene ontology (GO) categories that are most prominently influenced by PGC-1α and PGC-1α 2x9 related to energy metabolism. P values indicating the enrichment of these GO terms among the regulated genes were corrected for multiple comparisons.

to participate (Figure 3C). However, 39% of the upregulated genes are induced by PGC-1α 2x9 as well or better than PGC-1α, suggesting that these genes are primarily regulated by either ERRα or HNF4α. For 67% of the downregulated genes, PGC-1α 2x9 acts as a stronger corepressor. This may result from the fact that the effective pool of PGC-1α available to ERRα is greater, as other transcription factors cannot effectively compete for binding to the modified coactivator.

Analysis of the gene ontology (GO) terms associated with the differentially regulated transcripts revealed an enrichment of terms related to energy metabolism, including oxidative phosphorylation, tricarboxylic acid cycle, fatty acid beta-oxidation, lipid metabolism, lipid transport, and electron transport (Figure 3D). Most of these pathways have been shown previously to be targets of ERRα (Huss et al., 2004; Mootha et al., 2004; Sladek et al., 1997). Other categories such as mitochondrial transport, regulation of transcription, and amino acid metabolism did not show statistical significance in number, but the biological significance of the individual genes in each class should not be discounted (Figure S3).

The PGC-1α/ERRα Complex Is a Key Regulator of Rate-Limiting Enzymes Involved in Energy Metabolism

Potential ERRα targets identified in the array described above were validated in independent experiments by using qPCR after infection with a virus expressing PGC-1α. To differentiate between ERRα– and HNF4α-dependent targets, we compared the relative levels of mRNA of PGC-1α-induced genes in cells treated with an adenovirus expressing either a control (scrambled) or ERRα

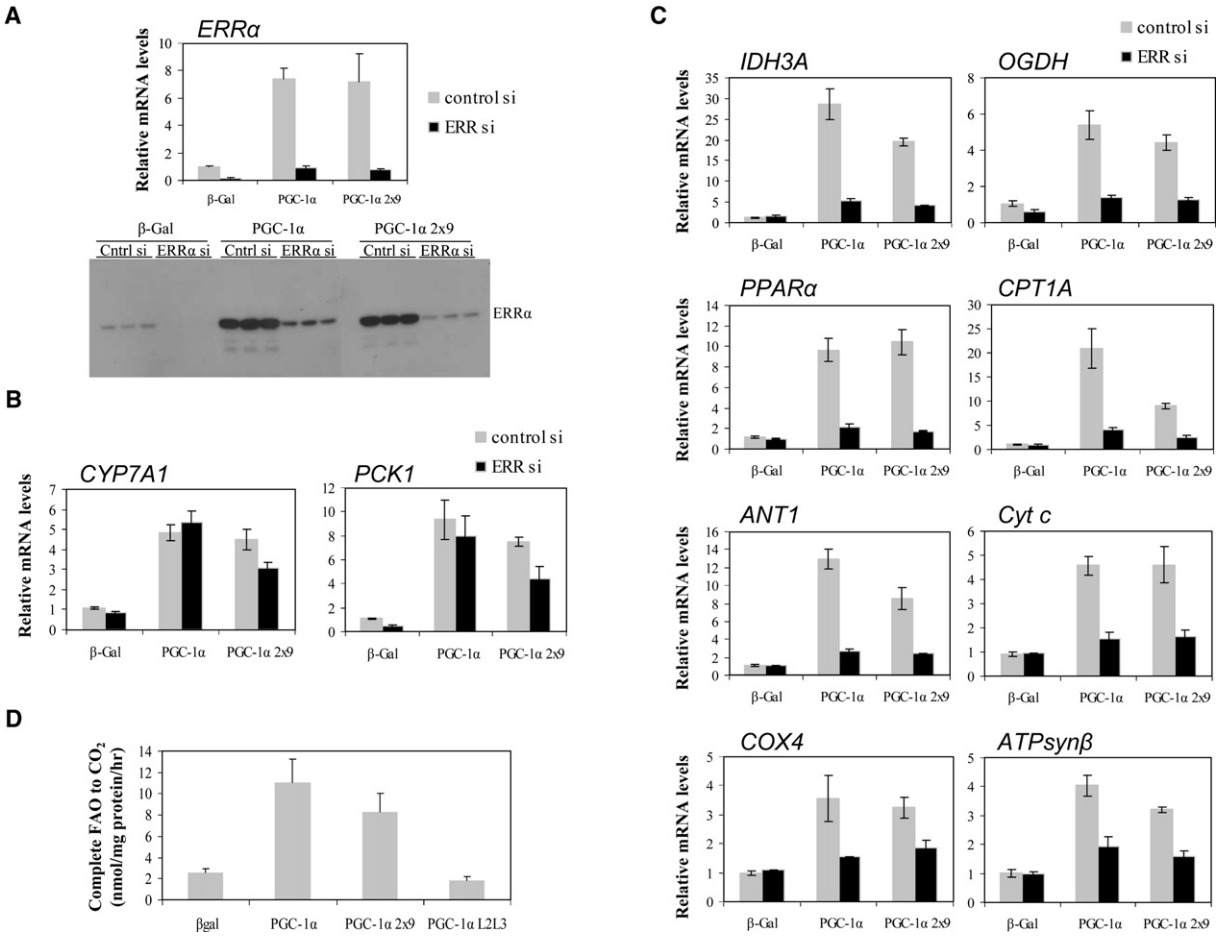


Figure 4. PGC-1α-Mediated Induction of Key Enzymes of Energy Metabolism Is Dependent on the Expression of ERRα

(A) Induction of ERRα mRNA and protein by PGC-1α or PGC-1α 2x9, and repression by the siRNA to ERRα.

(B) Genes not dependent on ERRα.

(C) ERRα-dependent genes.

Error bars in (A)–(C) represent SEM of three biological replicates.

(D) Fatty acid oxidation in HepG2 cells, normalized to protein content, with error bars indicating SEM of three independent experiments.

siRNA. The siRNA effectively inhibits the induction of ERRα mRNA and protein by PGC-1α as compared to a scrambled control siRNA (Figure 4A). Under these conditions, it was possible to distinguish known HNF4α-activated genes, such as *phosphoenolpyruvate carboxykinase 1* (*PCK1*), *cholesterol 7-alpha-hydroxylase* (*CYP7A1*), and *glucose-6-phosphatase* (*G6PD*) (Figure 4B and data not shown) from key genes of energy metabolism found to be dependent on ERRα, including *isocitrate dehydrogenase 3A* (*IDH3A*) and *oxaloglutarate dehydrogenase* (*OGDH*) of the tricarboxylic acid cycle, *peroxisome proliferator-activated receptor alpha* (*PPARα*) and *carnitine palmitoyltransferase 1A (CPT1A)* involved in regulating fatty acid oxidation, *ATP synthase β* (*ATPsynβ*, *cytochrome* c (*Cyt* c), and *cytochrome* c *oxidase 4 (COX4)* of oxidative phosphorylation and *adenine nucleotide translocator 1 (ANT1)* of mitochondrial transport. It is important to note that of all the genes we have studied thus far to be induced by PGC-1α 2x9 in HepG2 cells, *PCK1*, *CYP7A1*, and *G6PD* were the only genes shown to be dependent on a factor other than ERRα.

To validate this approach in other cell types, we tested induction of these ERRα target genes in AGS stomach cancer cells and U251 glioma cells and found that these genes were similarly induced by both PGC-1α and PGC-1α 2x9 in an ERRα-dependent manner (Figure S4 and data not shown). Additionally, we tested the robustness of the customizing process by exchanging a different ERRα-selective peptide, L28, for the two L9 peptides and observed that the ERRα target genes tested were induced by the PGC-1α 2x28 as well (Figure S5). Finally, to determine whether the PGC-1α 2x9 construct is functionally indistinguishable from PGC-1α in inducing one of the expected pathways, we measured fatty acid oxidation in HepG2 cells. We found PGC-1α 2x9 increases oxidation of oleic acid compared to cells expressing β-gal or PGC-1α L2L3M and recapitulates PGC-1α activity through ERRα (Figure 4D).

Discussion

It is unlikely that classical small molecule ligands will be found for all ONRs. Rather, it appears that coactivator availability or posttranslational modifications, such as phosphorylation, will emerge as primary mechanisms by which the transcriptional activity of some ONRs will

be regulated (Hermanson et al., 2002). ERRα appears to interact primarily with members of the PGC-1 and/or p160 classes of transcription factors (Lu et al., 2001; Schreiber et al., 2003). Because these cofactors interact with several NRs and with other unrelated transcription factors, it has been exceedingly difficult to define the biological responses in cells that are attributable to individual NR-CoA complexes. With this problem in mind, we developed an approach that has allowed us to genetically isolate ERRα/PGC-1α within cells and identify target genes that were regulated by this specific NR-CoA complex. This was accomplished by engineering the LXXLL NR-interacting motifs within PGC-1α such that they would interact with ERRα at the exclusion of other NRs. Although the LXXLL motifs within the known coactivators are relatively promiscuous, we were able to identify peptides that were highly selective for ERRα by screening large peptide libraries in which the amino acids flanking the core motif were randomized. We believe that this general approach to "customize" coactivators will be applicable to other NRs, as we have found it to be relatively easy to generate LXXLL-based peptides that display a high degree of receptor selectivity. Although it is unlikely that absolute specificity of the coactivator for the targeted receptor can be achieved with this technology, this problem also exists for natural ligands of the NRs. For instance, progesterone is an effective ligand not only of the progesterone receptor but also of the mineralocorticoid and glucocorticoid receptors. Thus, confirmation of an observed biological response to a small molecule "classic" agonist or a customized coactivator will always be required. To this end, we have used siRNA in this study to confirm the involvement of ERRα in the highlighted processes. Through the use of these two technologies, comparable to the use of classical agonists and antagonists, we believe that it will be possible to probe the biology of most any transcription factor-cofactor pair.

Experimental Procedures

Plasmids

Plasmids for pcDNA3-ERRα (Zuercher et al., 2005); VP16-ERα, VP16-ERβ, VP16-RARα, VP16-RXRα, pRST7-ERα, 3xERE-TATA-luciferase, and 5xGal4-luciferase (Chang et al., 1999); LRH-1 (Safi et al., 2005); and VP16-LXR and VP16-FXR (Hall et al., 2000) were described previously. cDNA encoding ERRα and PPARγ were excised from pcDNA3-ERRα and SG5-PPARγ, respectively, and ligated into the VP16 vector (Clontech). The following were gifts: SG5-PPARγ and 3xPPARE-luciferase (T. Willson), SHP-luciferase (S. Kliewer), DR1-luciferase and RSV-RXRα (R. Heyman), HIV A1-4-luciferase and pcDNA3-HNF4α (F. Sladek), Flag-PGC-1α (B. Spiegelman), VP16-VDR (W. Pike), VP16-TRβ (D. Moore), VP16-PRA (D. Wen), VP16-GR (J. Miner), VP16-AR (K. Marschke), and VP16-RORα-LBD (A. Means).

Generation of the PGC-1α 2x9 and L2L3M Mutants

PGC-1α 2x9 was generated by excising the L2 and L3 motifs of PGC-1α and ligating in their place the sequence of the L3-09 peptide (PSEDRGELWRLLSVTERQN). This resulted in the replacement of the L2 and L3 motifs with an ERR-selective peptide and the insertion of a total of seven amino acids corresponding to restriction enzyme sites. The PGC-1α L2L3M mutant, in which the leucines were mutated to alanines, was generated by using the QuikChange II mutagenesis kit (Stratagene). All mutants were sequenced to ensure the fidelity of the resulting constructs.

Generation of Adenoviruses

Adenoviruses used to overexpress PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M were generated by using the ViraPower Adenoviral Expression System (Invitrogen), purified with Adeno-x (BD Biosciences), and concentrated with a Centricon YM-50 Unit (Millipore).

The ERRα and control siRNA adenoviruses were generated as described above with the following changes: the ERRα siRNA oligonucleotides (described in Schreiber et al. [2003]) and control siRNA oligonucleotides (scrambled sequence) were ligated into pSuper (Oligoengine) and the H1-RNA promoter and the target sequence were inserted into the adenoviral backbone.

Mammalian Cell Culture, Transfections, and Adenovirus Transduction

Culture, transfection, and luciferase assays using HeLa (human cervical carcinoma) and HepG2 (hepatoma) cells were described previously (Chang et al., 1999). For assays involving hormone receptors, cells were treated with vehicle or the following concentrations of its respective hormone: 100 nM 17β-estradiol (ERα, ERβ), 100 nM progesterone (PR-A), 100 nM dexamethasone (GR), 1 μM 5α-dihydrotestosterone (AR), 100 nM 9-*cis*-retinoic acid (RARα, RXRα), 100 nM triiodothyronine (TRβ), 100 nM 1,25-dihydroxyvitamin D3 (VDR), 10 μM 22R-hydroxycholesterol (LXR), and 50 μM chenodeoxycholic acid (FXR). For transduction of protein using adenoviruses, HepG2 cells were infected at a multiplicity of infection (MOI) of 10–50 for 48–72 hr. In experiments with siRNA and PGC-1α infections, cells were infected with siRNA adenovirus 48 hr prior to a second infection with PGC-1α (or mutants).

Protein Immunoblotting

Whole-cell protein extracts were separated on an 8% SDS-PAGE and blotted onto PVDF (Millipore). PGC-1α protein was detected with a rabbit polyclonal PGC-1α antibody (Santa Cruz Biotechnology). ERRα protein was detected by using a monoclonal antibody (Gaillard et al., 2006).

Quantitative PCR

Total RNA was isolated by using the RNeasy kit with RNase-free DNase (Qiagen). One microgram of RNA was reverse transcribed by using the BioRad iScript cDNA synthesis kit. qPCR reactions (BioRad iCycler) were performed with 0.1 μl of cDNA, 10 μM specific primers, and iQ SYBRGreen supermix (BioRad). The sequences of the primers are listed in Table S1.

Fatty Acid Oxidation

HepG2 cells were infected at MOI 20 for 72 hr then incubated 3 hr at 37°C with 1 mM carnitine, 12.5 mM HEPES, 0.5% BSA, 500 μM sodium oleate, and 1.0 μCi/ml [1-^{14}C] oleic acid (MP Biomedicals). Oxidation end product ^{14}C-CO_2 released was measured as described previously (Koves et al., 2005).

Microarray Statistical and Gene Ontologic Analyses

Microarray gene profiling experiments were performed with the Human Genome U133 Plus 2.0 Array chips (Affymetrix). RNA was collected 24 hr after infection. Target preparation, hybridization to the Affymetrix HG-U133 plus 2.0 arrays, and scanning were performed by the Duke Microarray Center. The entire experiment was repeated three times. The probe intensities were extracted from CEL files by utilizing the Affymetrix Input Engine from the SAS Microarray Solution software (SAS Institute Inc., Cary, NC). After normalization, the mixed model was applied by running Mixed Model Analysis from the SAS Microarray Solution (Chu et al., 2002, 2004). Significant genes were determined by conducting t tests based on the estimated parameters from this model. The specific tests consisted of differences between β-gal control and three PGC-α variants treatment groups (WT, 2x9, and L2L3M). A Bonferroni correction was applied across all tests to control the probability of false positives to be <0.05. Enrichment in GO terms was detected by using the Onto-Express application (Draghici et al., 2003).

Supplemental Data

Supplemental Data include five figures and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/24/5/797/DC1/.

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Accession Numbers

Microarray .cel and .chp files can be found on the NURSA microarray database (http://www.nursa.org/datasets.cfm?doi+10.621/datasets.02004) and the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE5968.

Chapter 19

Nuclear Receptor Cofactor Interactions as Targets for New Drug Discovery

Linda L. Grasfeder and Donald P. McDonnell

19.1 Introduction

In basic models of steroid hormone receptor action, unoccupied receptors are proposed to reside in target cells in an inactive form. Upon agonist binding, the biochemical properties of the steroid receptor (SR) are altered to allow the interaction of a receptor dimer with specific DNA sequences within the promoters of target genes. The DNA bound receptor can then exert either a positive or negative effect on target gene transcription. With respect to the pharmacological actions of SR ligands, these simple models predict that agonists function merely as "switches" that facilitate the conversion of the SRs from an inactive to an active form, whereas antagonists function solely by competitively inhibiting the binding of agonists. Thus, when corrected for affinity, all agonists for a given receptor would be qualitatively the same, as would all compounds classified as antagonists. Not surprisingly, the majority of existing drugs that act on SRs were discovered using simple *in vitro* receptor binding assays with subsequent *in vivo* assays used to distinguish agonists from antagonists. In recent years, however, it has become increasingly clear that there is an unmet medical need for NR-interacting drugs that manifest positive activities in a tissue-restricted manner (especially for long-term treatment of chronic diseases). In particular, there are specific needs for (a) a glucocorticoid that exhibits

anti-inflammatory actions without causing metabolic disturbances or osteoporosis; (b) an estrogen capable of treating the climacteric symptoms associated with long-term estrogen deprivation but inactive in the breast and uterus; and (c) an androgen that improves lean body mass and bone density without liver toxicity and prostatic hypertrophy. However, within the confines of the classical models of NR pharmacology, it was difficult to understand how absent approaches that relied on differential ligand pharmacokinetics. It was going to be possible to develop receptor modulators with clinically useful selectivity. Interestingly, a retrospective analysis of the early preclinical studies of the estrogen receptor (ER) modulator, tamoxifen, revealed that it could function as an agonist or an antagonist in a manner that differed between tissues in the same species, and in the same target organ in different species.[1,2] Although these data clearly indicated that the pharmacological definitions "agonist" and "antagonist" did not adequately describe the pharmacology of ER ligands, it took nearly 30 years before the clinical importance of these seminal findings were appreciated. A further 10–15 years of research was needed to define the likely molecular basis for the tissue selectivity of this ER modulator.

Using the insights that have emerged from recent studies, it has been possible to construct a good first draft of the pathways and processes that impinge on and modulate the pharmacological activities of different ER-ligand complexes. Importantly, the information obtained from the study of the molecular pharmacology of ER ligands appears to translate to other receptors. Thus, it is not surprising that we are in the midst of a paradigm shift in NR ligand discovery programs away from classical "grind-and-bind" approaches to the exploitation of the complexity of NR signaling using mechanism-based functionally predictive screens. This review will discuss the seminal findings that have helped to shape our understanding of NR pharmacology and will consider where the field is going and what new types of pharmaceuticals are likely to emerge in the future.

19.1.1 *The "tamoxifen paradox": A clinical observation that reinvigorated interest in nuclear receptors as therapeutic targets*

It was not clear until the early 1990s that it would be possible to develop NR ligands with clinically useful selectivity. However, the

findings of a seminal study conducted by Love *et al.* demonstrated that, at least with respect to ER, this goal could be accomplished. Specifically, these investigators examined the impact of adjuvant tamoxifen treatment of two years duration on the skeleton in postmenopausal breast cancer patients.[3] It had been established that longer duration of treatment with tamoxifen had a positive impact on disease-free and overall survival in breast cancer patients. However, given its antiestrogenic properties and the morbidity associated with osteoporosis, it was important to determine the effects of long-term treatment on the skeleton. The surprising result of these studies was that tamoxifen, rather than functioning as an antiestrogen in the lumbar spine, actually significantly increased BMD in a manner similar to that expected of an estrogenic compound.[4,5] Indeed, were it not for the fact that tamoxifen was also shown to exhibit estrogenic activities in the endometrium, it is likely that this drug would have had the utility as an antiresorbtive for use in the treatment and prevention of osteoporosis. This result, referred to as the "tamoxifen paradox", indicated that although this drug can function as an antiestrogen in the breast, it is a significant estrogen in bone. This led to the reclassification of tamoxifen as a selective estrogen receptor modulator (SERM), as opposed to an antiestrogen, to reflect the fact that its relative agonist/antagonist activities manifested in a tissue-dependent manner.[6] The generality of the SERM concept was established in additional studies which demonstrated that another previously described ER antagonist, keoxifene (now called raloxifene), also exhibited a favorable estrogenic activity in bone, but unlike tamoxifen, it functioned as an antiestrogen in the endometrium.[7,8] Not surprisingly, given this selectivity, this SERM has been registered for the treatment and prevention of osteoporosis. It was determined to exhibit comparable activity to tamoxifen, as a breast cancer chemopreventative, in the recently completed STAR trial.[9] Thus, the clinical utility of this drug is likely to expand in the near future. Although tamoxifen and raloxifene exhibit similar pharmacological activities in most target organs, their dramatically different activities in the uterus have served to define the SERM concept. From a practical point of view, these are important findings as they provide the impetus to develop selective nuclear receptor modulators (SNRMs) which, by acting through their targeted receptors, are more clinically useful that classical agonists or antagonists.

19.2 The Molecular Determinants of ER Pharmacology

The identification of SERMs has represented a major advance in the pharmacotherapy of breast cancer and other endocrinopathies. In addition, studies of their molecular mechanism of action has led to the determination that among the most important determinants of NR ligand pharmacology are (a) receptor isoform and subtype expression in target tissues; (b) the impact of ligands on the structure of the receptor; and (c) the ability of differently conformed ligand-receptor complexes to recruit functionally distinct coregulators. A discussion of how these processes influence ER pharmacology will serve to highlight the generality of these concepts.

19.2.1 *The role of receptor subtypes in ER ligand pharmacology*

The biological actions of ER ligands are manifest through high affinity nuclear receptors located within the target cell nuclei. Until relatively recently, it was considered that all the biological actions of ER modulators were manifest through a single receptor that was biochemically identical in all cells. However, the discovery in 1996 of a second ER significantly increased the biological complexity of estrogen.[10] Unexpectedly, Kuiper and coworkers identified a novel receptor cDNA in the prostate of male rats which encoded a protein that bound 17β-estradiol with an affinity equivalent to that of the previously identified "breast/uterus" estrogen receptors.[10] A human homologue of this novel ER, now called ERβ, was subsequently cloned.[11] We now know that the biological action of these two receptor subtypes is not equivalent, and although a discussion of this aspect of ER signaling goes beyond the scope of this review, it is important to note two general themes that have emerged: (1) ERα and ERβ are not functionally redundant, with each being able to regulate a distinct set of biological processes, and[2] in cells where both receptors are expressed, ERβ functions as a transdominant inhibitor of ERα signaling.[12–14] It is clear that most of the known receptors have more than one functional isoform or subtype. Not surprisingly, there is a high level of interest in developing small molecules that target these receptor variants as a means of generating more selective drugs.

There has been considerable progress in identifying specific ERβ ligands, and the preliminary studies of these agents *in vivo* have suggested that there are potentially useful clinical outcomes that will emerge by selectively activating each receptor. Two important studies have been published which emphasize this point. Firstly, it has been shown by Harris and colleagues that a specific ERβ agonist, ERB-041, exhibits potent anti-inflammatory activities in rodent models of rheumatoid arthritis and inflammatory bowel disease.[15] These activities can be inhibited by the ER pan-antagonist ICI182,780, confirming the involvement of ERβ. However, these potent anti-inflammatory activities are not as pronounced in animals treated with estradiol, a non-selective ER agonist. This raises the possibility that the anti-inflammatory actions of ERβ ligands may represent a pharmacological activity of this class of compounds that may not occur when the receptor is activated by natural ligands. A second series of ERβ agonists have recently been described that reduce ventral prostate size in rodents.[16] This may relate to the fact that ERβ is a negative regulator of androgen receptor (AR) expression. In discussions at scientific meetings, it appears that the activities of the available ERβ agonists are not equivalent, leading to the suggestion that the existing compounds represent the founding members of a new class of ligands, the selective estrogen receptor beta agonists (SERBAs).[16] Thus, although there is considerable interest in developing novel ERβ agonists, the therapeutic (disease) targets and the profile of the desired drug remains to be determined.

19.2.3 *Ligand induced changes in NR conformation as a determinant of pharmacology*

Initially, it was considered that the selectivity of SERMs like tamoxifen and raloxifene could be explained by differential activation of ERα or ERβ. However, with minor exceptions, the affinity of the SERMs for both receptors has been shown to be equivalent.[17,18] Furthermore, it has been shown that the biological activity of some SERMs can differ among tissues or cells that express the same ER subtype. For instance, using ERα responsive transcription systems reconstituted in a variety of cells, it has been shown that tamoxifen can function in some backgrounds as an antagonist whereas in others, it can manifest partial agonist activity.[19,20] Furthermore, in those cells where tamoxifen manifests partial agonist activity, raloxifene, droloxifene and idoxifene can function

as pure ERα antagonists.[21] This suggests that there are differences in the way each ER is able to respond to different SERMs in different cells. Considerable insight into the molecular basis of this selectivity has come from studies aimed at understanding how information flows from the unique chemical structure of each SERM to the transcription apparatus, and how this translates into a different phenotypic response.

According to the classical models of NR action, ERα is a ligand-regulated transcription factor that remains inactive when associated with a heat-shock protein complex until an activating ligand binds. This activity promotes the displacement of the receptor from the inhibitory complex, allows receptor dimerization, and facilitates its interaction with specific estrogen response elements located within target gene promoters. Depending on the cellular and promoter context, the DNA bound promoter can either positively or negatively regulate target gene transcription.[20] In the simplest interpretation of this model, the antiestrogenic activities of SERMs, like tamoxifen, would reflect a displacement of estradiol and the retention of the receptor in an inactive state. Clearly, this was not the correct interpretation. The first departure from this model describing antagonist action came from the studies of Allan *et al*. that used protease digestion to map the conformational changes in the progesterone receptor (PR) which occur upon ligand binding.[22–24] Specifically, these studies demonstrated that agonists and antagonists induce distinct conformational changes in PR upon binding and, importantly, both conformations were distinct from apo-receptor. This implied that antagonists do not merely freeze the receptor in an inactive conformation but facilitate the formation of a uniquely structured complex. A similar series of studies were later performed with ERα and revealed that the overall conformations of the ERα-tamoxifen and ERα-estradiol complexes were different and distinct from apo-receptor.[25,26] Subsequent analysis of the crystal structure of the ERα ligand binding domains complexed with either an agonist or an antagonist confirmed the role of ligands in determining the overall shape of the receptor.[27–29] Furthermore, these latter studies provided insight as to the mechanisms by which agonist-activated receptors physically interacted with nuclear receptor coactivators. Specifically, ligand-induced alterations in the receptor enabled the formation of a hydrophobic cleft that was capable of interacting with the receptor interaction domains of coactivators. This domain is coincident with the previously defined "activation function-2 (AF-2)" of the receptor.[30] In the presence of

SERMs, like tamoxifen and raloxifene, this coactivator-binding pocket is not formed, providing a mechanistic explanation for the antagonist activity of these compounds. However, although these structural studies provided an explanation for the agonist activity of molecules, like estradiol, and for the antagonist activity of SERMs, they did not provide insight into the mechanisms underlying the tissue selective agonist activities of different SERM-ER complexes.

19.2.4 *SERMs enable the presentation of specific cofactor interaction surfaces that are required for tissue selective agonist activity*

One interpretation of the results of the assays of receptor conformation was that a specific agonist-induced structure of ER (or any NR) was required for transcriptional activity and that all other conformations were incompatible with activation. This provided a simple model of agonism and antagonism but did not provide an explanation for SERM activity. We proposed that although the canonical coactivator binding pocket was disabled in the SERM-ER complexes, there are additional surfaces presented upon binding these ligands that enable cofactor binding. We further posited that the availability of cofactors that could interact with these surfaces determined the relative agonist/antagonist activities of SERMs. To test this idea, we mapped the protein-protein interaction surfaces on ERα that were presented upon its interaction with different ligands, and then defined the roles of these surfaces in the pharmacological actions of the bound ligand. The complete details of these experiments have been published previously.[21,31–33] In brief, we used combinatorial phage display to screen libraries of random peptides of 15–19 amino acids in length that interacted with purified ERα. We did this in the presence of different ligands. The high-affinity interacting phage identified in this manner were then used in a phage ELISA assay as a means of profiling the surfaces presented on the receptor in the presence of different ligands. The results of one of the most informative assays are presented in Fig. 1. One class of peptides, represented by α/β I, interacted with ERα in the presence of estradiol but not other ligands tested. A second class of peptide, represented by α/β V interacted with tamoxifen-activated ERα but not with that activated by estradiol. Neither peptide interacted with the apo-receptor. These important findings confirmed the results of both the protease digestion

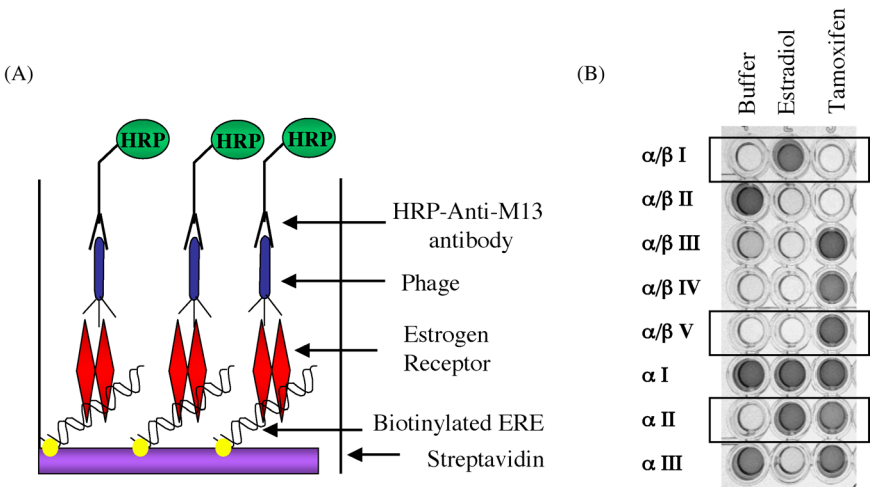

Fig. 1. Probing ligand-dependent changes in the conformation of ERα using combinatorial peptide phage display. Bacteriophage expressing peptides which is capable of interacting with ERα in the presence of different ligands were identified and purified as described previously.[32,33] These were then used to probe the conformational changes in the receptor that occurred subsequent to its interaction with different ligands. (A) A diagram of the features of the Phage ELISA assay used for these studies. (B) The ability of selected phage, expressing ERα interacting peptides, to interact with the receptor in the presence of different ligands was assessed using a phage ELISA assay. Those peptides that are discussed specifically in the text are highlighted.

and crystallography studies, but went further in that they revealed that there are distinct protein-protein interaction surfaces presented on ERα in the presence of estradiol and SERMs. In support of this conclusion, we observed obvious sequence conservation, with distinct consensus motifs apparent, for both the ERα-estradiol and the ERα-tamoxifen interacting peptides. For instance, most, if not all, the peptides that interacted specifically with estradiol-activated ERα contained the sequence LXXLL, a motif commonly found in the receptor interacting surfaces of coactivators. A strong, though unrelated, consensus sequence was also apparent in the peptides that interacted with tamoxifen-activated ERα. We concluded from these results that, in addition to serving as surrogate markers of ER structure, these peptides highlight surfaces on the receptor that are important for ligand pharmacology.

The functionality of the protein-protein interaction surfaces mapped using phage display was tested by assessing the ability of the expressed peptides (α/β I and α/β V) to inhibit the agonist activity of estradiol and tamoxifen in cells where both compounds can function as agonists. Specifically, using a reconstituted ERα-responsive transcription system in HepG2 cells, it was demonstrated that both estradiol and tamoxifen were capable of activating transcription of a C3-luciferase reporter. Interestingly, when the α/β I peptide was expressed in cells, estradiol but not tamoxifen agonist activity was inhibited, whereas the converse was true when the tamoxifen specific peptide α/β V was expressed (Fig. 2).[33] We believe that these results indicate that the agonist activity of estradiol and tamoxifen do not occur in the same manner, but rather each compound enables distinct cofactor interactions by presenting different protein-protein interaction surfaces on the receptor. The ERα/α/β V complex has not yet been crystallized and thus it has been difficult to assess how "different" the protein-protein interaction surfaces are on the receptor following activation with estradiol or tamoxifen. This is an important issue to resolve as it will enable us to determine if the tamoxifen-ERα complex manifests agonist activity by interacting with a subset of the cofactors with which the ERα-estradiol complex interacts or if the tamoxifen-ERα complex interacts in an ectopic manner with an as yet to be defined cofactor. A working model of our current understanding of some of the basic differences in estradiol and tamoxifen pharmacology is outlined in Fig. 3. In the presence of estradiol, a conformational change occurs that induces the formation of the AF-2 coactivator interacting pocket and the subsequent interaction of the receptor with a requisite coactivator (CoA). Upon binding tamoxifen, however, a surface other than AF-2 is presented. This surface is unable to engage a coactivator that utilizes a functional AF-2 and, in most circumstances, this results in tamoxifen functioning as an antagonist. However, in some environments, the surfaces presented on the tamoxifen-activated ERα can engage a cofactor that permits it to manifest agonist activity. Thus, differential expression of the putative coactivator, designated CoA(X), determines the relative agonist/antagonist activity of tamoxifen. Formal proof of this hypothesis will require the identification and functional analysis of proteins that can interact with the tamoxifen-activated receptor and whose knockdown prevents the manifestation of tamoxifen agonist activity.

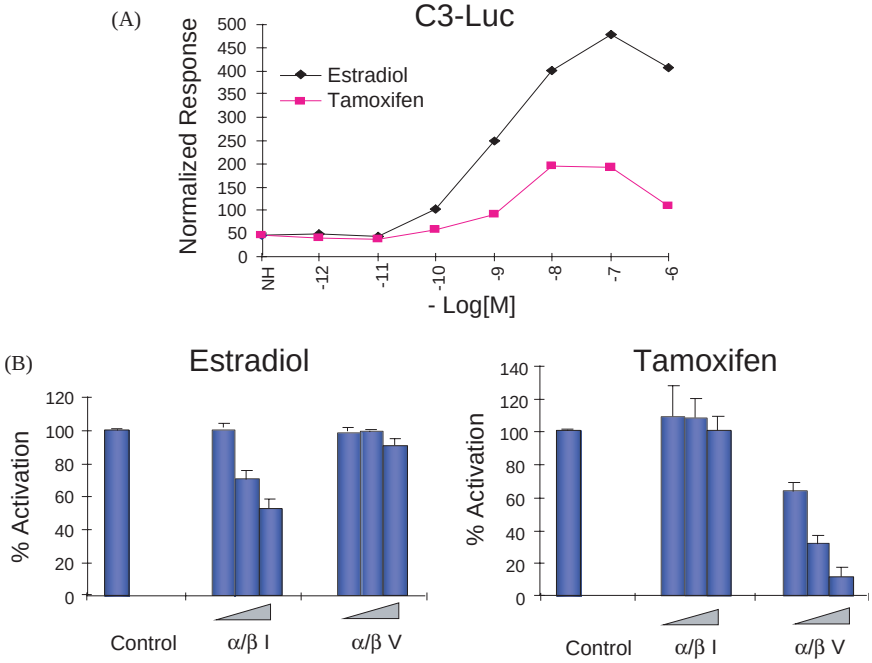

Fig. 2. Tamoxifen and estradiol facilitate the presentation of distinct protein-protein interaction surfaces on ERα. (A) HepG2 cells were transfected with the estrogen-responsive C3-Luc reporter gene along with expression vectors for ERα and a normalization plasmid (β-Gal). Cells were treated with either estradiol or tamoxifen as indicated and analyzed for luciferase and β-Gal activity. NH, no hormone. (B) HepG2 cells were transfected as in (A) except that expression vectors for peptide-Gal4 fusions were included as indicated. Control represents the transcriptional activity of estradiol or tamoxifen activated ERα in the presence of the Gal4-peptide fusion as shown with the resulting transcriptional activity presented as percentage of activation of control. Data are averaged from three independent experiments (each performed in triplicate) with error bars representing SEM.

19.3 Differential Cofactor Interaction Assays as a Means to Identify Novel ER Regulators

Resistance (both *denovo* and acquired) is a significant clinical issue that limits the efficacy and duration of response to tamoxifen in breast cancer.[34] Although resistance is likely to be a multifactoral process, it is

clear that the ability of tamoxifen to manifest agonist activity is a key contributing factor. It has been shown in breast cancer cell xenografts propagated in mice that tamoxifen initially functions as an antagonist and then "switches" to functioning as an agonist.[35,36] There were early anecdotal reports of "tamoxifen withdrawal" responses in women who progressed while on drug, a finding that is consistent with that observed in animal studies.[37–39] Additionally, the results of the NSABP-B14 trial revealed that although short-term adjuvant treatment with tamoxifen (<5 years) was clearly beneficial, longer duration of exposure (5–9 years) was associated with an increased risk of breast cancer recurrence.[40,41] This suggested that there was either a selection for cells in the breast that exhibited an innate ability to recognize tamoxifen as an agonist or that some epigenetic event occurred in breast tumor cells that permitted this activity. Given the model we proposed to explain the agonist activity of tamoxifen (Fig. 3), we posited that by screening against the presentation of cofactor interaction surfaces on ERα that are apparent upon binding estradiol or tamoxifen, it would be possible to identify a new class of antagonist that would have utility in the treatment of tamoxifen refractory breast cancer. The details of the successful screen that led to the identification of GW5638 (now called IOS974), a compound that inhibits tamoxifen agonist activity *in vitro* and *in vivo*, have been published previously.[35,42] Considering the manner in which it was identified, it is not surprising that we were able to show using peptide profiling and crystallography that this new compound permitted ERα to adopt a novel structure.[21,33,43] This drug is currently being evaluated in the clinic as a second line intervention for patients with tamoxifen refractory disease. The successful outcome of this project highlights the utility of mechanism-based screens in the search for novel NR modulators.

It is clear that ligand-induced alterations in ERα structure are important determinants of cofactor recruitment and downstream biology. A question that has arisen from these studies of ER action is whether or not endogenous ligands exist which manifest SERM activity. In this regard, it has been demonstrated recently by the Mangelsdorf laboratory that the oxysterol metabolite 27-hydroxycholesterol (27HC) exhibits SERM activity *in vivo*.[44] We have confirmed the SERM properties of 27HC in cellular models of estrogen action and demonstrated using peptide profiling that this compound permits ERα to adopt a conformation distinct from other ligands.[45] Interestingly, although 27HC has a relatively low affinity for ERα, it is present in high concentrations

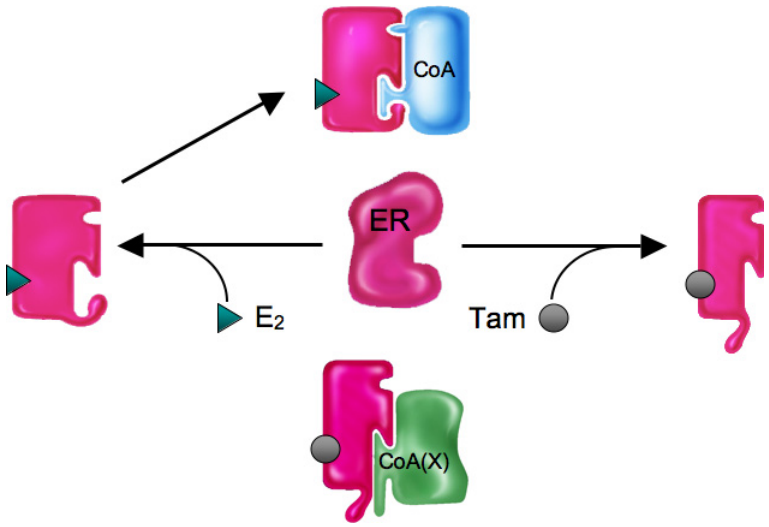

Fig. 3. The mechanisms by which estradiol and tamoxifen manifest agonist activity are dissimilar. Using peptide antagonists that inhibit specific protein-protein interactions, it has been possible to show that the mechanisms by which estradiol and tamoxifen manifest agonist activity are dissimilar. Estradiol binding enables ERα to adopt a structure that is compatible with the binding of the p160 class of coactivators. Tamoxifen binding, on the other hand, induces a unique alteration in receptor structure that permits an ectopic interaction of the receptor with an unidentified coactivator. The existence of this coactivator is supported by the fact that peptides of the α/β V class will inhibit tamoxifen, but not estradiol-mediated transcriptional activity when expressed in target cells. Tamoxifen functions as an antagonist in situations where it is unable to facilitate the recruitment of a coactivator to ERα.

within atherosclerotic plaques and is produced at high levels in various tissues by infiltrating macrophages. It remains to be determined if 27HC or similar endogenous SERM-like molecules serve as physiological regulators of ERα (or ERβ), or if they only come into play in pathological conditions.

19.4 Translating Insights from the Study of Serms to Other Nuclear Receptors

One of the unifying themes that has emerged from the study of SERMs is that receptor conformation is a primary determinant of selectivity.

This important observation has suggested that it may be possible to develop selective modulators of other nuclear receptors. Among the most advanced programs are those aimed at developing selective progesterone receptor modulators (SPRMs), selective androgen receptor modulators (SARMs) and selective glucocorticoid receptor modulators. A review of the literature, however, indicates that the SNRM descriptor is applied loosely and has been used to describe both compounds that achieve selectivity as a result of a unique action at the level of the receptor as well as those that achieve selectivity as a result of differential pharmacokinetics.

19.4.1 *Selective progesterone receptor modulators*

The major clinical uses of progestins to date relate to their ability to oppose estrogen action in the reproductive tract. Thus, progestin-containing medicines have seen widespread use as hormone therapy (HT), contraceptives and as third line therapies in ER-positive breast cancers. However, the currently used progestins have significant side effects creating a niche for molecules with improved therapeutic selectivity. The desired molecules would (a) exhibit antiproliferative activity in the endometrium and in breast tissue; (b) be neutral in the CNS; (c) have no impact on the cardiovascular system; and (d) does not negatively impact estrogen action in bone. There is also a high level of interest in developing PR antagonists for the treatment of uterine fibroids and endometriosis.[46] Much of this interest comes from the seminal findings of Sam Yen who first demonstrated that the antiprogestin RU486 could be used as a medical intervention in fibroids.[47,48] However, since this molecule was also a potent antiglucocorticoid, it was unclear as to what activity of RU486 was required for this therapeutic activity.[49] The positive clinical studies that have been published with new and more selective PR modulators that has validated PR as the appropriate therapeutic target. One of the most interesting drugs, asoprisnil (J867), has shown to display agonist, anti-proliferative effects on the endometrium, while functioning as an antagonist of progesterone-driven growth of uterine fibroids.[50–52] Therefore, this molecule exhibits the pharmacological characteristics of a selective progesterone receptor modulator (SPRM). Indeed, in a preliminary study of the molecular basis for its distinct pharmacological actions, it was determined that the PR-asoprisnil complex is capable of binding both coactivator (TIF2) and corepressor

(NCoR) peptides with equal affinities in fluorescent binding assays, whereas the corepressor peptide binds with 10-fold greater affinity to RU486-bound PR.[53] Additionally, the crystal structure of PR bound to asoprisnil and an NCoR peptide indicates that the receptor assumes a unique conformation.[53] Thus, the relationship between the structure of the ER-ligand complex and function that explains SERM pharmacology appears to extend to SPRMs.

Finally it is worth mentioning that there are multiple isoforms of PR (PR-A, PR-B, and PR-C), which have distinct biological activities in the female reproductive system. However, since all isoforms are encoded by the same gene, with PR-A having its N-terminal domain truncated, and PR-C additionally missing part of its AF-2 domain; the ligand binding pockets of the receptors are identical. Thus, absent drug-induced allosteric effects on the PR-ligand binding domain that are manifest differently in the context of the three isoforms, it is difficult to see how these receptor isoforms can be exploited differentially. It is possible, however, that they have different cofactor requirements, and that targeting these interaction surfaces may yield drugs with additional selectivity.

19.4.2 *Selective androgen receptor modulators*

There is a high level of interest in developing selective androgen receptor modulators (SARMs) that exhibit androgenic activity in bone and muscle while exhibiting neutral or antagonist activity in the bone.[54–60] Several classes of new molecules have emerged that exhibit these properties in rodent models and are currently being evaluated in the clinic. It is unclear if these compounds are truly functioning as SARMs, or if they are weak/partial agonists that achieve selectivity as a consequence of differential sensitivity of target tissues to agonists. Under normal circumstances, dihydrotestosterone is the androgen that is primarily responsible for AR-dependent prostate growth. Thus, the conversion of testosterone to DHT through the actions of 5α-reductase, amplifies the androgenic stimulus to the prostate. In the presence of a 5α-reductase inhibitor, testosterone has reduced activity in the prostate but retains its anabolic activities. This has led to the suggestion that the selectivity of the currently available SARMs is probably due to the fact that they have sufficient potency/efficacy to mimic the actions of testosterone in bone/muscle but they cannot maximally activate AR in prostate.[61] This

would explain why it is relatively easy to develop AR ligands with an improved therapeutic window (prostate versus muscle/bone). One concern, however, from lessons learned from studies of ER pharmacology is that over time the prostatic epithelial cells may adapt and compensate for the reduced androgenic stimulus by increasing the expression of AR, or one of its coactivators, or through a hyperactivation of a signaling pathway that increases cellular sensitivity to androgens. Thus, it would appear to be more useful to develop SARMs that function by facilitating differential cofactor recruitment as opposed to functioning as partial agonists.

Whereas most of the currently available SARMs were discovered in an empirical manner, we have undertaken to develop mechanism-based screens to identify new AR ligands. The goal of these studies was to identify compounds that had different effects on AR structure and to determine if they exhibited SARM-like properties. Specifically, we used AR-interacting peptide probes to select for compounds that altered the structure of the AF-2 pocket in different ways.[62] We reasoned that this might impact the interaction of AR with its cofactors. Using this approach, coupled with modeling and combinatorial peptide phage display, we were able to generate a series of compounds that were either partial agonists or neutral with respect to gene transcription. However, both classes of compounds were fully efficacious as agonists in cell proliferation assays.[62,63] Although clearly not the profile of the desired modulator, this study revealed that as with SERMs, it was possible separate the biological functions of AR using ligands that had a different effect on AR structure. We are currently expanding these efforts with a view to developing an understanding of the relationship between AR structure and function. This is a first step in the rational development of SARMs.

19.4.3 *Selective glucocorticoid receptor modulators*

The anti-inflammatory properties of glucocorticoids have been utilized in the clinics for many years for the treatment of various inflammatory diseases, including asthma, rheumatoid arthritis, and autoimmune diseases. However, the side effect profile of these compounds including hypertension, muscle atrophy, osteoporosis and glucose intolerance, severely limits their long term use. Many of the undesired effects of GR relate to its ability to function as positive activator of gene transcription

while its anti-inflammatory properties are likely to result from its ability to "transrepress" the expression of pro-inflammatory genes regulated by NF-κB and AP-1. Not surprisingly, therefore, the search for selective glucocorticoid receptor modulators (SGRMs) has focused on identifying compounds that permit transpression at the expense of classical transcriptional responses. Significant progress in developing SGRMs with this favorable pharmacological profile have emerged in recent years. For instance, several novel arylpyrazole compounds have been synthesized that behave different from classical agonists in terms of their activities on target gene transcription, GR nuclear localization, GRE occupancy, and cell proliferation and differentiation.[64–66] While all of these compounds bind GR with strong affinity and selectivity, they have dramatically different effects on receptor–promoter interactions and on gene expression profiles. Thus, as with other SNRMs, there exists a strong relationship between receptor structure and function.

19.5 Development of Novel NR Modulators that Function Outside of the Classical Ligand Binding Pocket

While the classical ligand binding pocket of NRs has been the primary target for pharmacological modulation of NR pathways, there is growing evidence that supports the idea that alternative NR surfaces can be targeted. In this regard, two new approaches appear to hold promise: (a) generation of short peptides that exhibit antagonist activity by inhibiting NR-cofactor interactions; and (b) developing small molecules that bind on the receptor surface and allosterically regulate coactivator function.

19.5.1 *Identification of antagonists that function by directly interfering with NR-cofactor interactions*

As described above, it has been possible to identify short, high affinity, peptides that interact with and inhibit NR transcriptional activity in a specific manner (Fig. 2).[31,33,67–69] Whereas these peptides are useful tools with which to probe NR pharmacology *in vitro*, they will require significant modifications and/or formulation to make them useful for studies *in vivo*. However, several groups have made progress in efforts aimed at

making these peptides more "drug-like". In one study, it was shown that a macrolactam ring could be used to constrain a peptide from the NR box of GRIP1, a modification that allows the peptide to assume a partial α-helical conformation.[70] This constrained structure exhibited a higher affinity for the TRβ/T_3 complex than the uncyclized parent molecule and efficiently blocked the interaction of the receptor-ligand complex with GRIP1. In a similar manner, this group created a library of macrolactam constrained peptides that interacted selectively with ERα, ERβ, or TRβ.[71] Other groups created peptide-derived antagonists using both disulfide and thioether bridges to create stabilized, cyclical peptides that are also able to block interaction of the SRC-1 NR box 2 with ERα or ERβ.[72–74] The extent to which these modifications have improved the pharmaceutical properties of the peptide-derived antagonists remains unclear.

Small molecules have several advantages over peptides in terms of drug delivery, stability, and permeability. Not surprisingly, therefore, several groups have attempted to isolate compounds that directly displace cofactor-AF-2 interactions. To this end, a structure-based design approach was used by John Katzenellenbogen and colleagues to identify small molecule inhibitors of NR-coactivator interactions.[75] Using the crystal structure of the LBD of ERα complexed with diethylstilbestrol and a 13 amino acid peptide from GRIP1, they identified key points of contact between the peptide and the receptor and designed compounds using a variety of scaffolds that allowed them to mimic the relevant interactions. Their most successful compound had a pyrimidine core with branched alkyl substituents mimicking the leucines of the coactivator NR-box. This compound was able to displace a coactivator peptide in a fluorescence anistrophy assay, and thus provided the first direct evidence that small molecules can be designed to target the coactivator binding pocket (Rodriguez, 2004).

Using a classical small molecule screen, Guy and Fletterick's groups identified compounds that inhibit TRβ-coactivator interactions.[76,77] The compounds identified in this manner did not compete with T_3 for binding to the LBD of TRβ. Instead, the receptor serves as a surface for catalyzing a reaction that releases an active, unsaturated ketone that covalently binds to the receptor and inhibits coactivator association. These compounds are able to completely block TRβ activity in cell-based transcription assays.[76] A subsequent report describing the crystal

structure of TRβ complexed with the active compound, aromatic β-enone 1-(4-hexylphenyl)-prop-2-en-1-one (HPPE), confirmed that it binds within the AF-2 region and led to the identification of the specific residues in this domain with which it interacts.[77] Thus, while the utility of targeting the AF-2 coactivator binding surface was initially demonstrated using peptide antagonists, it is likely that it will be possible to develop small molecules that directly target this surface on the receptor. Drugs that inhibit ER and AR in this manner would have utility in the treatment of breast and prostate cancer where complete inhibition of the receptor is desirable.

While the AF-2 is an attractive alternative target for drug design, there are hints that some NRs display additional surfaces (pockets) that might be amenable to pharmacological manipulation. For instance, peptides have been found that interact with regions of nuclear receptors other than the AF-2 that inhibit their transcriptional activity. One such peptide, αII peptide, was discovered using combinatorial peptide phage display that interacts with ERα in the presence of several different ligands, and inhibits its ability to activate transcription (Fig. 1).[33] The crystallization of this peptide in a complex with OHT-bound ERα led to the identification of a previously unidentified protein-protein interaction surface on the β-hairpin face of the LBD, located on the opposite side of the receptor from AF-2 domain.[78] Small molecules that interact with this pocket would be expected to exhibit antagonist activity.

The orphan nuclear receptor Nurr1 has no classical ligand binding pocket, as bulky side chains of the amino acids lining the putative pocket consume all the space of the pocket. Since this receptor appears to be in an active conformation, the question arises as to how the activity of this receptor is regulated. Additionally, the transcriptional activity of this receptor is not affected by LxxLL-containing coactivator. Structural modeling of the receptor identified a potential alternate coactivator binding pocket, and site-directed mutagenesis of the putative region decreased transcriptional activity of the receptor.[79] Pockets of this nature represent potential targets for new drug discovery.

19.5.2 *Identification of NR modulators that function through protein allosterism*

In addition to directly targeting coactivator interaction surfaces, some recently published work from the Fletterick laboratory has validated

"allosteric sites" on NRs as targets for drug discovery. Specifically, they identified a novel surface on AR when screening for compounds that dissociate an LxxLL peptide from DHT-bound AR.[80] Using this approach, they identified several molecules which when crystallized with AR were found in a novel pocket that they termed the BF-3 region, a domain distal to AF-2. Interestingly the mutation of various residues within this region altered the activity of the receptor, and furthermore many natural mutations within this region have been identified in patients with prostate cancer or androgen insensitivity syndrome. Clearly, this and other studies have provided the impetus to probe novel ways of manipulating NR activity that may yield drugs that work in place of, or in combination with, current modulators.

19.6 Exploiting Receptor-Cofactor Interactions for New Drug Discovery

The definition of a relationship between overall receptor conformation, differential cofactor recruitment and pharmacological activity has led to the emergence of the "selective nuclear receptor modulator (SNRM) hypothesis". One of the central tenets of this hypothesis is that by screening for small molecules that favor one NR-cofactor interaction at the expense of another, it will be possible to develop compounds that exhibit a high degree of process selectivity. Although supported by the available data, the SNRM hypothesis remains to be formally proven. It has been shown that the pharmacology of SNRMs (SARMs, SERMs and SPRMs) can be manipulated by overexpression of certain coactivators (i.e. SRC-1/3 or PGC-1α) or inhibition/knockdown of corepressors (NCoR or RTA).[81–83] Thus, there is little doubt that imbalances in cofactor expression, such as it occurs in certain pathological states, affects the pharmacology of NR ligands. However, it remains to be demonstrated that the normal variations in cofactor expression that exist between cells (or tissues) are key determinants of NR ligand pharmacology. This will require the identification of compounds that engender specific NR-cofactor interactions and the subsequent demonstration that these ligands exhibit predictable biology. An example of how this hypothesis applies to ER-ligand pharmacology is shown in Fig. 4. To date, attempts to develop SNRMs have generally been performed in an empirical manner with studies of differential NR-cofactor interactions being used in a retrospective manner to provide an explanation for

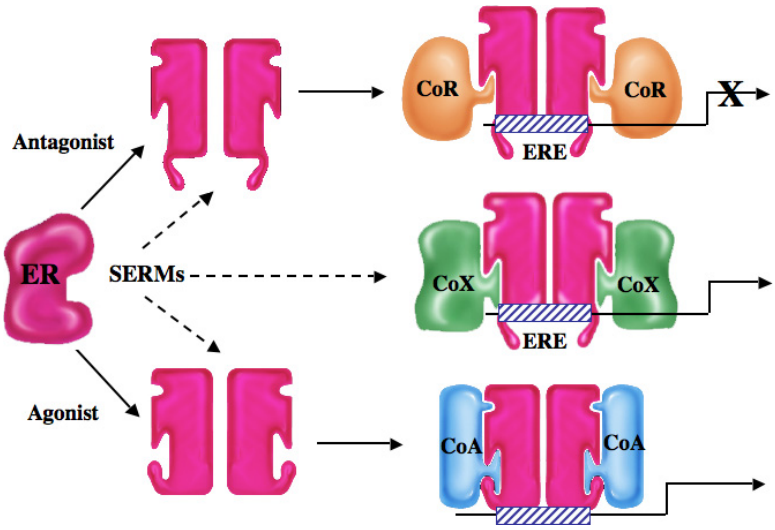

Fig. 4. An updated model of ER action may help to explain the activity of ER modulators. Upon binding an agonist or an antagonist, ER undergoes a conformational change that permits its spontaneous dimerization and facilitates the subsequent interaction of the dimer with estrogen response elements (ERE) located within target genes. Two genetically distinct ERs have been identified, ERα and ERβ, which have the potential to form homodimers or heterodimers in cells where both subtypes are expressed. It has recently been determined that different ligands can have different effects on ER structure. The functional consequences of different ligand-induced conformational changes were revealed with the discovery of receptor coactivators (CoA) and corepressors (CoR). Coactivators interact with agonist-activated ER and facilitate transcriptional activation, whereas corepressors interact with antagonist-activated receptor and help to maintain it in a quiescent state. SERMs permit the receptor to adopt a structure that is intermediate between that observed following the binding of agonists or antagonists. Thus, the relative agonist/antagonist activity of SERMs is a relectionreflection of the ability of differently conformed ERα-ligand complexes to engage coactivators or corepressors. It is also possible that some SERMs allow ERα to interact in an ectopic manner with coactivator/corepressorsreressors (CoX) that would not interact with ERα in normal physiological circumstances.

specific pharmacological attributes. However, this field will take a major leap forward when the NR-cofactors involved in specific biological processes are developed and used in mechanism-based screens for new compounds discovery. It is clear that although NRs are established and

well validated targets, there remains a significant opportunity to exploit the complexities of their signaling pathways for new drug discovery.

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Fasting-induced hepatic production of DHEA is regulated by PGC-1α, ERRα and HNF4α.

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Abstract

The transcriptional coactivator PGC-1α is involved in the coordinate induction of changes in gene expression in the liver that enable a homeostatic response to alterations in metabolic state, environmental cues and nutrient availability. In exploring the specific pathways under PGC-1α regulation in the liver, we have made the surprising observation that this coactivator can induce the expression of CYP11A1 and CYP17A1, key rate limiting enzymes involved in the initial steps of steroidogenesis. Both of these enzymes function to produce C_{19}-steroids, converting cholesterol into pregnenolone, and then to DHEA. ERRα mediates PGC-1α's induction of *CYP11A1* and binds within the first intron of the CYP11A1 gene. Both ERRα and HNF4α are required for PGC-1α-mediated induction of *CYP17A1* and specific binding sites for these receptors have been identified in the regulatory regions of this gene. The potential physiological significance of these observations was highlighted in rats where fasting induced hepatic expression of *PGC-1α* and *CYP17A1* and was associated with an increase in hepatic levels of DHEA. These data suggest that DHEA could be playing a role as an intracellular signaling molecule involved in modulating hepatic activity in response to fasting conditions.

Introduction

Homeostatic control of nutrient levels in the body is a primary function of the liver, which modulates circulating levels of sugars, lipids and proteins in response to hormones and other internal and external cues. Fasting causes a metabolic switch in liver that includes activation of glycogenolysis and gluconeogenesis, and increases in fatty acid oxidation to accommodate increased mobilization of free fatty acids from adipose stores. In the fed state these processes are usually repressed, however in pathological conditions such as type 2 diabetes, elements of this control are lost and hepatic glucose production persists in spite of elevated glucose levels (1). Sustained imbalances in glucose levels as well as distinct metabolic defects that influence lipid handling can cause fatty acids and triglycerides to accumulate in the liver, leading to hepatic steatosis (2).

The transcriptional coactivator PGC-1α is a key integrator of many of the signaling pathways that are induced in the liver and muscle upon fasting (3). Notably, glucagon and glucocorticoids induce the expression of PGC-1α in the liver through CREB (4), and the expressed coactivator serves to integrate signals from afferent signaling pathways that influence its ability to regulate transcription. Insulin suppresses expression of gluconeogenic enzymes partly through AKT/PKB-mediated phosphorylation and inactivation of PGC-1α (5). In addition, acetylation of PGC-1α by GCN5 reduces its activity (6), while SirT1, an NAD^+ sensor, deacetylates and increases its activity (7).

When expressed and active, PGC-1α acts as a potent transcriptional activator and can interact with a number of transcription factors to provide metabolic control at the transcriptional level. It interacts with the orphan nuclear receptor HNF4α to induce expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, key enzymes involved in gluconeogenesis (3, 8). It also interacts with HNF4α to increase hepatic synthesis of apolipoproteins A-IV, CII and CIII (9). ERRα is another key partner for PGC-1α, providing direct and indirect control of numerous genes involved in oxidative phosphorylation (10, 11), fatty acid oxidation (12) and the tricarboxylic acid cycle (13). Finally, PGC-1α can also interact with and coactivate several other nuclear receptors, most notably the PPARs, as well as other transcription factors including NRF-1, Gabpa/b, MEF2C, and FoxO1 (reviewed in(14)).

We have previously performed an extensive analysis of the changes in gene expression that occur in response to PGC-1α expression in hepatic cells (13) and discovered that the mRNAs corresponding to several of the rate limiting enzymes in steroidogenesis are dramatically and unexpectedly induced. Under normal circumstances steroidogenesis takes place in the adrenals and gonads, where cytochrome p450 hydroxylases and steroid dehydrogenases modify cholesterol to form the different steroid hormones. The initial enzymatic step is catalyzed by cytochrome p450 cholesterol side-chain cleavage enzyme, CYP11A1, which oxidizes cholesterol at the 20 and 22 carbon positions and cleaves the side chain between these carbons to produce pregnenolone. The enzyme CYP17A1 catalyzes the next steps, converting

pregnenolone to DHEA through its 17α-hydroxylase and 17,20 lyase activities. Additional enzymes can then modify DHEA to form sex steroids or pregnenolone and 17α-OH pregnenonlone to generate glucorticoids and mineralcorticoids (reviewed in (15) and (16)). While the adrenal and gonads secrete the majority of hormones in circulation, a number of tissues are capable of locally synthesizing steroids. In postmenopausal women, for instance, the concentration of estrogen in breast tumors has been reported to be significantly higher than circulating plasma levels (17). This elevated concentration is thought to be due to local conversion of androgens to estrogens by the ectopically expressed aromatase enzyme (CYP19) in tumor stromal cells (reviewed in (18)). In the brain, most steroids [neurosteroids] are thought to be synthesized locally, with both neurons and glia expressing many of the enzymes and transporters necessary for steroidogenesis from cholesterol (reviewed in (19) and (20)). Finally, and of direct relevance to the studies described below, it has been determined that human fetal liver expresses most of the steroidogenic enzymes, but at levels considerably lower than those found in primary steroidogenic organs (21). Fetal rat liver expresses CYP17A1 mRNA, and at birth, expression in liver is comparable to that in testis. These levels rise and peak after 8 days, and then steadily decline to undetectable levels after puberty (22). Additionally, expression analysis of the genes induced in the liver of fasted mice found that CYP17A1 was robustly induced by a 24-hour and 48-hour fast (23). However, it has not been determined whether or not these enzymes are expressed and functional in human liver or how their expression fits within the metabolic functions of the liver.

In this study, we report that CYP11A1 and CYP17A1, rate-limiting enzymes in steroidogenesis, are functionally induced in human hepatic cells by PGC-1α. While these genes have previously been shown to be regulated by cAMP and the orphan nuclear receptor SF-1 (reviewed in (24)), we show in this study that ERRα and HNF4α are novel regulators of these genes, mediating their induction in hepatic cells by PGC-1α. Interestingly, steroidogenesis in PGC-1α-expressing hepatic cells does not progress beyond DHEA to classical sex steroids, suggesting that this steroid or an unidentified metabolite could be involved in novel hepatic signaling pathway in response to fasting conditions.

Results

Induction of CYP11A1 and CYP17A1 expression in hepatic cells is regulated by PGC-1α.

The transcription coactivator PGC-1α has been shown to interact with and modulate the transcriptional activity of several nuclear receptors and unrelated transcription factors. This has made it difficult to link specific aspects of PGC-1α biology to its activity on an individual transcription factor. To circumvent this specificity problem, we previously developed a modified PGC-1α construct, PGC-1α 2x9, which interacts selectively with ERRα and HNF4α. Expression of this modified coactivator in HepG2 cells enabled us to use microarray-based approaches to define the target genes that are regulated by the PGC-1α-(ERRα/HNF4α) axis (13). A gene ontology analysis of the target genes regulated by both PGC-1α and PGC-1α 2x9 led to the identification of a number of genes involved in steroid metabolism, including Cytochrome p450 11A1 (CYP11A1) and Cytochrome p450 17A1 (CYP17A1).

To confirm that CYP11A1 and CYP17A1 are regulated by PGC-1α, HepG2 cells were treated with adenoviruses expressing β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M, a mutant form of PGC-1α in which the NR-interacting domain has been disrupted (11), (13). Expression of the mRNAs encoding these enzymes was analyzed using quantitative PCR, revealing that both *CYP11A1* and *CYP17A1* are robustly induced by either PGC-1α or PGC-1α 2x9, but not β-gal or PGC-1α L2L3M **(Figure 1A)**. A similar response was also observed in other human hepatic cells, including Hep3B cells **(Figure 1B)**, and in primary human hepatocytes **(Figure 1C)**. While *CYP17A1* was induced in primary rat hepatocytes (Supplemental figure 1), *CYP11A1* was not found to be expressed in rat livers in any of the conditions we tested (data not shown). We completed this survey of PGC-1α responsiveness by analyzing CYP11A1 and CYP17A1 expression in a series of cells that were not of hepatic origin. In this manner we observed that PGC-1α did not induce the expression of either gene in H295R adrenal cells (Supplemental figure 1) or MCF7 breast cancer cells (data not shown). However, PGC-1α did induce expression of *CYP11A1* in U251 cells, and *CYP17A1* in AGS stomach carcinoma cells (Supplemental figure 1). This data suggests that PGC-1α induces elements of the steroidogenic pathway in a limited set of cell types.

PGC-1α increases the functional activity of CYP11A1 and CYP17A1.

Both CYP11A1 and CYP17A1 require additional proteins and redox partners for their enzymatic activity. Specifically, CYP11A1 requires ferridoxin reductase (FDXR) and ferridoxin while the p450 oxidoreductase (POR) and cytochrome b5 are obligate partners of CYP17A1 (reviewed in (25)). Interestingly, we found that POR is induced by 3 to 3.5-fold in the PGC-1α expressing cells ($p < .001$) in the original array (13). Levels of FDXR were unaffected by PGC-1α expression. Therefore, we considered whether the observed increases in the mRNA expression of these enzymes results in increased functional activity when PGC-1α is expressed. To this end, [^{3}H]pregnenolone was incubated with HepG2 cells for 6 hours expressing either β-gal, PGC-1α, PGC-1α 2x9 or PGC-1α L2L3M. The steroids in the

cells at the end of the incubation period were extracted and separated using high performance liquid chromatography. Cells expressing either PGC-1α or PGC-1α 2x9 displayed increased synthesis of a product that co-migrates with standards for 17α-OH pregnenolone and dehydroepiandrosterone (DHEA) **(Figure 2A)**. Notably absent on the chromatograph were peaks corresponding to other sex steroids including androstenedione, which migrates on this column at 23.2 min, estrogen at 27.3 min, testosterone at 31.3 min, or progesterone at 50.2 min (data not shown). This observation suggests that the steroidogenic pathway induced by PGC-1α does not appear to progress beyond DHEA. Additionally, this data reflects our inability to detect significant mRNA expression of 3β-hydroxysteroid dehydrogenases 1 and 2, CYP21, and CYP11B1 in HepG2 cells, the encoded proteins of which are the enzymes required to convert DHEA to either sex steroids or glucocorticoids (Supplemental figure 2). Some additional differences between the chromatograms derived from the PGC-1α expressing cells and the control cells were apparent, particularly in the rapidly migrating polar products which are likely to be metabolites of DHEA. The percent conversion of pregnenolone to DHEA and 17α-OH pregnenolone was calculated by determining the area under the curves in each sample. As illustrated in the bar graph **(Figure 2A),** 14% and 8% of the pregnenolone was converted to 17α-OH pregnenolone in PGC-1α and PGC-1α 2x9 expressing cells, respectively, and 31% and 56% of the pregnenolone was converted to DHEA.

Additional confirmation that the product identified was DHEA was obtained by a radioimmunoassay to specifically measure this steroid in the media derived from HepG2 cells incubated with pregnenolone for 6 hours. This analysis confirmed that DHEA is synthesized in HepG2 cells expressing either PGC-1α or PGC-1α 2x9 **(Figure 2B)**. Finally, since we observed that CYP11A1 was also expressed in HepG2 cells, we examined whether PGC-1α expression would permit DHEA synthesis *de novo* from a CYP11A1 substrate. To test this idea, the cell-permeable cholesterol metabolite 22(R)-OH cholesterol was used as a substrate, and DHEA synthesis was measured by RIA. HepG2 cells expressing either PGC-1α or PGC-1α 2x9 showed increased synthesis of DHEA **(Figure 2C),** indicating that CYP11A1 was also functionally active following the expression of PGC-1α in these cells.

PGC-1α-dependent induction of CYP11A1 and CYP17A1 requires ERRα

We have determined that CYP11A1 is induced by both PGC-1α and PGC-1α 2x9, but not a mutant PGC-1α L2L3M that is incapable of interacting with NRs. This result implicates ERRα and/or HNF4α, in the regulation of this gene. Thus, we next examined the impact of siRNA-mediated knockdown of each of these receptors on *CYP11A1* expression in HepG2 cells. The results of this analysis, shown in **Figure 3A**, indicate that reducing the expression of ERRα substantially abrogated the induction of *CYP11A1* by PGC-1α and PGC-1α 2x9.

To examine regulation of CYP11A1 by HNF4α, we designed an siRNA to knockdown HNF4α expression. This construct was able to substantially reduce protein expression of HNF4α **(Figure 3B)** as well as reduce

expression of known HNF4α target genes (Supplemental figure 3). However, reduced expression of HNF4α had little or no effect (**Figure 3B**) on the expression of *CYP11A1*.

Using the consensus site and matrix previously described for ERRα (26), the sequence surrounding the CYP11A1 gene was scanned for putative ERRα response elements using TESS (27). This analysis identified 18 putative sites within the gene and the 10kb surrounding region **(Figure 3C).** Chromatin immunoprecipitation (ChIP) was used to determine if an ERRα binding site could be identified in the region surrounding CYP11A1. ERRα has previously been described to be subject to autoregulation by a defined binding site in its own promoter (28), and this site was used as a positive control for ERRα binding. PGC-1α expression further increases ERRα binding to its own promoter, as also described. We tested ERRα binding to each of the putative response elements around the CYP11A1 gene by designing primers to amplify each region and measuring the response by quantitative PCR. ERRα was found to associate most strongly with a site in the first intron of CYP11A1 **(Figure 3D** and Supplemental figure 4). PGC-1α is required for ERRα to bind to this site, as almost no binding is observed in cells expressing β-gal. ERRα exhibited very minimal binding to all other putative sites, including a site at -873bp **(Figure 3D),** and three additional sites that are perfect matches to the ERRα consensus (Supplemental figure 4). We conclude from these studies that ERRα is both required for PGC-1α-mediated induction of CYP11A1 expression and that this likely results from its interaction with a specific ERRE within the gene.

We next evaluated the role of ERRα in PGC-1α-mediated induction of CYP17A1 gene expression. Again using the siRNA-mediated knockdown approach, we observed reduced expression of *CYP17A1* by PGC-1α and PGC-1α 2x9 in cells in which ERRα expression was reduced **(Figure 4A).** Interestingly, however, we routinely found that even with substantial knockdown of ERRα, we only achieved approximately a 50% decrease in CYP17A1 expression, a result that implicated either HNF4α or a non-NR target of PGC-1α in the regulation of this gene (see below). Scanning the region 20kb around the CYP17A1 gene with the same method that we used for CYP11A1, 14 putative ERRE sites were identified **(Figure 4B**). ERRα association with each of these putative EREs was tested using ChIP (Supplemental figure 5). ERRα was found to be associated most strongly with an element 22kb downstream of the transcriptional start of CYP17A1 (**Figure 4C**), and as in the case of the ERRE in CYP11A, PGC-1α expression was required for this interaction, as no binding was observed in cells expressing β-gal alone. This site is contained within the gene region of the hypothetical protein C10orf26; however, expression of PGC-1α or siERR has no effect on the expression of this transcript (data not shown). Notably, ERRα does not bind to any significant degree to any of the other sites identified by this method, including the site 1294bp downstream from the transcription start site **(Figure 4C).** Thus, we have identified a potential site through which the ERRα/PGC-1α complex could regulate expression of CYP17A1.

HNF4α is required for maximal induction of CYP17A1 by PGC-1α.

Given the incomplete inhibition of PGC-1α-mediated CYP17A1 expression in ERRα knockdown cells, we next queried the role of HNF4α in the regulation of this enzyme. The results of these analyses revealed that knockdown of HNF4α protein (**Figure 3B**) was able to significantly impair PGC-1α-dependent induction of *CYP17A1* **(Figure 5A)** (~50%), indicating that HNF4α is also involved in regulating the expression of CYP17A1. Since HNF4α binds a direct repeat with 1 base pair spacing (DR-1 element) (29), NHR scan (30) was used to identify DR-1 elements in the genome within CYP17A1 or in its surrounding 20kb region. This scan identified six putative sites (**Figure 5B**), and we tested the association of HNF4α with each site using ChIP. Previously, CYP7A1 has been shown to be a direct transcriptional target of HNF4α (31), and therefore was used as a positive control for the ChIP analysis in this study. HNF4α was found most strongly associated with the DR-1 site most proximal to transcriptional start of CYP17A1 **(Figure 5C),** and may additionally bind the site at -6837 (Supplemental figure 6). At both sites, PGC-1α expression appears to increase HNF4α binding.

The functionality of the -878 site was next examined. To this end we used a reporter construct containing approximately 3.2kb of the promoter region immediately upstream of the start of the CYP17A1 gene (32). Two DR-1 elements are within this region of the promoter, and both half-sites of each element were mutated to examine binding. A VP16 activation domain was fused to HNF4α and SF-1, a well characterized regulator of CYP17A1. While mutation of either DR-1 site did not affect activation of the reporter by VP16-SF-1, mutation of the most proximal DR-1 site at -878bp significantly reduced the activity of VP16-HNF4α (**Figure 5D**). Mutation of the more distal DR-1 site at -2032bp had no effect on the reporter activity, indicating that HNF4α binds the proximal DR-1 site in the CYP17A1 promoter.

Fasting induces CYP17A1 and increases hepatic DHEA concentration.

All the studies presented thus far have involved the transient ectopic expression of PGC-1α or its derivatives in cells, with a subsequent analysis of CYP11A1 and CYP17A1 expression. We felt therefore that it was necessary to recapitulate this regulation in a system where the physiological significance was more apparent. Previously, PGC-1α expression has been shown to be induced in hepatocytes during fasting (3). We confirmed that the expression of PGC-1α mRNA is significantly induced in rat livers following a 14-16 hr fast **(Figure 6A)**. More importantly however, we observed that CYP17A1 gene expression is also elevated in rat livers under the same conditions (Figure 6A). These data highlight the physiological relevance of the regulation studies that we previously performed in cultured cells.

Since DHEA is a product of CYP17A1 activity and is a predominant steroid resulting from overexpression of PGC-1α in HepG2 cells (**Figure 2A**), hepatic DHEA levels were examined in fasted and fed rats. This analysis revealed a small, but significant increase in DHEA in the livers of the fasted rats (**Figure 6B**). Preliminary data indicates that serum levels of DHEA do not

appear to be altered by fasting conditions (data not shown), suggesting that increased hepatic DHEA concentration results from local synthesis. However, application of more sensitive methods is required to fully investigate this issue, and these are being developed. Thus, as was observed in cultured cells, fasting induced expression of CYP17A1 leads to an increase in hepatic production of DHEA.

DHEA reduces the concentration of free amino acids in hepatic cells.

DHEA's role in metabolism has been controversial, but several studies have shown that DHEA can reduce abdominal visceral fat and insulin levels in humans and animal models (33, 34). However, for the most part these studies have used concentrations of the steroid that we would not expect to achieve under the conditions we have studied to date. Thus, we were faced with defining a biological process in hepatic cells that could occur following the administration of DHEA at the levels we have determined to result from fasting-induced expression of steroidogenic enzymes. To this end, we asked whether low concentrations of DHEA might have an impact on any relevant metabolic markers in hepatic cells. Specifically, HepG2 cells were treated with 1nM DHEA and MS/MS was used to measure various metabolic parameters of the cells, including acylcarnitines and amino acids. No changes in acylcarnitines were evident (data not shown). However, while glycine, serine, proline, and aspartate levels did not change in treated cells, we reproducibly observed that the levels of alanine, arginine, valine, leucine, isoleucine, tyrosine, phenylalanine, methionine and histidine levels were reduced about 50% by 1nM of DHEA (**Figure 7**).

We have not yet been able to account for the loss in amino acids and do not know if they are incorporated into new proteins, exported or subject to catabolism. In addition, since a specific receptor or target for DHEA has not yet been characterized, we are unable to directly link PGC-1α-mediated induction of DHEA synthesis to the changes in amino acid biology. Regardless, the robust effects of nanomolar concentrations of DHEA puts the PGC-1α-mediated regulation of the synthesis of this hormone in liver into some physiological context and sets up future studies on this enigmatic steroid.

Discussion

PGC-1α has emerged as a key integrator of signaling pathways that regulate metabolism in a variety of tissues. In this study, we present evidence that PGC-1α regulates production of steroids in the liver, most notably DHEA. Our studies provide definitive evidence to show that PGC-1α can induce gene expression of *CYP11A1* and *CYP17A1* in both hepatic cell lines and in primary cultures of hepatocytes. Importantly, the necessary auxiliary proteins required for the functionality of these enzymes are expressed at a sufficient level, or are co-induced by PGC-1α, as we have observed conversion of either pregnenolone or 22(R)-OH cholesterol to DHEA in human hepatic cell models. The physiological significance of these observations is underscored by the observation that hepatic levels of DHEA are increased in fasted rats. A model describing our view of the relationship between fasting, PGC-1α, and steroidogenesis is presented in **Figure 8**.

Previously, Bauer et al have reported that *CYP17A1* is induced in livers of fasted mice (23). Our studies extend this previous work by providing a firm understanding of the mechanisms by which fasting leads to the increased synthesis of this enzyme as well as CYP11A1. While these enzymes are expressed predominantly in the adrenals and gonads where they are involved in mediating the initial steps in steroidogenesis, these studies show that they are also induced and functional in hepatic cells. Although the increase in hepatic DHEA we have observed in whole livers is relatively small, we have demonstrated in culture that this is sufficient to affect a wholesale change in the intracellular concentrations of amino acids. This latter activity highlights the potential physiological significance of PGC-1α-induced increases in DHEA synthesis, and we are currently investigating its role in the fasting response.

Regulation of the expression of steroidogenic genes by PGC-1α, ERRα and HNF4α.

In the classical steroidogenic tissues (adrenals and gonads), gene expression of steroidogenic enzymes is regulated by the hypothalamic-pituitary axis through cAMP-mediated pathways. The precise mechanisms regulating the coordinated expression of these genes have not been fully delineated, however, the nuclear receptor SF-1 has been shown to bind multiple regions of each promoter and regulate their expression (reviewed in (16)). Interestingly, the ERRE within the ERRα gene responsible for its auto-induction is almost identical to the canonical SFRE. Furthermore it has been shown that ERRα can activate SFRE-containing reporters in transient transfection assays (35). We found that ERREs are overrepresented in the region around the CYP11A1 and CYP17A1, genes. However, we noted that the binding of ERRα is very selective, interacting in a PGC-1α-dependent manner with only 1 of 18 and 1 of 14 putative ERREs located with the CYP11A1 and CYP17A1 genes respectively. This highly selective response suggests that other factors or mechanisms are also involved in regulating ERRα's binding and transcriptional activation of these genes in hepatic cells.

While these studies identify putative ERRα regulatory sites, ERRα could bind additional sites that contain a

more degenerate consensus site, or ERRα could regulate expression through other transcription factors.

HNF4α is a key transcriptional regulator of many hepatic processes, being reported to associate with the promoter regions of up to 12% of the genes expressed in hepatic cells that are represented on a Hu133K array (36). It was not surprising, therefore, that we were able to show that HNF4α is involved in regulating expression of *CYP17A1* in hepatic cells. However, whether or not the activities of these transcription factors can be regulated by other coactivators, permitting an uncoupling of the response observed in the presence of PGC-1α, remains to be determined.

A Possible role for DHEA in regulating metabolic function in hepatocytes.

Our results indicate that DHEA is synthesized in HepG2 cells when PGC-1α is expressed. However, downstream conversion of this steroid to androgens, such as androstenedione or testosterone, was not observed. This was in agreement with our inability to detect significant expression of 3β hydroxysteroid dehydrogenase 1 or 2 in unmanipulated cells or those expressing PGC-1α. It is therefore tempting to speculate that the primary purpose of these enzymes during fasting is to produce DHEA, and that it has an important role in the regulation of liver metabolism.

DHEA administration has been shown to have dramatic effects on metabolism in animal models, reducing hyperglycemia and/or hyperinsulinemia of diabetic db/db mice (37), stretozotocin-induced diabetes (34), Zucker rats (38), and obese ob/ob mice (37). Administration of DHEA to rats increases absolute and relative liver weight, protein, DNA, RNA, lipid and mitochondrial content within 7 days (39), indicating that DHEA can impact liver metabolism in rodents.

DHEA, or one of its yet to be identified metabolites, could have a role as an intracellular signaling molecule in hepatic cells during fasting. However, this hypothesis is difficult to test since its biological role is still unclear beyond that of an androgen precursor. A specific receptor for DHEA has not been identified, although this steroid has been reported to be able to modulate the activity of other nuclear receptors, such as the estrogen receptor (40, 41) and an unidentified membrane-bound receptor (42).

Intriguing data also suggests that DHEA can protect endothelial cells from apoptosis via a $G\alpha_i$ receptor-mediated induction of the phosphotidylinositol 3-kinase/Akt-mediated pathway (43). In fact, these protective effects were also observed using nanomolar concentrations of DHEA (0.1-10nM), and could be functioning in hepatic cells to protect them from an elevated oxidative state brought on by fasting. Alternatively, induction of the Akt pathway in hepatic cells has been shown to inhibit the activity of PGC-1α (5). Therefore, PGC-1α's induction of DHEA may constitute a component of a negative feedback loop that controls the gluconeogenic activity of PGC-1α in extended periods of fasting. Further work to explore these possibilities is currently underway.

Regulation of cholesterol homeostasis--Generation of other ligands.

Whereas we have observed a significant increase in the production of DHEA in cells expressing activated

PGC-1α, it is possible that CYP11A1 and CYP17A1 may induce the synthesis of other molecules which could be important in regulating the fasting response. For instance, it has been shown that CYP11A1 catalyzes three different modifications on cholesterol, generating cholesterol metabolites hydroxylated at the 20 and 22 carbons (25). These oxysterols are known ligands for LXRs, which regulate cholesterol homeostasis and lipid metabolism (44). Additionally, CYP17A1 has been described to have monooxygenase activity on squalene, a cholesterol precursor, producing squalene epoxide (45). Intriguingly, this squalene epoxide can be shunted into an alternative pathway to produce 24(S),25 epoxicholesterol which is also a potent LXR ligand (46). Therefore, induction of both of these enzymes leads to the production of LXR ligands by two independent pathways, potentially providing another mechanism by which fasting and PGC-1α can regulate LXR activity.

Regulation of steroidogenesis in other tissues

While these studies highlight the ability of PGC-1α, ERRα, and/or HNF4α to regulate the steroidogenic pathway in hepatic cells, it raises the possibility that they could also regulate this process in other tissues. Classical steroidogenic tissues such as the adrenals and gonads should be considered since cAMP induces expression of both PGC-1α and most of the steroidogenic enzymes.

Another possibility is that PGC-1α could be regulating a steroidogenic program in the brain. Neurosteroids are thought to be primarily synthesized *de novo* in both neurons and glia where they have a role in signaling and neuroprotection (20). PGC-1α has also been shown to have a neuroprotective role in the brain, resulting in part from its induction of radical oxygen scavengers (47). We have observed that PGC-1α is able to induce expression of CYP11A1 in the U251 glioma cell line (Supplemental figure 1), suggesting that synthesis of neurosteroids could contribute to PGC-1α's neuroprotective role in the brain.

Conclusion

While the functional significance of PGC-1α-mediated induction of functional CYP11A1 and CYP17A1 enzymes in the liver is still under investigation, these results have revealed a potential role for DHEA in regulating hepatic metabolism under conditions of fasting.

Materials and Methods

Plasmids

Lentiviral vectors pLL5.0, VSV-G, Rev, Gag/Pol were a gift from Dr. Jim Bear (UNC Chapel Hill). Hairpins were designed as described in Cai et al (48) to include a 19mer siRNA sequence. siRNA for ERRα was constructed using the sequence described in Schrieber et al (49), and the oligo used for cloning is listed in Supplemental table 1. siRNA for HNF4α was designed using Oligo engine, and the oligos used for cloning are listed in Supplemental table 1. Oligos were inserted into Hpa1 and Xho1 sites in pLL5.0.

The -3.2kb-uas-cyp17 reporter construct (32) was a gift from Dr. Walter Miller (UCSF). Mutations of the DR-1 elements of the CYP17 promoter were made by transferring the promoter region to pENTRT7 using Xho1 and HindIII sites, and using excite mutagenesis with the primers listed in Supplemental table 1. The mutated promoter regions were cloned back into the original vector using Xma1 and NcoI sites surrounding the DR1 at -2032, and using AgeI and Xho1 sites surrounding the DR-1 site at -878. The accuracy of these mutated clones was verified by sequencing. HNF4α was PCR amplified from HepG2 cell RNA using primers with restriction sites for EcoRI and XbaI on the 5'and 3' ends respectively, then cloned into sites in pENTR3C (Invitrogen). HNF4α was recombined into pVP16-GW, a Gateway (Invitrogen) compatible destination vector modified from pVP16 (Clontech). VP16-SF1 was subcloned from pcDNA3.1-Zeo-SF1 (kind gift from Keith Parker) into pVP16 (Clontech) at EcoR1 and BamH1 sites.

Cell Culture

Culture, transfection, infection and reporter assays using HepG2 (hepatoma), Hep3B (hepatoma), and HeLa (human cervical carcinoma) cells were maintained at 37^{o}C with 5% CO_2 in minimal essential media (Invitrogen) supplemented with 8% FBS, 0.1mM non-essential amino acids, and 1mM sodium pyruvate. HepG2 cells were cultured on dishes coated with 0.1% gelatin. Primary human hepatocytes (Lonza) were cultured in hepatocyte basal medium supplemented with HCM SingleQuots containing Ascorbic acid, BSA-FAF, Transferrin, Insulin, hEGF, and GA-1000 (Lonza).

For transient transfections, cells were plated on 24-well plates 24 hrs prior to transfection with lipofectin. A total of 3ug of plasmid DNA was used per 24-well triplicate in transfections, and CMV-β-gal was used for normalization. Luciferase and β-gal readings were measured as described previously (50).

Adenoviruses expressing β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M were constructed as described previously (13) and were amplified and purified by CsCl2 centrifugation (a kind gift from John Bisi at GlaxoSmith Kline). Cells were infected at a multiplicity of infection (MOI) of 5-50 for 48-72 h.

siRNA was delivered using lentiviruses which were grown in 293FT cells as described in Cai et al (48). Media was filtered through a 0.45um syringe filter and applied to cells with 4ug/ml polybrene. Fresh culture media was applied after 16 hours, and cells were cultured for 2-3 days, then plated on 6-well plates at 400,000 cells/well. Adenoviral infection followed immediately as described above.

qPCR

Total RNA was collected using either Qiagen, Sigma or BioRad RNA purification columns and using DNase from either Ambion, Sigma, or BioRad. One microgram of RNA was used to generate cDNA using iScript (BioRad). cDNA was diluted 1:50, using 5ul/well in a 13ul PCR reaction with iQ SYBRGreen supermix (BioRad) and 0.3uM of each primer. The sequences of the primers used for these studies are included in Supplemental table 1. Data was analyzed by the standard curve method ((51), normalizing expression to 36B4.

Western Blots

Cells were lysed in 20mM Tris, pH 8.0, 137mM NaCl, 10% glycerol, 1% NP40, 2mM EDTA. 30ug of protein was loaded on 8% SDS-PAGE gels, transferred onto nitrocellulose membranes, blocked with 5% milk in PBS with 0.1% Tween. PGC-1α antibody (H-300) (Santa Cruz Biotech), ERRα antibody(52), HNF4α antibody (C-19) (Santa Cruz Biotech sc-6556), or GapDH (V-18) (Santa Cruz Biotech sc-20357) were used to detect protein expression.

Steroid analysis

HepG2 cells were plated on 6-well dishes coated with 0.1% gelatin at 250,000 cells/well and infected at MOI 20-30 with adenoviruses expressing β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M. After 24 hrs, media was changed to white MEM with 8% charcoal filtered serum, 0.1mM non-essential amino acids, and 1mM sodium pyruvate for another 24 hrs. For HPLC analysis, cells were incubated with 200nM [7-^{3}H]pregnenolone (0.5uCi/ml) (Perkin-Elmer) for 6 hours. Media was collected, any cell debris was pelleted by centrifugation, and supernatant was transferred to fresh tubes and snap frozen. Steroids were extracted from the media with 3:2 ethyl acetate:hexane. This solvent was evaporated and samples were reconstituted in 100ul of methanol, vortexed, and 40 ul was injected onto a Breeze model 1525 HPLC binary pump system equipped with model 717 plus autoinjector (Waters Corp., Milford, MA) and a Waters Symmety C18 4.6 X 150mm, 5um reverse phase column. A methanol gradient was used starting at 50% methanol and holding this concentration for 20 minutes. This was followed by a gradient to 60% methanol for 22 minutes, then a gradient to 87% for 7 minutes. At 49 minutes, the concentration of methanol returns to the initial 50% for 10 minutes before the next injection. The column effluent was analyzed with a model 2487 dual-wave length UV detector set to 280 nm and a β-RAM model 3 in-line radioactivity detector (IN/US Systems, Inc., Tampa FL). [^{3}H]DHEA, [^{3}H]17OH pregnenolone, and [^{3}H] pregnenolone (NEN) were used as standards.

For radioimmunoassays measuring DHEA, cells were plated and infected as described for HPLC experiments, but were incubated with 200nM pregnenolone (Sigma) or 50uM 22(R)-OH cholesterol (Sigma) in 2ml media for 6 hrs. Media was collected and centrifuged to remove any cell debris. DHEA was measured directly using a RIA kit (MP Biomedicals or Diagnostic Systems Laboratories). For measurements of DHEA in rodents, liver tissues were harvested, sectioned into approximately 400mg pieces, weighed, then homogenized in equal volumes of solvent (3:2 mixture of ethyl acetate to hexane). Mixture was left at room temp for 16 hours and then centrifuged. The

organic layer was removed and evaporated under nitrogen gas and residue was resuspended in 110ul of PBS containing 5mg/ml BSA. 100ul was used in RIA for DHEA. Values were normalized to starting tissue mass.

Chromatin Immunoprecipitation

HepG2 cells ($4x10^6$) were plated on 15cm plates coated with 1% gelatin and infected the next day with adenoviruses expressing β-gal or PGC-1α for 48 hrs. Cells were washed with PBS with 1mM $MgCl_2$, and fixed with PBS +1mM $MgCl_2$ + 1% formaldehyde for 10-15 minutes. Glycine was added to 125mM, cells were washed 3x with PBS, then scraped off plate and snap-frozen. Pellet was resuspended in sonication buffer (50mM Hepes (pH 7.8), 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS) and sonicated at 50% power using a Misonic XL-2000 sonicator for 10s followed by 10s on ice, repeated 10x. Cell debris was removed by centrifugation, 100ul of 50% protein A/G beads containing 200ug/ml salmon sperm DNA and 500ug/ml BSA were used to preclear lysate. 1% of lysate was reserved for input samples. 2.5ul of ERRα polyclonal antibody (a kind gift from Vincent Giguere) or 5ug of normal mouse IgG (sc-2025, 200ug/0.5ml) was used to test for ERRα binding. 5ug of HNF4α (either sc-6556 or sc-8987 from Santa Cruz Biotechnology) or IgG control (goat IgG sc-2028 or rabbit IgG sc-2027, respectively) was used to test for HNF4α binding. Antibodies were incubated with 1ml of lysate for 4 hours at 4°C, followed by addition of 100ul 50% Protein A/G beads overnight. Beads were washed 2x with sonication buffer, 2x with wash A (50mM Hepes (pH 7.8), 500mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS), 2x with wash B (20mM Tris (pH8.0), 1mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate), and 2x with TE (10mM Tris (pH 8.0), 1mM EDTA). Samples were eluted in 50mM Tris (pH8.0), 1mM EDTA, 1% SDS at 65°C for 20 minutes. Crosslink was reversed using 0.2M NaCl and incubating at 65°C overnight. Samples and reserved input were incubated with EDTA (4.2mM) and proteinase K at 42°C for 1 hour, purified on PCR purification columns (Qiagen), eluted in 40ul 10mM Tris, and diluted 1:7 with water for analysis by qPCR. Data was analyzed as % of input. DNA shearing quality was monitored to ensure that fragment sizes were around 500bp. Putative ERREs were identified using the matrix described by Sladek et al (26), used in TESS (27) at 90% stringency. Putative HNF4 sites were identified using NHR scan (http://www.cisreg.ca/cgi-bin/NHR-scan/nhr_scan.cgi?rm=advanced) (30). Positive control primers for ERRα binding to its own promoter were described by Laganiere (28). Primers were designed using Genscript's real-time PCR primer design tool (https://www.genscript.com/ssl-bin/app/primer) to amplify a region within 300bp of each putative response element. List of primers are provided in Supplemental table 1.

Animal studies

Animal studies were conducted in accordance with IACUC standards according to protocol A-212-05-07 at Duke University. Male Wistar rats weighing between 225-250g were fed standard chow and housed in a controlled environment with 12hr light and dark cycles. Fasted rats had their food removed in the evening prior to the experiment and remained without food

for 14-16 hrs prior to sacrifice. Animals were sedated with Nembutal (~0.1cc/100g), serum was collected from the portal vein, and livers were harvested and flash frozen in liquid nitrogen.

Amino Acid profiling

HepG2 cells were plated at a density of 500,000 cells/well in 6-well dishes coated with 0.1% gelatin. After 24 hours, cells were dosed with DHEA or vehicle (ethanol) at the indicated concentration. DHEA media was replaced each day for 2-3 days. Cells were washed then lysed in 300ul water, sonicated, and debris was pelleted by centrifugation. Supernatant was transferred to fresh tubes and flash frozen in liquid nitrogen. Amino acids and acylcarnitines were analyzed using stable isotope dilution techniques. Measurements were made by flow injection tandem mass spectrometry using sample preparation methods described previously (53). The data were acquired using a Micromass Quattro MicroTM system equipped with a model 2777 autosampler, a model 1525 HPLC solvent delivery system and a data system controlled by MassLynx 4.1 operating system (Waters, Millford, MA).

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We would like to thank David Vance and Richard Auchus at UT Southwestern for their help and expertise with HPLC.

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Figure legends

Figure 1. PGC-1α and PGC-1α 2x9 induce gene expression of *CYP11A1* and *CYP17A1* in hepatic cells. Gene expression of *CYP11A1* and *CYP17A1* was measured by quantitative PCR in (A) HepG2 cells, (B) Hep3B cells, or (C) primary human hepatocytes infected with adenoviruses expressing β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M. Gene expression was normalized to 36B4 expression. Error bars represent SEM of three replicates, and each graph is representative of at least three independent experiments.

Figure 2. PGC-1α and PGC-1α 2x9 increase the functional activity of CYP11A1 and CYP17A1. A. HepG2 cells were infected with adenoviruses expressing β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M, then were incubated with 200nM [^{3}H]pregnenolone for 6 hrs. Steroids were extracted and separated by high performance liquid chromatography. Bar graph represents area under the curve. B-C. HepG2 cells were infected as above and incubated with 200nM pregnenolone (B) or 10uM 22(R)-OH cholesterol (C), and DHEA was measured using a radioimmunoassay. Error bars represent SEM of three replicates, and each RIA is representative of three independent experiments.

Figure 3. ERRα mediates PGC-1α induction of *CYP11A1*. A-B. HepG2 cells were transduced with lentiviruses expressing siRNA to ERRα (A) or HNF4α (B), followed by adenoviral delivery of β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M. Gene expression of *CYP11A1* was measured by qPCR, normalized to 36B4 expression, and relevant protein expression is shown by western blot. C. Schematic of the putative ERRE sites around the CYP11A1 gene tested by ChIP. Regions not bound by ERRα are indicated by boxes with black hash-marks. White box indicates the site identified by ChIP scanning in D. Numbering is relative to the start site. D. Chromatin immunoprecipitation of ERRα and amplification of putative ERREs by qPCR, where the ERRα promoter was used as a positive control.

Figure 4. *CYP17A1* is regulated by PGC-1α through ERRα. A. HepG2 cells were transduced with lentiviruses expressing siERRα, followed by adenoviral transduction of β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M. Gene expression of CYP17A1 was measured by qPCR, and normalized to 36B4 expression. B. Schematic of the putative ERRE sites found in and around the CYP17A1 gene region. Regions not bound by ERRα are indicated by boxes with black hash-marks. White boxes indicate the position of sites identified by ChIP scanning in (C). Numbering is relative to the start site. C. Chromatin immunoprecipitation of ERRα and amplification of putative ERREs by qPCR, where the ERRα promoter serves as a positive control.

Figure 5. *CYP17A1* is regulated by PGC-1α through HNF4α. A. HepG2 cells were transduced with lentiviruses expressing siHNF4α, followed by adenoviral transduction of β-gal, PGC-1α, PGC-1α 2x9, or PGC-1α L2L3M. Gene expression of *CYP17A1* was measured by qPCR and normalized to 36B4 expression. B. Schematic of the DR-1 sites found in and around the CYP17A1 gene region. Regions not bound by HNF4α are

indicated by boxes with black hash-marks. White box indicates the position of site identified by ChIP scanning shown in (C). C. Chromatin immunoprecipitation of HNF4α and amplification of regions by qPCR, where HNF4α binding to the CYP7A1 promoter served as a positive control. D. Activity of a reporter gene fused to 3.2kb promoter region upstream of CYP17A1 tested in combination with HNF4α fused to a VP16 activation domain. VP16-SF-1 is a positive control. Each DR1 site was mutated to examine binding activity.

Figure 6. Fasting induces *Cyp17A1* expression and function. A-B. Rats were fasted for 14-16 hours. RNA was collected for gene expression analysis by qPCR, gene expression was normalized to cyclophilin (A), and liver samples were collected for steroid extraction and analysis of DHEA concentration by RIA (B). Boxes represent the interquartile range (25-75th percentile), with median value in the center. Whiskers mark the 10th and 90th percentile, and dots represent measurments <10th percentile or >90th percentile. Statistical significance was calculated by a student's t-test.

Figure 7. DHEA reduces the concentration of free amino acids in HepG2 cells. HepG2 cells were treated with 1nM DHEA. Cell lysates were collected for analysis of free amino acid concentrations and normalized to total protein concentration. Error bars represent the standard error of 3 replicates, and graph is representative of 3 independent experiments. Asterisks indicate significant difference ($p<0.05$) by independent Student's t-test.

Figure 8. Model for fasting induced expression of steroidogenic enzymes and synthesis of hepatic steroids.

Figure 1

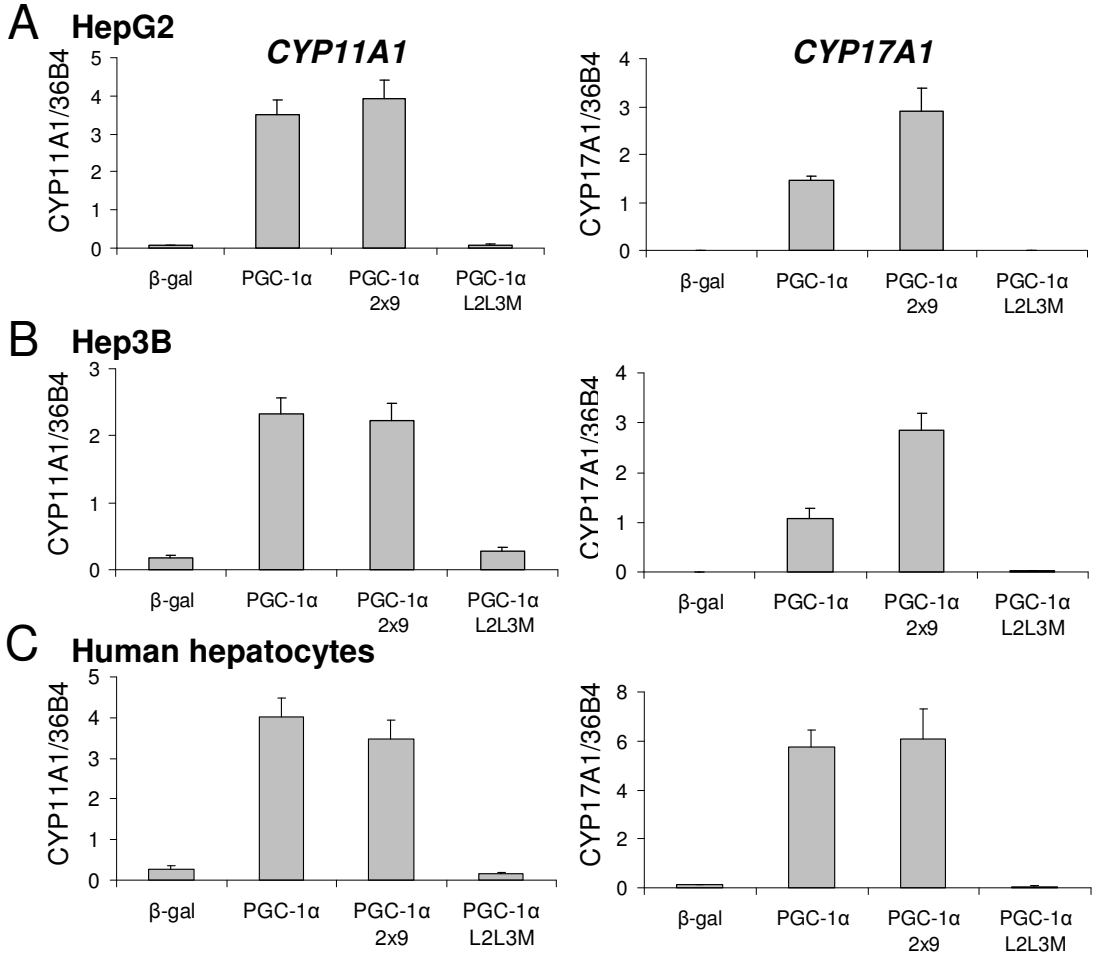
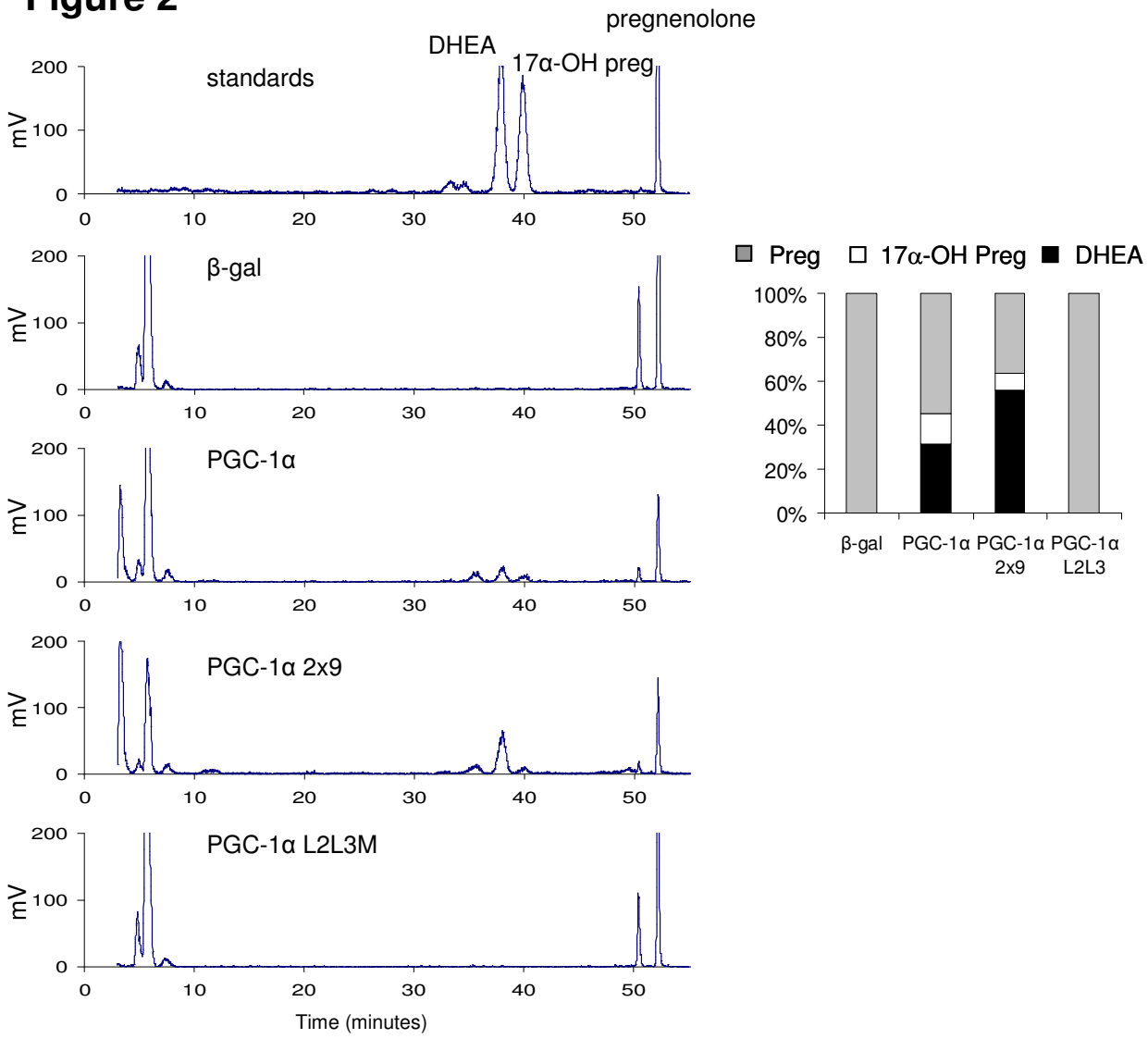

A HepG2
CYP11A1
CYP17A1
CYP11A1/36B4
CYP17A1/36B4
β-gal
PGC-1α
PGC-1α 2x9
PGC-1α L2L3M
B Hep3B
C Human hepatocytes

Figure 2


pregnenolone
DHEA
17α-OH preg
standards
β-gal
PGC-1α
PGC-1α 2x9
PGC-1α L2L3M
mV
Time (minutes)
Preg
17α-OH Preg
DHEA
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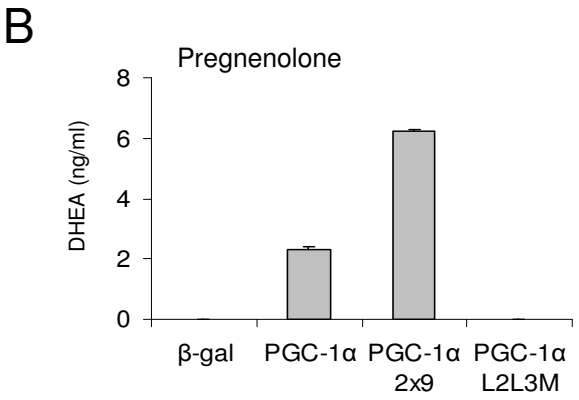
PGC-1α L2L3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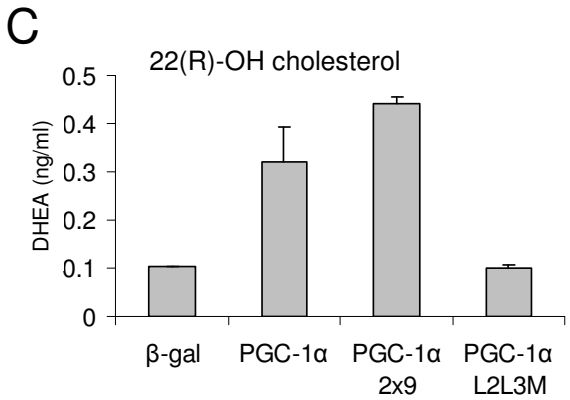
DHEA (ng/ml)
β-gal
PGC-1α
PGC-1α 2x9
PGC-1α L2L3M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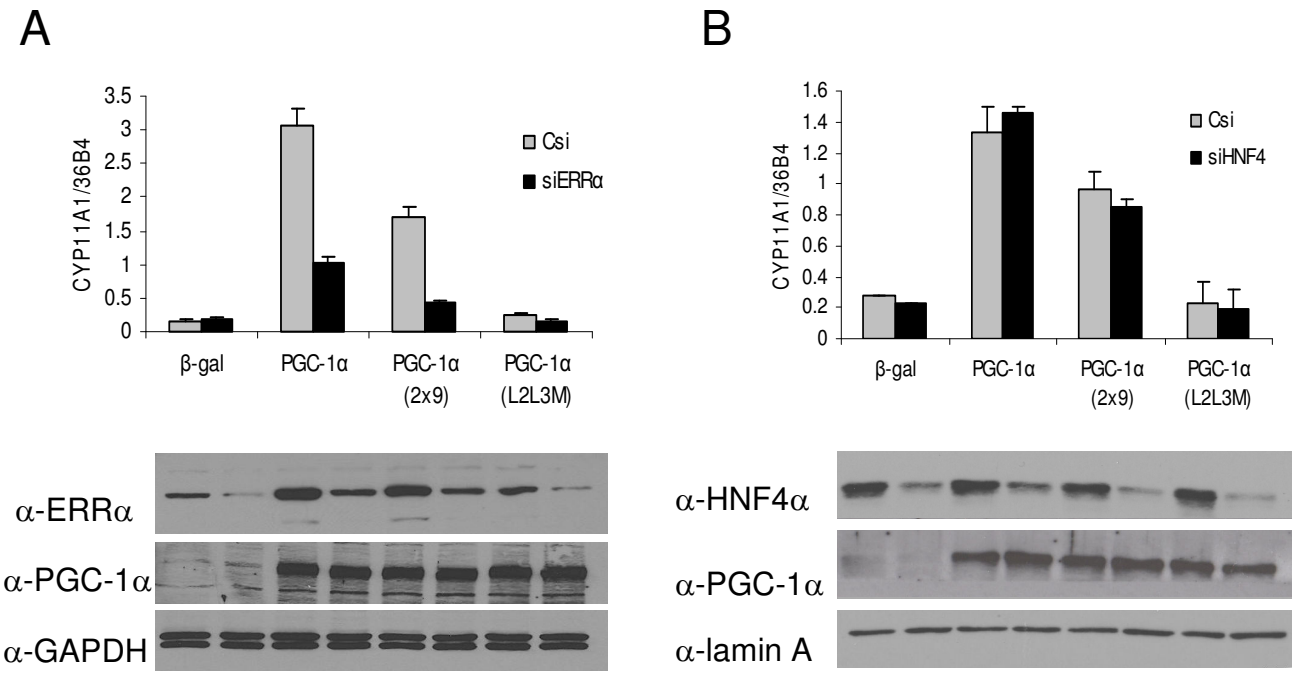
DHEA (ng/ml)
β-gal
PGC-1α
PGC-1α 2x9
PGC-1α L2L3M

Figure 3


A
B
CYP11A1/36B4
Csi
siERRα
siHNF4
β-gal
PGC-1α
PGC-1α (2x9)
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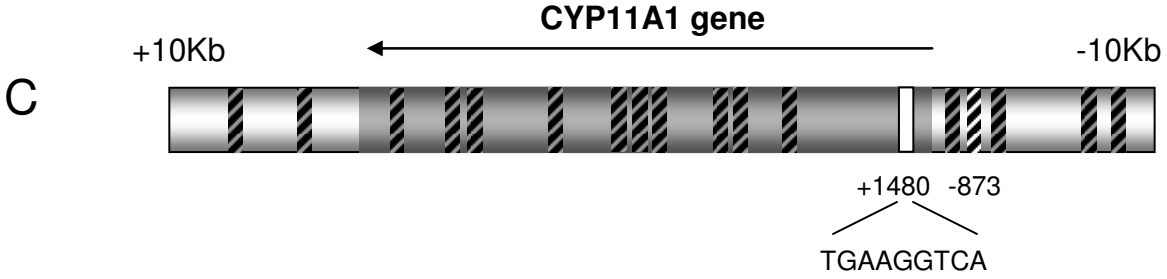
α-PGC-1α
α-GAPDH
α-HNF4α
α-lamin A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-10Kb
+1480
-873
TGAAGGTCA

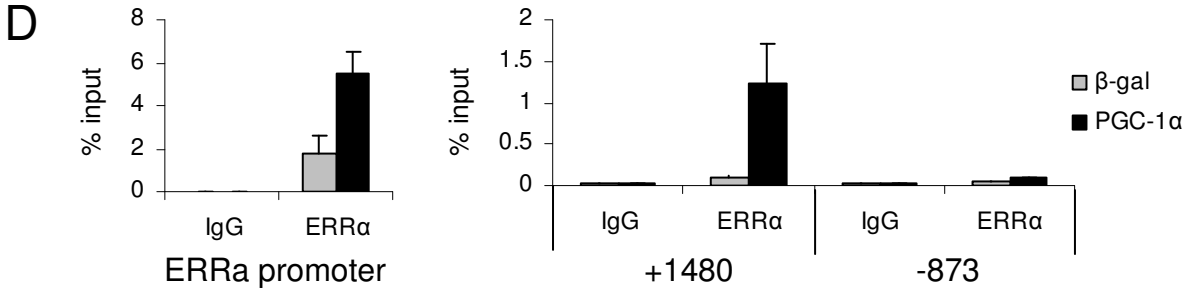

D
% input
IgG
ERRα
ERRa promoter
β-gal
PGC-1α
+1480
-873

Figure 4

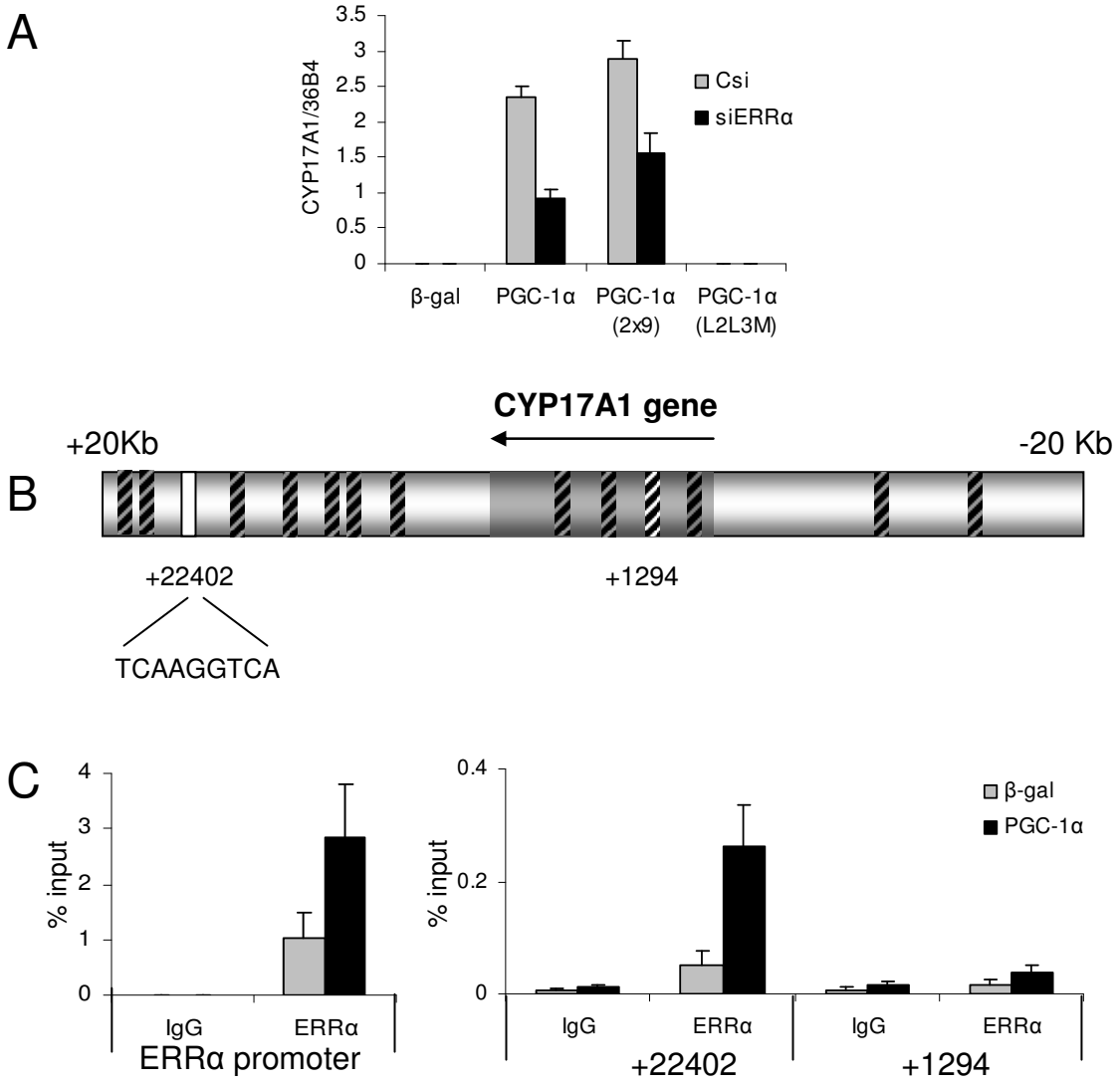
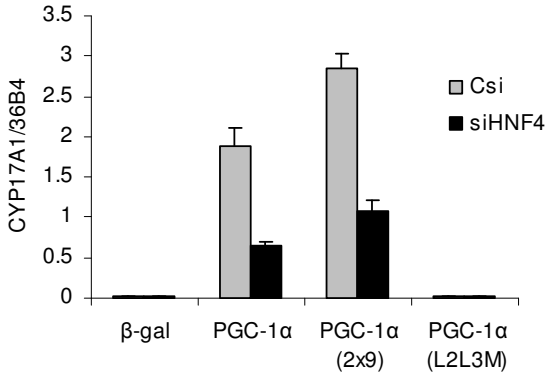
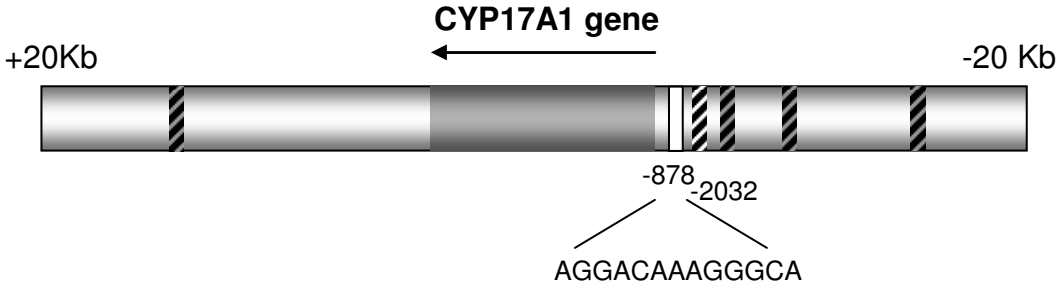

A
CYP17A1/36B4
3.5
3
2.5
2
1.5
1
0.5
0
Csi
siERRα
β-gal
PGC-1α
PGC-1α (2x9)
PGC-1α (L2L3M)
B
CYP17A1 gene
+20Kb
-20 Kb
+22402
+1294
TCAAGGTCA
C
% input
4
3
2
1
0
IgG
ERRα
ERRα promoter
0.4
0.2
0
β-gal
PGC-1α
+22402
+1294

Figure 5

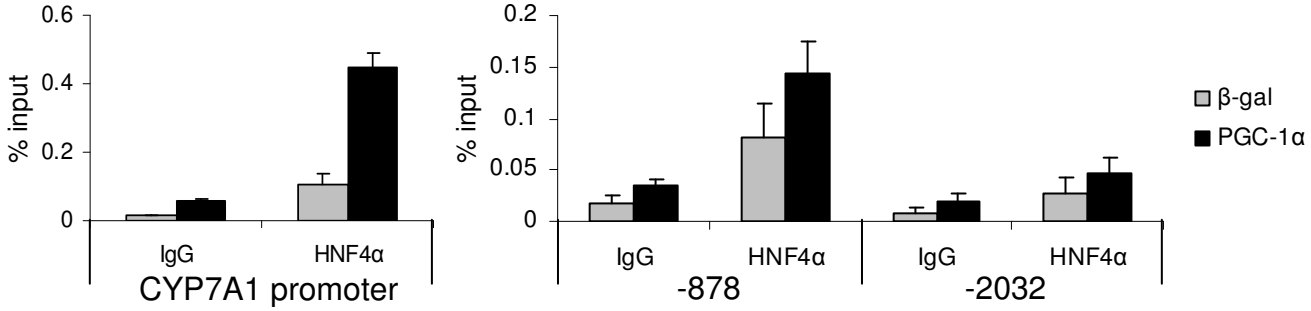
A



B



C



D

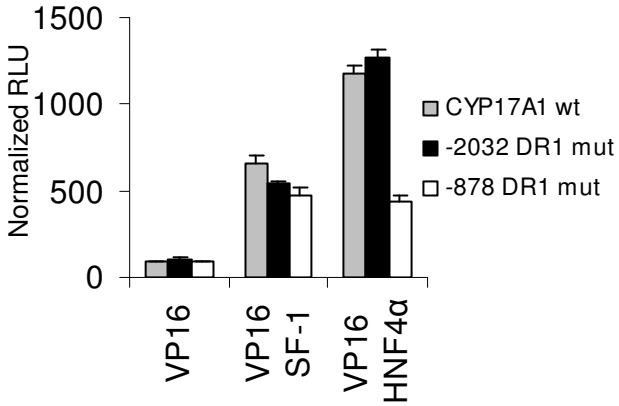
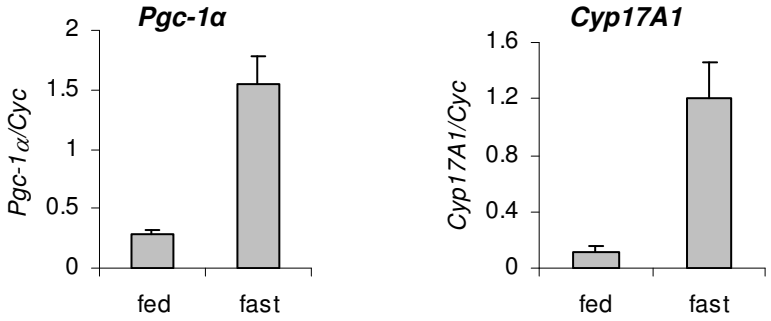


Figure 6

A


Pgc-1α
Pgc-1α/Cyc
2
1.5
1
0.5
0
fed
fast
Cyp17A1
Cyp17A1/Cyc
1.6
1.2
0.8
0.4
0
fed
fast

B

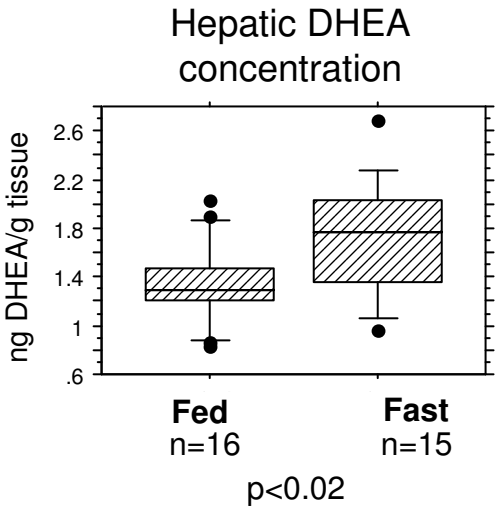

Hepatic DHEA concentration
ng DHEA/g tissue
2.6
2.2
1.8
1.4
1
.6
Fed
n=16
Fast
n=15
p<0.02

Figure 7

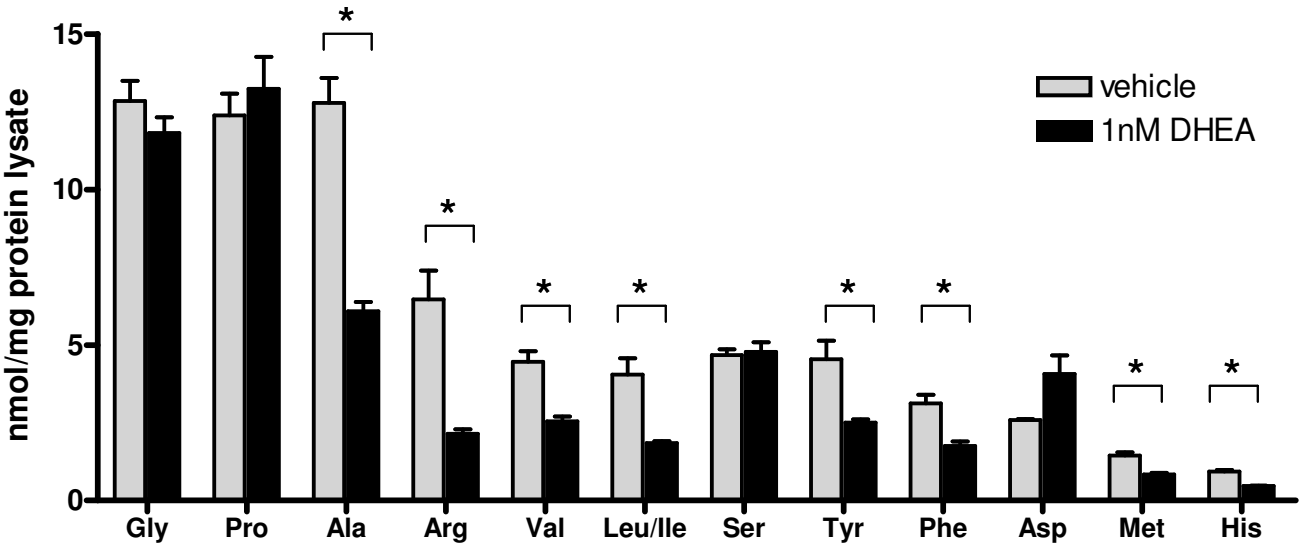
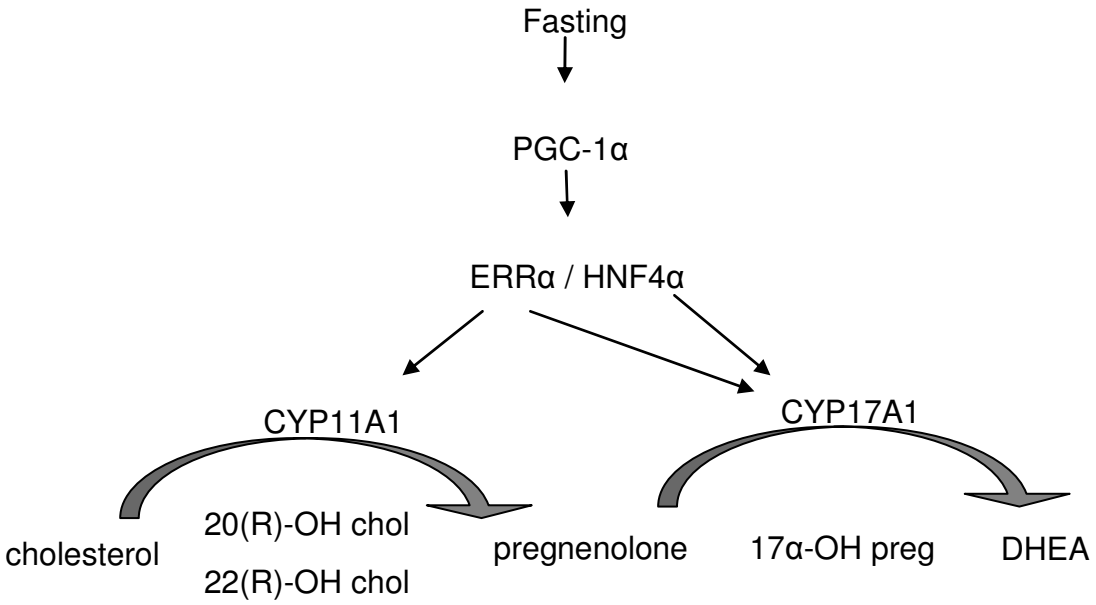

15
10
5
0
nmol/mg protein lysate
vehicle
1nM DHEA
*
*
*
*
*
*
*
*
Gly
Pro
Ala
Arg
Val
Leu/Ile
Ser
Tyr
Phe
Asp
Met
His

Figure 8


Fasting
PGC-1α
ERRα / HNF4α
CYP11A1
CYP17A1
cholesterol
20(R)-OH chol
22(R)-OH chol
pregnenolone
17α-OH preg
DHEA