RAPID COMMUNICATION

Liver receptor homologue-1 is expressed in human steroidogenic tissues and activates transcription of genes encoding steroidogenic enzymes

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Abstract

In the current study we test the hypothesis that liver receptor homologue-1 (LRH; designated NR5A2) is involved in the regulation of steroid hormone production. The potential role of LRH was assessed by first examining expression in human steroidogenic tissues and second by examining effects on transcription of genes encoding enzymes involved in steroidogenesis. LRH is closely related to steroidogenic factor 1 (SF1; designated NR5A1), which is expressed in most steroidogenic tissues and regulates expression of several steroid-metabolizing enzymes. LRH transcripts were expressed at high levels in the human ovary and testis. Adrenal and placenta expressed much lower levels of LRH than either ovary or liver. To examine the effects of LRH on steroidogenic capacity we used reporter constructs prepared with the 5′-flanking region of steroidogenic acute regulatory protein (*StAR*), cholesterol side-chain cleavage (*CYP11A1*), 3β hydroxysteroid dehydrogenase type II (*HSD3B2*), 17α hydroxylase, 17,20 lyase (*CYP17*), 11β hydroxylase (*CYP11B1*) and aldosterone synthase (*CYP11B2*). Co-transfection of these reporter constructs with LRH expression vector demonstrated that like SF1, LRH enhanced reporter activity driven by flanking DNA from *StAR*, *CYP11A1*, *CYP17*, *HSD3B2*, and *CYP11B1*. Reporter constructs driven by *CYP11A1* and *CYP17* were increased the most by co-transfection with LRH and SF1. Of the promoters examined only *HSD3B2* was more sensitive to LRH than SF1. The high level of ovarian and testicular LRH expression make it likely that LRH plays an important role in the regulation of gonadal function.

Journal of Endocrinology (2002) **174,** R13–R17

Introduction

Liver receptor homologue-1 (LRH; NR5A2) is an orphan nuclear hormone receptor that has recently been shown to play an important role in bile acid biosynthesis (Galarneau *et al.* 1996, Nitta *et al.* 1999, Repa & Mangelsdorf 1999). LRH is closely related to steroidogenic factor 1 (SF1; NR5A1) and both proteins recognize the same canonical DNA motif. The degree of similarity leaves open the possibility that these nuclear receptors might share certain target genes. Recently, we demonstrated low levels of LRH transcript expression in the human adrenal gland (Wang *et al.* 2001). The expression of LRH has also been shown in the rodent and horse ovary suggesting that this transcription factor may play a role not only in bile acid production but also steroidogenesis (Repa & Mangelsdorf 1999, Boerboom *et al.* 2000). A recent report also suggests that LRH regulates aromatase gene transcription (Clyne *et al.* 2002). Herein, we demonstrate that human steroidogenic tissues, particularly the ovary, express LRH and that this orphan nuclear receptor is able to activate transcription of genes encoding the enzymes involved in steroid hormone biosynthesis. These data strongly support the hypothesis that LRH plays a critical role in the regulation of both bile acid and steroid hormone biosynthesis.

Journal of Endocrinology (2002) **174,** R13–R17 Accepted 25 July 2002
0022–0795/02/0174–R13 *Printed in Great Britain*

Online version via http://www.endocrinology.org

Materials and Methods

Preparation of reporter constructs and expression vectors

The 5′ flanking DNA from the human genes for *StAR* (-1300) (Sugawara *et al.* 1996), *CYP11A1* (-4400 bp) (Chou *et al.* 1996), *CYP17* (-1124 bp) (Hanley *et al.* 2001), *HSD3B2* (-963 bp) (Leers-Sucheta *et al.* 1997), *CYP11B1* (-1102 bp) (Wang *et al.* 2001) and *CYP11B2* (-1521 bp) (Clyne *et al.* 1997) were inserted upstream of the firefly luciferase gene in the reporter vector pGL3-Basic (Promega, Madison, WI). For all transfections, empty pGL3-Basic was used as the control vector to measure basal activity. The coding region of mouse LRH (provided by Dr. David Mangelsdorf, UT Southwestern) and human SF1 (Ramayya *et al.* 1996) were inserted into pcDNA3·1 zeo (Invitrogen, Carlsbad, CA) eukaryotic expression vector.

Cell culture and transfection assay

HEK-293 cells were cultured in Dulbecco's modified Eagle's/Ham F12 (DME/F12) medium (GIBCO BRL) supplemented with 5% NU Serum (Collaborative Biom, Bedford, MA) and antibiotics. For transfection experiments Fugene 6 (Roche) was used to transfect 1 μg of reporter plasmid and the indicated amounts of expression vectors. pcDNA3·1 zeo empty vector was used to assure constant amounts of DNA per well for each transfection. To normalize luciferase activity cells were co-transfected with 50 ng/well of Renilla plasmid (Promega). Cells were assayed for reporter activity using the Dual Luciferase assay system (Promega) 18–24 h after transfection.

RNA extraction, cDNA synthesis and real-time RT-PCR

Normal adrenals, testis and livers were obtained through the Cooperative Human Tissue Network (Philadelphia, PA, USA). Human ovaries were obtained at the time of hysterectomy and were determined to be normal. The use of these tissues was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas, Texas. Total RNA was extracted from tissues as previously described (Freije *et al.* 1997) and DNase treated. Purity and integrity of RNA was checked spectroscopically and by gel electrophoresis prior to use. Four μg of total RNA was reverse transcribed in a final volume of 100 μl using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) and stored at –20 C.

Primers for the amplification were based on published sequences for the human LRH and SF1. The primers sequences used were: LRH (GenBank accession number: NM_003822) Forward: 5′-TACCGACAAGTGGTACA TGGAA-3′ (exon 6), Reverse: 5′-CGGCTTGTGATGC TATTAT GGA-3′ (exon 7) that gave an 89 bp fragment; SF1 (NM_004959) Forward: 5′-GGAGTTTGTCTGC CTCAAGTTCA-3′ (exon 6), Reverse: 5′-CGTCTTT CA CCAGGATGTGGTT-3′ (exon 7) that gave an 80 bp fragment. PCR reactions were performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a total volume of 30 μL reaction mixture following the manufactures recommendations using the SYBR Green Universal PCR Master Mix 2X (Applied Biosystems) and 0·1 μM of each primer using the dissociation protocol. Standard curves were prepared using the human SF1 and LRH expression vectors. Negative controls contained water instead of first-strand cDNA. Each sample was normalized on the basis of its 18S ribosomal RNA content.

The 18S quantification was performed using a TaqMan Ribosomal RNA Reagent kit (Applied Biosystems) and using the method protocol provided in the TaqMan Ribosomal RNA Control Reagent kit (Applied Biosystems).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using STATPAC software (Minneapolis, MN, USA).

Results and Discussion

Human steroidogenic tissues express LRH mRNA

The first objective was to determine if human steroidogenic tissues expressed LRH and compare the level of expression to that seen for SF1. To accomplish this goal RNA from human adrenal, placenta, testis, and ovary were used to quantify transcript levels using real-time RTPCR (Fig.1). LRH transcripts were in high abundance in the ovary (0·07 attmol/μg18S), and testis (0·02 attmol/μg18S), but relatively low in adrenal and placenta. Expression of LRH was highest in the liver (0·5 attmol/μg18S), where LRH has been shown to play a role in the regulation of bile acid biosynthesis (Repa & Mangelsdorf 1999). The relatively high expression of LRH in the human ovary extends previous reports demonstrating ovarian expression of LRH in mouse and equine ovaries by northern analysis (Repa & Mangelsdorf 1999, Boerboom *et al.* 2000). It was shown in the equine ovary that LRH expression was highest in the corpus luteum. As we are using whole ovarian cortex composed mainly of follicles and stroma, it is likely that levels of LRH will be even higher in corpus luteum. As compared with previous reports using northern analysis, the use of quantitative real time RTPCR allowed for the comparison of transcript expression levels of SF1 vs. LRH. SF1 expression was high in adrenal (4·8 attmol/μg18S), testis (0·56 attmol/μg18S), and ovary (0·54 attmol/μg18S).

However, as shown previously the level of SF1 mRNA in the placenta is extremely low suggesting that steroido-

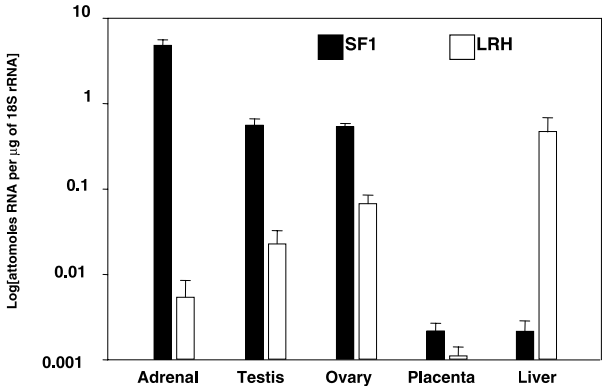

Figure 1 Quantification of liver receptor homologue-1 (LRH) and steroidogenic factor 1 (SF1) transcript levels in human steroidogenic tissues. Real-time RT-PCR was used to quantify the level of LRH and SF1 mRNA in adrenal, testis, ovary, placenta and liver as described in Materials and Methods. Data represent the mean ± S.E.M. of at least three independent RNA samples and are expressed as attomoles of mRNA per µg of 18S ribosomal RNA. Note that the data are presented with a log scale. The quantity of LRH and SF1 mRNA were significantly different within each tissue ($P \leq 0{\cdot}001$).

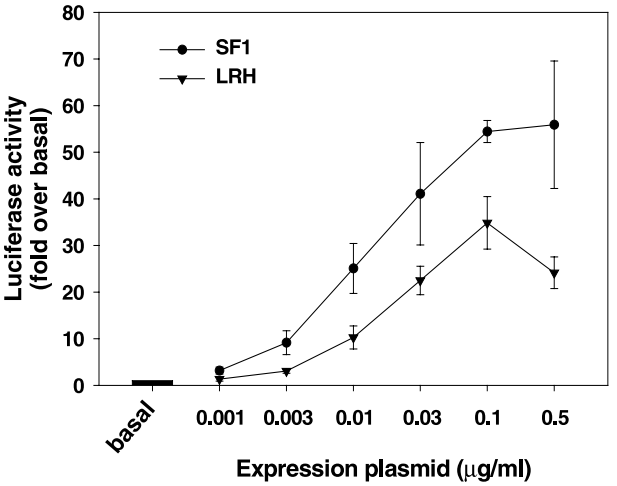

Figure 2 Concentration-dependent effects of LRH and SF1 on *CYP11A1* reporter gene activity. HEK-293 cells were transfected with luciferase reporter constructs containing the *CYP11A1* promoter construct (1 µg/well). Cells were co-transfected with empty pcDNA3·1 zeo expression vector or the indicated amounts of LRH or SF1 expression plasmid. Following recovery for 24 h, cells were lysed and assayed for luciferase activity. Data were normalized to co-expressed renilla luciferase and expressed as percentage of the basal. Results represent the mean ± S.D. of pooled data from 3 to 4 experiments. All the concentrations of SF1 except 0·001 were significantly different from vector alone ($P \leq 0{\cdot}001$). Concentrations of LRH greater than 0·003 µg/ml were significantly different from vector alone ($P \leq 0{\cdot}001$).

genic enzymes in the placenta do not require SF1 or LRH for expression (Ramaya *et al.* 1997). As can be seen in Fig. 1, adrenal expression of SF1 was greater than 900-fold that seen for LRH suggesting that in the adrenal SF1 would have a much greater impact. This explains the inability to detect LRH in adrenal RNA by northern analysis and the requirement to use RT-PCR to detect transcripts (Repa & Mangelsdorf 1999, Wang *et al.* 2001). Liver transcript levels of LRH were 200-fold higher than seen for SF1 confirming that SF1 does not play a key role in the liver.

One limitation that remains with real-time RT-PCR is the inability to take into consideration the dilution of steroidogenic cells by non-steroidogenic cells within a complex tissue. When one considers that a much smaller portion of the cells in the ovarian cortex and testis are steroidogenic, it is likely that the actual amount of SF1 and LRH in the steroid producing cells of these glands is likely to be significantly higher. With this in mind, the intra-glandular localization of LRH and the comparison to SF1 is still an important aspect that needs to be examined.

LRH enhances transcription of the genes involved in steroid hormone biosynthesis

To better determine the role of LRH in the regulation of steroidogenesis, we examined its effects on the transcription of the genes encoding the enzymes involved in steroid hormone production. For these experiments, expression vectors were prepared with the coding region of LRH and SF1. To minimize competition with inherent expression of these transcription factors, HEK-293 cells, which expressed low levels of both LRH and SF1 (Data not shown), were used for transfection studies.

The rate-limiting reaction in steroidogenesis is the conversion of cholesterol to pregnenolone and this reaction is carried-out by the enzyme CYP11A1. *CYP11A1* transcription is regulated in part by SF1 (Monte *et al.* 1998). Co-transfection of HEK-293 cells demonstrated that both LRH and SF1 were able to increase luciferase reporter driven by the *CYP11A1* promoter in a concentration-dependent manner (Fig. 2). Maximal stimulation of reporter activity was observed using 0·1 µg/well of either vector. Maximal induction of the *CYP11A1* reporter construct, however was higher using SF1 (54-fold) compared with that observed with LRH (34-fold). StAR protein controls the movement of cholesterol to the CYP11A1 enzyme and the acute regulation of StAR protein synthesis represents the rate-limiting step in steroid hormone biosynthesis (Stocco 2001). As has been previously shown (Boerboom *et al.* 2000, Reinhart *et al.* 1999), SF1 co-expression significantly increased reporter activity of promoter constructs prepared with the *StAR* promoter (23-fold) (Fig. 3). LRH also increased *StAR* reporter construct activity but only by 7-fold. These data suggests that, like SF1, LRH can enhance *StAR* and

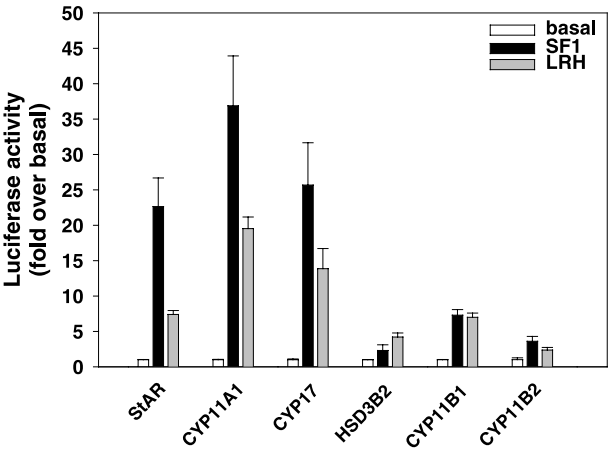

Figure 3 Effects of LRH and SF1 on transcription of various steroidogenic enzymes. HEK-293 cells were transfected with the indicated luciferase promoter constructs (1 μg/well). Cells were co-transfected with either empty vector or 0·5 μg/well of SF1 or LRH expression plasmid. 24 h after transfection cells were lysed and assayed for luciferase. Data were normalized to co-expressed renilla luciferase and expressed as percentage of the basal reporter activity. Results represent the mean ± S.D. of data from at least 3 experiments. SF1 enhanced transcription was significantly larger than LRH for *StAR, CYP11A1* and *CYP17* ($P \leq 0{\cdot}001$). LRH enhanced transcription was significantly larger than SF1 for *HSD3B2* ($P \leq 0{\cdot}001$).

CYP11A1 gene transcription and thus the rate-limiting step and reaction involved in steroidogenesis.

Pregnenolone produced by mitochondrial CYP11A1 can be metabolized by either CYP17 to 17α hydroxypregnenolone or by HSD3B2 to progesterone. Transcription of both *CYP17* and *HSD3B2* is enhanced by SF1 (Hanley *et al.* 2001, Leers-Sucheta *et al.* 1997). Co-transfection of HEK-293 cells with LRH stimulated *CYP17* reporter activity by 14-fold while SF1 increased reporter activity by 26-fold. LRH also significantly increased reporter activity of the *HSD3B2* construct. Interestingly, the activity of the *HSD3B2* reporter construct was increased more by LRH (4-fold) than was seen for SF1 (2-fold). Mutation of the SF1/LRH binding cis-elements in the *HSD3B2* promoter blocked the stimulation of both factors (Data not shown).

CYP11B1 and CYP11B2 carry out the final reactions in the synthesis of cortisol and aldosterone, respectively. We have previously shown using steroidogenic cells that *CYP11B1* transcription is enhanced by SF1, but that transcription of *CYP11B2* is not (Bassett *et al.* 2002). LRH and SF1 expression vectors increased *CYP11B1* reporter construct activity to a similar degree (7-fold) (Fig. 3). Interestingly LRH and SF1 caused a small but significant increase in *CYP11B2* driven reporter activity (Fig. 3), which was not seen when transfections were done in steroidogenic cells (Bassett *et al.* 2002). While LRH appears to enhance expression of the *CYP11B* genes, the low expression of LRH in the adrenal questions a physiologic role in the adrenal physiology.

In summary, LRH is able to regulate transcription of the genes encoding the enzymes involved in steroid hormone biosynthesis. This coupled with high expression in the human ovary and testis suggests that LRH may play an important role in the expression of the genes required for the production of steroid hormones.

Acknowledgments

Support for this study was provided by grants from the National Institutes of Health grants HD11149 and DK43140 to WER and HD17481 and HD17481 to DMS as well as funds from the Robert A Welch Foundation to DMS. An Italian CNR Short Term Mobility Grant supported V Pezzi.

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