Involvement of Sp1 and SREBP-1a in transcriptional activation of the LDL receptor gene by insulin and LH in cultured porcine granulosa-luteal cells

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Sekar, Natesampillai, and Johannes D. Veldhuis. Involvement of Sp1 and SREBP-1a in transcriptional activation of the LDL receptor gene by insulin and LH in cultured porcine granulosa-luteal cells. Am J Physiol Endocrinol Metab 287: E128–E135, 2004—Luteinizing hormone (LH) and insulin stimulate transcriptional activity of the porcine low-density lipoprotein (LDL) receptor (LDLR) promoter supraadditively in primary cultures of granulosa-luteal cells. The mechanistic basis of this bihormonal interaction is unknown. The pig LDLR gene promoter includes three putative Sp1/Sp3-binding sites and one sterol response element (SRE) site 5’ upstream to the transcriptional start site. To assess the role of SRE-binding protein (SREBP) in LDLR gene regulation, swine granulosa-luteal cells were cotransfected with CMV/SREBP-1a or SREBP-2 and the pLDLR1076 luc promoter. SREBP-1a and SREBP-2 stimulated LDLR gene transcription eight- and fourfold, respectively. LH alone augmented transcription by SREBP-1 twofold. Conversely, cotransfection of a dominant-negative mutant form of SREBP-1a repressed basal and hormonally stimulated LDLR promoter activity by >80% (P < 0.01). Mutation of the SRE −167 ATCACCCTCATG −157 to −167 ATCACCACATG −157 bp decreased basal expression by 50% and LH + insulin + LH + IGF-I-stimulated transcriptional activity by 80% and >90%, respectively (both P < 0.01). Mutations within each of the three flanking putative Sp1/Sp3 sites at −216 to −211, −201 to −196, and −151 to −146 bp in the LDLR gene promoter also reduced basal activity (by >85%) and hormonal responsiveness (>95%, P < 0.05). EMSA confirmed that presumptive SRE-1 and Sp1/Sp3 elements bind respective peptides. Muthuramn, an inhibitor of Sp1/Sp3 protein(s) binding, blocked hormonally induced LDLR promoter expression by 80%. Basal transcription and supra-additive stimulation of porcine LDLR gene transcription by LH and insulin in granulosa-luteal cells require SREBP-1a and Sp1/Sp3-binding elements.

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FOLLICLE-STIMULATING HORMONE (FSH) and luteinizing hormone (LH) stimulate Graafian follicle development and attendant steroidogenesis (38). Together, insulin and peptide mimetics determine gonadotropin action. For example, in the human, insulin-dependent diabetes mellitus is marked by an increased risk of primary amenorrhea, delayed menarche, anovulation, reduced pregnancy rates, and earlier menopause (36). In swine, streptozotocin-induced diabetes and insulinopenia imposed by feed restriction suppress follicular steroid production (2, 14, 32); in mice, transgenic inactivation of the insulin receptor and insulin receptor substrate-2 genes impairs ovarian follicle development (8, 9). Clinical states of insulin resistance increase the risk of subfertility and hyperandrogenism (28, 35). Under in vitro conditions, insulin and insulin-like growth factor I (IGF-I) enhance FSH-induced aromatase activity and LH-stimulated progesterone biosynthesis by human, rodent, and porcine granulosa and theca cells (11, 15, 19, 24, 42, 54).

Steroidogenesis requires coordinated regulation of the cellular uptake, transport, and utilization of cholesterol in committed enzymatic steps (33). Blood-borne low-density lipoprotein (LDL) cholesterol taken up by the cognate membrane receptor is the primary source of intracellular sterol substrate in the human, pig, monkey, and cow (10, 25, 29, 48, 53). In these species, LDL receptor (LDLR) expression increases in preovulatory granulosa-luteal cells and remains elevated in the corpus luteum (10, 18, 53). In vitro, FSH, LH, insulin, and IGF-I drive LDLR gene and protein expression and concomitant progesterone production (22, 25, 30, 42, 51, 52).

The LDLR gene is controlled by transcriptional signals, which include repression by intracellular cholesterol (26). In the human gene, key regulatory elements comprise (nonexclusively) three imperfect direct repeats of 16 bp localized within 100 bp upstream of the transcriptional start site (47). Repeat 1 (R1) and repeat 3 (R3) contain Sp1-binding sites that support basal transcriptional activity. Repeat 2 (R2) confers negative-feedback responsiveness via a sterol response element (SRE), a 10-bp sequence 5’ ATCACCACATG −3’ that associates with an SRE-binding protein (SREBP) in the basic helix-loop-helix leucine zipper family (7). SREBPs are synthesized in the endoplasmic reticulum and nuclear envelope and are released to the nucleus by sterol-sensitive proteolysis (39). Among the three major isoforms, SREBP-1a is a potent transcriptional activator of the LDLR gene and certain genes that encode enzymes in the biosynthesis of cholesterol and unsaturated fatty acids, SREBP-1c selectively stimulates fatty acid synthase, and SREBP-2 drives cholesterol biosynthetic genes (3). Recent studies show that interaction of SREBP with SRE in the human promoter enhances Sp1 binding to R3 and promotes transcription synergistically (5).

In the swine, LH and insulin induce LDLR gene transcription via partially independent (additive or supra-additive) mechanisms (44). The present studies examine the transcriptional roles of SRE and three putative Sp1 cis-acting 5’ upstream sequences in the porcine gene in mediating the facilitative interaction between LH and insulin on LDLR promoter activity in primary ovarian culture.

MATERIALS AND METHODS

Reagents. Ovine LH (NIH-oLH-26; potency 2.3× NIH-oLH-S1) and FSH (NIH-oFSH-19; potency 94× NIH-oFSH-S1) were

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obtained from the National Hormone and Pituitary Program (National Institutes of Health, Bethesda, MD); porcine insulin, human recombinant IGF-I, forskolin, 8-bromo-cAMP, rapamycin, and mithramycin from Sigma Chemical (St. Louis, MO); Eagle’s MEM, penicillin-streptomycin, gentamicin, fetal bovine serum, trypsin-EDTA, and Lipofectamine reagent from Life Technologies (Grand Island, NY); [γ-32P]ATP from NEN, Perkin-Elmer Life Sciences, (Boston, MA); anti-Sp1 (human) from Upstate Biotechnology (Lake Placid, NY); anti-Sp3 from Santa Cruz Biotechnology (Santa Cruz, CA); and the dual-luciferase reporter assay system, wherein pRL-TK contains the cDNA encoding Renilla reniformis (sea pansy), from Promega (Madison, WI). Oligonucleotides were synthesized by OperON (Operon Technologies, Alameda, CA).

Granulosa cell culture. Ovaries from prepubertal (60-70 kg) gilts were collected at an abattoir and transported in iced saline. Granulosa cells were isolated from small- and medium-sized (1- to 5-mm) antral follicles by fine-needle aspiration under sterile conditions and washed three times by low-speed centrifugation (3,000 rpm) in Eagle’s MEM. Approximately 5 × 10^6 viable granulosa cells were plated in 12-well culture dishes (Corning) containing bicarbonate-buffered MEM, 3% fetal bovine serum + insulin (1 μg/ml), estradiol (0.5 μg/ml), and FSH (5 ng/ml) to permit cell anchorage and partial lutein-like maturation (42–44 h). Cells were allowed to attach to culture dishes for 48 h at 37°C in 5% CO2.

Transient transfection of porcine granulosa-luteal cells. In transient transfection analyses, we utilized a 1,076-bp 5'-upstream regulatory fragment (−1,076 to +11 bp) of the porcine LDLR and 5'-nested deletion constructs driving a cytologically targeted firefly luciferase cDNA. After granulosa cells were allowed to attach for 48 h (see above), the medium was sequentially replaced after an additional 24 and 48 h. Cells were incubated in serum-free MEM without antibiotics for 20–30 min before transfection. Transfection medium (1 ml/well) consisted of serum-free MEM without antibiotics with 2 μg of total plasmid DNA (1.9 μg of pLDLR/luc and 0.1 μg of pRL-TK/luc) and 12 μl of Lipofectamine. On the basis of preliminary time-course experiments, the medium was replaced after 6 h with serum-free MEM containing antibiotics and the indicated hormone(s), inhibitors, or vehicle. For granulosa-luteal cells, expression was allowed to proceed over 4 h, which was optimal for definition of transcriptional effects of LH/cAMP and/or insulin/IGF-I. Where indicated, cells were pretreated for 30 min with 100 nM rapamycin or for 60 min with 100 nM mithramycin before exposure to LH/forskolin and/or insulin/IGF-I at the indicated concentrations in serum-free MEM. To quantitate responses, cells were rinsed once at room temperature with Dulbecco’s PBS, lysed in 100 μl of 1X lysis buffer (dual-luciferase assay system), and stored at −70°C until later assay. Transfection efficiency was monitored by cotransfection of pRL-TK/luc, a vector expressing Renilla luciferase. Data are expressed as the ratio of firefly to Renilla luciferase activity. Luciferase activity was measured using 100 μl each of firefly and Renilla luciferin substrate (Promega) per 20 μl of cellular lysate in a luminometer (model TD-20e, Turner Designs, Sunnyvale, CA).

Transfections of mutant LDLR sequences were performed with equimolar concentrations of DNA. A promoterless luciferase construct, p0luc, exhibiting no significant activity in response to any intervention was used to adjust total DNA to 2 μg. In cotransfection studies, 1.8 μg of full-length LDLR promoter (pLDLR1076/luc) were added with 0.03 μg of pCMV/SREBP-1 or 0.03 μg of pCMV/SREBP-2 and pCMV empty vector (obtained from Dr. Timothy F. Osborne, Dept. of Molecular Biology and Biochemistry, University of California, Irvine, CA). In separate studies, full-length LDLR promoter (pLDLR1076/luc) was added with expression vectors encoding dominant-negative mutant of pCMV/SREBP-1a-del (34) (0.03 μg) or pCMV/SREBP-2-del (41) (0.03 μg) or pCMV empty vector and pRL-TK/luc.

Site-directed mutagenesis within full-length pLDLR1076/luc was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sequences of the mutagenic primers (sense) for putative Sp1- or SRE-binding sites are shown in Table 1. The methylated, parental DNA templates were digested with DpnI at 37°C for 2 h. Specific mutations were confirmed by DNA sequence analysis.

Nuclear protein isolation and EMSA. The general procedure outlined by Dignam et al. (12) was followed with selected modifications as follows. Granulosa-luteal cells were washed twice with cold PBS and detached by scraping, recovered by centrifugation at 500 g for 5 min at 4°C, resuspended in ice-cold PBS, pH 7.4, and pelleted at 12,000 g for 20 s at 4°C. Cells were lysed in ice-cold buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 10 μl of freshly added protease inhibitor mixture (Sigma)] and mixed by pipetting. After incubation on ice for 10 min, Nonidet P-40 was added to a final detergent concentration of 0.05%, and the solution was mixed by pipetting before centrifugation at 12,000 g for 20 s. The nuclear pellet was suspended in 100 μl of ice-cold buffer B [20 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 0.4 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 25% glycerol, and 5 μl of

Table 1. Sense oligodeoxynucleotide strands used as templates to introduce mutations in 5'-upstream region of porcine LDLR gene

| pLDLRΔSRE-1 | 178-5-GATATTTGAAA TCACCCCGATG GAATCTTCTC CC-3-145 (wild-type) |
| pLDLRΔSp1-R3 | 178-5-GATATTTGAAA TCACCCCGATG GAATCTTCTC CC-3-145 (mutant) |
| pLDLRΔSp1-R5 | 165-5-GACCGCATCG AAACGTCTCG TCTACGAGA AACG-3-132 (wild-type) |
| pLDLRΔSp1-R5 | 165-5-GACCGCATCG AAACGTCTCG TCTACGAGA AACG-3-132 (mutant) |
| pLDLRΔSp1-R3 | 222-5-GGAATTCCT CCTCGGAGAA TCTCTTCTG TCTAGATGAGG-3-183 (wild-type) |
| pLDLRΔSp1-R5 | 222-5-GGAATTCCT CCTCGGAGAA TCTCTTCTG TCTAGATGAGG-3-183 (mutant) |
| pLDLRΔSp1-R1 | 222-5-GGAATTCCT CCTCGGAGAA TCTCTTCTG TCTAGATGAGG-3-183 (mutant) |
| pLDLRΔSp1-R5 | 233-5-GATACGGCGGT TGCAAACTCC TCTCGGAGAA AACTCTCC-3-196 (wild-type) |
| pLDLRΔSp1-R5 | 233-5-GATACGGCGGT TGCAAACTCC TCTCGGAGAA AACTCTCC-3-196 (mutant) |
| pLDLRΔSp1-R3 | 292-5-GGCCTTGGGA AAAGACTTGA ATCCCGTGCA GGCTCTCAAG-3-253 (wild-type) |
| pLDLRΔSp1-R5 | 292-5-GGCCTTGGGA AAAGACTTGA ATCCCGTGCA GGCTCTCAAG-3-253 (mutant) |

Uppercase letters represent wild-type and lowercase letters denote substituted base pairs. LDLR, LDL receptor; R1, R1a, and R3, repeats 1, 1a, and 3.
protease inhibitor mixture). The suspension was placed on ice for 30 min with occasional gentle shaking and then centrifuged at 12,000 g for 15 min at 4°C to obtain the nuclear extract (supernatant). The latter was stored at −70°C. Protein concentrations were measured by the Bradford method (Bio-Rad, Hercules, CA).

EMSA reactions were performed in 20 µl of 10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 50 µg/ml poly(dI-dC), 32P-labeled oligonucleotide probe (Table 2; 20,000 cpm), and 10 µg of nuclear protein for 30 min at room temperature. Antibodies or cold oligonucleotides were preincubated with nuclear proteins at 4°C for 1 h and 30 min, respectively. To block Sp1 binding, oligonucleotides were preincubated for 1 h at 4°C with 20 or 200 nM mithramycin (4). Protein-DNA complexes were separated from free probe by 5% non-denaturing polyacrylamide gel electrophoresis at 200 V for 2.5 h. The gels were subjected to autoradiography overnight at −80°C.

Statistical methods. Values are means ± SE of ≥3 independent experiments, in which separate batches of 200–300 ovaries were used on each occasion to confirm the reproducibility of results. Data were subjected to two-way ANOVA. Means were contrasted by the post hoc Tukey multiple comparison test. P < 0.05 was considered significant.

**RESULTS**

Functional identification of LH-, insulin-, and IGF-I-responsive sequences. Deletional analysis of pLDL1076/luc provisionally localized a region conferring supra-additive stimulation by LH and insulin/IGF-I to the proximal −255 bp (30, 44). Comparisons of the latter sequence among the mouse (Z19520), rat (M94388), hamster (M94387), human (L29401), and pig (AF022657) revealed maximal DNA sequence homology in a canonical SRE (−167 ATCACCCCCATG −157 bp) located in R2 (Fig. 1). A single upstream region consistent with R1 (−216 TCCTCC −211 bp) and a downstream R3 sequence (−151 TCCTCC −146 bp) exhibited potential Sp1/Sp3-binding sites. In addition, the porcine LDLR gene uniquely contains Sp1/Sp3-like-binding sites (−201 TCCTCC −196 bp) located between SRE (−167 to −157 bp) and R1, Sp1. The super-numerary putative Sp1/Sp3-binding site in the porcine LDLR promoter is denoted here as repeat 1a (R1a).

Supra-additive activation of porcine LDLR promoter by LH and insulin in granulosa-luteal cells. In this study and our previous study, time course analyses in granulosa-luteal cells established that 4 h of incubation allowed optimal stimulation of transcriptional activity of the full-length LDLR promoter sequence (−1,076 to +11 bp relative to the transcriptional start site) by LH (P < 0.01) and supra-additive interactions between LH and insulin or IGF-I (P < 0.01 vs. LH alone; Fig. 2). The effect of insulin or IGF-I alone required 24 h and was 1.7-fold basal (P < 0.01; see Figs. 6 and 7). Further studies were conducted at 4 h for LH and/or insulin/IGF-I stimulation and at 24 h to monitor insulin/IGF-I action alone.

Transcaltivational effects of SREBP-1a and SREBP-2. SREBP-1a and SREBP-1c are expressed in the human ovary (46), and SREBP-1a and SREBP-2 regulate human LDLR gene expression in nonsteroidogenic cells. Greater activation of LDLR promoter by SREBP-1 than by SREBP-2 is recognized in other systems (see introductory section). Accordingly, Sp1/Sp3-like-binding sites (−201 TCCTCC −196 bp) located between SRE (−167 to −157 bp) and R1, Sp1. The super-numerary putative Sp1/Sp3-binding site in the porcine LDLR promoter is denoted here as repeat 1a (R1a).

Table 2. Oligonucleotide (sequences) used in EMSA analysis

<table>
<thead>
<tr>
<th>Oligonucleotide (sequences)</th>
<th>GenBank Accession Number</th>
<th>Description</th>
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<tbody>
<tr>
<td>pLDLRSRE-1</td>
<td>174-TTGGAAAAATCGACCGATGAAACTG-3-150 (sense, wild-type)</td>
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</tr>
<tr>
<td>pLDLRSRE-2</td>
<td>174-TTGGAAAAATCGACCGATGAAACTG-3-150 (sense, mutant)</td>
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<tr>
<td>pLDLSp1-1</td>
<td>156-CAAAACTCCTGCCTCTCCGAAACCTCACA-3-127 (sense)</td>
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<tr>
<td>pLDLRASp1-1</td>
<td>207-5-GGACAGCCTCTCCCTGAGTtgAG-3-184 (sense)</td>
<td></td>
</tr>
<tr>
<td>pLDLRSp3-1</td>
<td>226-5-GCTTGGAGAACCTTCTCCGGAAAC-3-202 (sense)</td>
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Fig. 2. Impact of enforced expression of sterol response element (SRE)-binding protein (SREBP)-1a and SREBP-2 on transcriptional activity of a porcine LDLR promoter. Firefly luciferase construct, pLDL1076/luc, in granulosa-luteal and Hep G2 cells. Lipofectamine-mediated transfection was allowed to proceed for 6 h with pLDL1076/luc (1.9 µg/well), a plasmid encoding Renilla luciferase (50 ng/well), and an expression vector (30 ng/well), pCMV/SREBP-1a (aa 1–490), pCMV/SREBP-1a del (aa 91–490), or pCMV/SREBP-2 (aa 1–481), or pCMV/SREBP-2 del (aa 31–481). After transfection, cells were exposed to effector for 4 h and lysed, and luciferase activity was determined. Data are normalized to Renilla luciferase activity. LH, luteinizing hormone; IGF-I, insulin-like growth factor I. Values are means ± SE of 3–5 independent experiments, each performed in duplicate. Within each transfection set of 6 stimuli, a and b differ (P < 0.05) and c differs from both a and b (P < 0.01). *Significantly different from cognate intact (nondeleted) SREBP-1a or SREBP-2 sequence (P < 0.01).
SREBP-2 by about twofold (\(P < 0.01\)) (Fig. 3). Cotransfection of Sp1/Sp3 expression vectors (Dr. Robert Tjian, Howard Hughes Medical Institute, Dept. of Molecular and Cell Biology, University of California, Berkeley, CA) with the porcine LDLR reporter plasmid did not stimulate luciferase activity basally or after hormone exposure in granulosa-luteal or Hep G2 cells (data not shown). These observations could mean that endogenous Sp1/Sp3 availability per se is not required for or is not limited to transcriptional activity but do not exclude possible posttranslational control of Sp1/Sp3. To examine the latter consideration, selected mutants of full-length pLDLR1076/luc were created: \(-151 T\) TCCCTC \(-146\) to \(-151 T\) Ccag \(-146\) bp, designated pLDLRSp1–148mut/luc and, analogously, pLDLRSp1–151mut/luc. To identify a possible gonadotropin-responsive region, we tested the effect of LH (at 4 h) on expression of the full-length porcine LDLR reporter and full-length mutants of individual Sp1/Sp3-binding motifs. Insulin and IGF-I were inactive at this time point in all mutant/deletional reporters. As summarized in Fig. 3, LH failed to stimulate luciferase activity under the control of pLDLRSp1–151mut/luc, pLDLRSp1–215mut/luc, or pLDLRSp1–215mut/luc in granulosa-luteal cells. In contrast, LH caused a 2.1- to 3.0-fold increase in luciferase activity driven by R3 pLDLRSp1–148mut/luc and \(R_{1a}\) pLDLRSp1–

Functional role of SRE-, Sp1-, and Sp3-binding sites. In ovarian cells, LDLR promoter expression is repressed by LDL and oxysterols (30, 44). To determine whether repression is mediated via motifs in the SRE (\(-167\) to \(-157\) bp), a mutant reporter construct was prepared bearing a C-to-G transversion at \(-161\) bp in \(R_{2}\) pLDL\(\Delta SRE\)/luc. This point mutation reduced basal transcription in granulosa-luteal cells at 4 h by 50\% (\(P < 0.05\)) compared with that of wild-type pLDLR1076/luc and attenuated the stimulatory effects of LH alone by 80\% and of LH + insulin/IGF-I (both \(P < 0.01\)) by 80–90\% (Fig. 3).

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Fig. 3. Effect of individual mutation of presumptive SRE sequence and each of 3 flanking putative Sp1/Sp3-binding elements in 5'-upstream regulatory region of porcine LDLR gene reporter. Wild-type and mutant pLDLR1076/luc plasmids were transfected into granulosa-luteal cells. Effectors were added for 4 h. Values are means \(\pm\) SE of 3–5 independent experiments, each performed in duplicate. Within each transfection set of 6 stimuli, \(a\) and \(b\) differ (\(P < 0.05\)) and \(c\) differs from both \(a\) and \(b\) (\(P < 0.01\)). *Significantly different from cognate intact (nondeleted) SREBP-1a or SREBP-2 sequence (\(P < 0.01\)).

To explore the biological role(s) of endogenous SREBPs, granulosa-luteal cells were cotransfected with pLDLR1076/luc plasmid and expression vectors encoding a dominant-negative mutant of SREBP-1a or SREBP-2. The mutant proteins bind, but do not \(\text{trans-}\)activate, SRE-containing genes because of NH\(_2\)-terminal deletion of 90 amino acids (SREBP-1a) and 31 amino acids (SREBP-2) (34, 41, 50). Cotransfection of individual inhibitory constructs with pLDLR1076/luc significantly attenuated basal and hormonally stimulated luciferase expression (\(P < 0.01\)) in granulosa-luteal cells (Fig. 2).

Fig. 4. Binding of recombinant human SREBP-1a (rhSREBP-1) to putative porcine SRE sequence in 5'-upstream region of LDLR gene. A double-stranded DNA fragment corresponding to nucleotides \(-174\) to \(-150\) bp 5'-upstream of the transcriptional start site was end labeled with \([\text{\textsuperscript{32}}P]\text{ATP}\) and incubated with nuclear proteins extracted from granulosa-luteal cells (lanes 2–7), recombinant SREBP-1a (lanes 9–12 and 14), or protein from \textit{Escherichia coli} transformed with empty glutathione \(-\text{transferase vector extract (lane 13)}\) before gel electrophoresis. Competition analysis was performed with increasing concentrations (10- to 1,000-fold molar excess) of unlabeled wild-type (lanes 3–7 and 10–12) or mutant (C-to-G transversion at \(-161\) bp; \(lane\ 14\)) SRE sequences. Arrow, migration of SREBP-1a-SRE standard (see \textit{MATERIALS AND METHODS}). Data are representative of 3 independent analyses.
198mut/luc (P < 0.05). Thus partial gonadotropin responsiveness persisted, despite markedly impaired basal expression (4 h). Insulin exerted no detectable effects on any of the three mutant Sp1/Sp3 constructs in granulosa-luteal cells. IGF-I was also inactive (data not shown).

**SREBP and Sp1/Sp3 bind respective motifs.** To investigate putative protein binding by SRE sequences, EMSA was carried out utilizing granulosa-luteal nuclear extracts, recombinant SREBP-1a, and selected oligodeoxynucleotide probes (Table 2). Extracted nuclear protein and recombinant SREBP-1 formed complexes with 32P-labeled double-stranded probe containing the wild-type SRE (Fig. 4). Increasing concentrations of the unlabeled wild-type SRE sequence reduced binding, whereas point-mutated SRE (C-to-g transversion at −161 bp) failed to do so (Fig. 4, lane 14).

EMSA was extended to include each of the three possible Sp1/Sp3-binding sites (R1, R1a, and R3; Fig. 5, A–C). The porcine LDLR promoter TCCTCC motif formed a complex

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**Fig. 5.** EMSA of granulosa-luteal cell nuclear protein extracts using each of 3 presumptive Sp1/Sp3-binding oligodeoxynucleotide sequences flanking SRE in porcine LDLR gene. Specific wild-type DNA sequences correspond to −226 to −202 bp (A), −207 to −184 bp (B), and −156 to −127 bp (C). D: EMSA data for consensus Sp1/Sp3-binding oligonucleotides (lane 2). Competition analysis used 20- or 200-fold molar excess of unlabeled wild-type probe (lanes 3 and 4). To identify DNA-binding proteins, antibody to Sp1 or Sp3 was incubated with nuclear proteins before addition of radiolabeled DNA probe (lanes 5 and 6). Putative Sp1/Sp3 binding to DNA was further examined by preincubation with 30 and 300 nM mithramycin (lanes 7 and 8). Arrows, migration of distinct DNA-protein complexes. Each radiogram is representative of 3 separate EMSA experiments. R1, R1a, and R3, repeats 1, 1a, and 3.
with nuclear protein on PAGE similar to that of a consensus GC box probe (Promega; Fig. 5D). Nuclear extracts yielded three distinguishable DNA-protein complexes associated with each of R1, R1α, and R3. Preincubation with 20- and 200-fold molar excess of unlabeled cognate probe decreased and abolished, respectively, the signal intensity of each complex. To distinguish Sp1- and Sp3-binding activities, nuclear extracts were preincubated with specific antibody to each protein. Sp1 antibody decreased signal intensity in the lowest-mobility band only, whereas Sp3 antibody eliminated the intermediate band. Positive controls (GC box consensus oligonucleotides) responded analogously (Fig. 5D). Coincubation with a putative inhibitor of Sp1/Sp3 protein binding to DNA, mithramycin A (30 or 300 nM), blocked formation of presumptive DNA-Sp1 and DNA-Sp3 complexes but not that of the highest-mobility (indeterminate) band.

Incubation of transfected granulosa-luteal cells with 100 nM mithramycin A for 4 h inhibited basal and LH-induced porcine LDLR-mediated luciferase activity by 70% (P < 0.05) and eliminated the supra-additive interaction between LH and insulin/IGF-I (P < 0.01; Fig. 6). Extending mithramycin A exposure to 24 h suppressed basal reporter activity significantly (P < 0.05) and abolished stimulation by insulin and IGF-I.

**Signaling linkages.** LH and insulin (or IGF-I) upregulate LDLR transcriptional activity supra-additively in porcine granulosa-luteal cells. Relevant intracellular signals may include PKA, phosphatidylinositol 3-kinase (PI3K), and MAPK (44). To explore possible involvement of the PKB pathway, granulosa-luteal cells were incubated with rapamycin, a highly specific inhibitor of mammalian target of rapamycin associated with p70 S6 kinase activation (1, 21). Exposure to 100 nM rapamycin did not significantly alter LH- or LH + insulin/IGF-I-stimulated luciferase activity at 4 h (data not shown) but completely inhibited the effects of insulin and IGF-I in granulosa-luteal cells at 24 h (P < 0.01; Fig. 7). These results could reflect a requirement for protein synthesis in delayed promoter stimulation via PKA or insulinomimetic pathways.

**DISCUSSION**

The accompanying studies extend mechanistic concepts of LDLR transcriptional control in granulosa-luteal cells (17, 30, 42, 44). In conjunction with earlier analyses, we infer that LH and insulin jointly amplify LDLR gene expression by way of intracellular mechanisms that include (nonexclusively) PKA, PI3K, and ERK1/2 signaling, along with key transcriptional factors SREBP and Sp1/Sp3. The present inferences are supported by enforced expression of wild-type and dominant-negative SREBP-1a and SREBP-2, EMSA of granulosa-luteal cell nuclear-protein extracts (with or without recombinant SREBP-1a- and Sp1- vs. Sp3-specific antibodies), and in situ inhibition or augmentation of hormone action by relevant pharmacological probes.

A novel feature of the porcine LDLR gene is three canonical Sp1/Sp3 DNA-binding regions located −255 to −139 bp 5' upstream of the transcriptional start site. Two such elements are represented in the human and other mammalian genes (Fig. 1) (40). The role of the third repeat sequence (here designated R1α) flanking the SRE is not known. However, the present studies demonstrate nuclear-protein binding to each element, suggesting biological relevance.

Insulinomimetic peptide stimulation of the porcine LDLR reporter construct was abolished by point mutation of the SRE (Fig. 2). The ability of insulin or IGF-I to enhance LH action was also eliminated by the single base-pair transversion. However, LH still stimulated luciferase activity by twofold over basal. These findings predict distinguishable molecular control of the swine LDLR gene by insulin/IGF-I- and cAMP-dependent pathways. Under in vivo conditions, human chorionic gonadotropin stimulates accumulation of mature SREBP-1a protein in the rat ovary (31). Gonadotropic hormones and other activators of cAMP/PKA signaling also enhance transcriptional activity of steriodogenic acute regulatory protein, HDL receptor, and LDLR promoters, the effects of which appear to require SREBP-dependent mechanisms (44, 45). In all eukaryotic promoters, multiple DNA-binding transcription factors must assemble on the promoter, together forming a transcriptionally active complex. We show that overexpression of SREBP-1a stimulates transcriptional expression of the LDLR

![Fig. 6. In situ incubation with mithramycin inhibits LH/forskolin- and insulin/IGF-I-induced transcription of a porcine LDLR gene promoter construct in granulosa-luteal cells. Analyses were carried out as defined in Fig. 2 legend, except for posttransfection exposure to 100 nM mithramycin for 60 min before and during incubation with effectors for 4 or 24 h. Values are means ± SE (n = 3 independent experiments each performed in duplicate). Within each transfection set of 6 stimuli, a and b differ (P < 0.05) and c differs from both a and b (P < 0.01). *Significantly different from corresponding response of non-drug-exposed granulosa-luteal cells at the same time (P < 0.05).](image)

![Fig. 7. Effect of rapamycin on LH/forskolin- and insulin/IGF-I-stimulated activity of pLDL1076/luc in porcine granulosa-luteal cells. Data are presented as defined in Fig. 6 legend, except cells were exposed to 100 nM rapamycin for 30 min before and during incubations. Values are means ± SE (n = 3 independent experiments each performed in duplicate). Within each transfection set of 6 stimuli, a and b differ (P < 0.05). *Significantly different from corresponding response of non-drug-exposed granulosa-luteal cells at the same time (P < 0.05).](image)
gene in vitro in steroidogenic ovarian cells. Conversely, transfection of a dominant-negative SREBP-1a vector blocks LH-driven LDLR reporter expression in granulosa-luteal cells. In other contexts, SREBP-1a physically interacts with cAMP-response element-binding protein (CREB) and activates the 3-hydroxy-3-methylglutaryl-CoA synthase gene (13). Such observations could point to cooperative effects of SREBP with cAMP/PKA-responsive transcription factor (CREB) or other regulators of the swine LDLR gene.

Our analyses of transcriptional control of the porcine LDLR promoter are consistent with individual and combined upregulation by SREBP-1a and Sp1/Sp3 proteins in granulosa-luteal cells (Fig. 3). In particular, site-directed mutagenesis data indicated that integrity of each of the three putative Sp1/Sp3-binding sequences flanking the SRE in the swine LDLR gene is necessary to support basal as well as LH- and insulin/IGF-I-stimulated transcription. On the other hand, transfection of constitutively active Sp1 and Sp3 probes in granulosa-luteal cells did not alter pLDLR1076/luc expression. The foregoing collective results are consistent with non-rate-limiting availability of Sp1/Sp3 proteins in ovarian cells but do not exclude hormonal activation of endogenous Sp1 and Sp3.

By way of preliminary functional analyses, EMSA experiments showed that granulosa-luteal cell nuclear-protein extracts form complexes with each of the three putative Sp1/Sp3 cis-DNA sequences flanking the SRE (Fig. 6). Complex formation was competitively antagonized by excess molar amounts of corresponding wild-type Sp1/Sp3 oligodeoxynucleotide sequences and abolished by in vitro exposure to mithramycin A, a chemical inhibitor of Sp1/Sp3 binding (37). Incubation of granulosa-luteal cells with mithramycin after forskolin and insulin stimulation in Hep G2 cells. These observations point to a possible functional role of ovarian cell nuclear Sp1 and/or Sp3 proteins in hormonal upregulation of the porcine LDLR gene.

LH- and insulin/IGF-I-dependent stimulation of the swine LDLR gene appears to require PKA, PI3K, and MAPK signaling (44). The data suggest involvement of the PKB pathway also in granulosa-luteal cells. In particular, rapamycin, an inhibitor of mammalian target of rapamycin-dependent activation of p70 S6 kinase (6), impedes transcriptional enhancement of pLDLR1076/luc by LH and insulin without inhibiting basal promoter activity. FSH may also trigger PKB-dependent effects in granulosa cells (23). Relevance of the current findings is suggested, in that Sp1/Sp3 and SREBP-1a can be phosphorylated by PKB (16).

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