Effects of galectin-1 on regulation of progesterone production in granulosa cells from pig ovaries in vitro

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The detection of galectin-1 (gal-1) in pig granulosa cell lysates by immunoblotting and its cytotoxic as well as membrane-associated localization prompted us to study its effects on cell proliferation and regulation of progesterone synthesis. The lectin stimulates the proliferation of granulosa cells from pig ovaries cultured in serum-free medium. Gal-1 inhibits the FSH-stimulated progesterone synthesis of granulosa cells. This inhibitory effect was strongly reduced by the disaccharidic competitor lactose at 30 mM. The absence of inhibitory effects on dibutyryl-cAMP (db-cAMP), forskolin, and pregnenolone-hydroxysteroid dehydrogenase/isomerase (3\beta-HSD) indicates that gal-1 exerts its inhibitory effect on steroidogenic activity of granulosa cells by interfering with the hormone–receptor interaction resulting in decreased responses to FSH stimulation.

Key words: galectin-1/granulosa cells/pig ovaries/progesterone synthesis

Introduction

Galectin-1 (gal-1) is a member of a family of structurally related proteins that share a highly conserved amino acid sequence motif in the carbohydrate recognition domain (CRD) for the binding of \(\beta\)-galactosides (Barondes et al., 1994; Kasai and Hirabayashi, 1996). Gal-1, a proto-type galectin, forms noncovalently associated homodimers under physiological conditions with two CRDs and a binding preference for N-acetyllactosamine (LacNAc) residues present on all complex N-linked and many O-linked glycoproteins (Hirabayashi, 1993; Leffler and Barondes, 1986). Therefore the lectin can serve as a homobifunctional cross-linker for cell surface glycoconjugates containing the structural motif Gal\(\beta\)1-4GlcNAc.

All galectins studied so far have the characteristics of cytoplasmic proteins, such as acetylated N-termini, the absence of a disulfide bond in spite of the presence of several cysteine residues, and a lack of glycosylation although a potential glycosylation site is available (Drickamer, 1993; Hirabayashi, 1993). In general, galectins apparently are synthesized without any signal peptide sequence (Couraud et al., 1989; Ohyama et al., 1986) suggesting that they are designed as intracellular proteins. Although devoid of signal peptide, cytosolic gal-1 can be externalized from cells by a mechanism independent of the classical secretory pathway (Cooper and Barondes, 1990; Lindstedt et al., 1993) and is then bound by appropriate glycoepitopes on cell surface or extracellular matrix glycoproteins and glycolipids in an autocrine manner.

The binding of gal-1 to cell surface glycoconjugates suggests that the lectin is implicated in diverse cellular functions, such as cell adhesion to the endothelium (Allen et al., 1997; Barondes et al., 1994), cell growth regulation (Adams et al., 1996; Kopitz et al., 2001; Wells and Mallucci, 1991), immunosuppression (Offner et al., 1990; Rabinovich et al., 1999), and signaling events (Maeda et al., 2003; Rabinovich et al., 2000; Walzel et al., 2002). Gal-1 is synthesized and quantitatively externalized by Chinese hamster ovary cells and associates with cell surface glycoconjugates where the carbohydrate-binding activity is stabilized (Cho and Cummings, 1995). Gal-1 mRNA is abundantly expressed in mouse reproductive organs, such as uterus and ovary (Choe et al., 1997). Uterine expression of gal-1 mRNA is differentially regulated by ovarian steroids progesterone and estrogen and correlated with the implantation process. The synthesis of gal-1 in the trophoectoderm of expanded blastocyst immediately prior to implantation (Poier et al., 1992) suggests that it may be implicated in the interaction between the embryo and the extracellular matrix of uterine endometrium. Because gal-1 mRNA is abundantly expressed in mouse ovary (Choe et al., 1997), it is entirely conceivable that the lectin can influence fertility by changing granulosa cell–oocyte interaction (Hirschfield, 1997).

Progesterone is an important ovarian hormone to prepare the uterus for accepting the invading blastocyst/zygote for...
implantation and the subsequent maintenance of the pregnant stage (Hadley, 1995). Because granulosa cells secrete progesterone and estradiol (Campbell et al., 1996; Gutierrez et al., 1997), the study of granulosa cell function under defined conditions in vitro permits a detailed examination of the regulation of progesterone synthesis and its key enzymes by several trophic hormones and paracrine factors. Here we demonstrate the cytosolic and membrane-associated localization of gal-1 in granulosa cells from pig ovaries and provide evidence for the involvement of the lectin in regulation of progesterone synthesis of cultured ovarian granulosa cells. The gal-1-mediated decrease of follicle-stimulating hormone (FSH)-stimulated progesterone synthesis of granulosa cells and the absence of inhibitory effects on forskolin, dibutylryl cAMP (db-cAMP), and pregnenolone-enhanced progesterone production suggest that the lectin modulates the hormone receptor interaction.

Results

Characterization of confluent granulosa cells

The primary granulosa cell cultures form monolayers in ~2 days (Figure 1A) consisting of flat, spindle, and fibroblastic-like cells. Characterization of porcine granulosa cells by indirect immunofluorescence with monoclonal antibodies (mAbs) directed against cytokeratin and vimentin is summarized in Figure 1A and 1B. A mAb to cytokeratin, which identifies epithelial cells, did not stain confluent granulosa cells (Figure 1B). However, the vimentin mAb strongly stained granulosa cells (Figure 1A). The fluorescinated conjugate failed to stain granulosa cells when the primary antibody was omitted (not shown).

Confluent granulosa cells express gal-1: the lectin failed to recognize porcine FSH

For the detection of gal-1 in porcine granulosa cell lysates, the proteins were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose membranes. The binding pattern of the gal-1 polyclonal antibody (pAb) is shown in Figure 2, lane 1. The antibody generates one main protein band in the 14 kDa molecular mass range. An antibody binding pattern similar to granulosa cell lysates was recorded for purified human (lane 2) and bovine (lane 3) placental gal-1. Nonspecific binding reactions were excluded by probing the blots with rabbit IgG followed by incubation with goat anti-rabbit IgG–horseradish peroxidase (HRP) (lane 4). SDS-PAGE analysis of porcine (p) FSH (4.4 ng, 8.8 ng, 13.3 ng) followed by silver staining revealed one main band in the 32 kDa molecular mass range (data not shown).

As demonstrated in Figure 3 (lanes 3, 4, 5), gal-1 failed to recognize increasing concentrations of pFSH on blots. Strong immunochemical reactions were recorded for 4.4 ng gal-1 (lane 1, control). Consistent with the detection of gal-1 in cell lysates, fluorescence microscopic examination of confluent granulosa cells for the lectin with a gal-1 pAb by indirect immunofluorescence revealed the cytoplasmic and membrane-associated localization. As shown in Figure 4A, the antibody generated a uniform cytoplasmic immunofluorescence and immunochemical staining reactions of the plasma membrane. These data suggest that cytoplasmic gal-1 externalized by granulosa cells is bound to complementary cell surface glycoconjugates in an autocrine manner. According to the fluorescent image generated by costaining for gal-1 and the nuclei using propidium iodide, nuclear localization of gal-1 was not recorded.

Growth stimulation of granulosa cells by gal-1

We studied the effect of gal-1 on cell replication. Granulosa cells were maintained in serum-containing medium for 48 h and then in serum-free medium for further 48 h with 10 ng/ml FSH ± 30 mM lactose (controls, c), as well as
unexposed control cultures (100%). Lactose at 30 mM did not significantly reduce the stimulating effect of the lectin on cell proliferation.

Gal-1 inhibits the FSH-stimulated progesterone production of granulosa cells

To characterize the functional activity of cultured granulosa cells, we studied their steroidogenic activity and focused on the effects of gal-1 on FSH-stimulated progesterone synthesis. The basal level of progesterone production as measured by radioimmunoassay was 2.2 ± 0.5 ng/l × 10^5 granulosa cells. In the presence of FSH at 1 ng/ml, 10 ng/ml, and 100 ng/ml cellular progesterone secretion increased 2.7-, 5.6-, and 5.3-fold relative to basal levels.

To study whether the lectin interferes with FSH-enhanced progesterone production, granulosa cells were stimulated in serum-free medium with 10 ng/ml FSH in the presence of gal-1 at 3.3 μg/ml, 9.9 μg/ml, and 19.8 μg/ml. As demonstrated in Figure 6, gal-1 decreased the FSH-enhanced cellular progesterone production in a concentration-dependent manner. Gal-1 at 9.9 μg/ml and 19.8 μg/ml significantly reduced the cellular progesterone production to 39% and 28% relative to control cultures (c) stimulated with 10 ng/ml FSH (100%). In the presence of 30 mM lactose as a disaccharide competitor, the FSH-stimulated progesterone production was restored and approximately in the range of control cultures.

Gal-1 is without effects on db-cAMP, forskolin, and pregnenolone-enhanced progesterone synthesis

The FSH receptor signaling pathway is coupled via heterotrimeric G-proteins to adenylate cyclase. Because it is conceivable that gal-1 interferes with the hormone–receptor interaction leading to reduced cellular progesterone production, we studied the effects on transmembrane receptor independent stimulation of steroidogenic activity of granulosa cells by db-cAMP, forskolin, and pregnenolone. The effects of 19.8 μg/ml gal-1 on db-cAMP, forskolin, and pregnenolone-enhanced progesterone synthesis are summarized in Figure 7. Treatment of granulosa cell cultures in serum-free medium with 1 mM db-cAMP for 48 h increased progesterone accumulation in medium by twofold relative to basal levels. Gal-1 did not show an inhibitory effect on db-cAMP-enhanced progesterone production. The cell-permeable diterpene forskolin, a potent activator of adenylate cyclase, increased cellular progesterone production by eightfold versus basal levels. Gal-1 at 19.8 μg/ml was without effects on forskolin-enhanced progesterone accumulation in medium. Pregnenolone at 2.5 μM also stimulated the cellular progesterone production and was not found to be reduced by gal-1. Therefore the data provide evidence that gal-1 exerts its inhibitory effects on FSH-stimulated progesterone production of granulosa cells by modulating the hormone–receptor interaction.

Gal-1 inhibits mRNA transcription of P450SCC and 3β-HSD genes

To investigate if gal-1 affects the expression of the cytochrome P450-dependent cholesterol side chain cleavage enzyme (P450SCC) and 3β-hydroxysteroid dehydrogenase/isomerase with FSH and gal-1 ± lactose as indicated in Figure 5. Gal-1 at 3.3 μg/ml did not significantly increase the cell number, but 9.9 μg/ml and 19.8 μg/ml gal-1 significantly increased the cell number to 174% and 165%, respectively, relative to basal levels. Gal-1 at 9.9 μg/ml and 19.8 μg/ml significantly reduced the expression of P450SCC and 3β-HSD genes.
(3β-HSD) genes on the level of transcription, we determined concentrations of transcripts from both genes in porcine granulosa cell cultures stimulated with 10 ng/ml FSH under the influence of 19.8 μg/ml gal-1 with and without 30 mM of its disaccharidic competitor lactose. As demonstrated in Figure 8, the addition of the lectin significantly reduced concentrations of both transcripts to 35% and 16% of FSH stimulated controls in case of P450SCC and 3β-HSD, respectively. This inhibitory effect could be efficiently abolished by coincubation with lactose, although the addition of only lactose did not significantly affect transcript concentrations of both genes.

Gal-1 decreases cytochrome P450SCC expression of granulosa cells
To get evidence of whether down-regulation of enzyme expression is involved in gal-1-mediated inhibition of cellular progesterone synthesis, we concentrated on cytochrome P450SCC, which catalyzes the conversion of cholesterol to pregnenolone (Lahav et al., 1996). The effect of gal-1 on P450SCC expression of FSH-stimulated granulosa cells is illustrated in Figure 9. Immunoblot analysis revealed that granulosa cell cultures incubated in serum-free medium with 10 ng/ml FSH highly express the enzyme as indicated by a single band at 45 kDa. In the presence of gal-1 at 9.9 μg/ml and 19.8 μg/ml however, the FSH-enhanced cellular P450SCC expression was strongly reduced. When the cells were cultured with gal-1 in the presence of 30 mM lactose as a disaccharidic competitor to saturate the carbohydrate recognition domains, FSH-enhanced P450SCC expression was restored. Lactose at 30 mM did not show inhibitory effects on FSH-stimulated enzyme expression.

Discussion
The cytosolic and membrane-associated localization of gal-1 on granulosa cells suggests the secretion and binding
to cell surface glycoconjugates in an autocrine manner (Lutomski et al., 1997). By recognition and cross-linking of glycan ligands, gal-1 exerts distinct biological effects in various tissues and on cells, including cell migration, adhesion, differentiation, proliferation, and apoptosis (Cooper and Barondes, 1999; Goldring et al., 2002; Leffler, 2001; Perillo et al., 1998). The dimeric form of gal-1 facilitates cell surface receptor cross-linking believed to be essential for inducing signaling events when receptors are involved that are linked to downstream signal transduction pathways (Brewer, 2002; Dias-Baruffi et al., 2003; Leffler, 2001; Perillo et al., 1995). As demonstrated, gal-1 stimulates the proliferation of granulosa cells from pig ovaries cultured in serum-free medium. There is also evidence for induction of gal-1 accumulation in the stroma associated with ovary carcinoma cells where the lectin is involved in tumor cell proliferation and adhesion to laminin-1 and fibronectin (Van Den Brule, 2003).

LacNAc is the basic ligand recognized by conserved CRDs, however, gal-1 binds with increased avidity to multiple LacNAc sequences presented on branched N-linked or to poly-LacNAc chains on N- and/or O-linked glycans. The increased binding avidity for cellular glycoconjugates may be the reason that lactose nonsignificantly reduced the stimulating effects of gal-1 on cell proliferation. Gal-1 significantly reduced FSH-enhanced cellular progesterone synthesis in a concentration-dependent manner. Lactose as a disaccharide competitor decreased this galectin-mediated inhibitory effect, and FSH-stimulated progesterone production was restored. Therefore we conclude that by
recognition of low-affinity binding sites, gal-1 interferes the FSH–receptor interaction, leading to reduced FSH-enhanced cellular progesterone production. This assumption is supported by the data demonstrating no inhibitory effects of gal-1 when cellular progesterone production was enhanced by nonreceptor-mediated mechanisms.

Stimulation of cellular progesterone production with the cell-permeable derivative db-cAMP and forskolin believed to activate adenylate cyclase via its interaction with the catalytic subunit (Seamon and Daly, 1981) was not reduced by gal-1. On the other hand, binding of gal-1 to the glycosylated unique FSH β-chain, which confers biological specificity to thyrotropin, lutropin, and FSH could hinder receptor recognition. As demonstrated in Figure 3, gal-1 failed to recognize FSH on blots. Furthermore, granulosa cells respond with increased progesterone production when exogenous pregnenolone was added as a substrate, and gal-1 did not reduce the stimulatory effect. From the absence of effects on basal, db-cAMP, and forskolin-enhanced progesterone synthesis we conclude that the lectin did not reduce the uptake of cholesterol into the mitochondria, where progesterone synthesis takes place. It is also conceivable that gal-1 inducing down-regulation of FSH receptors by endocytosis is a possible mechanism of action at the cell surface. Gal-3 mediates endocytosis of β-1 integrins, advanced glycation end products, and acetylated low-density lipoproteins in Chinese hamster ovary cells. As an organizer of lipid rafts, a number of signaling molecules are sequestered in these raft domains (Ochieng et al., 2004).

Although nonsignificantly increased, there is evidence for gal-1 as an autocrine modulator because lactose in the absence of added gal-1 increased FSH-enhanced progesterone synthesis (Figure 6) and 3β-HSD gene transcription (Figure 8). The gal-1-mediated inhibition of FSH-induced P450SCC and 3β-HSD gene transcription identified the endogenous β-galactoside-binding protein as a negative regulator of steroid synthesis in ovaries. It has been demonstrated that cAMP stimulates P450SCC mRNA
accumulation in porcine granulosa cells (Picton et al., 1999; Urban et al., 1991). In human (Inoue et al., 1988) and bovine P450SCC genes (Ahlgren et al., 1990) upstream regulatory elements have been identified to be responsible for cAMP-dependent transcription. The FSH receptor signaling pathway is coupled via heterotrimeric G-proteins to adenylate cyclase that increases intracellular cAMP levels (Conti, 2002; Richards, 1994) leading to enhanced P450SCC gene transcription and progesterone synthesis in granulosa cells (Brentano et al., 1992). The data presented in Figure 8 that 3β-HSD transcripts exceed those of P450SCC and granulosa cells did respond to pregnenolone as an exogenous substrate with increased progesterone are consistent suggesting that conversion of cholesterol to pregnenolone catalyzed by P450SCC is considered to be the rate-limiting step for progesterone synthesis.

The data presented here contribute to define a role for gal-1 as a modulator of FSH-enhanced progesterone production in porcine granulosa cells.

Materials and methods

Materials

Tissue culture supplies, medium 199 (HEPES modification, tissue culture medium [TCM] 199), ABAM (penicillin [100 IU/ml], amphotericin B [250 ng/ml], streptomycin [100 μg/ml]), ITS (selenium [4 ng/ml], transferrin [2.5 μg/ml], insulin [10 ng/ml], forskolin, db-cAMP, asialofetuin, 3-amino-9-ethylcarbazole, protein A agarose, and pregnenolone were obtained from Sigma (Deisenhofen, Germany). FSH preparation: Ovaset lot no. 4782 T (39 mg pFSH/ampulla according to the NIH standard) was from CEVA (Libourne, France). CNBr-activated Sepharose 4B, NHS-activated Sepharose 4 Fast Flow, Resource Q column, Hybond ECL nitrocellulose membranes, and enhanced chemiluminescence (ECL) detection reagents were from Amersham Pharmacia Biotech (Freiburg, Germany). Vimentin and cytokeratin mAbs, rabbit anti-mouse IgG-fluorescein isothiocyanate (FITC) F(ab’)2 fragment, high pure polymerase chain reaction (PCR) product purification kit, and LightCycler-FastStart DNA master SYBR green I kit were ordered from Roche Diagnostics GmbH (Mannheim, Germany). Goat anti-rabbit IgG HRP was from Dako A/S (Hamburg, Germany), and the P450SCC pAb was from Chemicon (Hofheim, Germany); RNeasy mini kit was from Qiagen (Hilden, Germany); the GEM-T plasmid vector was from Promega (Mannheim, Germany); and goat anti-rabbit IgG F(ab’)2-FITC was ordered from Serotec (Düsseldorf, Germany).

Purification of gal-1 from bovine placenta

Gal-1 was prepared from bovine placenta by lactose extraction with ethylenediaminetetraacetic acid (EDTA)-Me phosphate buffered saline (PBS) (20 mM sodium phosphate, pH 7.2, 150 mM NaCl, 4 mM 2-mercaptoethanol,
2 mM EDTA) and purified by sequential affinity chromatography on asialofetuin Sepharose 4B (Hirabayashi and Kasai, 1984) followed by lactosyl agarose. The protein was purified to homogeneity by anion exchange chromatography on a Resource Q column (Walzel et al., 2000).

**Generation and purification of gal-1 pAbs**

Antibodies were produced in rabbits immunized with human placental gal-1. From serum the IgG fraction was separated by affinity chromatography on protein A–agarose. For separation of gal-1 specific antibodies, the IgG fraction was passed through gal-1 agarose prepared by coupling the lectin to NHS-activated Sepharose 4 Fast Flow according to the manufacturer’s instructions. The immunoaffinity-purified pAb cross-reacts with gal-1 from pig and bovine as detected by antibody binding to the proteins on blots as well as after spotting the lectins to nitrocellulose membranes (data not shown).

**Cell preparation and culture**

Tissue collection and isolation of pig granulosa cells were carried out as described previously (Tiemann et al., 1996). The ovaries derived from pigs of a commercial slaughterhouse were collected in PBS (pH 7.4). Then the cells were aspirated from nonatretic preovulatory follicles (≥3 mm in diameter) by means of a syringe and were flushed with PBS (pH 7.4). To disperse cell clusters, cell aggregates were resuspended several times through a Pasteur pipette. The cells were collected by centrifugation at 200 g for 2 min and resuspended in TCM 199 supplemented with 10% fetal calf serum (FCS) and 1% ABAM. Trypan blue exclusion and resuspended in TCM 199 supplemented with 10% fetal calf serum (FCS) and 1% ABAM. Trypan blue exclusion revealed that cell viability was about 70%.

The cells were plated in 24-well plastic plates. After 24 h, the culture medium was changed, and the cells were cultured in complete medium at 37°C in humidified 95% air–5% CO₂ for a further 24 h (50–70% confluency). Then the media were replaced with 1 ml/well serum-free medium supplemented with 1% ABAM and 1% ITS. The monolayers were incubated with either 10 ng/ml M forskolin, 2.5 μM P450SCC and 3.3 mF20 mAbs controls. For detection and localization of gal-1, granulosa cells were grown on 18-mm glass coverslips coated with human fibronectin (25 μg/ml TBS) overnight at room temperature. Cells were allowed to spread on coverslips for 2 days. Then the cells were treated as described and incubated with a gal-1 pAb at 1 μg/ml TBS containing 0.25% BSA and 0.005% Tween 20 for 60 min at room temperature. The cells were rinsed with TBS/Tween 20 (0.5% BSA, 0.05% Tween 20) and incubated with anti-rabbit IgG F(ab')₂–FITC (1:100 dilution in TBS/Tween 20) for 60 min at 37°C. After washing with TBS, coverslips were incubated with the DNA-binding dye propidium iodide at 1 μM for 20 min at room temperature. Then the coverslips were mounted in Mowiol, and the cells were analyzed for fluorescence using a Zeiss epifluorescence microscope. Incubations in the absence of gal-1 pAb with IgG-(Fab')₂–FITC (1:100 dilution) were used as controls.

**Detection of gal-1 in granulosa cell lysates by immunoblotting**

Granulosa cells cultured for 48 h in TCM 199 supplemented with 10% FCS and 1% ABAM (6 x 10⁶ cells) were lysed in 300 μl Laemmli sample buffer (Laemmli, 1970) by treatment for 5 min at 100°C. Cell lysates (10 μl) were separated by SDS–PAGE (12%) and electrophoretically transferred to Hybond ECL membranes. After blocking the blots with 4% BSA in PBS (pH 7.2, 1 M NaCl, 0.05% Tween 20) for 2 h, the blots were probed with 1 μg/ml anti-gal-1 antibody or 1 μg/ml rabbit IgG (control) in TBS/Tween containing 1% BSA for 16 h at 6°C. The blots were washed and incubated with goat anti-rabbit IgG-HRP conjugate (1:1000 dilution in TBS/Tween with 1% BSA) for 2 h. Staining was performed with a mixture of 10 mg 3-amino-9-ethylcarbazole in 1 ml acetone, 25 ml 50 mM acetate buffer, pH 5.0, and 15 μl H₂O₂ (30%).

**Detection of progesterone accumulation and cell monolayers**

For estimation of progesterone accumulation, the cells were plated in 24-well plates for up to 96 h. At the end of incubation, the cells were washed with Hank’s balanced salt solution (without Ca²⁺ and Mg²⁺) twice, and all remaining cells attached to the plate were regarded as viable cells, when they did not take up Trypan blue. Cells were detached with 500 μl trypsin–EDTA solution (0.02–0.05%) and the cell number was determined by a cell counter (Coulter-Multisizer, Krefeld, Germany). For counting, 100 μl cells were suspended in 9.9 ml 0.9% NaCl solution, and each sample was repeated twice.
Radioimmunoassay for progesterone

Progesterone concentrations were measured in culture medium by specific radioimmunoassay as previously described (Tiemann et al., 1996). Intra- and interassay precision were 4.8% and 13.2% for progesterone. The number of cells was counted in each well. Steroidogenesis is expressed as ng hormone/1 × 10^5 cells.

Analysis of granulosa cell P450SCC expression by immunoblotting

Granulosa cells were cultured with 10 ng/ml FSH in the absence and presence of 9.9 μg/ml and 19.8 μg/ml galectin-1 with or without 30 mM lactose for 48 h. For analysis of P450SCC expression the cells were detached with 500 μl trypsin-EDTA solution, washed with ice-cold PBS, and lysed at 1 × 10^6 cells/100 μl Laemmli sample buffer (Laemmli, 1970) by treatment for 2 min at 100°C. After separation the lysates on 12.5% SDS-PAGE slab gels, the proteins were transferred to polyvinylidene difluoride membranes. The membranes were saturated with 5% fat-free dry milk in TBS/Tween overnight at 4°C and, after washing with TBS/Tween, probed with a P450SCC pAb (1:1000 dilution in TBS/Tween with 5% BSA) for 3 h at room temperature. Then the blots were washed three times with TBS/Tween and incubated with a goat anti-rabbit secondary antibody conjugated to HRP (1:2000 dilution in TBS/Tween and incubation with a goat anti-rabbit secondary) and subsequently an HRP-conjugated secondary antibody (1:2000 dilution in TBS/Tween) and single-point fluorescence acquisition at 83°C for 6 s to avoid quantification of primer artifacts.

Immunoblotting

Quantification of mRNAs for P450SCC and 3β-HSD by real-time PCR

Total RNA was prepared with the RNeasy mini kit. Briefly, cells stored frozen at −80°C were lysed and collected in 300 μl of a guanidine isothiocyanate containing buffer (lysis buffer) and subjected to homogenization by using QiAshredder Homogenizers (Qiagen). Subsequently an equal volume of 70% ethanol was added to the lysates, and the RNA was extracted from the samples by adsorption to silica-gel spin columns. After three washing steps and elution in 30 μl deionized RNAse-free water (washing buffers and water provided by the kit) RNA was quantified in a GeneQuant II instrument (Pharmacia). Quality of RNA was monitored from randomly selected samples by denaturing agarose gel electrophoresis. GeneQuant II instrument (Pharmacia). Routinely dilutions of standards covering five orders of magnitude (5 × 10^{-16} to 5 × 10^{-12} μg DNA/μl) were coamplified during each run. Fluorescence signals, which were recorded online during amplification, were subsequently analyzed using the Second Derivative Maximum method of the LightCycler Data Analysis software. Copy numbers were calculated relative to the amount of initially transcribed RNA. To normalize for variations between individual LightCycler runs, one or two arbitrarily selected samples were coamplified during all runs for each gene.

Statistical analysis

Data are expressed as the means ± SEM of triplicate measurements from separate granulosa cell preparations. A one-way analysis of variance with Student-Newman-Keuls test was used to determine significant differences. A p-value of less than 0.05 was considered significant.

Abbreviations

3β-HSD, 3β-hydroxysteroid dehydrogenase; BSA, bovine serum albumin; CRD, carbohydrate recognition domain; db-cAMP, dibutyryl cyclic AMP; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; FCS, fetal calf serum; FSH, follicle stimulating hormone; HRP, horseradish peroxidase; mAb, monoclonal antibody; P450SCC, cytochrome P450-dependent cholesterol side chain cleavage enzyme; pAb, polyclonal antibody; PAGE, polyacrylamide
gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TCM, tissue culture medium.

References


