Luteinizing hormone receptor and cytochrome P450scc enzyme mRNA expression within the gonad of mice injected with Serine/threonine or glycine

Hüseyin Baki ÇİFTCİ

Department of Animal Science, School of Agriculture, Selçuk University, 42079 Kampüs, Konya-Türkiye

Abstract

The aim of this experiment was to test the effect of injecting serine/threonine or glycine on mRNA's for Luteinizing Hormone Receptor (LHR) and cytochrome P450scc enzyme ($P450_{ssc}$) expression in mouse testes and ovaries. Male and female mice (CD-1 strain) were injected with saline (S, control), serine/threonine (A, test) or glycine (G, test). Total RNA was extracted, transferred onto a nylon membrane and probed with ^{32}P-labelled cDNA probes. The expression of mRNA's for LHR and $P450_{ssc}$ were increased by serine/threonine and glycine injection in female while both of them were increased in male mice. The decrease in LHR and $P450_{ssc}$ mRNA expression, after serine/threonine or glycine injection, might be a result of changes in the function of PI3K or PKA enzymes and decreases in the phosphorylation of Sp1 and StAR proteins in the ovaries. The increases in LHR and $P450_{ssc}$ mRNA expression in testis indicating difference in regulatory mechanism between male and female.

Keywords: Enzyme, Gonad, Mice, P450scc, Receptor

Introduction

Luteinizing hormone (LH) is a glycoprotein secreted from the gonadotrophs of anterior pituitary. It is a heterodimer and consists of a common, 92 amino acid long, α-subunit linked non-covalently to the hormone specific, 121 amino acid long, β-subunit (Alevizaki and Huhtaniemi, 2002). Luteinizing hormone exerts its effect by binding to G-protein coupled to seven transmembrane receptors located on cell surface. The gene coding for LH receptor, has been characterised (Dufau et al., 1995, Abdennebi et al., 2002), and it has 11 exons and 10 introns. The last exon encodes the entire transmembrane and intracellular region and exons 2 and 9 encode for external domain of the receptors (Dufau et al., 1995; Abdennebi et al., 2002). In testis, LH receptors (LHR) are found in Leydig cell. In the ovary, LHRs are found in theca and granulosa cells of the preovulatory follicle, in the corpus luteum and in interstitial cells (Zeleznick et al., 1974; McFarland et al., 1989). Extragonadal expression of LH receptor has also been reported to be present in the bovine, porcine, rat, mouse, rabbit, and human uterus (Jensen and Odell, 1988; Sen, 1991; Bhattacharya et al., 1993; Mukherjee et al., 1994; Derecka et al., 1995; Friedman et al., 1995).

Cholesterol is the starting point for steroid production. The rate limiting step of steroid production is the transport of cholesterol from the outer to inner mitochondrial membrane which is primarily mediated by the steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996). The stimulation of steroid production is mediated by gonadotrophines (Amsterdam and Selvaraj, 1997). Binding of gonadotropins to their receptor stimulate the G proteins (Hai et al., 1999) which activates the membrane-associated adenylate cyclase, causing an elevation of intracellular cAMP level and consequent increases in the activities of StAR and P450 cholesterol side-chain cleavage enzyme (P450scc) (Cooke, 1999; Strauss et al., 1999; Stocco, 2000). Cholesterol side-chain cleavage enzyme converts the cholesterol to the pregnenolone in the inner mitochondrial membrane. The rate of pregnenolone synthesis also depends on the level and activity of P450scc enzyme (Rennert and Chang, 1993). Pregnenolone leaves the mitochondria and enters into smooth endoplasmic reticulum where it converted to progesterone by 3β-HSD enzyme. Progesterone is converted to androgens by cytochrome P450 enzyme 17α-hydroxylase (P450c17) in smooth endoplasmic reticulum. This androgen is then converted by cytochrome P450 aromatase (P450arom) to oestrogen (Gray et al., 1996).

Free serine and threonine amino acids are capable of phosphorylation (Lauren and Friedmann, 1971) by enzymes. These amino acids are taken up by intracellular proteins. In a study, non-phosphorylatable analogs of the serine and threonine were used to

determine the role of phosphorylation in the acute regulation of steroidogenesis in MA-10 mouse Leydig tumor cells. According to their results, substitution of the threonine analog into protein resulted in inhibition of hormone stimulated steroid production in these cells (Stocco and Clark, 1993). Although no a specific kinase has yet been specified, specifically phosphorylating free serine and threonine. The gonadotropin receptors possess serine and threonine residues in the cytoplasmic loop and the C-terminal tail of the receptor. Serine and threonine residues are the phosphorylation sites for protein kinases (Minegishi et al., 1989; Segaloff and Ascoli, 1993; Quintana et al., 1994).

Therefore, the objective of this study was to inject the mice with serine/threonine and glycine amino acids to see if there is any alteration in LH receptor and cytochrome P450scc mRNA expression in mouse gonads.

Materials and Methods

Nineteen and twenty one day old male (n=30) and female (n=30) mice (CD-1 strain) were injected (intra-peritoneal) daily between 10:00 and 12:00, for five days with saline, as a control or a mixture of Serine/Threonine (Containing 0.264 μg/200μl L-Serine, Cat; 21101-019, Life Technologies, Paisley, Scotland/ 0.132μg/200μl L-Threonine, Cat; 103053, ICN, Ohio, USA) or 38 ng Glycine (Cat; G-8898, Sigma, Steinheim, Germany) in 0.2 ml saline. After five days, the mice were killed by cervical dislocation, 60 minutes after the last injection, then testes and ovaries were removed. Removed testes and the ovaries were grounded in liquid nitrogen using RNAase-free pestle and mortar. Ground tissue (100 mg) was placed in 1ml Trizol reagent (Cat; 15596-026,100 ml, Life Technologies. Paisley, Scotland), in 2ml micro-centrifuge tube (Cat; 02-1420-2700, Fisher, Leicestershire, UK). The tubes containing the ground tissue and Trizol were left at room at room temperature for 5 minutes to permit complete dissociation of the nucleoprotein complexes. Then 0.2 ml of chloroform (0.2ml/1ml Trizol used) was added and the tube and was shaken vigorously for 15 seconds then centrifuged at 1200g for 15 min at 4°C. The top colourless phase was transferred to a fresh eppendorf tube. The contents of each tube were mixed with 0.5 ml, per ml of Trizol, isopropyl alcohol (Cat; 405-7, Sigma. St. Louis, MO, USA), incubated at room temperature for 10 min. and centrifuged at 12000 g at 4°C for 10 min. The supernatant was removed and the RNA pellet washed with 1 ml of 75% ethanol (Cat; 200-578-x, Hyman Limited. Essex, England) and RNA recovered by centrifuging again at 7500g for 5 min at 4°C. Finally, the supernatant was removed, the RNA pellet dried

briefly and then redissolved in 50μl nuclease free water which was prepared by adding 1ml diethyl pyrocarbonate (DEPC; Cat; D-5758, Sigma, Steinheim, Germany) to 1L dH$_2$O. Total RNA was quantified by using a RNA/DNA spectrophotometer (Mod; 80-2103-98, Ser; 66884, Pharmacia Biotech. Cambridge, England). Total RNA was converted to cDNA by reverse transcription by using Avantage RT-PCR kit (Cat; K1402-1, Clontech, Palo Alto, USA). Synthesized cDNA was amplified by using specific primers for LHR and cytocrom P450scc. Probes were obtained by Polymerase Chain Reaction (PCR), two tubes and two step procedures were applied. Primers for LHR and cytochrome P450$_{scc}$ kindly provided by Prof. PJ O'Shaughnessy (University of Glasgow Veterinary School Bearsden Rd, Glasgow G61 1QH). Samples were run in 1% ultra pure agarose (Cat; 15510-019, Life Technologies. Paisley, Scotland). One-gram agarose was placed in a 500ml-glass flask and 100ml TEA buffer was added then placed in an oven to dissolve the agarose and allowed to boil until the agarose was completely dissolved. Then, 5μl of a 10mg/ml solution of Ethidium bromide (Cat; E-7637, Sigma, St Louis, MO, USA) was added, mixed gently and cooled down and then poured into an electrophoresis tank. When the gel was solid, it was placed in 1XTris ethylene acetate (TEA) buffer made up to 50X by adding 252 g Tris base (Cat; T-1503, Sigma. St Louis, MO, USA), 57.1ml glacial acetic acid (Cat; 100001 8Q, BDH. Poole, England) and 100ml 0.5 M EDTA (Cat; E-4884, Sigma. St Louis, MO, USA) then autoclaved. The DNA ladder, size marker, 100bp (Cat; H0595, Perkin Elmer, Germany) was made up by mixing 60μl ladder with 10μl gel loading solution (Cat; G-2526, Sigma, Steinheim, Germany) from this 5μl was loaded. Both PCR product and DNA size marker were diluted in lauding buffer and run at 65V for 1.5h and then photographed under UV illumination. Probes were extracted by using quick gel extraction kit (Cat; 28704, Qiagen, Hilden, Germany).

Fifteen μg of total RNA from each experimental group was placed in 0.2 ml nuclease free micro tubes (Cat; AB-0337, Advanced Biotechnologies) and the volume made up to 15μl with sample buffer. The tubes, containing RNA plus sample buffer, were placed in a water bath at 61°C for 5 min and then immediately placed on ice, 1μl ethidium bromide (0.1mg/ml solution) was added. Fifteen μl was then loaded into the wells of the gel and run at 65V for 5h at room temperature. The gel was placed in saran film and photographed under UV illumination. The gel was then inverted and placed in a NaOH solution (2g NaOH in 1L DEPC water) for 40 min to produce sharper bands. A plastic platform was placed in a 25X15cm plastic nuclease free container, Then a piece of 3mm Whatman

filter paper was cut to the size of plastic platform and and immersed in transfer buffer (10X SCC). The wet filter paper was laid on the platform and pressed gently and slowly so that there were no bubbles between the platform and the filter paper. The paper covered the edges of platform, but did not touch the bottom of buffer container so as to facilitate upward transfer. Another piece of wet filter paper was placed on the top of platform without bubbles. The gel was taken out of the NaOH solution and placed on the filter paper that wells of the gel facing the platform. The edges of gel were protected from direct contact with the buffer. A piece of nucleic acid transfer membrane, Hybond-N+(Cat; I6435, Amersham, Buckinghamshire, England) cut to the size of the gel was placed on the gel. Another piece of wet filter paper was placed on membrane. To facilitate upward transfer of the buffer through the gel, by capillary movement, dry paper towels were stacked and a weight was placed on the gel and left approximately for 18 hours to transfer the RNA from the gel to the nylon membrane The membrane was sandwiched between Whatman filter paper and left at room temperature for 10 min to dry. The gel was taken to a dark room to visualise the transfer. The membrane was placed in an oven at 80°C for 2h to fix the RNA. The dried membranes were kept in a secure place for hybridisation. Fifteen ng of a DNA probe (for labelling) was dissolved in 16.27μl of distilled and filtered water, denatured at 100°C for 5 min and then chilled on ice and added with 2.4μl unlabelled dNTP, 3μl reaction buffer, 3μl primer solution, 3μl [α-^{32}P] dATP (3000CI/mmol), 1.2μl Klenow. To make a final volume of 30μl, 1.13μl water was added to reaction tube. The tube was mixed gently by pipetting up and down and then centrifuged to concentrate the contents at the bottom of the tube. The tube placed in a water bath at 37°C, the water bath was then switched off and left overnight for labelling. Labelled probe was extracted by using spin column. The membrane was removed from the filter cover and placed in a clear roller bottle in de-ionised water (100 ml) to wash out the agarose and salts from the membrane before pre-hybridisation. The washing was done by rolling the bottle by hand for 1-2 min and then pouring the water down the sink. The membrane was then placed in 10 ml hybridisation buffer preheated to 68°C and incubated in hybridisation oven for 50 min.

One ml hybridisation buffer was added to a screw cap tube, containing 30 μl labelled and denatured probe and 100 μl of denatured salmon sperm DNA (Cat; D-1626, Sigma, Steinheim, Germany). The tube was then mixed and the contents was transferred to a bottle and left to hybridise for 1h. One hour later the oven was opened and hybridisation buffer poured away. The blot was rinsed with 100 ml 2XSSC and 0.1X SDS (w/w)

and washed with a mixture of 100ml 0.1XSSC and 0.1XSDS for 10 min at 37 °C and washed again with 100ml 0.1XSSC and 0.1XSDS at 60°C for 20 min. After washing, the blot was wrapped with PVC and monitored for radioactivity and then placed in a case with the RNA side next to the film. The blot was exposed at -70°C for 24h. After 24h, the case was opened under the safe red light and developed using a Konica SRX-101 film processor (X-Ograph imaging systems, Gloucestershire, England). Quantification was done by using molecular analyst software (Copyright 1992-1995, Bio-Rad Laboratories, Hercules, USA). For each RNA transcript, background optical density was dismissed and the pipetting errors were corrected by dividing each transcript by β-actin which was hybridised on the same membrane used for the hybridisation of LH-R and P450scc enzymes.

Results

Both Luteinizing Hormone Receptor (LHR) and cytochrome P450scc mRNA are expressed in mouse testes and ovaries. Intra-perinonal injection of Serine/Threonine mixture or Glycine decreased Luteinizing LHR and cytochrome P450scc mRNA expressions within the ovaries whereas they were all increased in the testes. There were also parallel decreases and increases between LHR and cytochrome P450scc mRNA expression in both testes and ovaries (Figure 1).

Discussion

Threonine is essential amino acid while serine and glycine are not essential. Glycine is synthesized in the body from the amino acid serine. Serine and threonine both are bearing an OH group on which they are both phosphorylated by enzymes. An enzyme in *Propionibacterium shermanii* which uses inorganic pyrophosphate to phosphorylate L-serine have been reported (Cagen and Friedmann, 1972). However, a specific kinase phosphorylating serine and threonine have not been identified. Many cellular functions such as transcription, translation, ion transport, cell re-modelling, mitotic activity and the cell cycle are regulated by phosphorylation of serine and threonine residues (Potchinsky et al., 1997).

The requirement of phosphorylation on a threonine residue in the acute regulation of steroidogenesis in MA-10 mouse Leydig cell was studied by Stocco and Clark (1993). They used non-phosphorylatable analogs of the amino acids threonine and serine to determine the role of phosphorylation in the acute regulation of steroidogenesis. They have reported that substitution of the threonine analog into protein results in a inhibition
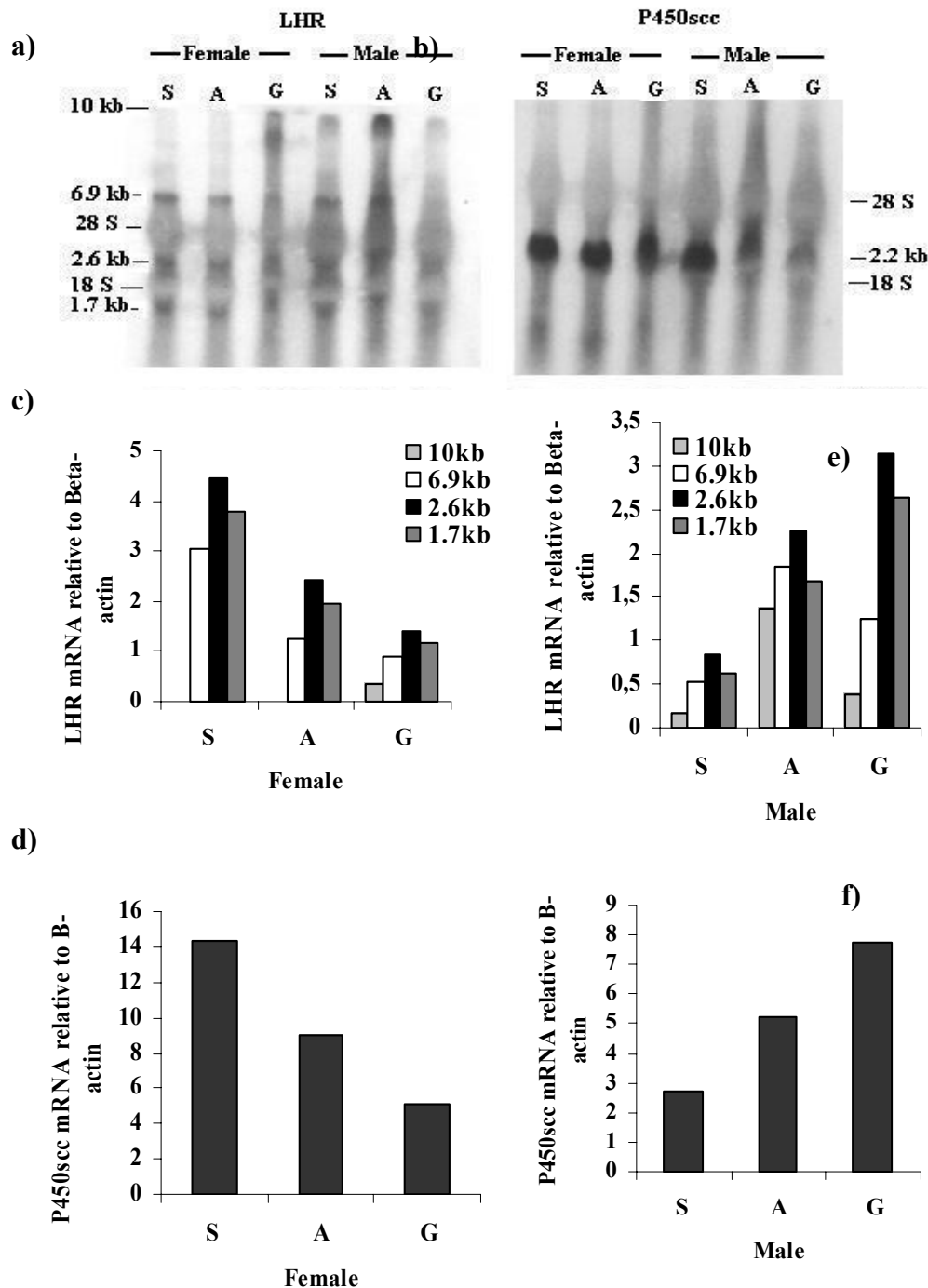
Fig. 1: Both Luteinizing Hormone Receptor (LHR) and stocrom P450scc mRNA are present in mouse testes and ovaries (a, b) Expression of mRNA for LHR was decreased in the ovaries of Serine/Threonine (A) and Glycine (G) injected female mice (c, d) while all were increased in the testis (e, f). There was only one type of mRNA for side chain cleavage enzyme in mice gonads. Its expression was decreased by amino acid (A or G) injection in female mice, while it was increased in male mice.

of hormone stimulated steroid production while none of the serine analogs employed displayed a similar inhibition. This shows the importance of serine and protein residues for phosphorylation and intacellular proteins taking role in cellular events.

The action of many transcription factors is regulated by phosphorylations (Jackson, 1992). Transcription factor Sp1 belongs to a specific subgroup of factors that are phosphorylated upon binding to promoter sequences. Finally, efficient phosphorylation of Sp1 requires both a functional DNA binding domain and a region containing the transcriptional activation domains (Jackson et al., 1990). It has been reported that phosphorylation of Sp1 mediated by the phosphatidylinositol 3-kinase (PI3K)/PKC pathway, which in turn causes the release of the p107 inhibitor from Sp1 and marked transcriptional activation of the LHR (Zang and Dufau, 2003).

The phosphorylation of serine-641 residue Sp1 protein by PI3K determines transcription of LHR receptor protein. Steroidogenic acute regulatory (StAR) protein is possessing multiple serine and threonine residues. Studies have identified two putative protein kinase (PKA) phosphorylation sites at Ser56/57 and Ser194/195, in murine and human StAR respectively. Phosphorylation of these residues increases function of StAR protein (Arakane et al., 1997; Manna et al., 2006).

The reason for the decrease in LHR and P450scc mRNA level in female mice might be result of changes in the function of kinase enzymes. Because kinase enzymes may mostly prefer free substrates (free serine and threonine) rather that serine and threonine residues in Sp1 and StAR proteins. Injection of serine and threonine caused increase LHR and P450scc mRNA level in male mice. This is because of sex difference. More work is required to see the changes in intracellular cAMP accumulation, PKA and PI3K expression after the serine and threonine injection. Injection of serine and threonine to mice may change the phosphorylation rate of intracellular proteins taking role in gene transcription and hormone secretion.

References

Abdennebi, L., Lesport, A.S., Remy, J.J., Grebert, D., Pisselet, C., Monniaux, D. and Salesse, R. 2002. Differences in splicing of mRNA encoding LH receptor intheca cells according to breeding season in ewes. *Reproduction*, 123: 819–826.

Alevizaki, M. and Huhtaniemi, I. 2002. Structure-function relationships of glycoprotein hormones; lessons from mutations and polymorphisms of the thyrotrophin and gonadotrophin subunit genes. *Hormones,* 1: 224-232.

Amsterdam, A. and Selvaraj, N. 1997. Control of differentiation, transformation, and apoptosis in granulosa cells by oncogenes, oncoviruses, and tumor suppressor genes. *Endocrine Reviews,* 18: 435-461.

Arakane, F., King, S.R., Du, Y., Kallen, C.B., Walsh, L.P., Watari, H., Stocco, D.M. and Strauss, J.F. 1997. Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. *Journal of Biological Chemistry,* 272: 32656-32662.

Bhattacharya, S., Banjerjee, A. and Manna, S.S. 1993. Human chorionic gonadotropin binding sites in the human endometrium. *Acta Endocrinologica,* 129:15-19.

Cagen, L.M. and Friedmann, H.C. 1972. Enzymatic Phosphorylation of Serine. *The Journal of Biological Chemistry,* 247: 3382-3392.

Cooke, B.A. 1999. Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis. *Molecular and Cellular Endocrinology*, 151: 25-35.

Derecka, K., Pietila, E.M., Rajaniemi, H.J. and Ziecik, A.J. 1995. Cycle dependent LH/hCG receptor gene expression in porcine nongonadal reproductive tissues. *Journal of Physiology and Pharmacology*, 46:77-85.

Dufau, M.L., Tsai-Morris, C.H., Hu, Z.Z. and Buczko, E. 1995. Structure and regulation of the luteinizing hormone receptor gene. *The Journal of Steroid Biochemistry and Molecular Biology*, 53: 283-291.

Friedman, S., Guevich, M. and Shemesh, M. 1995. Bovine cyclic endometrium contains high-affinity luteinizing hormone/human chorionic gonadotropin receptor. *Biology of Reproduction*, 52:1020-1026.

Gray, S.A. Manan, M.A. and O'Shaughnessy, P.J. 1996. Development of cytochrome P450 17α-hydroxylase (P450c17) mRNA and enzyme activity in neonatal ovaries of normal and hypogonadal (hpg) mice. *Journal of Molecular Endocrinology,* 17: 55-60.

Hai, M.V., De Roux, N., Ghinea, N., Beau, I., Loosfelt, H., Vannier, B., Meduri, G., Misrahi, M. and Milgrom, E. 1999. Gonadotropin receptors. *Annals of Endocrinology*, 60: 89-92.

Jackson, S.P.1992. Trends in Cell Biology. 2: 104-108.

Jackson, S.P., MacDonald, J.J., Lees-Miller, S. and Tjiean, R. 1990. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. *Cell,* 63: 155-165.

Jensen, J.D. and Odell, W.D. 1988. Identification of LH/hCG receptors in rabbit uterus. *Proceedings of The Society for Experimental Biology and Medicine,* 189:28-30.

Kash, J.C. and Menon, K.M.J. 1998. Identification of a hormonally regulated luteinizing hormone ∕ human chorionic gonadotropin receptor mRNA binding protein. Increased mRNA binding during receptor down-regulation. *The Journal of Biological Chemistry*, 273: 10658-10664.

Lauren, M.C. and Friedmann, H.C. 1971. Enzymic phosphorilation of serine. *The Journal of Biological Chemistry,* 247: 3382-3392.

Lu, D.L., Peegel, H., Mosier, S.M. and Menon, K.M.J. 1993. Loss of lutropin ∕ human choriogonadotropin receptor messenger ribonucleic acid during ligand-induced downregulation occurs post transcriptionally. *Endocrinology*, 132: 235-240.

Manna, P.R., Chandrala, S.P., King, S.R., Jo, Y., Counis, R., Huhtaniemi, I.T. and Stocco, D.M. 2006. Molecular Mechanisms of Insulin-like Growth Factor-I Mediated Regulation of the Steroidogenic Acute Regulatory Protein in Mouse Leydig Cells. *Molecu Endocrinology,* 20: 262-378.

McFarland, K.C., Spengel, R., Philips, H.S., Kohlet, M., Rosemblit, N., Nikolics, K., Segaloff, D.L. and Seeburg, P.H.1989. Lutropin–choriogonadotropin receptor an unusual member of the G protein-coupled receptor family. *Science*, 245: 494-499.

Minegishi, T., Delgado, C. and Dufau, M.I. 1989. Phosphorylation and glycosylation of the luteinizing hormone receptor. *Proceedings of the Nation Academy of Sciences, USA,* 86: 1470-1474.

Mukherjee, D., Manna, P.R. and Bhattacharya, S. 1994.Functional relevance of luteinizing hormone receptor in mouse uterus. *European Journal of Endocrinology,* 131:103-108.

Rennert H and Chang YJ, 1993. Intracellular cholesterol dynamics in sterodogenic cells. in: Adashi E.Y. (editor), The Ovary. Raven Press, New York, Pp: 147-164.

Segaloff, D.L. and Ascoli, M. 1993. The lutropin/choriogonadotropin receptor…4 years later. *Endocrine Reviews,* 14: 324-347.

Quintana, J., Hipkin, R.W., Sanchez-Yague, J. and Ascoli, M. 1994. Follitropin (FSH) and phorbol ester stimulate the phosphorylation of the FSH receptor in intact cells. *The Journal of Biological Chemistry,* 269: 8772-8779.

Sen, S. and Bhattacharya, S. 1991. Binding of radiolabeled luteinizing hormone to intact and ovariectomized rat uterus. *Indian Journal of Experimental Biology,* 30:972-976.

Strauss, J.F., Kallen, C.B., Christenson, L.K., Watari, H., Devoto, L., Arakane, F., Kiriakidou, M. and Sugawara, T. 1999. The steroidogenic acute regulatory protein (StAR): a window into the complexities of intracellular cholesterol trafficking. *Recent Progress in Hormone Res,* 54: 369-394.

Potchinsky, M.B., Weston, W.M., Lloyd, M.R. and Greene, R.M. 1997. TGF-β signalling in murine embryonic palate cells involves phosphorilation of CREB transcription factor. *Experimental Cell Research,* 231: 96-103.

Stocco, D.M. 2000. The role of the StAR protein in steroidogenesis: challenges for the future. *Journal of Endocrinology,* 64: 247-253.

Stocco, D.M., and Clark, B.J. 1996. Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews*, 17: 221-244.

Stocco, D.M. and Ckark, B.J., 1993. The requirement of phosphorylation on a threonine residue in the acute regulation of steroidogenesis in MA-10 mouse leydig cells. *The Journal of Steroid Biochemistry and Molecular Biology*, 46: 337-347.

Zhang, Y. and Dufau, M.L. 2003. Repression of the Luteinizing Hormone Receptor Gene Promoter by Cross Talk among EAR3/COUP-TFI, Sp1/Sp3, and TFIIB. *Molecular and Cellular Biology,* 23: 6958-6972.

Zeleznick, A.J., Midgley, A.R. and Reichert, L.E. 1974. Granulosa cell maturation in the rat increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. *Endocrinology,* 95: 818-825.