

Progesterone receptor-mediated effects on apoptosis in periovarian granulosa cells

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*Although much is known, much still remains
to be discovered, and much of what has been
discovered needs to be uncovered.*

R.V. Short
In *The ovary* (1977)

ABSTRACT

The most common fate of developing ovarian follicles is demise due to a process known as atresia. Regulation of atresia is dependent on the developmental stage of the follicles, resulting in a continuous reduction of the number of follicles as they differentiate and grow towards ovulation. The mechanism behind atresia of growing follicles is apoptosis of the granulosa cells. This thesis focuses on progesterone receptor (PR)-mediated regulation of granulosa cell apoptosis during the final phase of follicular development, the periovulatory interval.

By using two PR antagonists (RU 486 and the more specific Org 31710) we have shown that PR stimulation is important for the survival of periovulatory rat and human granulosa cells *in vitro*. PR regulated gene expression in rat periovulatory granulosa was characterised by microarray analysis, comparing the expression profiles after incubation *in vitro* with or without the addition of 10 μ M Org 31710. Close to 100 genes were found to be transcriptionally regulated in the presence of Org 31710. This included downregulation of several genes involved in cholesterol synthesis, and a decreased rate of cholesterol synthesis was verified by measuring the incorporation of 14 C-acetate into cholesterol, cholesterol ester and progesterone. Based on this we investigated the granulosa cell dependence on cholesterol synthesis and in particular the branch-point reactions supplying cells with prenylation substrates for post-translational lipid modification of proteins. Blocking the cholesterol synthesis with statins increased apoptosis, as did inhibitors of prenyl transferases. The increase in apoptosis after treatment with statins or PR antagonists was partially reversed by the addition of substrates for prenylation.

In conclusion, PR stimulation is important for the survival of periovulatory granulosa cells in both rats and humans. PR stimulation regulates the transcription of several groups of genes including cholesterol synthesis. The cholesterol synthesis also provides the cells with substrates for protein prenylation, which may be one of the factors regulating granulosa cell survival in periovulatory follicles.

LIST OF PUBLICATIONS

This thesis is based upon the following papers, which will be referred to in the text by their Roman numerals:

- I Progesterone receptor-mediated inhibition of apoptosis in granulosa cells isolated from rats treated with human Chorionic Gonadotropin.
Svensson ECh, Markström E, Andersson M and Billig H.
Biology of Reproduction (2000) 63: 1457-64
- II Progesterone receptor antagonists Org 31710 and RU 486 increase apoptosis in human periovulatory granulosa cells.
Svensson ECh, Markström E, Shao R, Andersson M and Billig H.
Fertility and Sterility (2001) 76: 1225-31
- III Progesterone receptor antagonists and statins decrease de novo cholesterol synthesis and increase apoptosis in rat and human periovulatory granulosa cells in vitro.
Rung E, Friberg PA, Shao R, Larsson DGJ, Nielsen ECh, Svensson PA, Carlsson B, Carlsson LMS and Billig H.
Biology of Reproduction (2005) 72: 538-45
- IV Depletion of substrates for protein prenylation increases apoptosis in human periovulatory granulosa cells.
Rung E, Friberg PA, Bergh C and Billig H.
Molecular Reproduction and Development (2006) 73: 1277-83

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ABBREVIATIONS

ADAMTS-1	a disintegrin and metalloproteinase with thrombospondin motifs-1
AR	androgen receptor
CAD	caspase-activated DNase
DBD	DNA-binding domain
dpm	decays per minute
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
EST	expressed sequence tag
FasL	Fas ligand
FGF	fibroblast growth factor
FOH	farnesol
FSH	follicle stimulating hormone
FTase	farnesyl transferase
FTI	farnesyl transferase inhibitor
GABA	gamma aminobutyric acid
GGOH	geranylgeraniol
GGTase	geranylgeranyl transferase
GGTI	geranylgeranyl transferase inhibitor
GnRH	gonadotropin-releasing hormone
GPI	glycosyl phosphatidylinositol
GR	glucocorticoid receptor
GTP	guanosine triphosphate
hCG	human chorionic gonadotropin
HDL	high density lipoprotein
HMG-CoA	hydroxymethylglutaryl co-enzyme A
ICAD	inhibitor of caspase-activated DNase
IGF-I	insulin-like growth factor I
IL	interleukin
IVF	in vitro fertilisation
KGF	keratinocyte growth factor
LBD	ligand-binding domain
LDL	low density lipoprotein
LH	luteinising hormone
MAS	meiosis activating sterol
MMP	matrix metalloproteinase
mPR	membrane progesterin receptor
PAC ₁	pituitary adenylate cyclase activating polypeptide receptor type 1
PACAP	pituitary adenylate cyclase activating polypeptide
PCR	polymerase chain reaction
PGRMC1	progesterone receptor membrane component 1
PMSG	pregnant mare serum gonadotropins
PR	progesterone receptor
PR-A	progesterone receptor isoform A
PRAKO	progesterone receptor isoform A knockout
PR-B	progesterone receptor isoform B

ABBREVIATIONS

PRBKO	progesterone receptor isoform B knockout
PRKO	progesterone receptor knockout
RLU	relative luminescence units
RPA	ribonuclease protection assay
RU 486	Roussel-Uclaf 38486; generic name mifepristone
SERBP1	serpine 1 mRNA binding protein
SRE	steroid response element
SREBP	sterol response element binding protein
StAR	steroidogenic acute regulatory protein
TLC	thin layer chromatography
TNF α	tumour necrosis factor α

POPULÄRVETENSKAPLIG SAMMANFATTNING

Äggstockarna har två huvuduppgifter: 1) att producera mogna, befruktningsbara ägg och 2) att frisätta könshormoner. I äggstockarna hos flickfoster bildas miljontals äggblåsor som består av ett centralt placerat ägg omgivet av stödjeceller. Av dessa äggblåsor kommer en bråkdel (hos människa maximalt ca 400 st) att nå hela vägen fram till ägglossning. Majoriteten av äggblåsorna tillbakabildas istället i en process som kallas atresi. Detta sker såväl under fosterlivet och barndomen som under den fertila delen av en kvinnas liv.

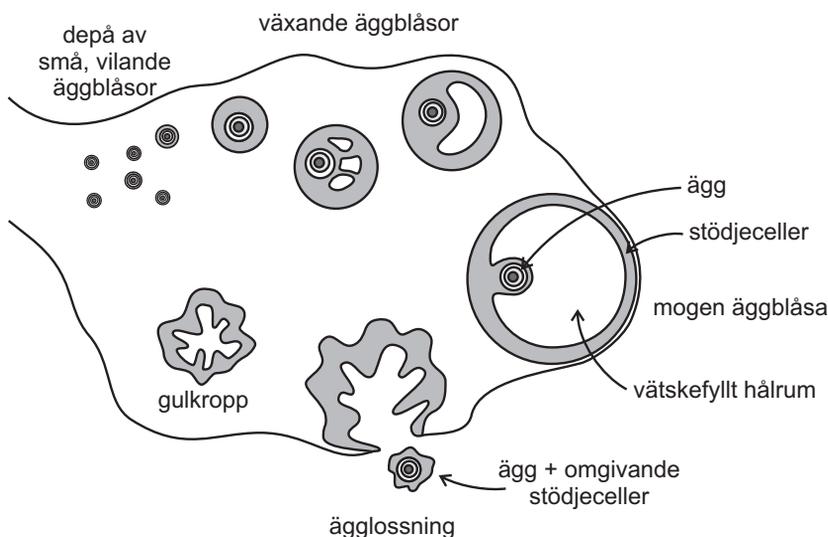


Illustration 1

Schematisk bild av en äggstock med äggblåsor i olika utvecklingsstadium.

Den bakomliggande mekanismen för atresi av växande äggblåsor är apoptos, även kallat programmerad celldöd, av äggblåsans stödjeceller. Apoptosen utförs av proteiner som redan finns färdiga i alla kroppens celler, därav begreppet programmerad celldöd. De olika stegen under apoptos åskådliggörs i illustration 2. Processen kan delas upp i tre steg: 1) beslut att dö, 2) genomförande av "självord", d.v.s. apoptos, och 3) avlägsnande av den döda cellen genom att den äts upp (fagocyteras) av andra celler. Jag har studerat vilka faktorer som avgör om äggblåsornas stödjeceller genomgår apoptos eller inte. Huvudsakligen har jag arbetat med isolerade stödjeceller som odlats i provrör.

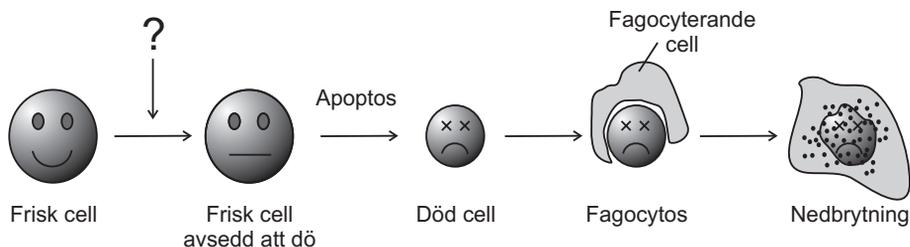


Illustration 2

Schematisk översikt över apoptos (programmerad celldöd).

Tillsammans med mina medarbetare i forskargruppen har jag kommit fram till att det kvinnliga könshormonet progesteron är viktigt för överlevnaden av de äggblåsor som är redo att genomgå ägglossning hos såväl råttor (arbete I) som människor (arbete II). Våra viktigaste verktyg i dessa studier var progesteronblockerare. Exempelvis använde vi substansen RU 486 (Mifegyne®) som i Sverige används som medicinsk alternativ till kirurgisk abort.

För att kartlägga förloppet har vi studerat vilka gener som påverkas av progesteron (arbete III). Detta har vi gjort med hjälp av microarray, en metod som ger möjlighet att samtidigt övervaka aktiviteten av tusentals gener. Progesteron påverkade aktiviteten av flera grupper av funktionellt sammanhörande gener. Baserat på resultatet av microarray-studien valde vi att studera sambandet mellan bildandet av kolesterol och cellöverlevnad. Det verkade vara en möjlighet att progesteron ökar kolesterolbildningen, vilket i sin tur indirekt påverkar stödjecellernas överlevnad.

I arbete IV försökte vi beskriva sambandet mellan bildandet av kolesterol och apoptos. För att blockera kolesterolbildningen använde vi statiner, en vanlig typ av läkemedel vid behandling av förhöjda blodfetter. Statinerna (t.ex. simvastatin/Zocord®) ökade apoptosen i äggblåsornas stödjeceller från råttor och människor. Den vetenskapliga litteraturen visar att kolesterolsyntesen även är viktig för produktion av vissa fetter som sätts fast på proteiner. Dessa små ”fettsvansar” hjälper proteiner att ankra till cellens membran och är därför viktiga för proteiners lokalisering inuti celler och för deras funktion. Våra resultat tyder på att denna process, som kallas prenylering, är betydelsefull för överlevnad av stödjecellerna och därmed äggblåsorna vid tiden för ägglossningen.

Det övergripande syftet med våra studier är att kartlägga regleringen av äggblåsornas överlevnad. Möjliga applikationer av resultaten finns såväl för framställande av nya preventivmedel som för behandling av vissa typer av ofrivillig barnlöshet.

INTRODUCTION

The mammalian ovary has two major functions: 1) the timely delivery of mature, fertilisable oocytes and 2) production of steroid hormones. The ovaries thus harbour, nurture and guide the development of the oocytes so that upon ovulation they are prepared for migration down the fallopian tube, fertilisation and eventual implantation in the uterus. Additionally, the ovaries secrete hormones necessary for transformation of the body from a prepubertal to a mature physique and for the onset of menstruation and its cyclic continuance.

Follicular development

During early foetal life, primordial germ cells migrate to the gonadal ridges where they proliferate and subsequently enter meiosis. As the oocytes commit to meiosis, they lose the capacity to proliferate. The oocytes arrest during meiosis and become surrounded by epithelial granulosa cells, forming a vast number of primordial follicles.

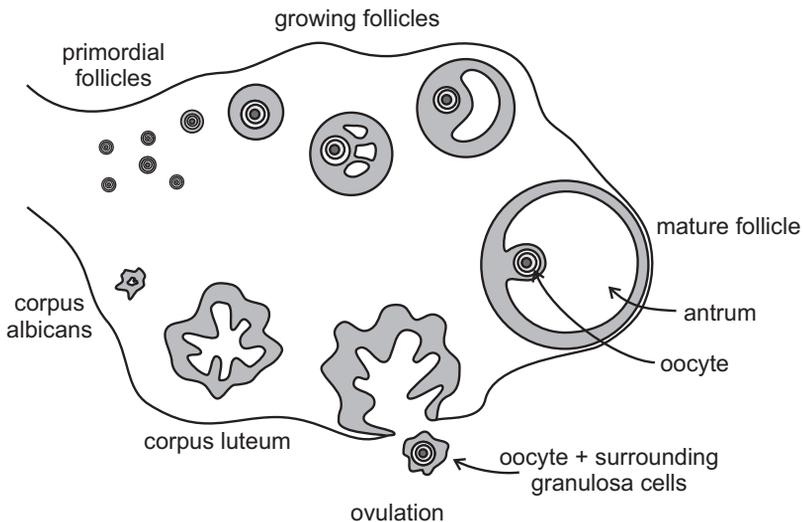


Figure 1
Schematic drawing of an ovary with follicles at different stages of development.

Folliculogenesis is the progressive development of the follicles during their growth towards ovulation and has recently been the subject of several comprehensive reviews (see Gougeon, 1996, Baird & Mitchell, 2002, Gougeon, 2004). Folliculogenesis is a long process that commences about a year before ovulation in humans.

The adult cycling ovary thus simultaneously contains follicles at various stages of development (Figure 1). The follicular development can be roughly divided into three stages: 1) initiation of growth of resting primordial follicles, 2) early follicle growth (preantral) and 3) selection and maturation.

The ovarian reserve of oocytes is deposited as resting primordial follicles which have not yet started to grow. These follicles consist of a central oocyte which is arrested in meiosis, a single layer of squamose granulosa cells and a thin basal lamina. The first sign of growth in resting follicles is the change of granulosa cell shape from squamose to cuboidal and onset of very slow proliferation. During or immediately after these events, the oocyte is activated and starts to enlarge. A follicle consisting of a growing oocyte and a single layer of cuboidal granulosa cells surrounded by a basal lamina is termed primary follicle. Primary follicles begin to express follicle stimulating hormone (FSH) receptors in the granulosa cell layer and gap junctions are formed between the oocyte and the surrounding granulosa cells as well as between individual granulosa cells. The oocyte markedly increases in size and starts to secrete an extracellular matrix, forming the zona pellucida. A secondary follicle consists of an almost fully grown oocyte with a practically complete zona pellucida and two to eight layers of granulosa cells. A theca cell layer now forms around the basal lamina. In addition, secondary follicles are connected to the vascular system and are thus exposed to the female endocrine environment for the first time. The follicle is termed tertiary when the oocyte has reached its full size and small fluid-filled cavities start to form within the granulosa cell layer. The theca cells now constitute two functionally different layers; the theca externa, which is a cell layer resembling smooth muscle, and the interstitial theca cells, which start expressing luteinising hormone (LH) receptors. This is the end of the slow process of early follicle growth.

The appearance of a fluid-filled antrum renders the follicle the name antral follicle. Each antral follicle is composed of several precisely positioned cell layers as outlined in figure 2. Antral follicles are characterized by a continuous rapid proliferation of granulosa cells and the follicles rapidly increase in size as ovulation draws nearer. Unlike earlier developmental stages, the follicles are now dependent on gonadotropin stimulation for their continued growth and development.

It has been an established dogma that the oocytes in the ovaries constitute a finite pool of female germ cells and thus limit reproductive capacity. As the primordial follicles are recruited into growth and development, the pool of oocytes is drained and ultimately emptied, leading to the onset of menopause and the end of the fertile period. However, this paradigm was recently challenged by a group of scientists, who claimed that renewal of oocytes in the adult mammalian ovary is a possibility (Johnson *et al.*, 2004a, 2005a, 2005b). This has, of course, attracted a great deal of

attention as well as criticism (Albertini, 2004, Gosden, 2004, Greenfield & Flaws, 2004, Byskov *et al.*, 2005, Telfer *et al.*, 2005).

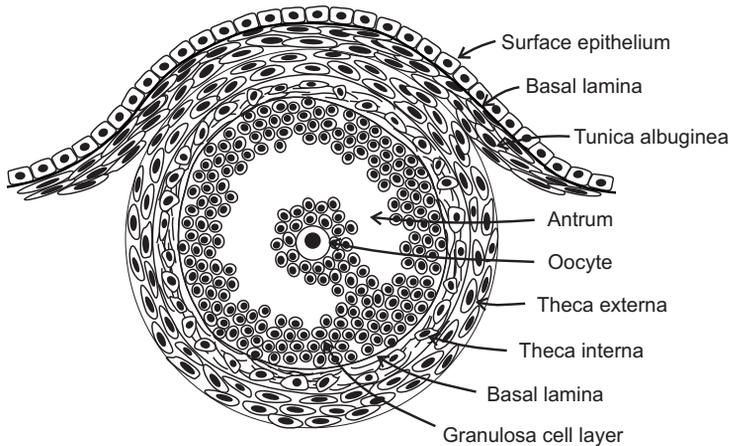


Figure 2

Schematic drawing of an antral follicle protruding towards the ovarian surface. Antral follicles are surrounded by innervated smooth muscle cells, known as the theca externa. The theca interna is composed of several cell layers and has a large capillary network. The theca layers are separated from the granulosa cells by a basal lamina, which functions as a barrier to the vascular tissue. The basal lamina surrounds granulosa cells and the oocyte, as well as the antrum.

Regulation of follicular development

Growth initiation is poorly understood and may be controlled by growth inhibitor(s), growth stimulator(s) or a combination of both. The studies on growth initiation are hampered by the fact that the early growing follicles are difficult to distinguish from resting primordial follicles and that early growth is a very slow, protracted process. Continued early growth is generally considered to be independent of stimulation by pituitary gonadotropins (FSH and LH). However, antral follicles develop an indispensable need for FSH stimulation. During each reproductive cycle, increased FSH levels lead to the recruitment of a cohort of antral follicles to participate in the run-up to ovulation of one or a few oocytes. It has been suggested that this process should be termed cyclic recruitment (McGee & Hsueh, 2000), as opposed to the initial recruitment of resting primordial follicles.

Ovulation and luteinisation

The ultimate goal of folliculogenesis is ovulation and luteinisation where the oocyte is extruded from the follicle and the remaining granulosa and theca cells transform into the corpus luteum. In mammals the ovulatory process begins with a surge of LH secreted from the pituitary and ends with follicular rupture. This periovulatory phase

lasts 36-38 hours in humans (Hanna *et al.*, 1994, Andersen *et al.*, 1995) and 12-15 hours in rodents (Tsafiriri & Kraicer, 1972). For more than 100 years, increased intrafollicular pressure was believed to be the cause of follicular rupture. Today, it has been established that intrafollicular pressure does not increase significantly before rupture. The current hypothesis is that LH initiates an acute inflammatory process accompanied by protease activity, thus leading to degradation of the connective tissue elements in the follicle wall (Espey & Richards, 2006).

Activity of several different proteases weakens the follicular wall until it eventually ruptures at the weakest point, the apex. Several families of proteases, including matrix metalloproteinases, ADAMTS-1 and serine proteinases such as plasmin and plasminogen activator, have been suggested to be involved in the degradation of the extracellular matrix in the follicle wall. However, the redundancy seems to be large, since disruption of single proteases in knock-out mice rarely affects ovulation (Ohnishi *et al.*, 2005).

Having nurtured the oocyte up to ovulation the follicle faces an important new task, namely transformation into corpus luteum and onset of progesterone production, which is essential for pregnancy. At this stage both granulosa and theca cells express LH receptors and are capable of responding to this hormonal stimulus triggering luteinisation. One prominent feature is increased vascularisation (Fraser, 2006). The vascular system is generally quiescent in adults, except during wound healing and some pathological conditions. The cyclic changes in angiogenesis in the ovaries are a unique process, which is regulated independently within individual follicles. The dominant follicle has high vascularity and flow velocity just before ovulation. In order to shift from oestrogen synthesis to large-scale progesterone synthesis there are changes in proteins that provide cholesterol as well as proteins involved in steroid synthesis (see Murphy, 2004).

Atresia

The path from primordial follicle to ovulation outlined above is not completed for the majority of the ovarian follicles. Instead, a degeneration process termed atresia is the most common outcome. During the reproductive period of a woman's life, a maximum of 400 follicles can be ovulated. However, at the 30th gestational week about 6 million follicles are present in the foetal ovary, at birth the number has declined to 2 million while at the onset of puberty they number 400,000 (Baker, 1963). At the end of the reproductive period the pool of functional follicles is empty. This means that more than 99.9% of the follicles are depleted by atresia. Atresia occurs at all stages of follicle development resulting in a continuous depletion of follicles (Gougeon, 1996). It has been shown that atresia is caused by apoptosis (Tilly *et al.*, 1991, Hsueh *et al.*, 1994), a form of programmed cell death.

Apoptosis

Apoptosis is a mechanism that allows cells to self-destruct and operates at all developmental stages and in all cell types (Raff, 1992). Apoptosis can be initiated for several reasons, such as when a cell is no longer needed or when it becomes a threat to the health of the organism. Controlled removal of cells is necessary in embryonic development as well as in the daily maintenance of a mature organism. For instance it has been calculated that, if cell proliferation was not balanced by apoptosis an 80-year-old person would have 2 tons of bone marrow and lymph nodes, and a 16 km long gut (Melino, 2001). The term apoptosis was suggested in 1972 by Kerr, Wyllie and Currie (Kerr *et al.*, 1972) to describe this natural and timely cell death. It is derived from a Greek word meaning “falling off” in the sense of leaves falling off the trees in autumn.

Apoptotic death requires co-ordinated activation and propagation of several sub-programs (Hengartner, 2000). The death mechanism is the same in all tissues, although the factors that trigger apoptosis appear to be tissue specific (Steller & Grether, 1994, Fraser & Evan, 1996, White, 1996). Two groups of proteins constitute the framework of the apoptotic program: the caspase family of proteases and the Bcl-2 family of regulatory proteins (Figure 3).

Caspases are the executioners of the apoptotic pathway (Hengartner, 2000) and function as proteases cleaving target proteins after an aspartic acid residue. There are at least three mechanisms for caspase activation, including 1) processing by an upstream caspase, 2) association with cofactors and 3) cleavage induced by a high local concentration of caspases, associated with activated death receptors (Figure 3). More than 100 proteins have been identified as caspase substrates, including lamins, causing nuclear shrinking and budding, and cytoskeletal proteins such as fodrin and gelsolin, which lead to the loss of overall cell shape (Hengartner, 2000). One function of caspases is to activate the endonuclease CAD (Caspase-Activated DNase). CAD and its inhibitory subunit ICAD are constantly expressed in the cells. Caspase-mediated cleavage of the inhibitory subunit results in release and activation of the endonuclease. The resulting internucleosomal DNA fragmentation is one of the classical hallmarks used for apoptosis detection.

The Bcl-2 family of apoptotic regulators comprises both anti- and pro-apoptotic proteins. Members of the Bcl-2 family can homodimerise or heterodimerise with other family members, thereby regulating each other's activity. The main function of the Bcl-2 family seems to be to regulate the release of pro-apoptotic factors, in particular cytochrome *c*, from mitochondria into the cytosol (Antonsson & Martinou, 2000). Many members of the Bcl-2 family have been isolated in the ovary, including BAD, Mcl-1 and Bok (Bae *et al.*, 2000).

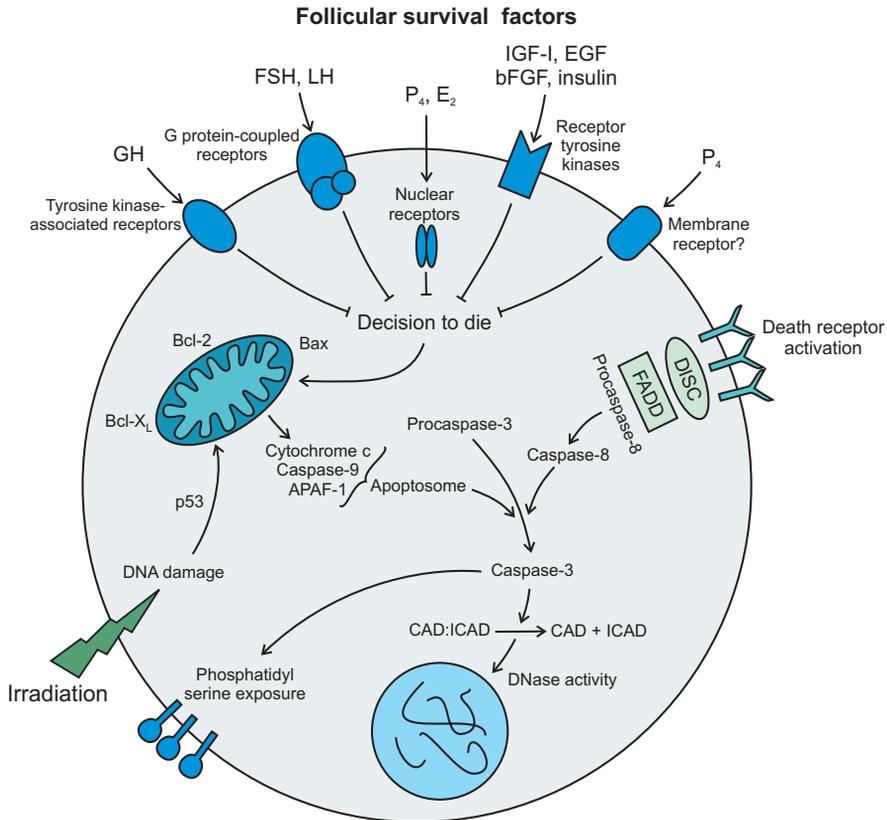


Figure 3

Schematic overview of the apoptotic process in follicular granulosa cells. A range of hormones and locally produced factors regulates the "decision to die" by means of their receptors. Examples of survival factors include the gonadotropins follicle stimulating hormone (FSH) and luteinising hormone (LH), the steroids progesterone (P_4) and oestrogen (E_2), as well as growth hormone (GH), insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin. As outlined in the figure the signalling of these factors is mediated by a range of different receptor types.

Execution of the apoptotic program converges on the mitochondria, where pro- and anti-apoptotic members of the Bcl-2 family interact at the surface. Excess of pro-apoptotic activity will cause the release of an array of molecules from the mitochondrial compartment. The most important of these is cytochrome c, which associates with APAF-1 and procaspase-9 to form the apoptosome complex. This complex subsequently activates downstream caspases, such as caspase-3. Downstream of caspase-3, the apoptotic program branches off into a multitude of subprograms, one of which is exposure of phosphatidyl serine on the cell surface. Another effect of caspase activation is cleavage of ICAD (Inhibitor of Caspase-Activate DNase) resulting in the release of CAD (Caspase-Activated DNase), the endonuclease responsible for internucleosomal DNA fragmentation.

Two other apoptosis induction pathways are also represented in the figure. One is DNA damage caused by, for instance, irradiation, which initiates the apoptotic process by way of p53. The second is the death receptor pathway, which has mainly been characterised in immune cells. Binding of ligand to these receptors (e.g. CD95/Fas, tumour necrosis factor receptor I) causes receptor clustering and the formation of a death inducing signalling complex (DISC). This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple copies of procaspase-8, resulting in caspase-8 activation.

Modified from Markström *et al.*, (2002).

INTRODUCTION

Mitochondria are crucial structures in the apoptotic machinery since they harbour several pro-apoptotic factors. When the apoptotic machinery is set in motion, these factors are released to the cytoplasm (Figure 3). One of the factors is cytochrome *c*, which is required for activation of caspase-9 in the cytosol. The question of how cytochrome *c* manages to cross the mitochondrial outer membrane is now beginning to be resolved, and the Bcl-2 family is closely involved in pore-formation for release of apoptotic factors (Armstrong, 2006). In addition to cytochrome *c*, other pro-apoptotic factors are stored in the mitochondria and released upon apoptosis induction. Examples include the apoptosis inducing factor (AIF) and several pro-caspases.

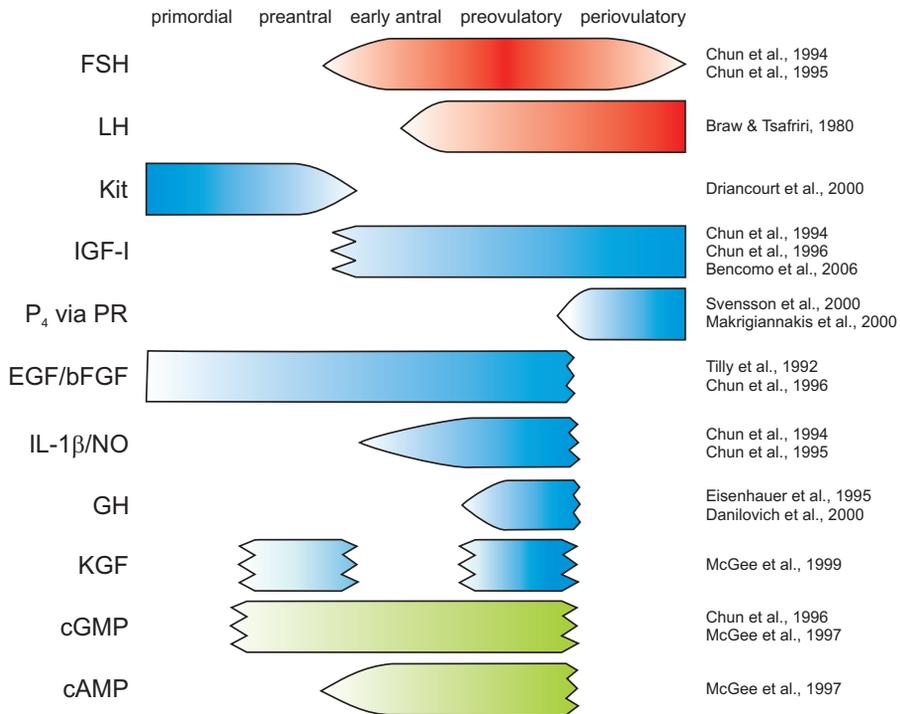


Figure 4

Examples of factors that regulate the stage-dependent survival of ovarian follicles. From top to bottom: gonadotropins (red) followed by other survival factors (blue) and intracellular mediators (green). Jagged ends indicate that studies dealing with earlier/later developmental stages have not been carried out. Examples of relevant references are provided to the right. Modified from Markström *et al.*, (2002).

Finally, the remains of the cell have to be removed by neighbouring or professional phagocytosing cells. On a molecular level, exposure of phosphatidyl serine on the outer side of the cell membrane is one signalling mechanism that occurs in apoptotic cells

(Figure 3). In the ovary, scavenger receptor class B type I on theca cells has been shown to mediate recognition and binding of apoptotic granulosa cells exposing phosphatidyl serine on the cell surface (Svensson *et al.*, 1999).

Regulation of ovarian apoptosis

The susceptibility to apoptosis, as well as the regulators of follicle survival, changes in the course of follicle development. This results in a gradual reduction of the number of follicles as they differentiate and grow towards ovulation. For a detailed review see Markström *et al.* (2002). The main physiological regulators of ovarian follicle survival are the gonadotropins, although a number of locally produced growth factors have also been demonstrated to affect follicle cell survival. Examples of survival factors include oestrogens, insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), fibroblast growth factor (FGF), interleukin-1 β (IL-1 β) and nitric oxide. Pro-apoptotic factors include androgens, gonadotropin-releasing hormone-like peptide (GnRH-like peptide) and interleukin-6 (IL-6) (Billig *et al.*, 1996). In order to understand the well-tuned balance between follicle growth and atresia, it is necessary to acknowledge the differentiation dependent regulation. Some of the factors promoting survival during the growth and differentiation of follicles are summarised in figure 4.

In primordial follicles, oocyte apoptosis is probably responsible for subsequent follicular degeneration. The phenomenon of oocyte apoptosis has recently been reviewed (Morita & Tilly, 1999, Reynaud & Driancourt, 2000), describing the importance of, for instance, Kit-Kit ligand interaction and the growth factors EGF and bFGF in rodents.

In growing ovarian follicles, apoptosis is mainly confined to somatic granulosa cells. Relatively little is known about the regulation of survival of preantral follicles in rodents and humans compared to later stages of development. Although FSH has the ability to enhance steroidogenic enzyme expression in preantral follicles (Dunkel *et al.*, 1994, Rannikki *et al.*, 1995) it has no effect on apoptosis in cultured rat follicles (McGee *et al.*, 1997). Locally produced survival factors include the keratinocyte growth factor (KGF), FGF and oestrogens (Billig *et al.*, 1993, McGee *et al.*, 1999).

Follicles that have differentiated to the antral stage or further express FSH receptors and are dependent on sufficient FSH concentrations for survival. Due to lack of FSH support, many follicles never pass this point in their development (see Hirshfield, 1991). In the adult human ovary, it has been suggested that the degree of atresia is highest in antral follicles of a diameter greater than 5 mm, 77% of which have been estimated to undergo atresia (Gougeon, 1986). During each reproductive cycle, increasing FSH concentrations rescue a cohort of developing follicles. Locally produced factors of importance for the survival of isolated early antral rat follicles include IGF-I, EGF, FGF, activin, and the cytokine IL-1 β (Chun *et al.*, 1996a). As in

preantral follicles, oestrogens have been shown to be of importance for the survival of early antral follicles in vivo in rats (Billig *et al.*, 1993). However, there is no report of oestrogens affecting the survival of isolated granulosa cells.

At the preovulatory stage of development, the ovarian follicles express LH receptors and are able to respond to the coming LH surge. Both FSH and LH suppress the degree of apoptosis in isolated preovulatory rat granulosa cells in a way that may be partially mediated by endogenously produced IGF-I (Chun *et al.*, 1994). Preovulatory follicles responding to the ovulatory LH surge seem to be rescued from the apoptotic pathway, since the number of corpora lutea roughly equals the number of preovulatory follicles. Blockage of the LH surge by removal of the pituitary (hypophysectomy) or by pentobarbital treatment results in massive atresia in rats (Talbert *et al.*, 1951, Braw & Tsafiriri, 1980). Recently, IGF-I has also been shown to be a survival factor after the LH surge in periovulatory follicles (Bencomo *et al.*, 2006).

Progesterone

The steroid hormone progesterone is a key component in the regulation of female reproduction and targets several organs. Its name derives from its vital supportive role during gestation. The word progesterone is etymologically related to the Latin root *gestare* - meaning to bear or carry - indicating the importance of this hormone in creating a fertile environment for conception and the continuing development of the embryo. The main functions of progesterone are 1) in the ovaries and uterus: ovulation, facilitation of implantation and maintenance of pregnancy by promoting uterine growth and suppressing myometrial contractility; 2) in the mammary glands: development of the ducts during pregnancy and suppression of milk protein synthesis before parturition; and 3) in the brain: mediation of signals for sexually responsive behaviour (Graham & Clarke, 1997). The main production site for progesterone is the corpus luteum and, during pregnancy, the placenta. Isolation and purification of progesterone was first reported in 1934 by four independent groups (Butenandt & Westphal, 1934, Hartmann & Wettstein, 1934, Slotta *et al.*, 1934, Wintersteiner & Allen, 1934).

Like all steroid hormones, progesterone is synthesised from cholesterol. The possible sources are cholesterol derived from the circulation, intracellularly stored cholesterol esters and *de novo* synthesis (Figure 5). Although the source of cholesterol for ovarian progesterone production has still not been fully elucidated, it is generally accepted that the major part is derived from circulating lipoproteins. The preference for different lipoproteins varies between species. Mice, rats and ruminants primarily utilise high density lipoprotein (HDL), whereas humans, rhesus macaques and pigs use low density lipoprotein (LDL) (see Christenson & Devoto, 2003).

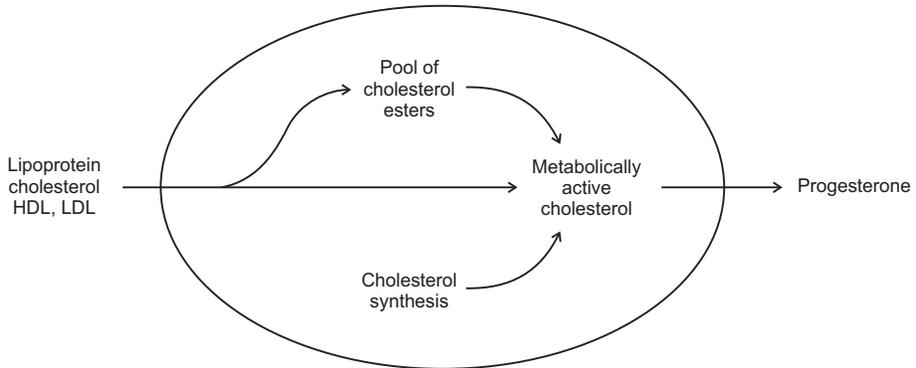


Figure 5

Schematic overview of the sources of cholesterol for progesterone production in a granulosa cell.

Synthesis of progesterone from cholesterol requires only two enzymatic conversions: 1) the conversion of cholesterol to pregnenolone by the enzyme P450 side chain cleavage and 2) conversion of pregnenolone to progesterone, catalysed by 3β -hydroxysteroid dehydrogenase. The first of these steps takes place at the inner mitochondrial membrane and the second at the smooth endoplasmic reticulum. Transport of free cholesterol to the inner mitochondrial membrane is the most regulated and rate limiting step in progesterone synthesis. Steroid acute regulatory protein (StAR) transports cholesterol from the cytoplasm to the mitochondria and peripheral-type benzodiazepine receptors are involved in transport from the outer to the inner mitochondrial membrane (see Niswender, 2002).

Just prior to ovulation progesterone synthesis is induced in luteinising follicles by LH stimulation. Increased progesterone serum levels can be observed a mere 30 minutes after the LH surge or hCG administration in rhesus macaques (Chaffin *et al.*, 1999a). This rapid response suggests that the enzymes necessary for progesterone synthesis are already present in the cells. The early increase in progesterone production is probably achieved by theca cells, since the enzymatic machinery is absent in granulosa cells in several species and the availability of LDL/HDL for granulosa cells is low as a consequence of limited vascularisation. Maximal progesterone secretion is reached several days after ovulation in humans, when the vascular network of the corpus luteum is fully developed. For recent reviews of corpus luteum progesterone synthesis, see Niswender (2002) and Christenson & Devoto (2003).

The progesterone receptor

Progesterone exerts its functions by interacting with a specific nuclear progesterone receptor (PR) protein (official nomenclature NR3C3 (Nuclear Receptors Committee, 1999)). The PR belongs to the superfamily of nuclear receptors. All members share structural domain organisation with a highly conserved DNA-binding domain

(DBD), a C-terminal ligand-binding domain (LBD) and a more variable N-terminal domain. The steroid receptors contain at least two transcription activation function domains that are important for interaction with coactivators. Upon ligand binding the receptor-ligand complex translocates to the nucleus and functions as a transcription factor, thereby regulating the expression of target genes. The receptor-mediated action of progesterone has been extensively characterised by O'Malley and colleagues (O'Malley *et al.*, 1991, Li & O'Malley, 2003).

Upon progesterone binding the PR undergoes conformational changes, causing dissociation from the multi-protein chaperone, dimerisation, increased phosphorylation, and binding to steroid response elements (SREs) within target gene promoters (see Gronemeyer, 1991, Tsai & O'Malley, 1994) as outlined in figure 6. The consensus sequence of the response element is 5'-TGTTCT-3', a semi-palindromic half-site usually separated by three base pairs. As each individual response element is weak, there are usually several SREs near the regulatory region of steroid controlled genes. Active PR interacts with coactivators that facilitate transcription in two ways; by interacting with the general transcription machinery and by promoting local chromatin remodelling (Edwards *et al.*, 2002).

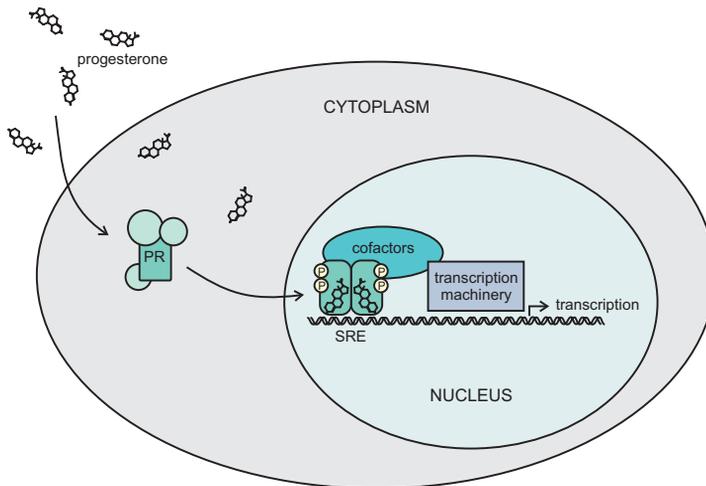


Figure 6

Schematic drawing of progesterone receptor activation and transcriptional regulation. Abbreviations: PR, progesterone receptor; SRE, steroid response element; P, phosphorylation.

A bewildering aspect of PR induced gene transcription is the fact that the PR response element in the promoters of regulated genes is identical to those of the glucocorticoid, androgen, and mineralocorticoid receptors (see Geserick *et al.*, 2005). Nevertheless, the different steroid hormone receptors exhibit distinct effects on gene expression within the same cell. Both the ligand and the SRE induce conformational

changes of the receptor, which affect interaction with different cofactors. Together the many liganded receptors bound to SREs within the regulatory region of a gene create a surface for interaction with cofactors and the transcription machinery. Fine-tuning of the interactions between steroid receptors and a multitude of different cofactors may form the basis for selective control of gene expression.

PR isoforms

In several vertebrate species, including humans (Horwitz, 1992, McDonnell, 1995), monkeys (Bethea & Widmann, 1998), rodents (Shyamala *et al.*, 1990) and chicken (Schrader & O'Malley, 1972), the PR is expressed in two isoforms, PR-A and PR-B. The human PR gene was sequenced in 1987 (Misrahi *et al.*). The two different isoforms (Figure 7) arise from the same gene and differ as a result of alternate transcriptional initiation (Kastner *et al.*, 1990, Kraus *et al.*, 1993). This is in contrast to e.g. the two oestrogen receptor isoforms, which arise from two different genes (Kuiper & Gustafsson, 1997).

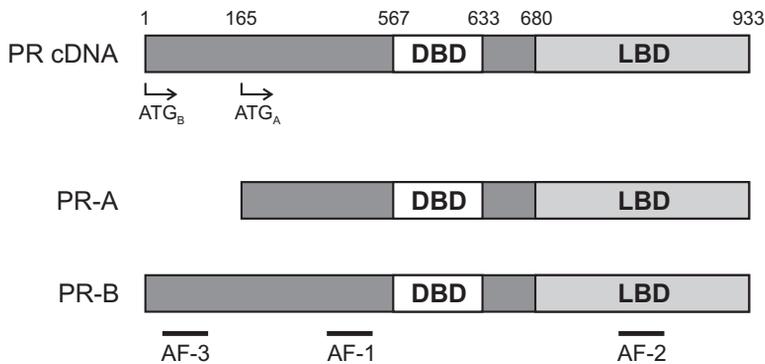


Figure 7

Schematic overview of the human progesterone receptor (PR) isoforms. The top part of the figure represents the PR cDNA and outlines the transcription initiation sites for PR-B and PR-A at codon 1 and 165, respectively. The numbers above indicate the codon number. The bottom part of the figure illustrates the two isoforms and the location of the DNA-binding domain (DBD), ligand-binding domain (LBD) as well as the three activation functions (AF).

The two PR isoforms exhibit quite different transcriptional activities, dependent on both target gene promoter and cell type. PR-B has been shown to function as a transcriptional activator of several PR-dependent promoters and in a variety of cell lines in which PR-A is inactive. In addition, PR-A can function as a dominant inhibitor of PR-B and other nuclear steroid hormone receptors in contexts where PR-A is inactive. It may thus facilitate cross-talk between different steroid receptor signalling pathways in the cell (see Graham & Clarke, 2002). The PR-B isoform

contains an additional amino-terminal sequence, which in humans consists of 164 amino acids. The PR-B unique region contains a third activation function domain, which probably accounts for at least part of the difference in transcriptional activity between the A and B isoforms. The ratio of PR-A / PR-B determines the cellular response to progesterone (see Graham & Clarke, 2002).

In addition, several other truncated or splice variants of PR have been described (Wei & Miner, 1994, Misao *et al.*, 1998, Hodges *et al.*, 1999, Hirata *et al.*, 2000, Misao *et al.*, 2000, Hirata *et al.*, 2002). Variants lacking the DNA-binding domain or the ligand-binding domain can modify the effects of the full-length PR-B.

Physiological roles of PR-A and PR-B

Both PR-A and PR-B have important and distinct physiological roles *in vivo*. Selective ablation of PR-A (PR-A knockout mice, PRAKO mice) shows normal mammary gland development, but defects in the uterus and ovaries (Mulac-Jericevic *et al.*, 2000). PR-B ablation (PRBKO) mice have normal physiology of the uterus and ovaries, but decreased pregnancy-associated mammary gland morphogenesis (Mulac-Jericevic *et al.*, 2003). Thus, PR-A is both necessary and sufficient to elicit the progesterone-dependent reproductive responses required for female fertility, whereas PR-B is required for normal proliferative progesterone-responses in the mammary gland (Conneely *et al.*, 2002).

PR expression

In the human female reproductive tract PR is expressed in the ovary, fallopian tube, uterus and cervix (Gadkar-Sable *et al.*, 2005). In the ovary PR is expressed in theca cells, surface epithelial cells and stroma (see Chaffin & Stouffer, 2002). It is also expressed in granulosa cells of the dominant follicle(s) after the LH surge. There is evidence that progesterone enhances the hCG induced rise in PR expression (Chaffin & Stouffer, 2002). However, PR is not under progestin control during the later stages of the periovulatory interval. In humans and other primates PR expression persists in the corpus luteum (Press & Greene, 1988, Iwai *et al.*, 1990). In contrast, PR expression in rats is transient (Park & Mayo, 1991, Natraj & Richards, 1993). PR expression in mouse granulosa cells has been reported by our group to be transient for both isoforms (Shao *et al.*, 2003), in the same way as in rats. However, others have reported that PR-B is also expressed at earlier stages of follicular development, as well as in the mouse corpus luteum (Gava *et al.*, 2004).

PR regulated events in the ovary

Progesterone was suggested in 1974 (Rondell, 1974) to play an intraovarian role during ovulation and in 1981 Rothchild presented the hypothesis that progesterone functions as an intraovarian luteotropin, promoting the formation of the corpus luteum (Rothchild, 1981). But it was not until after the discovery of PR expression in luteinising granulosa cells in the late 1980s that research on PR regulated events in the ovary received greater attention. Many studies have been conducted both *in vivo*

and in vitro to further characterise the intraovarian role of progesterone during ovulation and luteinisation in several species. The local role of progesterone in the ovary during the periovulatory interval was recently reviewed by Chaffin & Stouffer (2002).

Since the endogenous intraovarian concentration of progesterone is high, studies are usually performed by blocking progesterone action. Three main strategies have been employed: 1) inhibition of progesterone synthesis, 2) administration of PR antagonists to inhibit progesterone actions via PR and 3) studies of PR-null mice that do not express the PR-A isoform (PRAKO), the PR-B isoform (PRBKO) or either of the receptor isoforms (PRKO). Taken together these three experimental strategies irrefutably show that PR stimulation is essential for ovulation in all studied mammalian species. Several different progesterone synthesis inhibitors prevent ovulation both in vivo (Snyder *et al.*, 1984, Murdoch *et al.*, 1986, Hibbert *et al.*, 1996) and in in vitro perfusion systems (Brännström & Janson, 1989). PR antagonists, such as Org 31710 and RU 486, inhibit ovulation when administered in vivo (Rose *et al.*, 1999, Shao *et al.*, 2003) or in perfusion systems (Brännström, 1993). The various PR knock-out mice strains have clarified the roles of the different isoforms. The PRKO mice completely fail to ovulate (Lydon *et al.*, 1995, 1996), while the PRAKO mice exhibit decreased ovulation after stimulation (Mulac-Jericevic *et al.*, 2000). In contrast, the PRBKO mice are fertile with normal litter sizes (Mulac-Jericevic *et al.*, 2003).

PR has also been demonstrated to be important for luteinisation. The best characterisation has been presented by Stouffer and colleagues, using rhesus macaques as a primate model. PR stimulation influences the changes in steroidogenesis, the vascularisation, the morphological alterations and the terminal differentiation and cell cycle withdrawal of follicular cells (see Chaffin & Stouffer, 2002).

PR antagonists

PR antagonists, also termed antiprogestins, are widely used to study the mechanisms of PR function. All PR antagonists available today are based on a steroidal skeleton derived from 19-nor-testosterone. They compete with progesterone for the PR binding site but do not interact with exactly the same amino acids of the LBD as does progesterone (Leonhardt & Edwards, 2002). The two antagonists used in this thesis do not impair the receptor activation steps of dissociation from heat shock proteins, receptor dimerisation and binding to SREs. Instead, they induce a different receptor conformation, which inhibits coactivator interaction. More specifically, helix 12 in the C-terminal part of PR is dislocated (Tanenbaum *et al.*, 1998, Williams & Sigler, 1998). The steroidal PR antagonists exhibit higher potency than can be expected from their PR binding affinities. There are three reasons for this; 1) antagonist-bound PR can form transcriptionally inactive heterodimers with agonist-bound PR, 2)

antagonist-bound PR exhibit greater DNA-binding affinity than agonist bound PR, thus competing for SREs and 3) antagonist-bound PR recruits co-repressors that are not recruited by agonist-bound PR, which further diminishes transcriptional activity (see Leonhardt & Edwards, 2002).

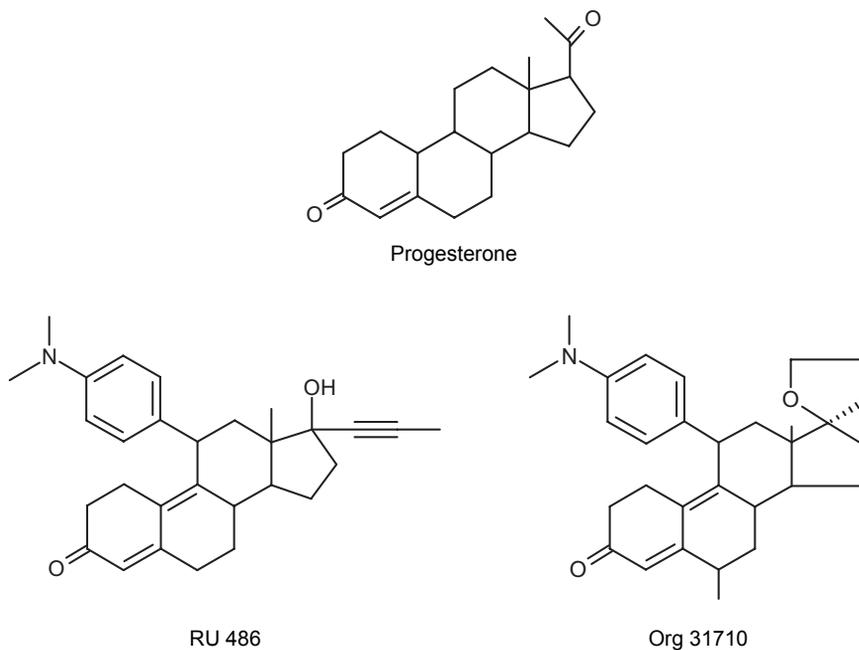


Figure 8
Chemical structures of progesterone and the RU 486 and Org 31710 progesterone receptor antagonists.

RU 486

RU 486 (Roussel-Uclaf 38486; generic name mifepristone) was the first PR antagonist to be used in clinical practise. It binds both the PR and the glucocorticoid receptor with high affinity and has a low, but demonstrable, affinity for the androgen receptor. It has no affinity for the mineralocorticoid or oestrogen receptors (see Cadepond *et al.*, 1997). PR with RU 486 as ligand binds to SREs in the target gene promoters, but does not induce transcription due to conformational changes (Baulieu, 1991).

Org 31710

Org 31710 (Organon 31710) is a highly selective PR antagonist with little antiglucocorticoid activity and no known other hormonal interactions except for weak androgenic and antiandrogenic activities. Org 31710 exhibit similar binding to PR as RU 486 and the interaction of the PR-antagonist complex with DNA is also

similar (Kloosterboer *et al.*, 1994, Hurd *et al.*, 1997). Furthermore, Org 31710 and RU 486 appear to induce similar conformational changes in the PR (Mizutani *et al.*, 1992). However, Org 31710 has less antiglucocorticoid activity than RU 486 due to a tetrahydrofuran ring at carbon 17 (Mizutani *et al.*, 1992).

Nongenomic PR signalling

In addition to well-documented genomic effects, steroid hormones also exert effects that are rapid and insensitive to transcription inhibitors, mimicked by steroids coupled to membrane-impermeable molecules and observed in cells that do not express classical nuclear receptors (Bramley, 2003, Losel *et al.*, 2003, Edwards, 2004, 2005). The rapid actions of steroid hormones that are independent of gene transcription have been termed nongenomic, in order to distinguish them from the direct (genomic) effects on gene transcription in the nucleus. Four types of receptor have been proposed to mediate rapid steroid signalling; 1) transmembrane receptors that are unrelated to the corresponding nuclear receptor, 2) modified nuclear receptors located at the plasma membrane, 3) conventional nuclear receptors associated with signalling complexes at the plasma membrane or in the cytosol and 4) neurotransmitters or peptide hormone receptors that are allosterically modified by steroids (see Edwards, 2005).

A novel transmembrane receptor for progesterone was recently characterised in fish (Zhu *et al.*, 2003c) and several other vertebrate species (Zhu *et al.*, 2003b). This is the first identified protein that fulfils the criteria of a plausible structure, specific tissue and membrane localisation, steroid binding characteristics of steroid and progestin receptors, coupling to second messenger pathways, regulation by steroid hormones and biological relevance. The receptor, termed membrane progestin receptor (mPR), is a seven membrane spanning G-coupled receptor that is expressed in three isoforms (α , β and γ). The human mPR α is localised to reproductive tissues, including ovary, placenta, testis and possibly uterus (Zhu *et al.*, 2003b). Our group has shown that all three isoforms are expressed in the rat ovary and are differentially regulated during the oestrous cycle (Nutu *et al.*, 2005, 2006).

Progesterone modifies the activity of oxytocin and GABA_A by interacting with their respective receptors. Thus, progesterone maintains the quiescence of the uterus during pregnancy by reducing oxytocin receptor activity (Grazzini *et al.*, 1998, Burger *et al.*, 1999, Bogacki *et al.*, 2002, Dunlap & Stormshak, 2004). The progesterone concentrations at which this occurs are unclear, but progesterone serum levels are high during pregnancy. Allopregesterone, a reduced progesterone metabolite, has been shown to modify the function of the GABA_A receptor (Lambert *et al.*, 2003, Reddy *et al.*, 2004). Allosteric modulation of GABA_A receptor activity in the brain mediates the sedative, analgesic and anticonvulsive effects of progesterone, which are retained in PRKO mice (Reddy *et al.*, 2004). However, no

progesterone binding site has been characterised on the GABA_A receptor (Lambert *et al.*, 2003).

In addition, progesterone can exert nongenomic functions via the classical nuclear receptors by interactions with other signalling pathways. Both PR isoforms have multiple phosphorylation sites (Lange, 2004). A total of 14 PR-B phosphorylation sites are known, of which 4 are basal sites that are phosphorylated in the absence of hormone, whereas others are induced by hormone binding and phosphorylated within 1-2 hours. The role of phosphorylation is poorly understood, but it influences ligand dependent and independent transcription, interaction with co-regulators and receptor turnover. Phosphorylation of serine 294 is required for rapid nuclear translocation, which may allow transcription in the absence of ligand via non-classical mechanisms or protect PR from degradation in the cytoplasm. The PR phosphorylation sites may also be of importance for integrating growth factor signals and other steroid responses. For instance, phosphorylated PR is sensitive to sub-physiological levels of progestins (Lange, 2004).

Cholesterol synthesis and the branch-point reactions

The results obtained in the course of the work on this thesis indicated that PR stimulation regulates cholesterol synthesis in periovulatory granulosa cells. This section describes the cholesterol synthesis and the branch-point reactions, with special emphasis on protein prenylation.

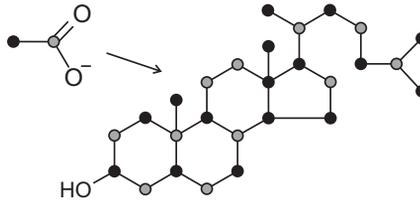
Cholesterol was first discovered in 1815. Since then no less than three Nobel prizes have been awarded to scientists working on cholesterol. In 1927 the Nobel Prize in Chemistry was awarded to H. Wieland for his work on cholesterol and cholic acid structure. K. Bloch was awarded the Nobel Prize in Physiology and Medicine in 1963 in recognition of his achievements in the area of cholesterol biosynthesis. Finally M. Brown and J. Goldstein were awarded the 1985 Nobel Prize in Physiology and Medicine for their research on the regulation of cholesterol biosynthesis (Vance & Van den Bosch, 2000).

Cholesterol synthesis is a well characterised pathway. As illustrated in figure 9A, all the 27 carbons that constitute the complex cholesterol molecule are derived from the simple two-carbon molecule acetate. Cholesterol synthesis requires more than 30 enzymes and can be performed by most mammalian cells. A simplified overview of the cholesterol synthesis pathway is presented in figure 9B. Apart from providing the cell with cholesterol it is also used for production of ubiquinone, heme a, dolichols and protein prenylation substrates (Grünler *et al.*, 1994). The common precursor of all these end products is farnesyl pyrophosphate. Cholesterol synthesis is mainly regulated by the activity of the hydroxymethylglutaryl co-enzyme A (HMG-CoA) synthase and HMG-CoA reductase enzymes. The key regulators are the sterol regulatory element binding protein (SREBP)-family. The SREBPs are activated

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when sterol levels are low and function as transcription factors that increase the transcription of several genes involved in cholesterol synthesis.

A



B

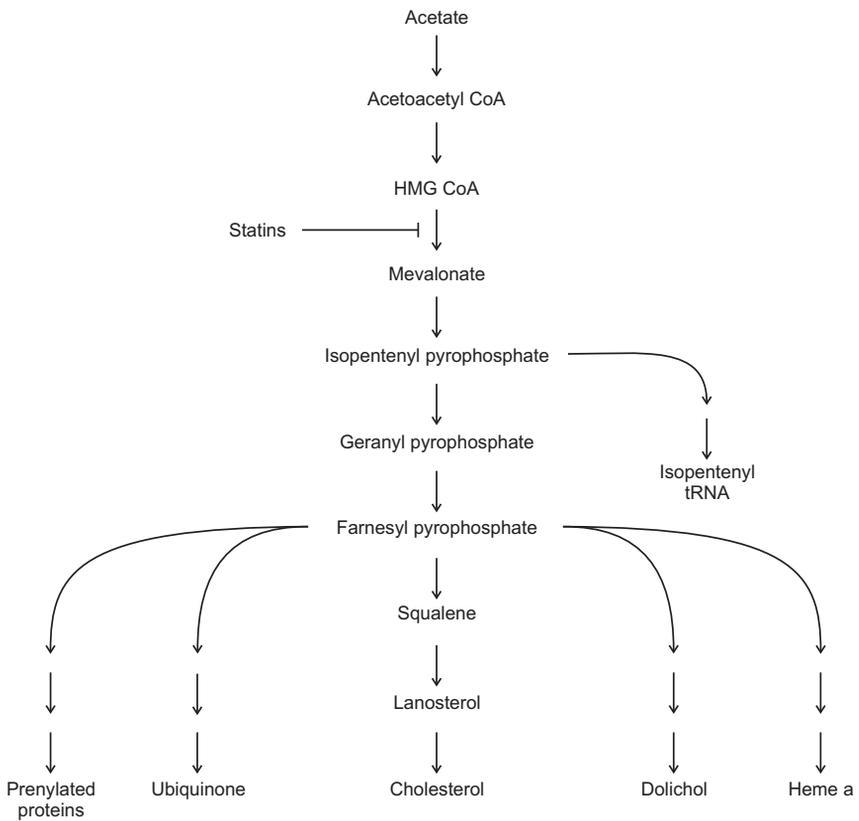


Figure 9

A) Schematic representation of the origin of cholesterol carbon atoms from acetate carbon atoms. **B)** Simplified overview of the cholesterol synthesis pathway including the branch-point to ubiquinone, dolichol, heme a and prenylated proteins.

Statins

The generic names for a group of drugs that function as inhibitors of cholesterol synthesis all end with “statin”, which is the reason why drugs belonging to this group are commonly referred to as “statins”. Statins are widely used to lower blood plasma cholesterol levels. The effectiveness of statins in reducing cholesterol synthesis is due to the fact that they target the HMG-CoA reductase enzyme (Figure 9B), which is generally considered to be rate limiting for cholesterol synthesis. In addition HMG-CoA, the last intermediate before the inhibition, is water soluble and degradable via other pathways, thereby preventing toxic accumulation of cholesterol precursors (Tobert, 2003).

In this thesis three different statins were used (Figure 10). Mevastatin (initially named compactin) is a natural product found in fermentation broth from *Penicillium citrinum*. Lovastatin (initially named mevinolin) is also a natural product derived from *Aspergillus terreus*. Simvastatin is a semisynthetic product derived from lovastatin.

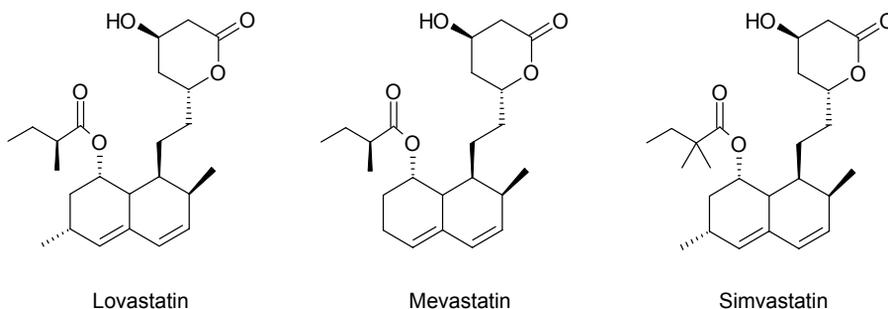


Figure 10

Chemical structures of lovastatin, mevastatin and simvastatin.

Prenylation

The majority of proteins in eukaryotic cells are posttranslationally modified and prenylation is one of the more recently discovered modifications (Gelb *et al.*, 1998). There are four known lipid modifications of proteins (see Casey, 1995); 1) Palmitoylation, which consists of a 16-carbon saturated fatty acyl group attached to cysteine residues. The palmitoyl chain can be replaced by other fatty acyl groups and the process is reversible. 2) Myristoylation, an attachment of a 14-carbon saturated acyl group to an N-terminal glycine residue. This modification has also been reported to be reversible. 3) The third group of reversible protein lipidation is attachment of a complex glycosyl phosphatidylinositol (GPI), i.e. a complete phospholipid with associated sugars and ethanolamine. Essentially all GPI-linked proteins are located on the outer cell surface. 4) Prenylation is a posttranslational lipid modification with

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either the 15 carbon moiety farnesyl pyrophosphate or the 20 carbon moiety geranylgeranyl pyrophosphate (Figure 11). It is not reversible, with the exception of the following methylation (see below).

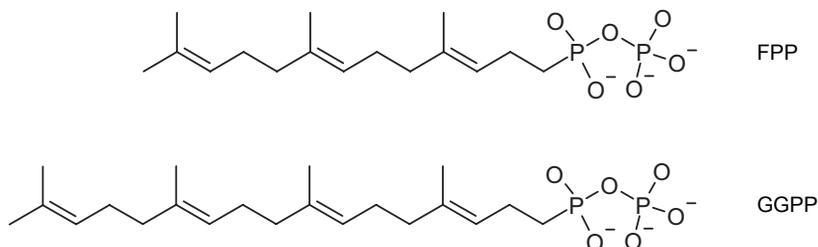


Figure 11

Structure of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), the two lipid moieties used for the prenylation of proteins.

Geranylgeranylation is more common than farnesylation (Rando, 1996). Studies have shown that geranylgeranylated cysteine residues are 4-10 times more common than farnesylated cysteine residues in tissues (Epstein *et al.*, 1991). However, farnesylation has received much attention as a potential target for cancer treatment, mainly due to the fact that Ras, a commonly mutated oncogene, is farnesylated (Zhu *et al.*, 2003a). Prenylated proteins include the Ras family of GTP binding proteins, the γ -subunits of all known heterotrimeric G proteins, the nuclear membrane associated lamin B and the fungus mating factor Rhodotorucine A (Gelb *et al.*, 1998). It is generally believed that prenylation functions as hydrophobic anchors which tether proteins to cell membranes (Figure 12). However, other roles, for instance in mediating protein-protein or protein-lipid interactions are also possible (Rando, 1996). Prenylated proteins are located to the plasma membrane, Golgi membrane, endoplasmatic reticulum or vesicles. Furthermore some prenylated proteins appear to be soluble rather than membrane associated, whereas some cycle between membranes and the cytoplasm as a result of interactions with specific soluble proteins (see Jackson *et al.*, 1997). The mechanism behind the specific targeting of prenylated proteins is unknown.

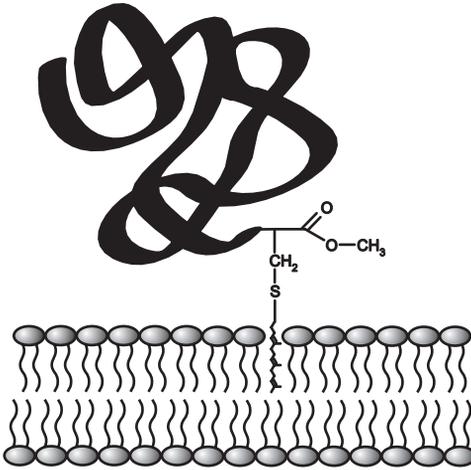


Figure 12
Schematic drawing of a farnesylated protein anchored at a cell membrane.

Prenylation mechanisms

Prenyl groups are transferred to proteins by three known prenyltransferases: protein farnesyl transferase (FTase) (Reiss *et al.*, 1990), protein geranylgeranyl transferase type I (GGTase-I) (Moomaw & Casey, 1992, Yokoyama & Gelb, 1993) and type II (GGTase-II) (Seabra *et al.*, 1992a, 1992b). FTase transfer of farnesyl pyrophosphate and GGTase-I transfer of geranylgeranyl pyrophosphate take place to a cysteine near the carboxy-terminal of a protein or peptide. GGTase-II transfers two geranylgeranyl groups to Rab proteins (see Gelb *et al.*, 1998). The rate-limiting step is product release and requires the presence of a new substrate prenyl group (Tschantz *et al.*, 1997). Protein prenylation is usually followed by methylation, the only step that is reversible. The prenylation mechanism is outlined in figure 13.

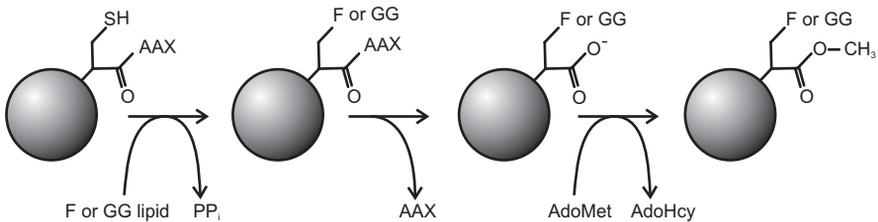


Figure 13
Schematic drawing of the three enzymatic processes in protein prenylation. First the prenyl group, either farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP), is covalently linked to the sulphur of a cysteine amino acid. This is followed by enzymatic removal of the three amino acids located N-terminal to the cysteine (AAX). In the third and final step the protein is methylated to further increase lipophilicity.

Prenylation and apoptosis

Inhibition of prenylation has been shown to lead to a range of cellular effects, including induction of apoptosis (Choi & Jung, 1999, Macaulay *et al.*, 1999, García-Román *et al.*, 2001, Blanco-Colio *et al.*, 2002), proliferation inhibition (Bouterfa *et al.*, 2000), migration inhibition (Bouterfa *et al.*, 2000) and inhibition of angiogenesis (Park *et al.*, 2002). It remains unclear whether global loss of prenylation or loss of a restricted substrate(s) is responsible for the apoptotic response (Wong *et al.*, 2002). Most studies on prenylation have been performed on cell lines *in vitro*. Knowledge of the effects *in vivo* is very limited, and even less is known about the possible physiological regulation of protein prenylation.

AIMS OF THIS THESIS

The specific aims developed during the work on this thesis were:

- ◇ To evaluate the effects of LH receptor stimulation on apoptosis sensitivity in granulosa cells from mature follicles.
- ◇ To determine whether or not progesterone affects apoptosis in rat and human granulosa cells at the time of ovulation.
- ◇ To investigate PR-mediated effects on transcription.
- ◇ To confirm PR-mediated effects on cholesterol synthesis.
- ◇ To investigate the link between cholesterol synthesis and apoptosis.

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in detail in the respective papers and the references therein. A general discussion of some of the methods is presented in the following.

Models

The factors that regulate apoptosis in the ovary vary between follicles at different stages of development, necessitating models in which homogeneous cohorts of follicles can be isolated at specific points of development. Experiments in this thesis were performed with periovulatory granulosa cells. The periovulatory interval is defined as the time from the onset of the endogenous LH surge (in natural cycles) or hCG administration (in artificial cycles) until follicle rupture (Chaffin & Stouffer, 2002). We used a rat and a human model, both of which included superovulation, i.e. hormonal stimulation to produce multiple mature follicles (Figure 14 and 15).

Human model

Patients scheduled for in vitro fertilisation (IVF) at Sahlgrenska University Hospital were asked to participate in the studies. No other inclusion or exclusion criterion was used. The women were treated according to the routines of the hospital, with a GnRH-agonist for 2-3 weeks in order to downregulate endogenous gonadotropin secretion. Recombinant FSH was then administered and follicular growth monitored by means of ultrasonography. When the follicles had reached an appropriate size, hCG was administered to induce final maturation of the follicles. Ultrasound-guided aspiration of oocytes, surrounding granulosa cells and follicular fluid was performed 34-36 hours later.

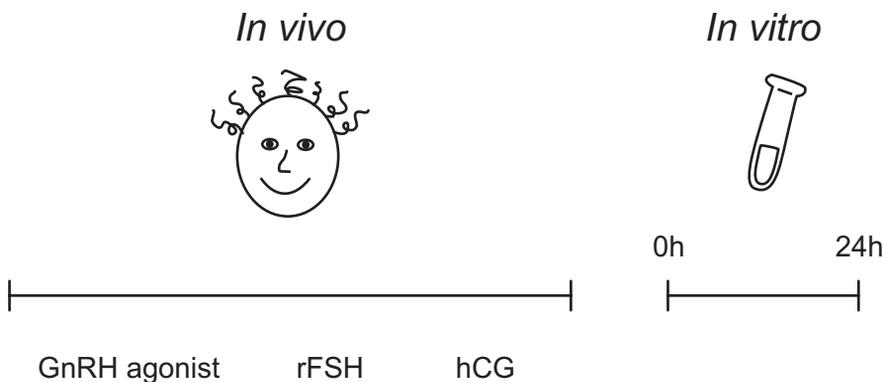


Figure 14

Schematic representation of the human model used in the studies presented in this thesis. Abbreviations: GnRH, gonadotropin releasing hormone; rFSH, recombinant follicle stimulating hormone; hCG, human chorionic gonadotropin.

Rat model

Rat studies were performed with immature, female Sprague-Dawley rats treated with eCG and hCG in order to obtain synchronous maturation of multiple follicles. eCG, also known as PMSG, is a gonadotropin with both FSH and LH activity (Christakos & Bahl, 1979, Hoppen, 1994). It has a longer half-life than FSH and can thus be administered as a single injection. FSH can also be used to induce follicle maturation in immature rats, but has to be administered every 12 hours by injection or as a continuous infusion with osmotic minipumps, which is more time consuming and expensive than intraperitoneal eCG injection (Popova *et al.*, 2002). Urinary hCG has been used for many years as a substitute for LH in stimulated cycles. hCG is closely related to LH and the two hormones share the same receptor.

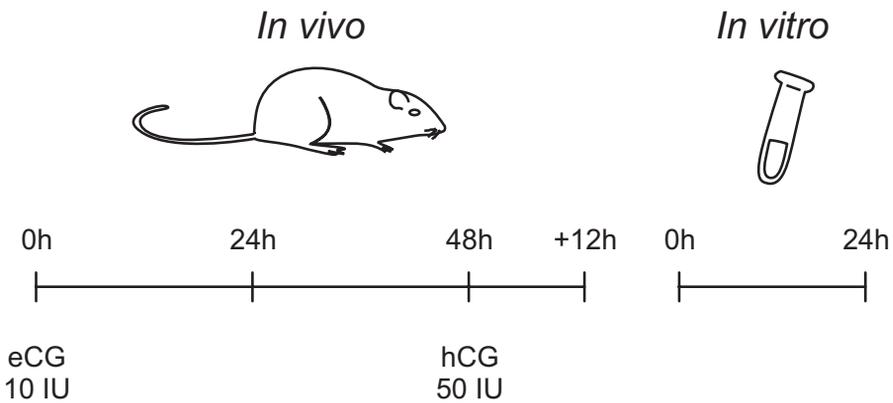


Figure 15

Schematic representation of the rat model used in the studies presented in this thesis. Abbreviations: eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin.

Isolation of granulosa cells and cell culture

Human granulosa cells were retrieved in connection with oocyte aspirations for IVF through trans-vaginal ultrasound-guided puncture of preovulatory follicles. Some follicles, in which the oocyte was not found after puncture, were rinsed with medium in order to retrieve it. After the IVF staff had collected all oocytes, granulosa cell were collected from the medium. In order to obtain a purer granulosa cell preparation the cells were subjected to isotonic Percoll centrifugation to reduce red blood cell contamination. Density gradient centrifugation with e.g. Percoll is the standard method for the preparation of human granulosa cells retrieved from IVF patients, although a simpler method has recently been proposed (Lobb & Younglai, 2006). Some degree of contamination, including leukocytes, macrophages and epithelial cells, is inevitable when working with human granulosa cells obtained by ultrasound-guided puncture and aspiration (Beckmann *et al.*, 1991). Obtaining a pure granulosa

cell preparation would result in very low yield and require extensive handling of the cells, possibly leading to cellular damage. With this in mind, we decided to allow some contamination by other cell types.

Rat granulosa cells were obtained by puncture of follicles. Dissected ovaries were placed in medium and carefully dissected free from the ovarian bursa, fallopian tubes and surrounding fat tissue before puncture of the follicles. Since the follicles and ovaries are complex tissues consisting of several different cell types, it is virtually impossible to obtain a pure granulosa cell culture with a satisfactory cell yield. In our laboratory we checked for the presence of theca cells in granulosa cells from mouse ovaries, which were obtained in the same way as the rat granulosa cells. However, no detectable amount was revealed (Shao, 2005).

Many models for studying follicular atresia have taken advantage of the fact that isolated follicles or granulosa cells undergo spontaneous apoptosis when cultured in the absence of serum (Tilly *et al.*, 1992). The addition of survival or pro-apoptotic factors to the incubation medium can affect the rate of spontaneous apoptosis. However, some factors that are known to affect granulosa cell apoptosis *in vivo*, e.g. oestrogens (Billig *et al.*, 1993), have no influence on isolated granulosa cells *in vitro*, which suggests that interaction with other cell types is necessary for their effect.

Methods for apoptosis detection

Investigating apoptosis presents an intrinsic problem of studying something that disappears. The apoptotic process ends with phagocytosis by surrounding cells, leaving no long-lasting traces for investigation. However, apoptosis is characterised by several morphological and biochemical events that can be used for apoptosis detection. Morphological changes include cell shrinkage, condensation of nuclear chromatin, fragmentation of the cell into membrane-enclosed apoptotic bodies and phagocytosis by neighbouring cells (Kerr *et al.*, 1972). The biochemical hallmarks of apoptosis include events such as the release of cytochrome *c* from mitochondria, exposure of phosphatidyl serine on the cell surface, internucleosomal DNA fragmentation and caspase activation (Saraste & Pulkki, 2000).

In this thesis we have used several assays to detect different end-points of the apoptotic process, thus strengthening the results. The choice of methods was based on several parameters. First, granulosa cells are highly interconnected with each other, forming a syncytium of cells (Kidder & Mhawi, 2002). Single cell analysis is difficult, since breaking up these connections requires quite harsh treatment of the cells, which can induce cell death. Second, the limited amount of cells available made it necessary to use assays that can function with relatively few cells. Third, it is valuable to use methods that do not involve much handling of the cells, since apoptotic cells are fragmented and difficult to handle. Finally, granulosa cells,

especially apoptotic granulosa cells, do not attach well to culture plates, making it difficult to use methods that include staining and washing.

DNA fragmentation

Internucleosomal DNA fragmentation is a typical sign of apoptosis (Wyllie, 1980, Billig *et al.*, 1993, Tilly & Hsueh, 1993) and has been demonstrated in a wide range of cell types (Bortner *et al.*, 1995). In this thesis, two different methods were used to study DNA fragmentation. Using a fluorospectrophotometric method, fragmented DNA was separated from intact DNA and the amounts measured with a fluorescence spectrophotometer after the addition of Hoechst's dye H33258 (Labarca & Paigen, 1980). This dye binds selectively to DNA by intercalating between the two DNA strands and fluoresces upon binding. A disadvantage of this method is that it does not distinguish between internucleosomal DNA fragmentation and the non-specific DNA fragmentation that occurs during necrosis. For this reason we complemented the method by a qualitative visualisation of the DNA using gel electrophoresis. Apoptotic, internucleosomal DNA fragmentation gives rise to a typical ladder pattern, representing DNA fragments of 180-200 basepairs and multiples thereof.

Caspase-3/-7 activity

Induction of caspase-3 activity is a well-known event during apoptosis and has been demonstrated in both rat and human granulosa cells (Boone & Tsang, 1998, Izawa *et al.*, 1998). Caspases are proteases that cleave a specific amino acid sequence after aspartic acid residues and the substrate specificity is determined by the four amino acids amino-terminal to the cleavage site (Hengartner, 2000). Caspase activity in apoptotic cells can be monitored in several ways, including cleavage of synthetic substrates, immunoblotting (Western blot) of the processing of pro-caspases into active caspases and detection of cleaved caspase substrates (Köhler *et al.*, 2002). Cleavage of a synthetic substrate upon incubation with lysates of apoptotic cells was first described in 1994 (Pennington & Thornberry, 1994). Today, there are several commercially available assays where cleavage of a substrate generates a shift in colour, fluorescence or luminescence. The intensity of the signal is proportional to the amount of cleaved substrate and thus to caspase activity and the percentage of apoptotic cells in a cell population. DEVD (aspartic acid – glutamic acid – valine – aspartic acid) is the amino acid sequence that determines cleavage by means of caspase-3 and caspase-7. Both caspase-3 and caspase-7 activity is related to apoptosis. However, caspase-3 is considered to be one of the main executioner caspases and probably contributes much more than caspase-7 to this activity. Several different synthetic DEVD substrates were used in this thesis, generating either fluorescence or luminescence upon cleavage.

cDNA microarray

cDNA microarray is used to detect the transcription levels of several thousand genes simultaneously. The method is based on traditional DNA/RNA hybridisation. There

are two main types of microarrays available, oligonucleotide arrays (commercial) and spotted cDNA microarrays (commercial or home-made) (Chittur, 2004). In this thesis we used a commercial oligonucleotide microarray manufactured by Affymetrix. Oligonucleotide arrays are more expensive than cDNA microarrays but provide higher density, thus allowing for multiple oligonucleotides representing each gene and the inclusion of mismatch probes. Hybridisation in the specific microarray used in this thesis occurs between sample RNA and 25 base pair long DNA oligonucleotides attached to a glass slide. Each gene on the array is represented by 15-20 oligonucleotides covering different parts of the gene. In order to reduce interference of unspecific hybridisation, each oligonucleotide is complemented by a second oligonucleotide that is identical except for a substitution of the middle nucleotide.

Microarrays representing large parts of the genome should mainly be used for hypothesis generating experiments. Results should be followed up by confirming changes in gene expression using e.g. quantitative PCR or ribonuclease protection assay (RPA), changes in protein levels using e.g. Western blot or changes in the activity of metabolic or signalling pathways.

Cholesterol synthesis measurements

Cholesterol synthesis is a multi-step conversion of acetate into cholesterol (Figure 9B). In order to study the formation of cholesterol, ^{14}C -labelled acetate was added to granulosa cell incubations followed by detection of ^{14}C -incorporation into cholesterol, cholesterol ester and progesterone. Lipids were extracted from granulosa cells and subjected to separation by thin layer chromatography (TLC). The samples were dissolved in chloroform and applied as narrow bands near the base of the TLC plates, which consisted of glass plates covered by a thin layer of silica. The TLC plate was then placed in a developing chamber with the bottom part submerged in solvent. The solvent, or mobile phase, slowly moves up the plate by capillary action and as it moves past the applied sample it carries the mixture of molecules with it. Since the various compounds in the sample differ in solubility in the mobile phase and in adsorption to the solid phase the distance they are carried will vary, causing separation to occur. The major disadvantage of this method is the poor resolution after separation of the radiolabelled lipids in our samples. Interfering bands representing other labelled lipids may distort the results. Optimisation of the mobile phase, which is usually done by trial and error, can increase the resolution (Fried & Sherma, 1999). Scanning the TLC plates by means of the phosphorimager technique enables visualisation of all labelled lipids present on the plate, including those that interfere with the bands of interest. We have recently started to use TLC plates with a concentration zone, which produces sharper bands.

SUMMARY OF RESULTS

This section summarises the results from papers I-IV and presents some relevant but as yet unpublished data.

Apoptosis sensitivity during late follicular development (Paper I)

Follicular atresia occurs during all stages of follicular development. The degree of follicular demise peaks when the follicles become dependent on gonadotropin stimulation during the antral stage. Only the follicles that reach this stage at the time of increasing FSH levels at the beginning of each oestrous cycle manage to pass through this bottleneck. In our studies we focused on periovulatory follicular development and, using rat as a model organism, demonstrated that the sensitivity of granulosa cells to apoptosis decreases after treatment with eCG and even more after treatment with hCG. The DNA fragmentation at different developmental stages of rat follicles is shown in figure 16A. The decrease after administration of hCG was further characterised by measuring DNA fragmentation with a spectrophotometric, quantitative method (Figure 16B). The effect of hCG was not due to selection of follicles during isolation of granulosa cells, since similar results were obtained with DNA from whole ovaries (data not shown).

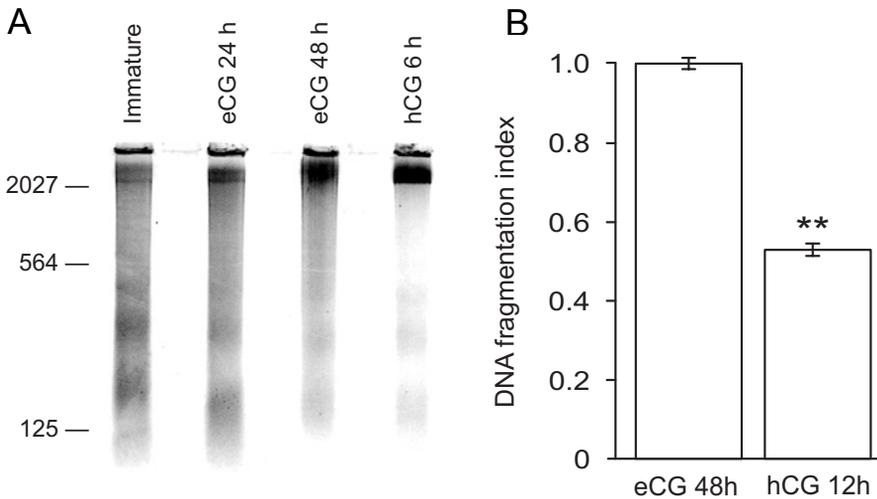


Figure 16

Effect of in vivo differentiation on internucleosomal DNA fragmentation. **A)** Representative figure showing gel electrophoretic separation of ^{35}S -labelled DNA isolated from granulosa cells from immature, untreated rats or rats treated with eCG for 24h, eCG for 48h or hCG for 6 h in addition to eCG priming. **B)** The degree of DNA fragmentation assayed by fluorospectrophotometry in granulosa cells isolated from rats treated with eCG for 48h or hCG for 12 h in addition to eCG priming.

Previously published in paper I.

SUMMARY OF RESULTS

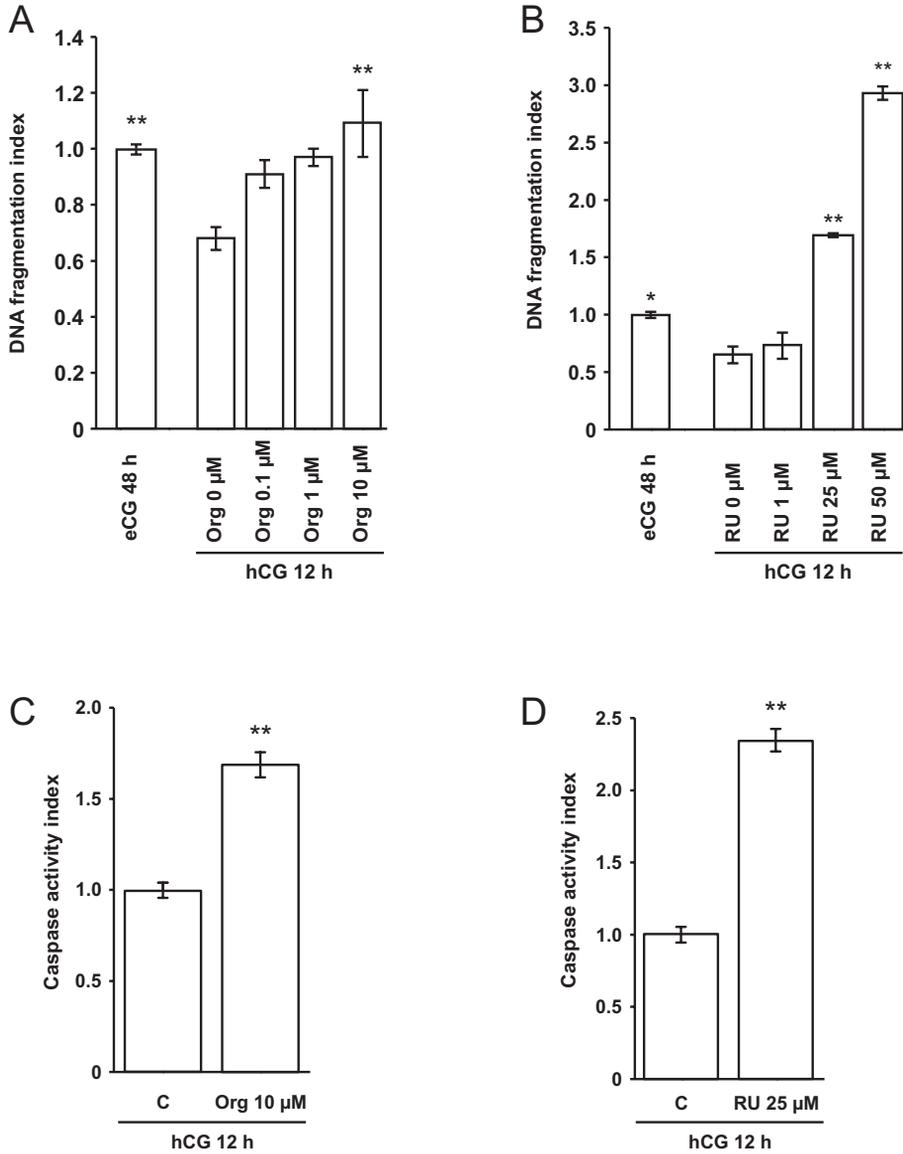


Figure 17

Effect of the progesterone receptor antagonists Org 31710 and RU 486 on apoptosis in rat periovulatory granulosa cell incubated in vitro for 24 hours. **A)** Effect of Org 31710 on DNA fragmentation. **B)** Effect of RU 486 on DNA fragmentation. **C)** Effect of Org 31710 on caspase activity. **D)** Effect of RU 486 on caspase activity.

Previously published in Paper I.

PR regulation of apoptosis (Papers I-IV)

A main characteristic of the periovulatory interval is the onset of progesterone production in the luteinising follicles and expression of PR in the granulosa cells. The PR expression is transient in rats, whereas in humans it remains in the corpus luteum. The appearance of PR, coinciding with induction of progesterone synthesis, prompted us to analyse the role of PR stimulation in the decreased apoptosis sensitivity observed in periovulatory granulosa cells.

In our rat model system we confirmed that PR mRNA expression increases dramatically after hCG treatment. The significance of PR stimulation for the survival of periovulatory granulosa cells was investigated by the addition of PR antagonists to the cell culture medium. Both RU 486 and the more specific antagonist Org 31710 increased the degree of DNA fragmentation in rat (Figure 17) and human (data not shown) granulosa cells as detected by fluorospectrophotometry and gel electrophoresis. Both antagonists also increased the caspase activity (Figure 17 / data not shown).

The specificity for PR was strengthened by the fact that Org 31710 had no effect on apoptosis in rat granulosa cells isolated from immature rats (Figure 18), in which PR is not expressed.

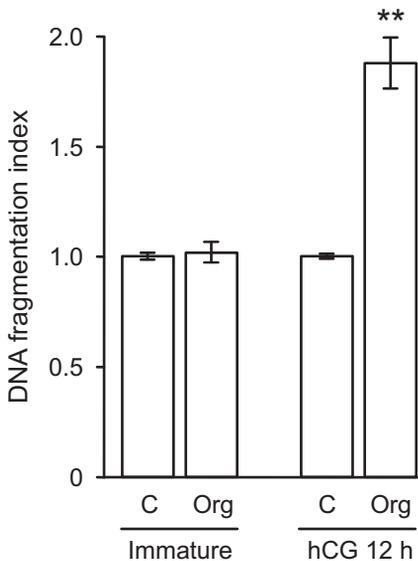


Figure 18

Effect of the progesterone receptor antagonist Org 31710 on rat granulosa cell DNA fragmentation in vitro. Org 31710 (10 μ M) was added to the culture medium during incubation of granulosa cells isolated from immature rats and from rats treated with eCG for 48 h followed by hCG-treatment for 12 h. The control group was set to 1.0 for both developmental stages (n=9 in each group).

** $P < 0.01$ compared to control
Previously published in Svensson (2000).

Another way to test the importance of PR stimulation is to add progesterone to the isolated granulosa cells. However, the addition of progesterone to the culture medium

did not markedly affect the degree of apoptosis in rat or human periovulatory granulosa cells. This probably reflects the high endogenous concentrations of progesterone. All PRs can be expected to be occupied by ligands. The effect of progesterone in granulosa cells is instead likely to be regulated by the level of receptor expression.

Although not physiological, blocking the endogenous production of progesterone may reveal the importance of PR stimulation for granulosa cell survival. Decreasing the endogenous progesterone synthesis by the addition of cyanoketone increased the degree of apoptosis in rat periovulatory granulosa cells. Supplementation of the progesterone-depleted medium with exogenous progesterone reversed the degree of apoptosis to the baseline level (Figure 19).

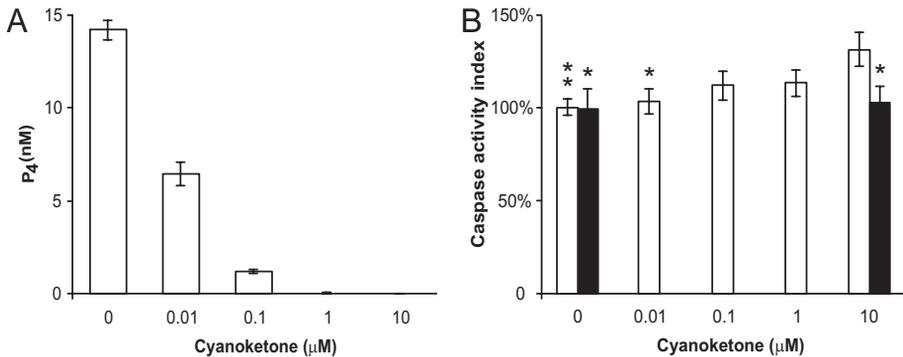


Figure 19

A) Effect of cyanoketone (0.01-10 μM) on the level of accumulated progesterone in spent medium after incubation of periovulatory rat granulosa cells for 24 hours ($n = 12-24$). Progesterone was undetectable in 10 out of 12 samples treated with 1 μM cyanoketone and in all 12 samples treated with 10 μM cyanoketone. **B)** Effect of cyanoketone (0.01-10 μM) on the degree of apoptosis measured as caspase-3/7 activity. Black bars represent the addition of 3 μM progesterone ($n = 24-36$).

* $P < 0.05$; ** $P < 0.01$ compared to treatment with 10 μM cyanoketone alone.

Previously published in paper III.

Effect of other receptor agonists / antagonists (Papers I-II)

In order to establish the specificity of PR stimulation as a survival factor, several other related hormones and compounds were tested in our rat and human systems.

It is well established that progesterone can interact with the GABA_A-receptor. While this receptor has been found in the ovary, there are to our knowledge no reports on ovarian effects of progesterone mediated by the GABA_A-receptor. However, it has

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been suggested that progesterone can interfere with a GABA_A-like receptor in the ovary and thereby reduce granulosa cell apoptosis at earlier stages of follicular development (Peluso & Pappalardo, 1998). Using the GABA antagonists picrotoxin and bicuculline and the GABA agonist muscimol we tested this hypothesis in our system. None of the three tested GABA receptor modulators affected the degree of DNA fragmentation in granulosa cells isolated from rat follicles after treatment with eCG for 48 h or hCG for an additional 12 h. Picrotoxin was also evaluated in human periovulatory granulosa cells, where it had no effect on DNA fragmentation or accumulation of progesterone in the culture medium.

RU 486 is a potent glucocorticoid antagonist, in addition to being a PR antagonist. To our knowledge there are no antagonists that are specific for the glucocorticoid receptor. Thus the agonist dexamethasone was added to reveal effects caused by interference with glucocorticoid receptors. Dexamethasone did not affect DNA fragmentation in human periovulatory granulosa cells when added in concentrations of 0.5-100 μ M. Nor did it affect the dose-dependent increase in DNA fragmentation after treatment with RU 486 (data not shown).

RU 486 has also been shown to interact with the androgen receptor (AR). The AR agonist dihydrotestosterone did not affect DNA fragmentation when added in concentrations of 1 nM to 25 μ M (Table 1). Org 31710 is a much more specific antagonist than RU 486 and has only been demonstrated to have a very weak interaction with AR (Kloosterboer *et al.*, 1994, Hurd *et al.*, 1997).

Table 1

Effect of dihydrotestosterone (DHT) on DNA fragmentation as determined by fluorospectrophotometry after serum-free incubation in vitro for 24 hours of isolated human periovulatory granulosa cells. N.S. = not significant compared to dihydrotestosterone 0 nM ($P > 0.05$). n = 9 in each group. Previously published in paper II.

DHT (nM)	DNA fragmentation index
0	1.00 \pm 0.03
1	1.00 \pm 0.05 ^{N.S.}
10	1.01 \pm 0.06 ^{N.S.}
50	1.08 \pm 0.05 ^{N.S.}
100	1.01 \pm 0.06 ^{N.S.}
1000	1.06 \pm 0.04 ^{N.S.}
10000	1.12 \pm 0.11 ^{N.S.}
25000	1.13 \pm 0.03 ^{N.S.}

Transcriptional regulation by PR (Paper III)

Ligand-bound PR functions as a transcription factor regulating gene expression. It is thus reasonable to assume that PR stimulation regulates the expression of genes that are important for apoptosis as well as other events in periovulatory granulosa cells. To better understand the regulation of apoptosis after PR stimulation of periovulatory granulosa cells we investigated the effect of Org 31710 on gene expression. The

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microarray technique offers the possibility to perform a genome wide screening of gene expression. Perioviulatory rat granulosa cells were incubated in vitro for 24 hours with or without the addition of 10 μ M Org 31710 to the incubation medium. Isolated RNA was hybridised to commercially available microarray chips manufactured by Affymetrix. The analysis revealed close to 100 regulated genes (Tables 2 and 3). A total of 8.800 gene sequences were represented on the array out of which approximately 3.400 were classified as present in the RNA-pool from control granulosa cells. Groups of regulated genes were identified as belonging to different metabolic or functional pathways. This included downregulation of cholesterol synthesis (see below), angiogenesis and proteases possibly involved in follicular rupture as well as increased stress responses.

Four of the downregulated genes were identified as being involved in cholesterol synthesis. These were the mitochondrial and cytosolic HMG-CoA synthases, mevalonate kinase and one EST sequence (GenBank accession number H33491), which was identified as *Rattus norvegicus* sterol delta 8-isomerase mRNA (GenBank accession number AF071501) after a BLAST homology search. The sterol carrier protein-2, which is involved in cholesterol transport, was also downregulated. Further analysis of the microarray results with focus on genes implicated in cholesterol synthesis revealed other genes that were transcriptionally decreased after incubation in the presence of Org 31710, but did not meet our primary, more stringent, criteria for change in transcription. Figure 20 illustrates the relative expression levels of all genes involved in cholesterol synthesis that were present on the microarray chip, showing that the cholesterol synthesis pathway as a whole is suppressed after treatment with Org 31710. Of the 29 transcripts present on the array 17 were downregulated to ≤ 75 % of controls and an additional 6 were downregulated to ≤ 90 % of controls.

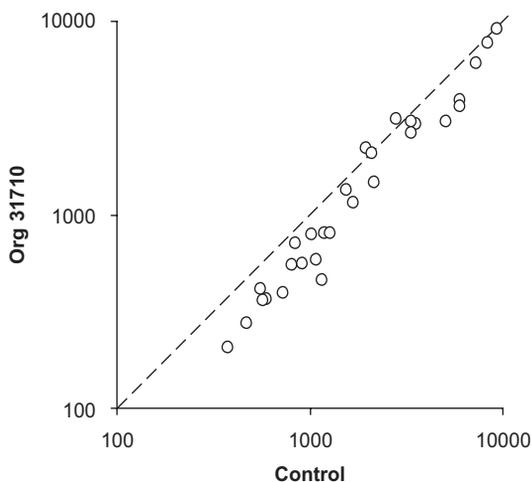


Figure 20

Relative expression levels of genes involved in cholesterol synthesis. Each circle represents one transcript on the array. Totally 29 different transcripts involved in cholesterol synthesis were present on the array. Some genes were represented by duplicate transcripts. Previously unpublished data.

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Table 2. Genes showing decreased expression in periovulatory rat granulosa cells after incubation for 24 h in the presence of Org 31710 (10 μ M) compared to control as revealed by DNA microarray analysis. Comparisons were made between duplicate control and Org 31710 DNA microarray chips, generating a total of 4 comparisons. Genes that were considered by the Affymetrix software's Diff Call parameter to be decreased (D) or marginally decreased (MD) in at least 3 out of 4 comparisons were considered to be down-regulated. Each gene is identified by GenBank accession number followed by the "Diff Call" parameter in the four comparisons, average fold change and finally a description of the gene derived from GenBank. Text in italics indicates the plausible identity of the genes after a BLAST homology search. NC = no change. Previously unpublished data.

GenBank #	Diff Call	Fold				Description derived from GenBank
		Change	Change	Change	Change	
D00036	D D D D	-7.5				pancreatic phospholipase A2
Z33403	D D D D	-6.9				chorionic gonadotropin receptor
AA874873	NC D D D	-3.6				cDNA clone UI-R-E0-ci-d-11-0-UI
U07560	MD D NC D	-2.9				ELK ligand LERK-2 (Eplg2)
AF035953	NC D D D	-2.9				kinesin-related protein KRP4 (KRP4)
M38135	D D D D	-2.9				cathepsin H (RCHII)
AI171268	D D D D	-2.7				EST217223 <i>similar to inhibitor of DNA binding 3 (Id3) (E=0.0)</i>
S52878	D D D D	-2.6				intestinal 15 kda protein=fatty acid-binding protein homolog
AA891255	D D NC D	-2.6				EST195058
J04629	NC D MD D	-2.5				(Na ⁺ , K ⁺)-ATPase-beta-2 subunit
M84719	D D D D	-2.5				flavin-containing monooxygenase 1 (FMO-1)
AA892775	MD D NC MD	-2.4				EST196578 <i>similar to lysozyme (Lyz) (E=0.0)</i>
AF030358	D D NC D	-2.4				chemokine CX3C
S53527	D D NC MD	-2.3				S-100 beta subunit
AA859902	D NC D D	-2.3				cDNA clone UI-R-E0-cg-a-06-0-UI
M29472	MD D MD MD	-2.2				mevalonate kinase
M15944	D D NC D	-2.2				enkephalinase (neutral endopeptidase)
AA945054	D D NC D	-2.2				EST200553 <i>similar to cytochrome b5 (E=0.0)</i>
AA891734	D D NC D	-2.2				EST195537
AI008815	D D D D	-2.2				EST203266 <i>similar to cytochrome c (E=0.0)</i>
AI230778	D D D MD	-2.2				EST227473 <i>similar to protein-tyrosine sulfotransferase 2 (E=0.0)</i>
L38483	D D D D	-2.1				jagged protein
M23697	D D D D	-2.1				tissue-type plasminogen activator (t-PA)
M33648	D D D D	-2.1				Rt mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase
AA874803	NC D D D	-2.1				cDNA clone <i>similar to chemokine (CXC motif) ligand 14 (E=0.0)</i>
AF076619	D D D D	-2.0				molecular adapter rGrb14 (Grb14)
M58040	D D NC D	-2.0				transferrin receptor
AA893235	D D D D	-2.0				EST197038 <i>similar to G0/G1 switch gene 2 (E=0.0)</i>
AI232078	D D D D	-2.0				EST228766 <i>similar to latent transforming growth factor beta binding protein 1 (Ltbp1) (E=0.0)</i>
J02585	D D D D	-1.9				stearyl-CoA desaturase
X07365	D D D D	-1.9				glutathione peroxidase
X08056	D D D D	-1.9				guanidinoacetate methyltransferase
AA858520	D D D D	-1.9				cDNA clone similar to follistatin
M62763	NC D D D	-1.9				sterol carrier protein 2
H33491	NC D D D	-1.9				EST109547 <i>similar to sterol delta 8-isomerase (E=0.0)</i>
V01227	D D D D	-1.9				alpha-tubulin
S68578	D D D NC	-1.8				gonadotropin-releasing hormone receptor
AJ000347	D MD MD NC	-1.8				3'(2'),5'-bisphosphate nucleotidase
L16532	D D D D	-1.8				2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPII)
AA893781	NC D MD D	-1.8				EST197584 <i>similar to centaurin, beta 1 (E=8e¹²²)</i>
AF016503	D D D D	-1.8				procollagen C-proteinase enhancer protein (PCPE)
J03914	D D MD D	-1.8				glutathione S-transferase Yb subunit
L01624	D D D D	-1.8				serum and glucocorticoid-regulated kinase (sgk)
AI014135	D D D D	-1.7				EST207690
U92469	D D D D	-1.7				gonadotropin-releasing hormone receptor
J03588	D D D D	-1.7				guanidinoacetate methyltransferase
X52625	NC D D D	-1.7				cytosolic 3-hydroxy 3-methylglutaryl coenzyme A synthase
AI237654	D NC D D	-1.6				EST234216 <i>similar to thioredoxin-interacting protein (E=0.0)</i>
M76110	D NC D D	-1.5				tartrate-resistant acid phosphatase type 5
M15114	D D NC D	-1.1				DNA polymerase alpha

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Table 3. Genes showing increased expression in periovulatory rat granulosa cells after incubation for 24 h in the presence of Org 31710 (10 μ M) compared to control. Text in italics indicates the plausible identity of the genes after a BLAST homology search. I = increased; MI = marginally increased; NC = no change. Previously unpublished data.

GenBank #	Diff Call				Fold Change	Description derived from GenBank
	I	MI	NC	I		
X77235	I	NC	I	I	5.1	ARL gene 4
M58495	I	I	I	I	5.1	NAD(P)H: quinone reductase
K00136	I	I	I	I	3.7	glutathione S-transferase Ya subunit
AA818604	I	NC	I	I	3.5	cDNA clone similar to Hsp70-2
AJ011969	I	I	I	I	3.5	growth/differentiation factor-15/macrophage inhibiting cytokine-1
AA893803	I	I	I	MI	3.5	EST197606
AA859509	MI	MI	I	I	3.1	cDNA clone <i>identical to proline-rich protein PRP2</i>
U95178	I	I	I	I	3.0	DOC-2 p59 isoform
D43623	I	I	I	I	2.9	carnitine palmitoyltransferase I like protein
U63923	I	I	I	I	2.8	thioredoxin reductase
AF020618	I	I	I	I	2.6	progression elevated gene 3 protein
AI639167	MI	I	I	I	2.6	rx01402s Rat mixed-tissue library
AA945737	I	NC	I	I	2.4	EST201236 <i>similar to CXC chemokine receptor (CXCR4) (E=0.0)</i>
AF001417	I	NC	I	I	2.4	zinc finger protein
AA891286	I	I	I	I	2.4	EST195089 <i>similar to thioredoxin reductase 1 (E= 2e⁻¹⁰⁸)</i>
U02553	NC	MI	I	I	2.4	protein tyrosine phosphatase
U90610	I	I	I	I	2.3	CXC chemokine receptor (CXCR4)
AF020618	I	NC	I	MI	2.3	progression elevated gene 3 protein, homologous to growth arrest and DNA damage-inducible gene gadd34
U30186	NC	I	I	I	2.3	growth arrest and DNA-damage-inducible protein GADD153
S74351	MI	I	MI	I	2.3	protein tyrosine phosphatase
X66494	I	I	I	I	2.3	CHOT1
AA859882	I	I	I	I	2.3	cDNA clone similar to ubiquitin carboxyl-terminal hydrolase PGP9.5
X89225	I	I	I	I	2.2	4F2 heavy chain (4F2hc)
Y08355	I	I	I	I	2.2	PKC-zeta-interacting protein
J02679	I	I	I	I	2.2	NAD(P)H:menadione oxidoreductase
D10699	I	I	I	I	2.2	ubiquitin carboxyl-terminal hydrolase PGP9.5
S45812	I	I	I	I	2.2	monoamine oxidase A
AI179610	I	I	I	I	2.2	EST223333 <i>identical to heme oxygenase (decycling) 1 (E=0.0)</i>
J05181	I	MI	I	I	2.1	gamma-glutamylcysteine synthetase
U90610	I	I	I	I	2.1	CXC chemokine receptor (CXCR4)
X62875	I	I	I	I	2.0	High Mobility Group Protein I (Y)
AA892248	I	I	I	I	2.0	EST196051 <i>similar to mitochondrial genome 12S RNA (E=0.0)</i>
AA894148	MI	I	NC	I	2.0	EST197951 <i>similar to apolipoprotein A-IV (E=8e⁻¹³⁸)</i>
D10699	I	I	I	I	2.0	ubiquitin carboxyl-terminal hydrolase PGP9.5
J02722	I	I	I	I	2.0	heme oxygenase
M96601	I	I	NC	I	2.0	taurine transporter
J02962	I	I	I	I	2.0	IgE binding protein
AA799406	I	I	I	I	2.0	EST188903 similar to cytochrome P450 like_TCB
X02904	NC	I	I	I	1.9	glutathione S-transferase P subunit
AB006007	I	I	I	I	1.9	steroidogenic acute regulatory protein
U72660	I	I	I	I	1.8	ninjurin1
AA892520	I	I	I	NC	1.8	EST196323 <i>identical to vesicle amine transport protein 1 homolog (T californica) (Vat1)</i>
M13100	I	I	NC	I	1.8	Rat long interspersed repetitive DNA sequence LINE3 (L1Rn)
AI007824	I	I	I	I	1.8	EST202275
AB001349	I	I	I	NC	1.8	steroidogenic acute regulatory protein
AB015433	I	I	I	I	1.8	4F2 heavy chain (4F2hc)
AI013194	I	I	I	NC	1.7	EST207869 <i>identical to eukaryotic translation initiation factor 5</i>
X07686	I	NC	I	I	1.6	Rat L1Rn B6 repetitive DNA element
AA874843	MI	I	NC	I	1.5	cDNA clone similar to scavenger receptor class B

SUMMARY OF RESULTS

The decrease in the cholesterol synthesis pathway was verified on the level of enzymatic activity. ^{14}C -labelled acetate was added during the last 5 hours of the 24-hour incubation of rat granulosa cells and incorporation into cholesterol, cholesterol ester and progesterone was detected by means of TLC and scintillation counting (Figure 21A). The PR antagonists Org 31710 and RU 486 exhibited a similar effect on the cholesterol synthesis rate in human periovulatory granulosa cells (Figure 21B).

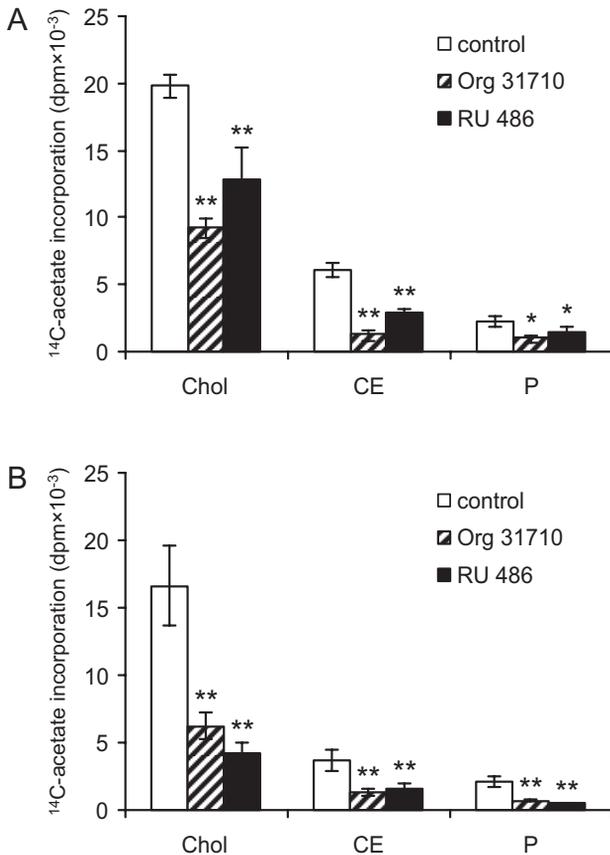


Figure 21

Effects of Org 31710 (10 μM) and RU 486 (10 μM) on the incorporation of ^{14}C -acetate into cholesterol (Chol), cholesterol ester (CE) and progesterone (P) in **A**) periovulatory rat granulosa and **B**) periovulatory human granulosa cells in vitro. Primary isolated periovulatory granulosa cells were incubated in serum free medium for 24 h and ^{14}C -acetate was added during the last 5 h (n = 9-14). * $P < 0.05$; ** $P < 0.01$. Previously published in paper III.

Cholesterol synthesis and apoptosis (Papers III-IV)

The results outlined above indicate that PR antagonists both increase apoptosis and decrease cholesterol synthesis. Three cause-effect relationships constitute plausible, alternative explanations for this result: 1) PR antagonists independently affect apoptosis and cholesterol synthesis, 2) PR antagonists increase apoptosis, which in turn decreases cholesterol synthesis and 3) PR antagonists decrease cholesterol

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synthesis, which in turn increases the degree of apoptosis in periovulatory granulosa cells isolated from rats or humans. We proceeded to elucidate which of the above explanations is the most likely.

If the increase in apoptosis causes a decreased cholesterol synthesis level, other inducers of apoptosis would also cause a decreased cholesterol synthesis rate. However, induction of apoptosis in periovulatory rat granulosa cells to an extent similar to Org 31710 by treatment with the TNF α signalling second messenger C6-ceramide (5 μ M) or the DNA damaging agent doxorubicine (2 μ M) had only minute effects on cholesterol synthesis (unpublished data), indicating that the decreased incorporation of 14 C-acetate is not secondary to apoptosis induction.

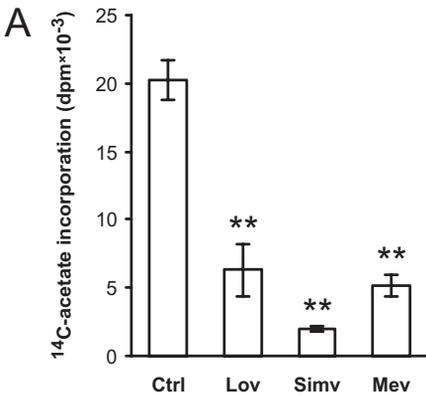
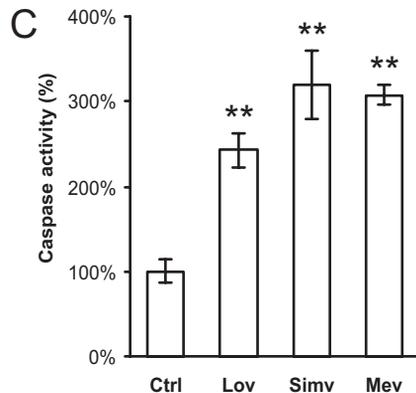
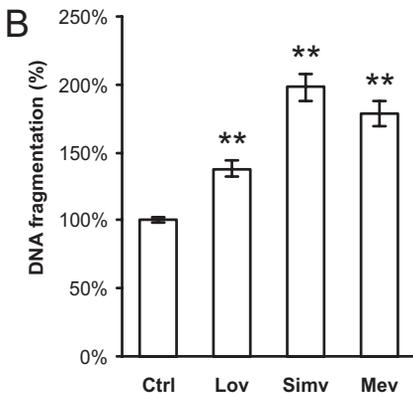


Figure 22

Effects of lovastatin, simvastatin or mevastatin (all 10 μ M) on periovulatory rat granulosa cells during 24 h incubation in serum free medium on **A**) incorporation of 14 C-acetate into cholesterol (n = 11-20), **B**) apoptosis measured as DNA fragmentation (control set to 100%) (n = 11-23) and **C**) apoptosis measured as caspase-3/7 activity (control set to 100%) (n = 4).

** $P < 0.01$

Previously published in paper III.



If the decreased cholesterol synthesis leads to increased apoptosis sensitivity then the degree of apoptosis would also increase after treatment with other cholesterol

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synthesis inhibitors. To test this we added statins, which function as inhibitors of HMG-CoA reductase (the rate-limiting enzyme of cholesterol synthesis), to the incubation medium. It was found that treatment of both rat and human periovulatory granulosa cells with statins increased apoptosis (Figure 22, results from rat granulosa cells). Thus, the decrease in cholesterol synthesis caused by PR antagonists may in turn increase apoptosis in periovulatory granulosa cells.

Furthermore, the addition of mevalonic acid, the product of HMG-CoA reductase, reversed the increase in apoptosis caused by simvastatin (Figure 23). On the other hand, mevalonic acid was unable to reverse the effect on apoptosis after treatment with RU 486. This was not surprising, since the microarray results indicated that many genes downstream from mevalonate were downregulated after treatment with Org 31710.

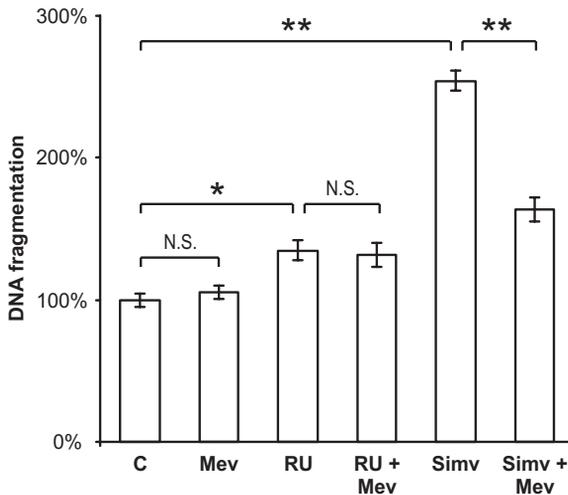


Figure 23

Effect of mevalonic acid (1.6 mM) (Mev) on the degree of apoptosis in periovulatory rat granulosa cells incubated with or without the addition of RU 486 (10 μ M) (RU) or simvastatin (10 μ M) (Simv). The degree of apoptosis was measured as DNA fragmentation (control set to 100%) (n =8-10).

N.S. = non-significant;

* $P < 0.05$; ** $P < 0.01$

Previously published in paper III.

Protein prenylation and apoptosis (Paper IV)

Cholesterol synthesis is also a pathway for the production of dolichols, ubiquinone and substrates for protein prenylation. There have been several studies performed on cell lines in vitro, which suggest that protein prenylation is vital for cell survival. In order to investigate the dependence of human periovulatory granulosa cells on protein prenylation, we treated isolated cells with two different prenylation inhibitors, a farnesyl transferase inhibitor (FTI R115777) and a geranylgeranyl transferase I inhibitor (GGTI 2147). Both inhibitors (10 μ M) increased the degree of apoptosis and combining them resulted in a further increase. This suggests that prenylation is vital for human periovulatory granulosa cell survival.

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The increase in apoptosis observed after obstruction of the cholesterol synthesis could be due to depletion of prenylation substrates. In order to test this hypothesis, the prenylation precursors farnesol (FOH) and geranylgeraniol (GGOH) were added to simvastatin treated cells. FOH, which is utilized as a substrate for protein farnesylation, partially reversed the increase in apoptosis induced by simvastatin. Likewise, GGOH, which is utilized for geranylgeranylation, also reversed the increase in apoptosis caused by simvastatin (Figure 24A). Administered separately, the highest dose of GGOH (30 μM) caused an increase in caspase activity whereas FOH had no significant effect on apoptosis (data not shown).

Blocking PR is a pleiotropic action, which has many effects on human periovulatory granulosa cells. Nevertheless, the effect on apoptosis could be due to lack of prenylation substrates. Both FOH and GGOH partially reversed the effect of Org 31710 (Figure 24B), measured as caspase activity.

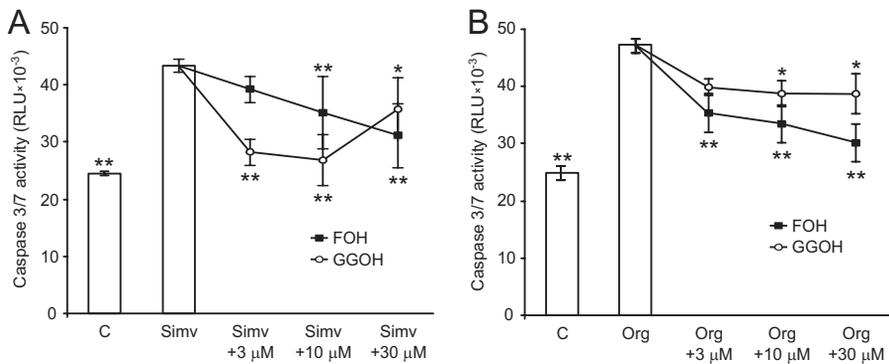


Figure 24

Effect of farnesol (FOH) and geranylgeraniol (GGOH) on apoptosis induced by **A**) simvastatin (50 μM) or **B**) Org 31710 (25 μM) in periovulatory human granulosa cells incubated in serum free medium for 24 hours. Apoptosis was measured as caspase-3/7 activity and is reported as relative luminescence units (RLU) ($n = 11-18$).

* $P < 0.05$, ** $P < 0.01$ compared to treatment with either simvastatin or Org 31710.

Previously published in paper IV.

DISCUSSION

Decreased apoptosis sensitivity after the LH surge

In paper I we mimicked the periovulatory interval in rats by treating immature 26 day old Sprague-Dawley rats with eCG followed by hCG. The observed decrease in DNA fragmentation both in vivo and after 24 hour incubation of isolated granulosa cells in vitro suggests that LH receptor stimulation decreases apoptosis sensitivity in periovulatory rat granulosa cells. Other studies have shown that mature rodent follicles undergo atresia in vivo when gonadotropin support is totally eliminated by hypophysectomy (Braw *et al.*, 1981) or when the LH surge fails to occur as a result of treatment with pentobarbitone (Braw & Tsafri, 1980, Shao *et al.*, 2004) or a GnRH antagonist (Durlinger *et al.*, 2000). In addition, granulosa cells isolated from cows 12 hours after the LH surge have been reported to be resistant to FasL-induced apoptosis, in contrast to those isolated before the LH surge (Porter *et al.*, 2001). In vitro, LH has been reported to reduce apoptosis in intact follicles isolated 48 hours after FSH receptor stimulation of immature rats (Chun *et al.*, 1994). It can thus be considered an established fact that LH receptor stimulation is important for providing resistance to atresia as the follicles enter luteinisation. In contrast, LH has not been demonstrated to reduce apoptosis in isolated granulosa cells in vitro. This probably reflects the importance of interactions with surrounding theca cells as well as dependence on other locally produced substances.

PR stimulation as a survival mechanism

One of the main effects of LH receptor stimulation of mature follicles is the onset of progesterone synthesis and PR expression in granulosa cells. We proceeded to examine the hypothesis that the reduction in apoptosis sensitivity after LH receptor stimulation is mediated by PR. Two different PR antagonists, RU 486 and Org 31710, increased the degree of apoptosis in isolated rat and human periovulatory granulosa cells. Furthermore, inhibition of progesterone synthesis in rat granulosa cells in vitro as a result of the addition of cyanoketone increased the degree of apoptosis. The increase was reversed by the addition of exogenous progesterone, demonstrating that it was specifically due to a lack of progesterone. Taken together, these results are in accordance with the idea that PR signalling is important for the survival of periovulatory granulosa cells. In contrast, only the highest dose of progesterone (1-100 μ M) significantly augmented the hCG induced reduction of DNA fragmentation by an additional 20%, compared to granulosa cells isolated from rats 48 h after administration of eCG. Due to the high endogenous concentration of progesterone it is likely that all PR are constantly occupied by ligand. The concentration of progesterone in follicular fluid has been estimated to remain in the micromolar range at all times (Fujii *et al.*, 1983), suggesting that the regulation of progesterone signalling is on the level of PR expression.

In addition to papers I and II, our group also investigated the effect of PR antagonists on periovulatory mouse granulosa cells (Shao *et al.*, 2003). RU 486 increased apoptosis *in vivo* when injected 4 hours before or at the same time as administration of hCG. Both RU 486 and Org 31710 increased the degree of DNA fragmentation when added to isolated periovulatory granulosa cells *in vitro* for a 24 hour period. Other groups have reported similar results. A study of human periovulatory granulosa cells reported that RU 486 (1 μ M) increases apoptosis over 24 hours of serum free incubation *in vitro* (Makrigiannakis *et al.*, 2000). Contrary to our results, they also reported that the addition of progesterone (0.01-1 μ M) to the culture medium decreases granulosa cell apoptosis *in vitro*. However, no further details about the cell incubations were provided in the article, which makes it difficult to discuss the possible reason(s) for this discrepancy. The LH surge in cows has been reported to decrease sensitivity to FasL-induced apoptosis in the dominant follicle (Porter *et al.*, 2001). This was reversed by the addition of the PR antagonist RU 486, suggesting that PR stimulation is a survival factor at this stage in bovine follicles (Quirk *et al.*, 2004). It has equally been described as a survival factor for bovine luteal cells (Rueda *et al.*, 2000). Inhibition of progesterone synthesis *in vivo* in rhesus macaques by injection of the 3β -hydroxysteroid dehydrogenase inhibitor trilostane increased the number of atretic follicles. The effect was reversed by the administration of the unmetabolisable progestin R5020 (Chaffin & Stouffer, 2000).

Since paper I was published, several studies by our group and others have concluded that PR stimulation is important for the survival of periovulatory granulosa cells in rodents, humans, monkeys and cows. When these studies are taken together it seems reasonable to consider this to be an established fact.

The mechanism behind reduced apoptosis sensitivity after the LH surge has been suggested to be withdrawal from the cell cycle (Quirk *et al.*, 2004). In that study GnRH was used to induce the LH surge in cows. Periovulatory granulosa cells were isolated for up to 12 hours after the LH surge and incubated in serum free medium. Apoptosis was not altered when RU 486 (0.5 μ M) was added to the incubation medium. However, RU 486 increased the sensitivity to FasL-induced apoptosis in granulosa cells isolated 12 h after the LH surge occurred. The granulosa cells were observed to withdraw from the cell cycle after LH receptor stimulation, which was counteracted by the addition of RU 486. Resistance to apoptosis increased when granulosa cells isolated from growing follicles were treated with the DNA replication inhibitor mimosine in order to block the progression through the cell cycle. When co-treated with mimosine, RU 486 was unable to increase FasL-induced apoptosis in granulosa cells isolated 12 h after the occurrence of the LH surge. The results indicate that terminal differentiation and withdrawal from the cell cycle may promote the long-term survival of granulosa-derived corpus luteum cells.

Specificity for the nuclear PR

The classical way in which the effects of progesterone are mediated is via nuclear PR, although several other ways have been reported. Outlined below are the different ways in which progesterone can affect the survival of periovulatory granulosa cells, and an attempt is made to elucidate the possible roles of the different signalling systems in increasing the survival of periovulatory granulosa cells. These signalling pathways are not exclusive. On the contrary, it is likely that progesterone activates several signalling pathways in the same cell. The signalling of the different receptor systems is regulated by the expression levels and progesterone affinity for the receptors, as well as the amount of progesterone available.

Several different experimental approaches have been used to establish that the observed effects are really mediated by the classical nuclear receptor. We tested two PR antagonists, each of which has a different specificity. The fact that the same results were obtained supports the theory that the effects are mediated by the classical nuclear PR (see figure 25).

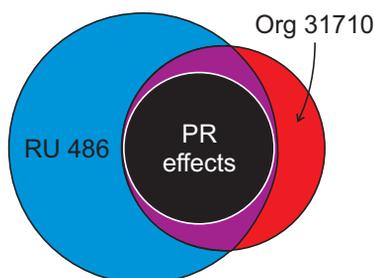


Figure 25

Schematic representation of the effects of two PR antagonists compared to real PR-mediated effects in a cell. The blue circle represents the effects of the PR antagonist RU 486 and the red circle those of the more specific PR antagonist Org 31710. Real PR effects (black circle) can mainly be expected in the overlapping, purple field, in which both RU 486 and Org 31710 operate. However, the possibility that some aspects of PR signalling are unaffected by RU 486 and / or Org 31710 can not be totally excluded.

If the apoptosis increase observed after treatment of periovulatory granulosa cells with RU 486 or Org 31710 is due to the inhibition of PR signalling, the effect should be reversed by the addition of progesterone in molar excess. This would rule out the possibility that Org 31710 and RU 486 produce the same effects by chance without affecting the progesterone receptor. In spite of the fact that granulosa cells produce a great deal of progesterone on their own, this experimental strategy has been successful in human granulosa cells (Makrigiannakis *et al.*, 2000). In the aforementioned study, the apoptosis increase observed after treatment with RU 486 was reversed by the addition of progesterone. This is in contrast to our results, where the addition of progesterone to the incubation medium did not affect the degree of

apoptosis induced by PR antagonists (paper II). In cow periovulatory granulosa cells the effect of RU 486 has been reported to be reversed by the addition of medroxyprogesterone acetate, which can function as a PR agonist (Quirk *et al.*, 2004).

The observed effects of Org 31710 and RU 486 on granulosa cell apoptosis also fit well with the expression pattern of PR. Org 31710 and RU 486 did not affect the degree of apoptosis in granulosa cells isolated from earlier differentiation stage follicles, where PR is not expressed.

Interaction with the glucocorticoid receptor

Progesterone has been shown to increase its own synthesis by means of the GR (Sugino *et al.*, 1997, Telleria *et al.*, 1999) in rat corpora lutea in which PR is absent. RU 486 is a known GR antagonist (Cadepond *et al.*, 1997), which raises the possibility that effects observed after RU 486 treatment are due to interaction with GR. However, Org 31710 does not interact with GR, but is a rather PR specific antagonist. Since RU 486 and Org 31710 exhibited similar effects on periovulatory granulosa cell apoptosis, the effect is likely due to interaction with PR rather than GR. Nevertheless, we have endeavoured to identify GR-mediated effects on apoptosis in periovulatory granulosa cells. The best way to do this would have been to use a GR specific antagonist, thus allowing a direct comparison with our results pertaining to PR antagonist treatment. However, to our knowledge there is no GR specific antagonist. We therefore attempted to identify GR-mediated effects on apoptosis by using the GR agonist dexamethasone. When added alone or in combination with RU 486 dexamethasone did not affect the degree of DNA fragmentation in periovulatory human granulosa cells. In a study on periovulatory cow granulosa cells, this experimental strategy was taken one step further by the inclusion of medroxyprogesterone acetate (Quirk *et al.*, 2004). The increase in FasL-induced apoptosis after treatment with RU 486 was not reversed by the GR agonist dexamethasone. In contrast, the apoptosis-increasing effect of RU 486 was reversed by medroxyprogesterone acetate, which functions as both a GR and a PR agonist. Taken together, our results as well as those reported by others strongly suggest that the increase in apoptosis sensitivity after treatment with RU 486 is not dependent on interaction with GR.

Interaction with the androgen receptor

Both RU 486 and Org 31710 can interact with AR, although their effects on it are weak (Bygdeman *et al.*, 1993, Kloosterboer *et al.*, 1994, Hurd *et al.*, 1997). However, if both PR antagonists affect signalling via AR, this could be misinterpreted as a PR-mediated effect (purple area in figure 25). In order to clarify the possible AR-mediated component of the response to PR antagonists, we treated cells with the AR agonist dihydrotestosterone. The addition of dihydrotestosterone did not affect the degree of apoptosis in periovulatory human granulosa cells. An apoptotic effect

caused by blocking AR signalling seems unlikely, since it has been shown that androgens antagonise oestrogen-induced survival of antral follicles in vivo (Billig *et al.*, 1993). Further experiments, for instance with AR antagonists, are needed to exclude the possibility that AR plays a role in the regulation of periovulatory granulosa cell survival.

Interaction with a GABA_A-like receptor

A few years before paper I was published, it was reported that progesterone can decrease apoptosis in granulosa cells of earlier differentiation stages by way of a GABA_A receptor-like protein (Peluso & Pappalardo, 1998). This was an early attempt to identify other mediators of progesterone signalling in granulosa cells and it seemed plausible, since it is well known that the progesterone metabolite allopregesterone signals via GABA_A receptors in the brain (Lambert *et al.*, 2003, Reddy *et al.*, 2004). However, it has now been recognised that GABA_A-like receptors are not involved in progesterone signalling in granulosa cells (Peluso *et al.*, 2002). In our hands the GABA receptor antagonists picrotoxin and bicuculline as well as the agonist muscimol did not affect the degree of apoptosis in periovulatory rat granulosa cells or granulosa cells isolated 48 hours after eCG treatment (paper I).

Interaction with the membrane progesterin receptor

The recently identified membrane progesterin receptor (mPR) (Zhu *et al.*, 2003b, 2003c) is expressed as three isoforms; mPR α , β and γ . Their expression patterns in rat granulosa cells during follicular development have been characterised in our laboratory (unpublished data). None of these isoforms display an expression pattern that mimics that of nuclear PR. Thus, there is no association between the expression of the mPRs and the effects on apoptosis after treating granulosa cells with PR antagonists. Since neither RU 486 nor Org 31710 interact with human mPR γ (Zhu *et al.*, 2003b), there is no indication that the increased apoptosis observed after treating periovulatory granulosa cells with RU 486 or Org 31710 is mediated by mPR or at least mPR γ .

Interaction with PGRMC1 and SERBP1

Finally, progesterone has also been reported to mediate effects in rat granulosa cells by means of the progesterone receptor membrane component 1 (PGRMC1) (Peluso *et al.*, 2006). In the aforementioned study it was reported that PGRMC1 is expressed throughout the ovary and in granulosa cells at different developmental stages as well as in luteal cells. Overexpression of PGRMC1 in spontaneously immortalised granulosa cells increased the capacity of the cells to bind progesterone and augmented the anti-apoptotic effect of progesterone. Furthermore, the anti-apoptotic effect of progesterone was eliminated by a PGRMC1 antibody.

The same researchers also reported that the SERBP1 protein (serpine 1 mRNA binding protein; also known as PAIRBP1 or RDA288) may co-operate with

PGRMC1 in mediating progesterone signalling (Peluso *et al.*, 2005). SERBP1 was first identified using an antibody targeting the ligand-binding domain of the nuclear progesterone receptor. It is localised in the extracellular surface of the plasma membrane, but does not possess a transmembrane domain, thus it is unlikely that it participates in signal transduction. SERBP1 antibodies attenuated the anti-apoptotic action of progesterone in immature rat granulosa cells.

The possible role of PGRMC1 and/or SERBP1 in reducing periovulatory granulosa cell apoptosis is still unknown. Spontaneously immortalised granulosa cells, which express PGRMC1 and SERBP1, specifically bind ^3H -progesterone, but RU 486 does not reduce ^3H -progesterone binding (Peluso *et al.*, 2001). It is unlikely that RU 486 affects progesterone signalling mediated by SERBP1/PGRMC1 (J.J. Peluso, personal communication).

Conclusions about PR specificity

When the results obtained by us and others are taken together it seems unlikely that the induction of apoptosis after treatment of periovulatory rat or human granulosa cells with RU 486 or Org 31710 is due to interaction with GR, GABA_A, mPR or PGRMC1/SERBP1. The data regarding the possible interaction with AR are weaker and do not justify exclusion. However, the effects are most likely to be mediated by PR.

The results from the knock-out mice lacking PR-A or PR-B have implicated PR-A as being important for ovarian physiology, whereas PR-B seems to have little influence. PR-A can function as a dominant inhibitor of other steroid receptors, which opens yet other possible routes for interaction with other receptor signalling systems.

Progesterone regulated genes in periovulatory granulosa cells

Since progesterone together with its (nuclear) receptor functions as a transcription factor, we proceeded to study the PR-mediated effect on gene transcription in periovulatory rat granulosa cells. We did this by applying the microarray technique to mRNA from granulosa cells incubated for 24 hours *in vitro* with or without the addition of 10 μM Org 31710. This technique enables simultaneous supervision of the expression of thousands of genes and is thus suitable for screening purposes. In addition to changes in gene expression related to apoptosis, PR can also be expected to be involved in ovulation and luteinisation.

During the last decade, interest in ovulation specific gene expression in ovarian follicles has resulted in the identification of several transcriptionally regulated genes. To date there are at least 85 known ovulation-related genes, i.e. genes that are considered to be involved in ovulation and/or luteinisation with direct or indirect evidence of induction as a result of the LH surge (Espey & Richards, 2006). Less attention has been devoted to PR regulated genes in the ovulatory process. There are

DISCUSSION

probably less than 20 genes that are known to be PR regulated during ovulation (table 4). In view of the relative lack of knowledge of PR regulated genes during ovulation, the gene expression analysis presented in this thesis (table 2 and 3) may be an important contribution to the understanding of PR regulated events during ovulation. Our data suggest that PR is involved in many processes, including increased angiogenesis, follicular rupture and regulation of apoptosis.

Table 4

Genes reported by others to be transcriptionally regulated by progesterone in periovulatory granulosa cells. Genes that were found to be regulated by the PR antagonist Org 31710 in this thesis are listed in table 2 and 3. ↔ indicates that progesterone prevents downregulation of the gene. Abbreviations: ADAMTS-1, a disintegrin and metalloproteinase with thrombospondin motifs-1; Ang-1, angiopoietin-1; cGK II, cGMP-dependent protein kinase, type II; Cox-2, cyclooxygenase-2; ER α , oestrogen receptor α ; ET-2, endothelin-2; GC-A, guanylate cyclase-A; GnRHII, gonadotropin-releasing hormone 2; MMP-1, matrix metalloproteinase-1; PAC1, PACAP receptor type 1; PACAP, pituitary adenylate cyclase activating polypeptide; PR, progesterone receptor; Runx1, runt-related transcription factor 1; SUMO-1, small ubiquitin-related modifier-1; TIMP-1, TIMP metalloproteinase inhibitor 1.

Gene	Regulation	Species	References
ADAMTS-1	↑	mouse rat pig	(Robker <i>et al.</i> , 2000) (Espey <i>et al.</i> , 2000, Doyle <i>et al.</i> , 2004) (Shimada <i>et al.</i> , 2004)
Ang-1	↑	monkey	(Hazzard <i>et al.</i> , 1999)
Cathepsin L	↑	mouse	(Robker <i>et al.</i> , 2000)
cGK II	↑	mouse	(Sriraman <i>et al.</i> , 2006)
Cox-2	↑	cow	(Bridges <i>et al.</i> , 2006)
ER α	↔	monkey	(Chaffin <i>et al.</i> , 1999b)
ET-2	↑	mouse	(Palanisamy <i>et al.</i> , 2006)
GC-A	↑	mouse	(Sriraman <i>et al.</i> , 2006)
GnRHII	↓	human	(Khosravi & Leung, 2003)
MMP-1	↑	monkey	(Chaffin & Stouffer, 1999)
PAC ₁	↑	rat	(Ko & Park-Sarge, 2000)
PACAP	↑	rat	(Ko <i>et al.</i> , 1999, Park <i>et al.</i> , 2000)
PR	↑	monkey	(Chaffin <i>et al.</i> , 1999b)
Runx1	↑	rat	(Jo & Curry, 2006)
SUMO-1	↓	mouse	(Shao <i>et al.</i> , 2004)
TIMP-1	↑	monkey	(Chaffin & Stouffer, 1999)

For instance, the known PR-mediated regulation of the proteases ADAMTS-1 and cathepsin L is complemented by our finding pertaining to PR regulation of cathepsin H and tissue-type plasminogen activator. The tissue-type plasminogen activator is known to be upregulated in follicles of several species immediately before ovulation (see Liu, 1999), but to our knowledge the increase in tissue-type plasminogen activator expression has not been demonstrated to be mediated by PR.

Another example is pancreatic phospholipase A2, which was the most downregulated gene in the microarray study (table 2). A few months ago it was reported that phospholipase A2 group IVA is upregulated in granulosa cells of ovulating bovine follicles in response to administration of hCG (Diouf *et al.*, 2006). Our result indicates that this may be mediated by PR. Phospholipase A2 is involved in prostaglandin synthesis by releasing arachidonic acid from membrane phospholipids. Arachidonic acid is then oxygenised by a cyclooxygenase. Indeed, cyclooxygenase-2 was recently demonstrated to be transcriptionally upregulated by progesterone in cow granulosa cells at the time of ovulation (Bridges *et al.*, 2006). Furthermore, prostaglandin F2 α and prostaglandin E are increased by PR in granulosa cells at the time of ovulation (Pall *et al.*, 2000, Bridges *et al.*, 2006). Prostaglandins are classically associated with inflammation and were first reported to be involved in ovulation in 1972 (see Espey & Richards, 2006).

PR-mediated regulation of cholesterol synthesis

A subset of the genes shown to be downregulated by Org 31710 in the microarray analysis is involved in cholesterol synthesis. This was confirmed by treating isolated rat and human periovulatory granulosa cells with Org 31710 or RU 486 and measuring the incorporation of ¹⁴C-labelled acetate into cholesterol, cholesterol ester and progesterone. Both antagonists significantly decreased ¹⁴C-acetate incorporation, demonstrating a downregulation of enzyme activity in the cholesterol synthesis pathway, rather than mere mRNA regulation. This suggests that PR stimulation causes increased de novo cholesterol synthesis in periovulatory granulosa cells. As outlined in figure 5, the possible sources of cholesterol for steroid synthesis in granulosa cells are plasma lipoproteins, stored cholesterol esters and de novo synthesized cholesterol. Most of the cholesterol is usually provided by circulating lipoproteins and the preference for different classes varies between species. However, LH receptor stimulation has also been reported to stimulate the incorporation of acetate into sterols and steroids in granulosa cells (Morris & Gorski, 1973, Andersen & Dietschy, 1978).

Several genes involved in cholesterol synthesis were found to be similarly regulated (Figure 20), suggesting a common regulator. A possible candidate is the SREBP (sterol regulatory element binding protein) family. In particular SREBP-2 has been implied in the transcriptional regulation of genes involved in cholesterol synthesis (Brown & Goldstein, 1997). SREBP-1a and -2 were not represented on our

microarray and the expression of ADD1, the rat homologue of SREBP-1c, remained unchanged. However, lack of evidence on the transcriptional level does not exclude SREBP family involvement, since its members are mainly post-translationally regulated.

Meiosis activating sterols

Progestins have previously been suggested to inhibit the cholesterol synthesis pathway, causing accumulation of meiosis activating sterols (MAS) (Lindenthal *et al.*, 2001). Two different types of MAS have been isolated from follicular fluid (FF-MAS) and testis (T-MAS), respectively. Both of these compounds are intermediates of the cholesterol synthesis pathway between lanosterol and cholesterol, i.e. at a late stage in the synthesis pathway. It has been suggested that resumption of meiosis in oocytes is mediated by MAS from surrounding granulosa cells. Although the function of these sterols is still a matter of debate (Byskov *et al.*, 2002, Tsafiriri *et al.*, 2002), it seems generally accepted that they are formed by the somatic compartment of the follicle after the LH surge.

Multidrug resistance P-glycoproteins

In addition to causing an accumulation of MAS, very high, supraphysiological concentrations of progesterone (100 μ M) and other steroids have been reported to inhibit cholesterol synthesis in several human cell lines as well as in primary isolated cells in vitro (Metherall *et al.*, 1996a, 1996b), causing an accumulation of lanosterol and some other unidentified cholesterol intermediates. The inhibition is not specific to progesterone but appears to be correlated to the hydrophobicity of the steroids and has been suggested to be mediated by multidrug resistance P-glycoproteins (Metherall *et al.*, 1996a).

Cholesterol synthesis and the LH receptor

An interesting relationship between LH and cholesterol synthesis has been presented by Nair & Menon (2004), who suggested that the cholesterol synthesis enzyme mevalonate kinase can function as an LH receptor specific mRNA binding protein. The studies have been extended to show that mevalonate kinase binding of LH receptor mRNA causes increased mRNA degradation and reduces translation into protein (Nair & Menon, 2005). In our model Org 31710 reduced the expression of many cholesterol synthesis genes, including mevalonate kinase. Reduced mevalonate kinase levels would then lead to decreased degradation of LH receptor mRNA, thus increasing LH receptor mRNA levels. However, in our microarray study LH receptor mRNA was the second most downregulated gene after treatment with Org 31710 (see table 2).

Cholesterol synthesis and apoptosis

Our results showed that PR antagonists both increased apoptosis and decreased cholesterol synthesis. In order to elucidate the possible link between apoptosis and

cholesterol synthesis we used statins to pharmacologically inhibit the cholesterol synthesis. As expected, the statins (lovastatin, simvastatin and mevastatin) reduced the incorporation of acetate into cholesterol, cholesterol ester and progesterone in both rat and human periovulatory granulosa cells. In addition, the statins increased apoptosis, supporting the idea that a decrease in cholesterol synthesis increases apoptosis.

Caspases and SREBP

There is at least one direct link between cholesterol synthesis and apoptosis. The SREBP family of cholesterol synthesis regulators has been reported to be cleaved and activated by caspase-3, an important protease during execution of apoptosis (Wang *et al.*, 1995, 1996). Recently, caspase-2 was reported to be transcriptionally induced by SREBP (Logette *et al.*, 2005a, 2005b). In our studies PR antagonists increased caspase-3/-7 activity. The results reported by Wang *et al.* imply that the increased caspase-3 activity should increase SREBP cleavage and activation, thus increasing the transcription of cholesterol synthesis genes. In contrast, we observed a decrease in mRNA expression of these genes.

Statins and fertility

The fact that statins clearly increased granulosa cell apoptosis raises the question of statin treatment and fertility. Statin treatment of women of fertile age is usually combined with contraceptives, since high doses of lovastatin (Minsker *et al.*, 1983) and atorvastatin (Henck *et al.*, 1998) have been reported to be teratogenic in rats. In humans there have been some cases of central nervous system and limb anomalies reported after exposure to statins during the first trimester of pregnancy (Edison & Muenke, 2004, 2005), but no controlled studies have shown teratogenicity in humans and unintentional exposure to statins during pregnancy is not considered a reason for termination (Kyle, 2006). Nevertheless, patients are advised to stop medication prior to conception, especially as elevated cholesterol levels during the relatively short period of pregnancy is believed to have little impact on the long-term outcome. However, recent data have suggested that maternal hypercholesterolemia is associated with the development of foetal atherosclerosis which continues to progress during early childhood despite normal cholesterol levels in the children. In view of this and the tentative human data suggesting that statins are not major human teratogens, today's recommendations may be revised in the future (Hosokawa *et al.*, 2003).

Statins are potent HMG-CoA reductase inhibitors, but less than 5% of an oral dose reaches the circulation as an active drug or metabolite, due to an extensive first-pass extraction by the liver. Cholesterol synthesis is effectively inhibited in the liver, and since such low doses reach the rest of the body it has been suggested that non-hepatic tissues may increase their cholesterol synthesis rate as a means of compensation (Witztum, 1996).

Several statins have been reported to have no adverse effects on reproduction or fertility, including e.g. the number of corpora lutea, in female rats (Tanase & Hirose, 1987, Wise *et al.*, 1990, Dostal *et al.*, 1996). Studies have reported that statin treatment does not affect the levels of circulating sex steroids in pre-, peri- or postmenopausal women (Bailey Merz *et al.*, 2002) or in men (Dobs *et al.*, 2000a, 2000b), nor the duration of the luteal phase (Plotkin *et al.*, 2002). Even if statins would increase the probability of atresia in growing follicles, it could be compensated by increased FSH levels due to negative feedback regulation and thus not affect fertility or steroid levels in serum. To date, the only major effect on fertility has been reported in the German cockroach, where statins have been suggested as potential insecticides (Zapata *et al.*, 2003).

Prenylation

In the field of reproductive endocrinology, cholesterol synthesis has mainly been viewed as responsible for the production of cholesterol for steroidogenesis. However, in addition to providing cholesterol for steroid synthesis, the *de novo* cholesterol synthesis pathway is also of importance for supplying the cell with dolichols, ubiquinone and substrates for protein prenylation (Grünler *et al.*, 1994). Functional prenylation has been shown to be important for cell survival (Perez-Sala & Mollinedo, 1994, Tanaka *et al.*, 2000). Indeed, inhibitors of prenyl transferases increased apoptosis in periovulatory human granulosa cells (paper IV).

We proceeded to study the hypothesis that the induction of apoptosis after blocking cholesterol synthesis in periovulatory granulosa cells is at least partially due to the lack of substrates for protein prenylation. Both the farnesylation substrate FOH and the geranylgeranylation substrate GGOH reversed the increase in apoptosis observed after treatment with either simvastatin or Org 31710. This suggests that Org 31710 and simvastatin increase apoptosis sensitivity in periovulatory granulosa cells by interfering with protein prenylation. Inhibition of protein prenylation as a consequence of statin-treatment has been shown to cause apoptosis in several cell lines *in vitro* (García-Román *et al.*, 2001, Blanco-Colio *et al.*, 2002, Johnson *et al.*, 2004b). *In vivo*, inhibition of protein prenylation has been shown to induce apoptosis in cancer cells (End *et al.*, 2001, Gordon *et al.*, 2002). Based on the data reported by others it seems likely that statins induce apoptosis by means of decreased protein prenylation. However, in order to establish that PR antagonists decrease prenylation it would be necessary to directly measure protein prenylation.

Depletion of prenylation substrates after inhibition of the cholesterol synthesis pathway indicates that a global downregulation of protein prenylation causes increased apoptosis in human periovulatory granulosa cells. However, it remains unclear whether the apoptotic response in cells is due to a global loss of prenylation or loss of a restricted substrate(s) (Wong *et al.*, 2002). To our knowledge there is

only one other study that suggests a regulation of prenylation on a substrate availability level (Gadbut *et al.*, 1997). In this study it is proposed that the induction of cholesterol synthesis in embryonic chick heart cells increases the availability of prenylation substrates, which in turn increases farnesylation and membrane association of Ras proteins.

Prenylated proteins are either farnesylated or geranylgeranylated. In human periovulatory granulosa cells inhibition of either FTase or GGTase-I increased apoptosis and both FOH and GGOH reversed the apoptosis seen after treatment with simvastatin or Org 31710. However, FTase and GGTase-I exhibits cross-specificity for some protein substrates and the activity of the target protein was at least partially retained (Trueblood *et al.*, 1993, Reid *et al.*, 2004). Thus, this does not warrant the conclusion that both farnesylated and geranylgeranylated proteins are necessary for the survival of periovulatory granulosa cells.

Physiological regulation of prenylation

Most studies on prenylation have been performed with cell lines in vitro. Although as many as 0.5-2% of all proteins have been estimated to be prenylated (Epstein *et al.*, 1991), very little is known about the possible physiological regulation. In this thesis we propose a PR-mediated upregulation of the availability of prenylation substrates in periovulatory human granulosa cells. In addition to our study, we are aware of two other studies that suggest a potential hormonal regulation of prenylation (Vicent *et al.*, 2000, Jiang *et al.*, 2001), but neither of them describe a truly physiological situation. Rather, geranylgeranyl pyrophosphate synthase is overexpressed in ob/ob mice, an animal model of obesity and insulin resistance, and upregulated during adipogenesis (Vicent *et al.*, 2000). In the second of these studies, farnesyl pyrophosphate synthase was reduced in the prostate during castration-induced apoptosis. Testosterone replacement, which stimulates rapid prostate regrowth, led to increased farnesyl pyrophosphate synthase expression levels (Jiang *et al.*, 2001).

Summary

Throughout the studies in this thesis the results in rats have been representative of those in humans, despite differences in the ovarian physiology. For instance, rats are multi-ovulatory whereas humans are generally mono-ovulatory, suggesting differences in the precise regulation of follicle survival during the final phases of growth and development. Another, for this thesis more specific, difference is the PR expression pattern. In rats, PR is expressed in a transient manner shortly after the LH surge, whereas it remains in the corpus luteum in humans.

When the results presented in this thesis are taken together with those reported by other groups, it seems reasonable to consider it an established fact that the LH surge reduces apoptosis in periovulatory granulosa cells and that PR stimulation plays an important role in this. Furthermore, the PR-mediated effects on gene transcription

DISCUSSION

reported here complement both earlier and ongoing studies, supporting the important role of PR in ovulation and luteinisation. The PR-mediated regulation of cholesterol synthesis was verified on the level of enzymatic activity of the pathway. It also seems likely that statins induce apoptosis in periovulatory granulosa cells by causing a depletion of the substrates for protein prenylation. The effect of PR antagonists on prenylation is more tentative.

CONCLUSIONS

Based on the studies presented here we have arrived at the following conclusions regarding periovulatory granulosa cells:

- ◇ LH receptor stimulation decreases apoptosis in granulosa cells in mature follicles in vivo.
- ◇ The reduction in apoptosis is at least partially mediated by PR stimulation.
- ◇ The PR antagonist Org 31710 regulates the transcription of many genes, including downregulation of genes involved in cholesterol synthesis.
- ◇ PR antagonists decrease cholesterol synthesis, as detected by the incorporation of ^{14}C -acetate.
- ◇ Cholesterol synthesis inhibitors (statins) increase apoptosis in addition to decreasing cholesterol synthesis.
- ◇ Protein prenylation inhibitors increase apoptosis in human periovulatory granulosa cells.
- ◇ Supplementation of the cell culture medium with prenylation substrates reverses the increase in apoptosis caused by statins or PR antagonists.

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