

OVULATION: Intra-ovarian mechanisms

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Cover illustration: My loves, by Farnosh Zakerkish Sørensen, featuring Tristan and Livas hands.

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Yesterday I was clever, so I wanted to change the world.

Today I am wise, so I am changing myself.

Rumi

ABSTRACT

Background Ovulation is the central biological process involved in the menstrual cycle of women. Specifically, ovulation involves the tissue remodelling of the preovulatory follicle to achieve the rupture of the exterior follicle wall with the extrusion of the oocyte. Other important facets of ovulation include the expansion of cumulus cells around the oocyte, the resumption of the meiotic arrest of the oocyte, and the functional/structural reorganization of the rupturing follicle into a corpus luteum. The ovulatory process involves many mediators that cooperatively and redundantly carry out changes that are necessary for ovulation, normal progression and natural conception. Increased knowledge of mammalian ovulation is important regarding many aspects of female fertility, such as the treatment of anovulation, ovarian stimulation in assisted reproduction, and the prevention of ovarian hyperstimulation. Another aspect is that it may lead to the development of new strategies for contraception.

Aims: The general aim of this study was to increase knowledge regarding the intra-ovarian regulation of ovulation, which was achieved via studies on protease expression, the expression and regulation of the protease inhibitor, the proteome profile in follicular fluid, the expression of osteoprotegerin (OPG), the receptor activator of the nuclear factor kappa B ligand (RANKL), and the effects of calcineurin inhibitors on ovulation.

Methods: Granulosa cells, theca cells, follicular fluid, and whole follicles were obtained from women at four different stages of the ovulatory process. Expression, proteome profile, and immunohistochemistry were performed. Granulosa lutein cells were used for the cell culture from women undergoing in vitro fertilisation (IVF). Immature Sprague-Dawley rats were primed with pregnant mare's serum gonadotropin to induce maturation and subsequent ovulation, that was triggered 48 hours later with human chorionic gonadotropin (hCG).

In vivo experiments in this animal model as well as in vitro experiments on its cells and tissues were conducted.

Expression patterns were studied via a quantitative, real-time polymerase chain reaction (RT-PCR) and a microarray. Proteins were quantified and identified by mass spectrometry isobaric tags for relative and absolute quantification (iTRAQ), and localization was performed with immunohistochemistry. Assays were also used for the assessment of plasmin activity, leukocyte distribution, steroid levels, and levels of mediators / pharmacological agents in the blood.

Results: Paper I indicate that an ovulatory trigger induces expression in the human granulosa and theca cells of certain proteases from the matrix metalloproteinase (MMP) as well as a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTs) family. Paper II presents data on the

increased expression of the protease inhibitor tissue factor pathway inhibitor 2 (TFPI2) in the ovulating follicle of the human and rat. Moreover, the downstream signalling pathways and effects on a large number of mediators were also characterized. Paper III use a modern proteomic technique to identify more than 500 proteins in the follicular fluid during ovulation, with 25 showing level changes during human ovulation.

Paper IV identifies OPG and RANKL as potential mediators in the intra-ovarian events of ovulation. Paper V demonstrates that cyclosporine-A, but not tacrolimus, negatively influences ovulation in the rat.

Conclusion: The results of the thesis provide information on the roles and functions of several new mediators in ovulation.

Key words: *A disintegrin and metalloproteinase with thrombospondin-like motifs, animal model, calcineurin, cyclosporine-A, follicle, follicular fluid, granulosa cell, human, human chorionic gonadotropin, immunohistochemistry matrix metalloproteinase, menstrual cycle, osteoprotegerin, ovary, ovulation, plasmin, protease, proteomic, rat, receptor activator of nuclear factor kappa B ligand, tacrolimus, theca cell, tissue factor pathway inhibitor 2*

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Rosewell KL, Al-Alem L, Zakerkish F, McCord L, Akin JW, Chaffin CL, Brännström M, Curry TE Jr. Induction of proteinases in the human preovulatory follicle of the menstrual cycle by human chorionic gonadotropin. *Fertil Steril* 2015;103:826-833
- II. Puttabytappa M, Al-Alem LF, Zakerkish F, Rosewell KL, Brännström M, Curry TE Jr. Induction of tissue factor pathway inhibitor 2 by hCG regulates periovulatory gene expression and plasmin activity. *Endocrinology* 2017;158:109-120
- III. Zakerkish F, Brännström M, Carlsohn E, Sihlbom C, Van der Post S, Thoroddsen A. Proteomic analysis of follicular fluid during human ovulation. Revision 1, submitted to *Acta Obstet Gynecol Scand*
- IV. Zakerkish F, Thoroddsen A, Dahm-Kähler P, Olofsson J, Brännström M. Expression patterns of osteoprotegerin (OPG) and receptor activator nuclear factor kappa B ligand (RANKL) in human follicles during ovulation. In manuscript
- V. Zakerkish F, Soriano MJ, Novella-Maestre E, Brännström M, Diaz-Garcia C. Differential effects of the immunosuppressive calcineurin inhibitors cyclosporine-A and tacrolimus on ovulation in a murine model. Revision 1, submitted to *Hum Reprod Open*

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ABBREVIATIONS

A2PLA2G4A	phospholipase cytosolic phospholipase A2
ACN	acetonitrile
ADAMTS	a disintegrin and metalloproteinase with thrombospondin-like motifs
ANGP	angiopoietins
AKT	phosphorylation of protein kinase b
APO	apolipoprotein
AR	androgen receptor
AREG	amphiregulin
BMP	bone morphogenetic protein
BTG	betacellulin
cAMP	cyclic adenosine monophosphate
CCL	chemokine (c-c motif) ligand
cDNA	complementary deoxyribonucleic acid
CID	collision induced dissociation
CMIA	automated chemiluminescent immunoassay
CNI	calcineurin inhibitors
COC	cumulus-oocyte complex
CEBP	ccat/enhancer-binding protein
CREB	cyclic adenosine monophosphate response element binding protein
Ct	comparative cycle threshold
Ctsl	cathepin
CXCL	chemokine (c-x-c motif) ligand
CyA	cyclosporine-a
CYP17A1	17 α -monooxygenase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eCG	equine chorionic gonadotrophin
ECM	extracellular matrix
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
ELANE	elastase neutrophil expressed
EO	early ovulatory
EREG	epiregulin
ERK	extracellular signal-regulated kinase
ESR	estrogen receptor
FF	follicular fluid
FKBP	fk506 binding protein
FSH	follicle stimulating hormone
FSK	forskolin
FOXO1	forkhead boxprotein O1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDF-9	growth differentiation factor 9
GnRH	gonadotropin releasing hormone
GPX	glutathione peroxidase
GST	glutathione s-transferase
HCD	highenergy collision dissociation
hCG	human chorionic gonadotropin
HIF	hypoxia-inducible factor
HRT	hormonal replacement therapy
HSD3B	3 β -hydroxysteroid dehydrogenase
IL	interleukin
ITI	inter- α -trypsin inhibitor
ITIHC	inter- α -trypsin inhibitor heavy chain
iTRAQ	isobaric tags for relative and absolute quantification
ITS	insulin transferrin selenium
IVF	in vitro fertilization
LH	luteinizing hormone
LHCGR	luteinizing hormone/human chorionic receptor
LO	late ovulatory
LUF	luteinized unruptured follicle

MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MEK	mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NSAID	non-steroidal anti-inflammatory drugs
NRIP	nuclear receptor interacting protein
OHSS	ovarian hyperstimulation syndrome
OPG	osteoprotegerin
OPTN	organ procurement and transplantation network
OSE	ovarian surface epithelium
PA	plasminogen activator
PAI	pa inhibitor
PAR1	protease-activated receptor 1
PI3K	phosphatidylinositol 3-kinase
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PGA	progesterone receptor a
PGB	progesterone receptor b
PGF2 α	prostaglandin F2 α
PGR	progesterone receptor
PGRMC	progesterone receptor membrane component
PKA	protein kinase a
PKC	protein kinase c
PLAT	tissue-type plasminogen activator
PLAU	urokinase-type plasminogen activator
PMA	phorbol-12-myristate-13-acetate
PMSG	pregnant mare serum gonadotropin
PO	preovulatory
PON	paraoxonase
PR	progesterone receptor

PSO	postovulatory
PTGER	prostaglandin e receptor
PTGES	prostaglandin e synthase
PTSGS2	peroxidase/cyclooxygenase prostaglandin-endoperoxide synthase 2
RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa B ligand
RBC	red blood cell
rhCG	recombinant human chorionic gonadotropin
RPLPO	large ribosomal protein
RT	reverse transcription
RT-PCR	real time polymerase chain reaction
RUNX	runt-related transcription factor
SHBG	sex hormone-binding globulin
siRNA	small interfering ribonucleic acid
sOPG	serum osteoprotegerin
sRANKL	soluble RANKL
StAR	steroidogenic acute regulatory protein
SXC	steric exclusion chromatography
TAC	tacrolimus
TGF	transforming growth factor
TFP12	tissue factor pathway inhibitor 2
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
TVU	transvaginal ultrasound
VEGF	vascular endothelial growth factor
v/v	volume-to-volume ratio

INTRODUCTION

During the fertile life of a woman, there exists several monthly cyclic events of the reproductive tract. The ultimate goal of these cyclic events in the woman is to develop a fertilizable oocyte, and then after fertilization by one sperm, provide optimal circumstances for embryo development, transport towards the uterus, implantation, placentation and further pregnancy. Within the ovary, ovulation is the central event after development of the oocyte and the ovarian follicle, and then culminates in the rupture of the follicle wall with the extrusion of a mature and fertilizable oocyte. The ovulatory follicle is invaded by leukocytes and functional as well as structural remodeling leads to formation of a corpus luteum. The present study deals with some of the intricate biochemical changes involved in the mechanism of ovulation.

FOLLICULOGENESIS

The mammalian primordial germ cells are derived from the embryonic ectoderm (Gardener et al., 1985) and are formed in the yolk sac wall. These germ cells migrate early in embryonic life into the evolving gonads, with continued mitotic divisions during migration and colonization of the primary gonad (Tam & Snow, 1981). In the primary gonad, the cells transform from ameboid into rounded shapes and are then named oogonia. Also, in this oogonial stage, there is a high mitotic activity (Beaumont & Mandl, 1962) with the cells located into clusters. The mitotic activity of this oogonial stage is exclusively during fetal life. Later on, onset of meiosis marks the transition of oogonia into oocyte and the initiation of this is by meiosis inducing substances (Andersen et al, 1981). Meiosis is arrested at the diplotene stage of the first meiotic division and this block of meiosis persists for variable periods of time, depending on when a follicle starts to grow. Around the periphery of a small-sized oocyte (9-25 μm), there is a single layer of flattened pregranulosa cells and around that a basement membrane. These pools of non-growing primordial follicles are situated in the outer cortex of the ovary. In early human fetal life, there are about 7 million primordial follicles in the two ovaries together. There is a continuous and genetically determined non-gonadotropin dependent reduction in the pool of primordial follicles, and there exist around 400 000-500 000 primordial follicles left at puberty (Gougeon, 1996). The development from this smallest follicle, the primordial follicle, into a preovulatory (Graafian) follicle is a long journey, with most of the primordial follicles undergoing apoptosis along the pathway towards the preovulatory follicle. The follicular development in human from a primordial follicle into a large preovulatory follicle takes about 120 days, as schematically shown in Fig. 1

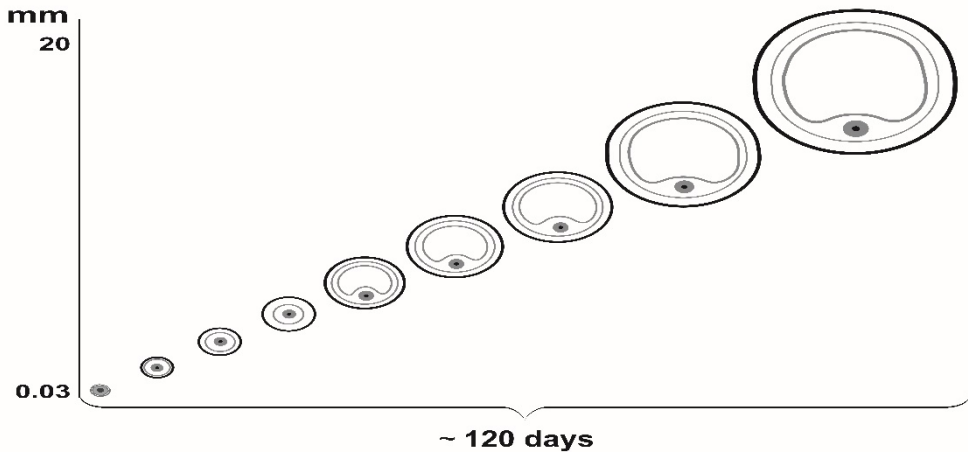


Fig. 1. Schematic overview of follicular development in the human from a primordial follicle into a preovulatory follicle.

The follicles are divided into preantral (all follicles from primordial until formation of a follicular antrum) and antral (follicles with fluid-filled antrum). The preantral follicles are characterized into three developmental stages (Lintern-Moore et al., 1974). The smallest is the primordial follicle (30-60 μm in diameter), that contains of a small oocyte (9-25 μm), which is surrounded by a layer of flattened pre-granulosa cells. The second type is the primary follicle (>60 μm in diameter), with also a small-sized oocyte (9-25 μm), but now with a change of pre-granulosa from flattened to cuboidal shape with proper granulosa cell appearance. The theca layer develops from interstitial stroma cells at the end of the primary follicle stage before transition into a secondary follicle. The third stage is the secondary follicle (≥ 120 μm in diameter), containing an enlarged oocyte (>60 μm) and with several layers of cuboidal granulosa cells, estimated to be > 500 in numbers and with some functional differentiation (Gougeon, 1996). These granulosa cells secrete mucopolysaccharides, which give rise to the zona pellucida (Chiquoine, 1960) of the oocyte and this facilitates gap junctional contact and transfer of biochemical information between granulosa cells and the oocyte. As the follicle grows, the theca externa forms. The appearance and the development of the theca layer are associated with follicular blood supply expansion. The overall development from primordial follicle to secondary follicle is gonadotropin independent, with influence from growth factors and cytokines (Findlay et al., 2002), such as transforming growth factor β (TGF β), bone

morphogenetic protein (BMP), and growth differentiation factor-9 (GDF-9) working in paracrine fashions (Liu et al., 2019).

The follicular development phase that converts secondary and preantral follicles into antral follicles (>2 mm in diameter) is called the tonic growth phase. The differentiating secondary follicles migrate from the periphery of the ovary into the medulla and thecal arterioles are acquired (Bjersing & Cajander, 1974c). Concurrent with the formation of capillaries, the theca-interstitial cells differentiate and start to express receptors for luteinizing hormone (LH) (Bacich et al., 1994) and show steroidogenic activity.

These theca interna cells are epitheloid-shaped and located close to the basement membrane. The more peripheral layer of theca externa cells keep their spindle-shaped form and show a gradual merger with the stroma cells. The increase of LH and follicle stimulating hormone (FSH) in the human cycle, leads to recruitment of several growing follicles from the pool of preantral follicles and 5-10 follicles per ovary are commonly recruited each cycle. There is an increase in follicular diameter and high mitotic activity of granulosa cells. The overall increase in follicular size is due to the proliferation of the granulosa cells and the development of the follicular antrum. When the follicle is about 0.2-0.4 mm in diameter, several sites of accumulated fluid and spots with aggregated extra cellular matrix (ECM) are seen among the granulosa (van Wezel et al., 1999). A focimatrix also develops as aggregates of basal lamina-like material between granulosa cells somewhat later during follicular development (Irving-Rodgers & Rodgers, 2005). The follicular antrum is then formed with accumulation of fluid and the follicle is transformed into a true antral follicle. In general, a preantral follicle develops into an antral follicle with formation of more granulosa cells and production of follicular fluid. The antral follicle will grow both by increase in volume of follicular fluid but also by increased number of granulosa cells. The follicular fluid contains also major blood proteins at approximately the same concentration as serum, but the relative proportion of proteins differs (Shalgi et al., 1973). These proteins reach the antrum by diffusion from the vascular spaces outside the basal membrane but there is also a large proportion of proteins that are produced within the granulosa cells, as elaborated on within the thesis. The development of the follicles and conversion into corpus luteum is outlined in Fig. 2

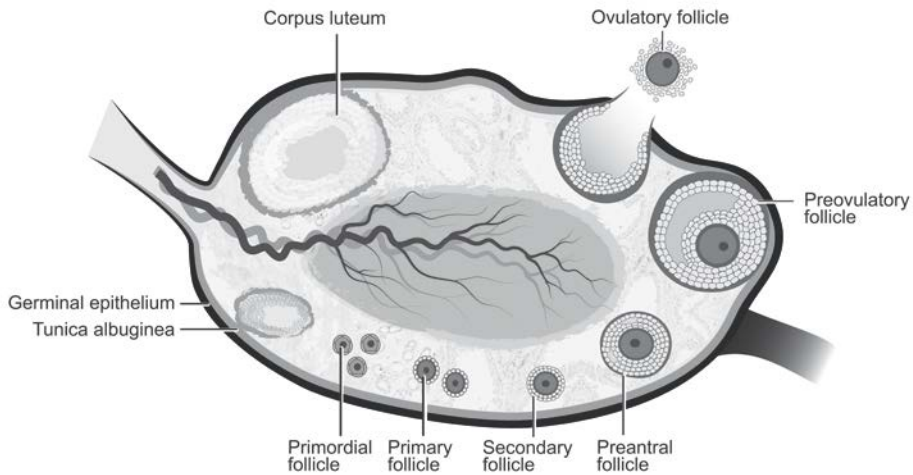


Fig. 2. Follicular development within the ovary.

The cellular and extracellular arrangement of the preovulatory follicle

The preovulatory follicle is made up of three separate functional units, which are the oocyte, the granulosa cell compartment, and the thecal cell compartment, as schematically shown in Fig 3 and described in detail below. The compartments contain different cell types and between that extracellular matrix (ECM), which supports the structure of the follicle but is also important for communication between cells within and between compartments.

There are three phenotypically different granulosa cells in the preovulatory follicle. All granulosa cells are steroidogenically active with estradiol being their main product, but the different types of granulosa cells of the preovulatory follicle show disparate profiles of paracrine and autocrine signaling. The granulosa-cumulus cells enclose and support the oocyte, the antral granulosa cells are positioned adjacent to the follicular antrum, and the mural granulosa cells are next to the basement membrane, which is the structural barrier between the compartment of granulosa and theca cells.

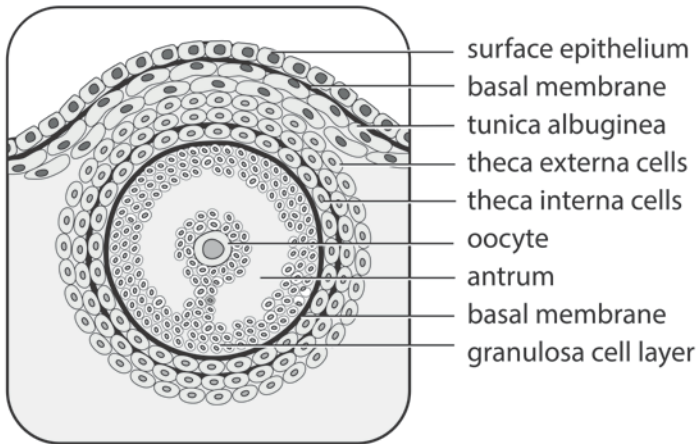


Fig. 3. The different cells and layers of the preovulatory follicle.

The theca cell compartment comprises a more centrally situated layer with theca interna cells, which are steroidogenically active and produce mainly the androgens, androstenedione and testosterone. In the functional unit of the preovulatory follicle, the two-cell cooperation is that of LH-stimulated androgen production in theca cells, diffusion of androgens into the avascular granulosa cell compartment, and conversion of androgens into estradiol, a process which is driven by FSH, stimulated induction of aromatase enzyme. The theca interna section has a thickness of around three to five cells in layers. There is also a vascular network of capillaries from the theca externa that reaches the theca interna. The more peripherally situated theca externa cells are non-steroid-producing cells. They are of two slightly different types. One sort resembles a fibroblast, and another is similar to a smooth muscle cell (Osvaldo-Decima, 1970; Fumagalli et al., 1971).

The theca externa is a collagenous stratum with up to eight layers of fusiform cells and with extensive networks of blood and lymph vessels. Concerning the ECM composition of the theca layer, collagen type III is present in both the theca externa and the theca interna but is lacking in the basement membrane separating the theca interna from the granulosa cell compartment. Collagen type IV is found in both the theca interna and in the thecal-granulosa basement membrane (Lind et al., 2006).

The tunica albuginea, outside the theca externa, is a dense layer of ECM that is positioned all around the periphery of the ovary. The tunica albuginea is composed of densely packed collagen fibers, surrounding a single layer of fibroblasts. The ECM of the tunica albuginea is composed of collagens type I,

type III, and type IV (Lind et al., 2006), and with collagen type I having concentric, network-like distribution.

Above the tunica albuginea there is a basement membrane comprised primarily of laminin, enacting, and heparin sulfate proteoglycans (Espey, 1980, Murdoch & McDonnel, 2002, Yang et al., 2004). The outermost layer covering the preovulatory follicle is the single layer of ovarian surface epithelium (OSE), with cells that are flat or cuboidal, depending on stage of the reproductive cycle and position in relation to the preovulatory follicle.

Structural and vascular alterations of the preovulatory follicle during ovulation

There exist some studies that have looked at the structural changes of the preovulatory follicle at ovulation. An early study in the rabbit, using preovulatory follicles of different times during the ovulatory interval, showed typical patterns (Espey, 1971). Around two hours prior to rupture, the cells on the apex of preovulatory follicle become flattened and lose their microvilli (Motta et al., 1971). In some areas of the apex, the cells disappear completely, and large droplets of viscous fluid material collect on the surface (Motta & van Blerkom, 1975). Fibroblasts in the tunica albuginea and theca externa transform from quiescent to motile cells as they get elongated in shape.

Further inside the follicle the mitotic activity among the granulosa cells decreases shortly after the LH surge (Boucek et al., 1967) and some of the granulosa cells come loose into the follicular antrum. Both theca cells and granulosa cells stop proliferating, enlarge in size and accumulate lipids into droplets that supply cholesterol for steroid hormone synthesis, as well as acquire of mitochondria with tubular cristae and large proportion smooth endoplasmic reticulum (Enders, 1973). In the theca externa there is a preovulatory increase in multivesicular structures in the fibroblasts and the possible release of enzymes from these may be related to the disintegration of the collagen fibers as ovulation approaches (Espey, 1971).

There are also vascular changes that take place during the ovulatory process, and that is primarily in the theca layers. Within minutes after the gonadotropin surge, there is a rise in ovarian blood flow, caused by arteriolar dilation (Janson, 1975).

Later there is an increase in vascular permeability (Kanzaki, et al., 1982). The increase in permeability is due to appearance of small fenestrations in the endothelium (Bjersing & Cajander, 1974b), and to the formation of gaps between the cells (Okuda et al., 1980).

In a recent study this was quantified as a 3-fold increase in number of large pores during ovulation in the rat (Mitsube et al., 2013) interstitial edema

develops and with extravasation of erythrocytes (Parr, 1974) and leukocytes (Bjersing & Cajander, 1974c). The blood cells accumulate in the theca layer. Due to disruption of the basal membranes around the vessels and between the layers of theca cells and granulosa cells (Bjersing & Cajander, 1974b) fluid also collects among granulosa cells to increase the volume of follicular fluid in the antrum. This focal disruption of the basement membrane under the granulosa cells basal lamina, with local production of factors that stimulate neoangiogenesis cause ingrowth of capillaries into the previously avascular granulosa cell layer. Vessels, that grow from the theca and stromal vessels and towards the center of the follicle pushes into the granulosa cell layer so that an intersecting network will eventually contact every granulosa-lutein cell (Brannian et al., 1991). Vasoconstriction caused by soluble molecules such as endothelins may cause reduced blood flow through apical vessels (Migone et al., 2016).

Along with expanding vasculature, increased blood flow and secretion of chemokines from theca and granulosa cells, induce a massive infiltration of leukocytes from circulating blood (Brännström & Enskog, 2002). The leukocytes with their machinery of active substances, including proteinases, will together with the resident cells of the preovulatory follicle cause a directed weakening of the follicular wall at the apex, leading to the rupture of the follicle when the gradually decreasing tensile strength of the follicle can no longer withhold the intra-follicular pressure (Matousek et al., 2001).

THE HUMAN REPRODUCTIVE CYCLE

The monthly reproductive cycle of the human is divided into the follicular phase, the luteal phase and between those the shorter ovulatory phase. Simultaneous changes occur in the endometrium and the ovary during the reproductive cycle. The two main phases of the endometrium are named the proliferative phase (corresponding to the ovarian follicular phase) and the secretory phase (corresponding to the ovarian luteal phase). The first day of menstruation (cycle day 1) is the start of the follicular phase and this phase usually has a length of 10-14 days, but with considerable inter-individual variations.

Deviations in total menstrual cycle length are most often due to variations in duration of follicular phase, since the duration of the luteal phase is fairly constant. Typically, the menstrual length becomes shorter towards the end of the fertile period. The hormonal changes during the follicular phase are shown in Fig.4.

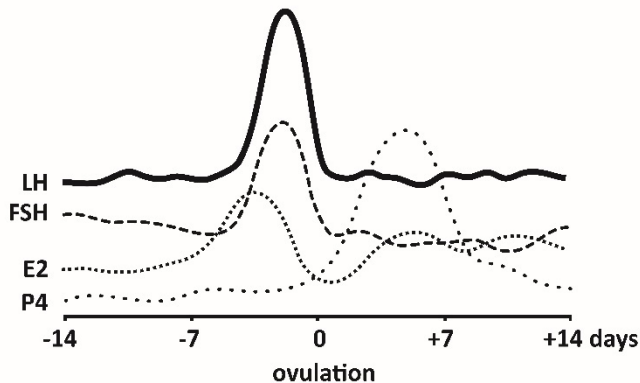


Fig. 4. Hormonal changes during the human menstrual cycle. E2 = estradiol; P4 = progesterone.

There are elevated FSH levels during the first days of the follicular phase and this helps to stimulate recruited follicles that are engaged into each cycle. These recruited antral follicles are identical in their morphological appearance (Goodman et al., 1977, Nilsson et al., 1982) and around cycle-day 5, the processes of selection and dominance takes place (Pache et al., 1990). One follicle will during the midfollicular phase produce more estradiol than the other follicles and become dominant. It will show increased sensitivity for FSH and will also later gain of LH receptors.

The dramatic increase of estradiol and inhibin A, produced by the dominant follicle, results in a negative feed-back regulation and FSH is decreased, which cause all the non-dominant follicles to go into atresia (Tilly et al., 1991). The dominant follicle is surrounded by theca cells that selectively bind more LH than the theca cells surrounding the non-dominant follicles (DiZerega et al., 1980, Zeleznik et al., 1981). The estradiol levels will continue to rise despite decreasing FSH levels due to high androgen availability from the theca cells, a high sensitivity for FSH because of increased receptor density, and since the absolute number of granulosa cells increase rapidly due to marked mitosis among the cells. Moreover, vascularity in the theca of the dominant follicle becomes more prominent in comparison to other follicles (Kanzaki et al., 1981)

and this leads to increased delivery of LH to theca cells and FSH to the granulosa cells, also supporting further follicular growth and rising estradiol levels. This final phase of follicle development is therefore highly dependent on gonadotropins. The rising estradiol levels will prime the pituitary for the gonadotropin surge, where LH is the signal for ovulation. The regulation and effects of the LH surge are described in more detail below.

The ovulation period is approximately 36 hours (h) long from the initiation of the LH surge until follicular rupture (Andersen et al., 1995, Hanna et al., 1994). There are prominent structural and functional changes in the follicle during this phase, as described above and below.

After ovulation, the luteal phase starts, and the dominant follicle transforms into a corpus luteum with secretion of mainly progesterone but also estradiol. The changes in the steroidogenic machinery that allows this are described further in the section below. The secretion of these steroids results in a bell-shaped concentration curve of progesterone and estradiol in peripheral blood, in the event of that implantation of an embryo does not occur. The duration of the non-pregnant luteal phase is fairly constant around 12-14 days duration and with luteolysis caused by prostaglandins (Dennefors et al., 1982, Vega et al., 1998) and further invasion of macrophages (Lei et al., 1991, Wang et al., 1992).

The LH surge

The midcycle surge of LH is the initiator of the ovulatory events. The LH secretion is driven by gonadotropin releasing hormone (GnRH), a decapeptide of the arcuate nucleus of the hypothalamus that is released in pulses into the hypophyseal portal circulation.

High and increasing blood levels of estradiol, as in the late follicular phase increase the frequency of GnRH pulses and prepare the gonadotropes (containing granules of LH) of the anterior pituitary to release large amounts of LH in response to each pulse of GnRH. This results in sustained high blood levels of LH for around 34 h in women (Casper, 2015). In the experimental setting and also commonly in vitro fertilization (IVF), human chorionic gonadotropin (hCG) is used as substitute for LH. It has a higher affinity to the LH/hCG receptor (LHCGR) than LH and is cleared more slowly from circulation, mainly because its higher content of saccharide chains (Norman et al., 2000).

There are some differences in regional responsiveness to LH. While theca and granulosa cells of the preovulatory follicles express LHCGR, highest levels are present in theca and the mural granulosa cells closest to the basal lamina (Peng et al., 1991, Yung et al., 2014). Studies also show that LHCGR may be

expressed in focal areas around the ovulatory follicle, but in low density around the follicle apex (Nguyen et al., 2012).

Second messengers of LH

The complexity of the LH signaling pathway has been elucidated in greater detail during the last decades, from the original concept that cyclic adenosine monophosphate (cAMP), with interactions on protein kinase A (PKA), was the sole and second messenger for further downstream signaling after LH coupling to the G protein-coupled membrane receptor LHCGR.

Thus other LH-induced intracellular signaling cascades in preovulatory follicle include protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), tyrosine kinase-mediated pathways, and their respective downstream mitogen-activated protein kinases (MAPKs) (Panigone et al., 2008, Richards et al., 1979).

The classical cAMP dependent way is by receptor activation of adenylate cyclase, causing increased levels of intracellular cAMP (Richards et al., 1979), leading to activation of the cAMP-dependent PKA to further activate the cAMP-response element-binding protein (CREB) (Richards et al., 1979). This intracellular signaling pathway is the primary pathway mediating LH action in the preovulatory follicle at ovulation. LH-activation of cAMP-PKA signaling pathway leads to very rapid activation of the epidermal growth factor receptor (EGFR)-tyrosine kinase pathway, as demonstrated by that hCG stimulates rapid and dramatic increases in (epidermal growth factor) EGF-like growth factors such as amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTG) and also induces phosphorylation of EGFR in the preovulatory follicle (Panigone et al., 2008, Park et al., 2004,). Experiments with mutant mouse models have demonstrated the obligatory role of the activation of EGFR and their key downstream kinases, extracellular signal-regulated kinase (ERK)1/2, in the ovulation, involving cumulus expansion, follicular rupture, and luteinization (Fan et al., 2009, Hsieh et al., 2007).

Another pathway activated by LH is the PI3K pathway, shown by an effect to increase phosphorylation of protein kinase B (AKT) (Fan et al., 2008) and forkhead box protein O1 (FOXO1) (Fan et al., 2008), both well-known signaling effectors downstream of the PI3K pathway. The binding of LH to LHCGR also activates member of the MAPK superfamily, including p38MAPK. Thus, hCG induced transient increases in p38MAPK phosphorylation in preovulatory rat follicles (Maizels et al., 2001) and there is a relationship with cumulus expansion since pharmacological inhibition of p38MAPK activity or genetic deletion of p38MAPK α isoform resulted in impaired meiotic resumption and cumulus expansion in porcine and mouse cumulus-oocyte complex (COCs) (Yamashita et al., 2009, Liu et al., 2010). It

has also been shown that LH increases inositol triphosphate levels in granulosa cells, suggesting activation of the PKC pathway (Davis et al., 1986).

Transcriptional factors and regulation of post second messengers by luteinizing hormone (LH)

Activation of the second messenger systems by LH, as described above, lead to activation/production of transcriptional regulators that directly control the transcription of downstream target genes. Concerning the classical nuclear progesterone receptor (PGR), it is known that two forms of PGR, denoted progesterone receptor A (PRA) and progesterone receptor B (PRB), are generated from the same gene via ribonucleic acid (RNA) splice variants (Kraus et al., 1993). In granulosa cells, expression of PRA predominates over PRB both before and after the LH surge (Shao et al., 2003). Distribution of PRA and PRB in theca cells has not been reported. The PGR is present in very low to nondetectable levels in granulosa cells of dominant follicles before the LH surge but expression increases rapidly after the LH surge (Shaffin et al., 1999, Hild-Petito et al., 1988).

Theca cells express modest levels of PGR before and after the surge (Horie et al., 1992). Membrane progesterone receptors also mediate progesterone action within the ovulatory follicle. These membrane receptors, progesterone receptor membrane component (PGRMC) 1 and PGRMC2, are progesterone binding proteins which cooperate with additional protein partners to generate a cellular response to progesterone (Peluso & Pru, 2014). The first indication of an obligatory role for PGR in ovulation was that progesterone receptor (*Pgr*) null mice fail to ovulate with oocytes remaining trapped within the newly formed corpus luteum (Robker et al., 2000). Early, it was found that the genes a disintegrin and metalloproteinase with thrombospondin-like motifs 1 (*Adamts1*) and cathepin (*Ctsl*) (Robker et al., 2000), coding for two proteases, are highly up-regulated by hCG, but markedly down-regulated in granulosa cells of ovulatory follicles in *Pgr* null mice. The proteases *Adamts1* and *Ctsl* can act on ECM proteins to aid the breakdown of the follicular wall and it was later shown that *Adamts1* deficient mice had compromised follicular development and ovulation (Shozu et al., 2005) and also effects on cumulus expansion. Recent gene profiling studies using *Pgr* null mice (Kim et al., 2008, Kim et al., 2009) have identified an array of PGR-downstream genes in granulosa cells of ovulatory follicles including the hypoxia-inducible factors *HIF1A*, *HIF2A*, and *HIF1B*.

There is also an increased expression of CCAAT/enhancer-binding protein beta (*CEBPB*) transcription factors by an ovulatory signal in granulosa cells of preovulatory follicles in both rat and mouse (Park et al., 1991, Garcia et al., 2012) and *CEBPB* null mice do not ovulate (Sterneck et al., 1997). Moreover,

administration of anti-sense oligos against *CEBPB* resulted in decreased ovulation rate in the in vitro perfused rat ovary (Pall et al., 1997). The use of conditional knockout mouse showed that the deletion of either CCAAT/enhancer-binding protein alpha (CEBPA) or CEBPB in granulosa cells resulted in reduced and deletion of both CEBPA and *CEBPB* induce complete blockade of the ovulatory events including cumulus expansion, the rupture of follicles, and luteinization (Fan et al., 2011).

Core binding factor (CBF) is a heterodimeric transcription factor complex composed of α and β subunits, with the subunit encoded by one of three runt-related transcription factor (*Runx*) genes (*Runx1*, *Runx2*, and *Runx3*) and the β subunit encoded by a single gene.

Expression of *Runx1* and *Runx2* is rapidly induced in granulosa cells of preovulatory follicles by LH in humans (Park et al., 2010). Studies using rat granulosa cell cultures with small interfering ribonucleic acid (siRNA) identified several genes regulated by RUNX1 or RUNX2 including specific ovulatory genes and luteal genes (Park et al., 2012).

Nuclear receptor interacting protein 1 (NRIP1) does not bind the deoxyribonucleic acid (DNA) directly but instead interacts with nuclear receptors to modulate transcriptional activity. The expression is highest in preovulatory follicles before the LH surge and nuclear receptor interaction protein 1 (*Nrip1*) null mice have defective ovulation and cumulus expansion, with effects on many genes involved in ovulation (White et al., 2000, Tullet et al., 2005).

PARACRINE MEDIATORS IN OVULATION

Steroids

Prior to the LH surge, the mural granulosa cells begin to express LHCGR, to become responsive to the stimulatory actions of LH (Peng et al., 1991). Follicular fluid levels of estradiol are high before the LH surge (Andersen et al., 2006). After LH, granulosa cells then rapidly accumulate cholesterol-containing lipid droplets, and there is enhanced expression of steroidogenic acute regulatory protein (StAR) and 3β -hydroxysteroid dehydrogenase (HSD3B1), which are involved in the early steps of steroidogenesis. Later, steps are low because of low expression of steroid 17α -monooxygenase (CYP17A1), so that conversion of progesterone to androgens and estrogens is severely limited (Chaffin et al., 1999, Weick et al., 1973, Wissing et al., 2014). Ovulatory follicles of humans produce both progesterone and 17α -hydroxyprogesterone, which are both present at high concentrations in serum and in follicular fluid (Amin et al., 2014). Circulating levels of progesterone are very

low prior to the LH surge. Within minutes of the LH surge, serum progesterone levels increase, and follicular fluid levels of LH rapidly rise from nM to μ M levels (Andersen et al., 2006). The action of progesterone and PGR activation on a large number of ovulatory mediators are described in other parts of the thesis.

Administration of a progesterone synthesis inhibitor in macaque reduced structural luteinization and vascular remodeling in response to an ovulation trigger (Chaffin & Stouffer, 2000) which was restored. Replacement of progestin activity restored follicular angiogenesis (Chaffin & Stouffer, 2000). Moreover, the PGR antagonist mifepristone (RU486) reduced vascular remodeling associated with ovulation in pigs (Mauro et al., 2014). Besides, this steroid also has direct action on smooth muscle of the vasculature of the ovary (Press et al., 1988, Snijders et al., 1992, Sahlin et al., 2006).

Androstenedione is the predominant androgen from the ovulatory follicle. Androstenedione most often serves as a substrate for local production of estrogens and testosterone, with high androgen receptor (AR) affinity. The high concentrations of androstenedione suggest that this is also ligands for ARs in the ovulatory follicle. AR are present in both theca and granulosa cells before and after the LH surge (Horie et al., Hild-Petito et al., 1991). Optimal androgen concentrations appear to be critical for successful ovulation, with both high and low androgen levels causing ovulatory dysfunction (Walters et al., 2018).

Androgens have also been implicated in control of microvascular dilation. Testosterone reduces the ability of subcutaneous vessels to dilate in response to endothelins (Wenner et al., 2013), but an effect of androgens on ovarian blood flow has not been directly demonstrated.

A role for estrogen in human ovulation remains controversial. In the ovary, the two classical estrogen receptors (ESR), ESR1 and ESR2 are expressed, with ESR2 being the main type in granulosa cells (Choi et al., 2001). Gonadotropin-driven ovarian follicular development, oocyte maturation, and fertilization were achieved in women with severely reduced estrogen synthesis due to specific enzyme deficits (Pellicer et al., 1991). However, reduction of nonhuman primate ovarian steroidogenesis by HSD3B inhibitor did not alter follicle development but caused ovulation failure and gave poorly fertilizable oocytes (Hibbert et al., 1996).

Prostaglandins and leukotrienes

Follicular levels of both prostaglandin E2 (PGE2) and prostaglandin F2 α (PGF2 α) increase in response to the LH surge (Duffy et al., 2005) and that is because increased expression of at least one form of every enzyme involved in

the synthesis of PGE₂, including the phospholipase cytosolic phospholipase A₂ (A₂ PLA₂G₄A), the peroxidase/cyclooxygenase prostaglandin-endoperoxide synthase 2 (PTGS₂), and the prostaglandin E synthase (PTGES) (Duffy, 2015). Ablate-and-replace studies in nonhuman primates, identified PGE₂ as the key ovulatory prostaglandin (Duffy, 2015) and the four receptors four PGE₂ receptors (PTGERs) are expressed by the primate ovulatory follicle, with an increase after LH (Kim & Duffy, 2016). The distribution is that of PTGER1 in mural granulosa cells and invading vascular endothelial cells, and of PTGER2 in mural granulosa cells near the rupture site, endothelial cells, the oocyte, and cumulus granulosa cells. The PTGER3 is found on mural granulosa cells and cumulus granulosa cells, while PTGER4 expression is limited but is detected in mural granulosa cells, the oocyte, and cumulus granulosa cells (Kim & Duffy, 2016). The role of prostaglandins in ovulation is mostly linked to regulation of ovarian blood flow (Murdoch & Myers, 1983) and in angiogenesis (Kim et al., 2014). This latter is mostly by PGE₂ action via PTGER1 and PTGER2 receptors, as shown in assays of endothelial cell migration and capillary stalk formation (Trau et al., 2016, Kim et al., 2014).

Leukotrienes are produced by the ovulatory follicle and follicular concentrations of leukotrienes increase rapidly after the LH surge (Espey et al., 1989).

Furthermore, a member of this class of eicosanoid is present in human follicular fluid (Heinonen et al., 1986). Administration of lipoxygenase inhibitors to rodents decrease ovulation rates in vivo (Gaytán et al., 2006) and in the in vitro perfused ovary (Mikuni et al., 1998).

Vasoactive substances

Bradykinin is a non-peptide, which together with other kinins are produced locally in the tissue from circulating kininogens. There is a preovulatory rise in kinin-forming enzymes, kallikreins, observed in the adult cycling rat (Smith & Perks, 1983b) as well as in the immature PMSG/hCG-primed rat (Espey et al., 1986). Moreover, addition of bradykinin markedly increases the number of LH-induced ovulations in the perfused rat ovary (Brännström & Hellberg, 1989).

There is a preovulatory decrease in the histamine concentration in rat ovaries (Szego & Gitin, 1964; Schmidt et al., 1988), which can be associated with a degranulation of ovarian mast cells (Jones et al., 1980). Further evidence for participation of histamine in ovulation is that histamine induces ovulations in the in vitro perfused rat (Schmidt et al., 1986) and rabbit (Kobayashi et al., 1983) ovary. Histamine effects are likely on the ovary.

Growth factors

The epidermal growth factor (EGF) family of proteins is essential for ovulation, most notably cumulus expansion (Russel & Robker, 2007)). The EGF family includes proposed ovarian mediators such as amphiregulin (AREG) and epiregulin (EREG), the LH-surge stimulates a rapid increase in follicular expression of both AREG and EREG, identifying these proteins as likely paracrine regulators of ovulation (Park et al., 2004). They activate epidermal growth factor receptors (EGFRs), located on both mural and cumulus granulosa cells (Park et al., 2004, Shimada et al., 2006).

Members of the vascular endothelial growth factor (VEGF) family of growth factors are structurally-related and utilize the same group of receptors to mediate vessel growth and permeability. Vascular endothelial growth factor A (VEGFA) seems to have a role in ovulation since neutralization of VEGFA action within the follicle significantly disrupted ovulation in non-human primates (Wullf et al., 2002, Hazzard et al., 1999) and since there is a rapid increase in follicular fluid VEGFA levels after ovulation triggering as measured in women (Gutman et al., 2008) and non-human primates (Hazzard et al., 1999, Gutman et al., 2008, Mauro et al., 2014, Chowdhury et al., 2010, Miyabayashi et al., 2005, Baskind et al., 2014). Furthermore, the LH surge increases VEGFA mRNA and protein in granulosa cells and follicular fluid, respectively (Miyabayashi et al., 2005, Baskind et al., 2014).

Rodents lacking VEGFA expression in granulosa cells showed reduced ovulation rates and litter sizes (Sargent et al., 2015). The effect of VEGFA is likely to be through modulation of ovarian blood flow and transition into a corpus luteum (Fraser et al., 2000, Hazzard et al., 2002).

A critical role for placental growth factor (PGF) in ovulation has also been demonstrated (Bender et al., 2018) and the effect by blockage of PGF action seems to be on endothelial cell proliferation and capillary lengthening (Herbert & Stainier, 2011). This growth factor is present in human follicular fluid (Gutman et al., 2008). Messenger ribonucleic acid (mRNA) for the angiopoietins (ANGPTs), ANGPT1 and ANGPT2 are detected in granulosa and theca cells and the proteins are detectable in follicular fluid (Miyabayashi et al., 2005, Nishigaki et al., 2011). The ratio of ANGPT1/ANGPT2 protein in human follicular fluid is lowest in the largest and most mature preovulatory follicles (Nishigaki et al., 2011), supporting the concept that this ratio favors ovulatory events. A functional role is indicated by that intrafollicular administration of a blocker of ANGPT2 reduced ovulation in the macaque (Xu et al., 2005).

Plasminogen activators

Plasminogen activators (PAs) are selective serine proteases which convert plasminogen, an inactive zymogen present in high concentrations in most extracellular fluids including follicular fluid (Beers, 1975), into plasmin.

The glycoprotein plasminogen is synthesized in the liver and plasminogen is enzymatically cleaved by PAs to form plasmin. Plasmin is a broad-spectrum serine protease that cleaves fibrin and fibrinogen as well as a variety of ECM proteins. These include collagen types III, IV, and VI, fibronectin, laminin, which are components of the ovarian follicle. Plasmin can also activate pro matrix metalloproteinases (pro-MMPs) by cleavage (Curry & Smith, 2006). There exist two main plasminogen activators, the urokinase-type plasminogen activator (PLAU) and tissue-type plasminogen activator (PLAT), being products of independent genes, but sharing similarities in their basic structures and physiological modes of action (Degen et al., 1986). Activation of the plasmin/PA system is started by release of PLAT or PLAU by cells in response to external signals such as hormones, growth factors, or cytokines and this leads to locally-restricted extracellular proteolytic activities as shown in ovulation in the macaque (Liu et al., 2004).

There are several indications that PAs, are critically involved in ovulation. A large (3- to 14-fold) preovulatory increase in PA activity in rat ovarian homogenates is seen just prior to follicular rupture (Espey et al, 1985; Canipari & Strickland, 1985; Liu et al., 1987) and a localized increase in fibrinolytic activity over the stigma region was seen prior to ovulation in the rat (Akazawa et al., 1983). Gonadotropins decrease the activity of PA inhibitors in granulosa cells (Ny et al., 1985). Streptokinase, an exogenous PA, induces ovulation in the perfused rabbit ovary (Yoshimura et al., 1987), while the product of PA action, plasmin, decreases the tensile strength of incubated strips of the bovine follicle wall (Beers, 1975).

Matrix metalloproteinases

There are major alterations in the ECM during ovulation with main breakdown of structural ECM components in the top of the follicle and reorganization of ECM around other parts of the follicle. The matrix metalloproteinases (MMPs) and their associated endogenous inhibitors control the site and extent of this ECM turnover in and around the follicle and are linked to the ovulatory process. The MMP family is made up of more than 20 related proteolytic enzymes (Nagase & Hideaki, 1996, Murphy et al., 1999), divided into the subclasses collagenases, gelatinases, stromelysins, and membrane type enzymes (MT-MMPs). The MMPs have several structural and functional similarities. There is a zinc in the active site of the catalytic domain. They are synthesized as inactive pro-enzymes with activation of the latent pro-MMP in

the extracellular space. They exhibit their action by site specific recognition by the catalytic domain of the enzyme, causing cleavage of a specific ECM protein. The MMPs are inhibited by both serum borne (macroglobulin) and tissue derived MMP inhibitors (TIMPs), and this action is important to restrict proteolytic action in time and in space. In the ovulatory follicle this will include early activation on the top of the follicle but protection from degradation at basal levels of the follicle.

The collagenases (MMP1, MMP8, and MMP13) cleave both fibrillar collagens (collagen types I, II, III, V, XI) and nonfibrillar collagens (collagen types IX, XII, XIV). At MMP cleavage of collagen the collagen protein will denature and form gelatin, which is further degraded by the gelatinases (MMP2 and MMP9) and stromelysins (MMP3, MMP7, MMP10, MMP11). The gelatinases and stromelysins have action on the major constituents of basement membranes including type IV collagen, laminin, and fibronectin. An important function of MMPs in relation to ovulation is that they also exhibit activity on growth factors, and cytokines. The MMPs can cleave growth factors and cytokines of extracellular domains and thereby modulate their bioavailability during the ovulatory process. There exist four members, with tissue inhibitor of metalloproteinase (TIMP)1 preferentially binding MMP9 and TIMP2 inhibiting MMP2. These two TIMPS are freely mobilized in the extracellular space, in divergence with TIMP3 which is bound to the ECM. In addition to the classical role to regulate MMP action, the TIMPs also have roles in regulation of steroidogenesis (Fassina et al., 2000), of embryo development (Sato et al., 1994), and of angiogenesis agents (Johnson et al., 1994). There are several lines of evidence that MMPs are active in ovulation. The first scientific results on the action of MMPs in ovulation came from experiments that was conducted 30 years ago and included the methodology of the in vitro perfused ovary. Specific blocker of MMPs were able to significantly reduce the number of LH-induced ovulations in a rat ovary that was perfused ex vivo for several hours (Butler et al., 1991, Brännström et al., 1988). The concept of MMP action in the ovary has since then been expanded to several animal species including the primates (Peluffo et al., 2011). Action and role of MMPs and other proteases is one focus of the present thesis and this will be discussed further in the Discussion section.

Proteases of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)

The ADAMTS family are a class of 19 related and secreted metalloendopeptidases and are divided into seven subgroups based on similarities of action of the targeted substrates (Porter et al., 2005). The ADAMTS are synthesized as inactive pre-proenzymes and secreted. They have

diverse actions including degradation of proteoglycans such as aggrecan, brevican, and versican. Moreover, they can activate procollagens to collagen and can control the basement membrane remodeling (Blelloch et al., 1999, Nishiwaki et al., 2000). TIMPs can also regulate ADAMTS in the extracellular space as exemplified by the role of TIMP3 (Kashiwagi et al., 2001). There are studies in rodents that have clearly found a role for ADAMTS, especially ADAMT-1 in ovulation (Boerboom et al., 2003, Russel et al., 2003).

Recent studies in the human have shown increased expression in of ADAMTs granulosa cells during ovulation and possible relationship with fertilization capacity (Yung et al., 2010)

Leukocytes and chemokines in ovulation

Several studies point towards the importance of invasion of leukocytes into the follicle at ovulation. Early studies showed addition of leukocytes to the in vitro perfused ovary increased LH-induced ovulation rate (Hellberg et al., 1991) and a depletion of neutrophils (Brännström et al., 1995) or macrophages (Van der Hoek et al., 2000) in vivo decreases ovulation rate in rats and mice, respectively. Studies in the rat could show a periovulatory increase in several leukocyte subsets in the follicle (Brännström et al., 1994) and this could later be verified in the human (Brännström et al., 1994).

The leukocyte migration is via the classical four steps of rolling, activation, adhesion, and transmigration into the ovarian tissue. In the ovary, interleukin (IL)8 synthesis is induced in granulosa cells and theca cells to a large extent and rapidly after LH stimulation (Runesson et al., 1996). Increased IL8 stimulates neutrophils to infiltrate the ovarian vascular permeability (Murayama et al., 210). Treatment with neutralizing antibodies to IL8 reduces ovulation and neutrophil invasion (Ujioka et al., 1998). Other important chemokines are chemokine (c-c motif) ligand (CCL)20 and chemokine (c-x-c motif) ligand 12 (CXCL12), which are induced in human granulosa cells and theca cells at ovulation (Al-Alem et al., 2015). A recent study in the rat details the sequential pattern of leukocyte influx into the ovulatory ovary (Oakley et al., 2010). By flow cytometry temporal changes in leukocyte populations in the ovaries of naturally cycling adults and immature rats during ovulation was characterized showing a massive influx of leukocytes at around half of the duration of the ovulatory interval (Oakley et al., 2010). Other important chemokines in the ovary are monocyte chemoattractant protein-1 (MCP1), which is a potent chemoattractant for monocytes and it also effectively recruits macrophages and T-lymphocytes. Studies have shown that inhibiting the production of monocyte chemoattractant protein-1 results in reduced ovulation (Cohen et al., 1997). Another chemokine which is likely to be active in

ovulation is CCL25, which inhibited leukocyte infiltration to the ovary and resulted in complete inhibition of ovulation in mice (Zhou et al., 2009).

Ovarian CCL25 expression is firmly regulated by gonadotropins (Foster et al., 210), and inhibition of ovulation failure was attributed to the lack of infiltration of a rare T cell population (Zhou et al., 2009, Zhou et al., 2005).

The action of leukocytes in ovulation is most likely mediated through proteases. Leukocytes secrete MMPs in the preovulatory ovary (Fedorcsák et al., 210) and granulosa cells and theca cells produce both MMPs and their tissue inhibitors (TIMPs) (Shalev et al., 2001, Stamouli et al., 1996, Goldman & Shalev, 2001). Collectively, this leads to digestion and weakening of the follicular wall, thereby contributing to rupture of the follicle.

AIMS

The overall aim of this thesis was to study the mechanisms of intra-ovarian regulation at ovulation in order to increase the basic knowledge of this biologically important process.

THE SPECIFIC AIMS WERE:

PAPER I

To characterize the expression patterns of certain matrix metalloproteinase (MMPs) and the disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS) in the human follicle compartments of the theca and granulosa cells during human ovulation.

PAPER II

To assess the expression profile and regulation of tissue factor pathway inhibitor 2 (TFPI2) during ovulation in the human and the rat, as well as exploring the downstream effects on multiple ovulation-associated genes.

PAPER III

To identify for the first time by modern proteomic technique, which proteins in the human follicle, as mirrored by levels in follicular fluid are differentially regulated during the natural menstrual cycle.

PAPER IV

To explore the expression patterns of osteoprotegerin (OPG) and receptor activator nuclear factor kappa B ligand (RANKL) in granulosa and theca cells during human ovulation of the natural menstrual cycle.

PAPER V

To explore the effects of calcineurin inhibitors on ovulation rate, invasion of ovarian immune cells, and on expression of intraovarian modulators of ovulation in the rat.

PATIENTS AND METHODS

Animal and human collection of samples

Patients for obtainment of cells/tissue/fluid during ovulation of the natural menstrual cycle

All women participating in the studies of papers I, II, III, and IV were monitored for obtainment of cells, tissue, and follicular fluid during ovulation of the natural menstrual cycle according to the schematic overview, in fig 5. Fifty women were initially included during the study period which extended over 36 months. Due to the strict inclusion criteria, 12 patients were excluded, reducing the number of participants to 38 premenopausal women undergoing surgery for tubal ligation. Of the 38 women participating in the study, 28 of the samples were used for analysis of expression patterns, profiles, and regulations of the intended study object, and 12 samples were used for immunohistochemistry. Twenty-eight women in study I were also included in papers II and IV and, of these, 15 were also included in paper III.

Monitoring and follicle collection of cells/tissue/fluid of women during ovulation of the normal menstrual cycle

Collection of follicular cells, tissue, and fluid in papers I, II, III, and IV were collected from women with previously proven fertility, regular menstrual cycles, and who had not undergone hormonal treatment for at least 3 months. To enable follicle collection at one of four phases, that is, pre-, early, late, or postovulatory, the women were monitored with repeated transvaginal ultrasounds for an average of two cycles.

For those participating the pre-ovulatory phase, the surgery was performed before the luteinizing hormone (LH) surge when the dominant follicle was $\geq 14\text{mm}$ and $\leq 17.5\text{mm}$. To mimic the endogenous LH surge, an injection of recombinant human chorionic gonadotropin (rhCG) was given, and surgery was performed at varying lengths of time after the injection: early ovulatory phase (12 to ≤ 18 hours), late ovulatory phase (>12 to ≤ 34 hours) and postovulatory phase (>44 to ≤ 70 hours). This is illustrated in fig 6.

The entire dominant follicle was excised from the ovary using scissors and placed inside a laparoscopic sac, which was retrieved through a suprapubic trocar incision.

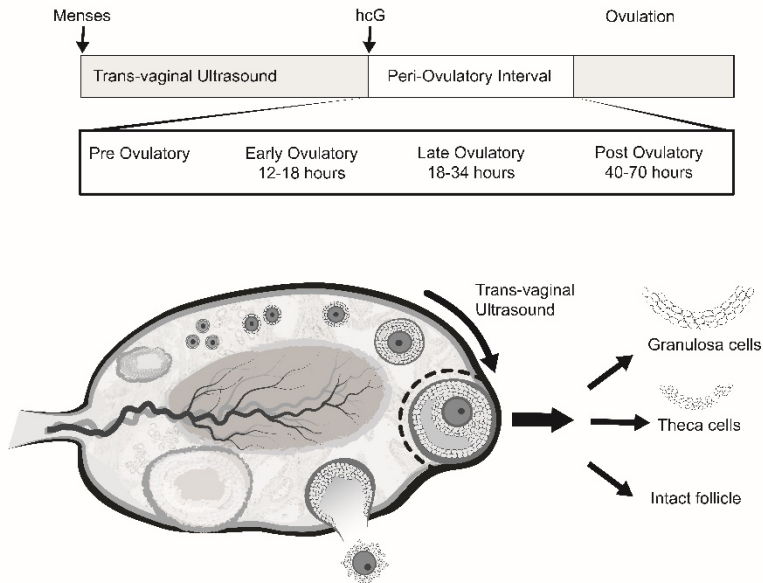


Fig 6. Illustration of monitoring and follicle collection

Follicle collection for cells/tissue/fluid of women during ovulation when undergoing in vitro fertilization (IVF)

In paper I, women undergoing IVF were treated with gonadotropin-releasing hormone (GnRH) agonist and given recombinant follicle stimulating hormone (rFSH) to induce follicular growth. Recombinant human chorionic gonadotropin (rhCG) was administered when the two largest follicles had an average diameter of $\geq 18\text{mm}$ and, thereafter, granulosa-lutein cells were collected 34–36 hours post-rhCG administration by ultrasound-guided aspiration.

Peripheral blood collection of women

In paper IV, peripheral blood samples were collected from three groups of women to investigate possible variations. The subgroups were women with regular menstrual cycles, breastfeeding women, and postmenopausal women. Blood samples were retrieved every third day for 4 weeks, starting on the first day of menstruation in the group of women with normal menstrual cycles, and less frequently in the other two groups.

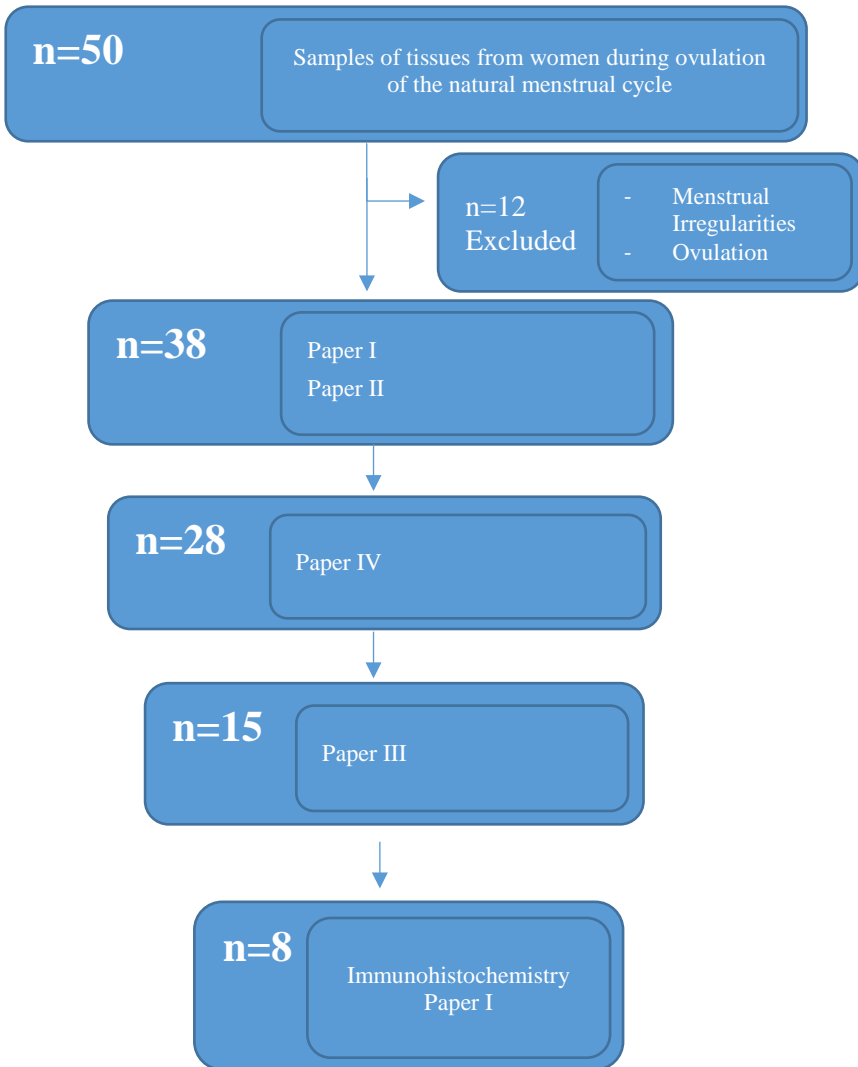


Figure 7. Flow-chart of exclusion of study participants in paper I, II, III and IV.

Rat ovarian tissue/cells and blood collection

All the rats in the studies were maintained at room temperature with food and water provided ad libitum.

In paper II, immature female Sprague-Dawley rats were purchased from Harlan, Indianapolis, IN. When the rats were 24 to 25 days of age, they were administered 10 IU of pregnant mare serum (PMSG). To induce ovulation, 5 IU of human chorionic gonadotropin (hCG) was administered 48 hours after the PMSG injection. The rats were then euthanized at varying intervals after hCG treatment (0, 4, 8, 12, and 24 hours) for tissue collection. Whole, intact ovaries were used, as well as isolate granulosa cells and residual ovarian tissue. The residual tissue represents the ovarian tissue left behind after granulosa cell collection and is a heterogeneous tissue. The ovaries collected at defined time points after hCG were punctured with a 26-gauge needle to release the granulosa cells for collection. The residual ovarian tissue left over was removed, and granulosa cells were partially purified by filtration through a 40- μ pore size nylon filter and pelleted by centrifugation at 300 x g. This was done to remove tissue debris and cumulus-oocyte complexes (COCs). Tissues collected for messenger ribonucleic acid (mRNA) analysis were frozen and stored at -70°C for analysis.

In paper V, immature female Sprague-Dawley rats were purchased from Harlan, Horst, Netherlands, weighed at the age of 21 days, and allocated to three different groups: group 1 control with sodium chloride (0.9% NaCl), group 2 cyclosporin A (CyA), and group 3 tacrolimus (TAC).

When the rats were 24 to 25 days of age, they were administered 10 IU of equine chorionic gonadotropin (eCG). To induce ovulation, 10 IU of hCG was administered 48 hours after eCG injection. The rats were then euthanized 20 hours after hCG treatment for tissue collection.

Calcineurin inhibitors administration

In paper V, mini-osmotic pumps were filled with a solution tacrolimus (TAC), cyclosporine-A (CyA), or control according to each animal's weight. Two different pump models were used with identical external composition. The pumps were primed in NaCl at 37°C 12 hours prior to insertion, according to the manufacturer's instruction. Postnatal day 21, under isoflurane anesthesia, a skin incision was made in the back of each animal caudally to the neck and the pump placed subcutaneously in a parasagittal position. The skin was subsequently closed with two polyglactin stitches.

Cell cultures and cell experiments

In vitro fertilization (IVF) granulosa-lutein cells

In paper I, the oocyte was removed from the follicular aspirate in women undergoing IVF, and follicular aspirates were pooled from multiple follicles of the same patient and placed in OptiMEM I media. To separate the red blood cells from the granulosa-lutein cells, Percoll gradient centrifugation was used. The aspirates were pelleted by centrifugation and resuspended in 1ml of OptiMEM I media. The isolated granulosa-lutein cells were then cultured in OptiMEM media containing 10% fetal Bovine serum for 6–7 days. The media was changed every 24 hours. This allowed the cells to regain responsiveness to human chorionic gonadotropin (hCG) after being desensitized by the ovulatory dose of hCG. These cells were serum starved for 1 hour and then treated with or without hCG and collected at 6, 12, and 24 hours to examine expression of matrix metalloproteinase (MMP) and the α disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTs), in human granulosa-lutein cells from women undergoing IVF.

HGL5 cell experiment

HGL5 cell line in paper I is a virally transformed luteinized granulosa cell line that overcomes cell variability in human granulosa cells. HGL5 cells were used to examine the regulation of the desired proteins, such as matrix metalloproteinase (MMP) and the α disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTs) in the study. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium and supplemented with 10% ultralow IgG fetal bovine serum, 1% insulin-transferrin-selenium (ITS), 1% Pen-Strep and 10 mg/mL gentamicin. The HGL5 cells were then plated in 12-well plates at a density of 1.5×10^5 cells/mL/well, serum starved for 1 hour, and then treated with or without forskolin (FSK) plus phorbol-12-myristate-13-acetate (PMA) and collected at 6, 12, and 24 hours.

Cell viability

In paper II, granulosa cell viability was assessed by using a colorimetric method for determining the number of viable cells. The assay was performed with addition of CellTiter Aqueous One Solution Reagent directly to culture wells and incubated for 4 hours before quantification, according to the manufacturer's instructions.

Rat granulosa cell culture

In paper V, immature rats were treated with pregnant mare serum (PMSG). 48 hours after the treatment, the ovaries were collected and punctured. Preovulatory granulosa cells were isolated by follicular puncture of the ovaries and used for cell culture experiments. Isolated granulosa cells were cultured at 5×10^5 cells/well in a 12-well tissue culture plate in OptiMEM media containing insulin-transferrin-selenium (ITS) and gentamicin. Thereafter, the cells were treated with or without human chorionic gonadotropin (hCG) for different durations of time (0, 4, 8, 12 and 24 hours). Both the conditioned culture media and cells were collected for hormone assay or ribonucleic acid (RNA) analysis.

Chemical laboratory analysis

Microarray analysis

In paper II, granulosa cells from rats were cultured for small interfering ribonucleic acid (siRNA)-mediated knockdown of tissue factor pathway inhibitor 2 (TFPI2). Ribonucleic acid (RNA) from rat granulosa cells was transfected with TFPI2 siRNA or negative control siRNA, and then treated with forskolin (FSK) and phorbol-12-myristate-13-acetate (PMA) for 6 hours before microarray analysis. The integrity of the riboprobe was confirmed by gel electrophoresis.

The Affymetrix GeneChip Rat Gene 2.0 ST oligonucleotide array was hybridized, washed, and scanned according to the manufacturer's protocol. The changes observed by deoxyribonucleic acid (DNA) microarray analysis was confirmed by real-time polymerase chain reaction (PCR) for a select subset of genes.

Steroid immunoassay

In paper II, progesterone (P) in the conditioned media was assayed using an Immulite kit on an IMMULITE 1000, which uses enzyme amplified chemiluminescent technology. In this process, a stable chemiluminescent substrate is hydrolyzed by alkaline phosphatase and results in the production of the unstable adamantyl dioxetane anion that gives rise to emission of light,

which is quantified by using an Immulite luminometer. Assay sensitivity was 0.2ng/ml, and the intra-assay and inter-assay coefficients of variations were 6.3% and 9.1% respectively.

Enzyme-linked immunosorbent assay (ELISA)

In paper IV, the levels of osteoprotegerin (OPG) and receptor activator nuclear factor kappa B ligand (RANKL) in serum were measured by use of commercially available polyclonal antibody-based sandwich ELISA technique according to the manufacturer's instructions. The assay utilizes the two-site sandwich technique with two selected antibodies that bind to human serum OPG and RANKL. Briefly a monoclonal anti-human antibody was coated onto the inner surface of polystyrene microtiter wells, then a biotin-labeled polyclonal anti-OPG or anti-RANKL antibody was added. An enzyme-labeled streptavidin was added to the wells after overnight incubation, in order to selectively bind to the complexed biotin. A chromogenic substrate was used to develop color, and the absorbance noted in a microtiter plate reader. The intra-assay and inter-assay variability were 8% and <10% for serum OPG, and 0.9% and 9.3% for serum RANKL.

Proteomics

As opposed to analyzing the entire proteome, the functional proteomics allow for the analysis of target proteins. Protein identification and characterization has meaningful outcomes, as these are usually driven by a specific biological question. In paper III, 50 ml of all follicular fluid (FF) samples were filtered. BCA assay was used to determine the concentration of proteins. The Qproteome Albumin/immunoglobulin G (IgG) Depletion Kit depleted human albumin and IgG. Thereafter, BCA assay was used again. ProteoExtract® Protein Precipitation were used to remove non-protein impurities.

Pellets were then dissolved in iTRAQ® (Isobaric Tags for Relative and Absolute Quantification) dissolution buffer with addition of 1µl 2% sodium dodecyl sulfate and the samples reduced, alkylated, and digested with trypsin. The patient samples were then labeled with the iTRAQ reagents following the manufacturer's instructions. The iTRAQ sets were then acidified with 10% formic acid and diluted with steric exclusion chromatography (SCX) 25mM ammonium formate, pH 2.8, 20% acetonitrile (ACN), and loaded onto a SCX column.

The SCX chromatography and fractionation were carried out on a purifier system at 0.25mL/min flow rate. Fractions were collected at 0.5mL intervals and dried under vacuum while ultraviolet absorbance at 254 and 280 nm was monitored. The peptide containing fractions were desalted on PepClean C18 spin columns according to the manufacturer's instructions.

The fractions were reconstituted into 0.1% formic acid and analyzed on an LTQ-Orbitrap XL with an in-house constructed LC setup. 2 μ L sample injections were made with an HTC-PAL autosampler and connected to an Agilent 1200 binary pump. The peptides were trapped on a precolumn and separated on a reversed phase column. Both columns were then packed with 3 μ m Repronil-Pur C-AQ particles. The flow through the analytic column was reduced to 100 nl/min. One mass spectrometry scan was followed by CID (collision induced dissociation) and HCD (high energy collision dissociation) MS2 scans were performed of the three most abundant doubly or triply protonated ions of each scan.

Proteome Discoverer version 1.1 was used for relative quantification and identification of all 10 SCX fractions for each 4-plex iTRAQ sets. Mascot was used for database searches of all sets and performed against Swiss-Prot version 57.15. The identified proteins were grouped based on the same sequences to minimize redundancy, and the protein identification threshold was set to 95% confidence.

To calculate fold changes between samples, iTRAQ raw data sets from the ratios of iTRAQ reporter ion intensities in MS/MS spectra were used. Ratios were derived by using the Proteome Discoverer version 1.1. Only peptides that were unique for a given protein were used for relative quantification as seen in fig 7.

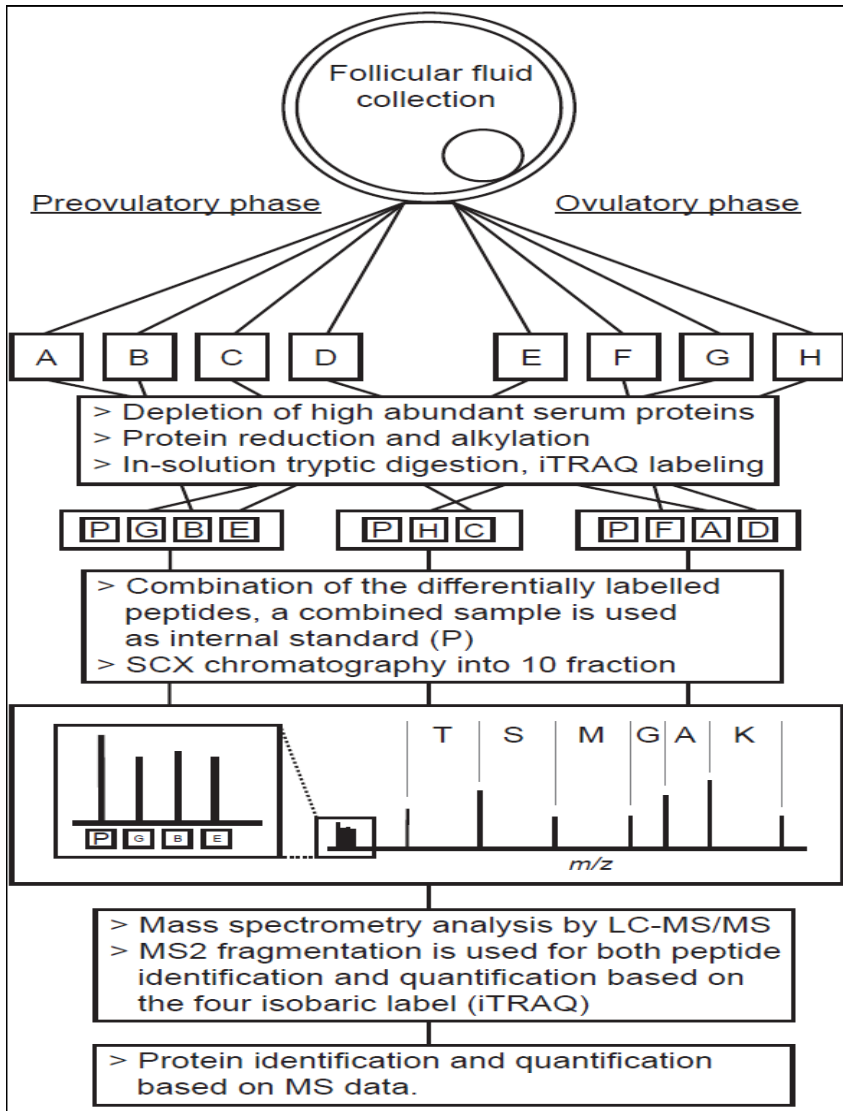


Figure 7. Experimental overview of the mass spectrometry (MS) based method using the iTRAQ technology.

Plasmin assay

In paper II, two different methods were used to assess plasmin activity; one method was by fluorimetric activity, and the other method was employed by utilizing the ability of the plasmin to cleave fibrinogen. Conditioned media from rat granulosa cell cultures were concentrated by centrifugation at 7,500g for 20 min and used in the activity assay. The total protein in the conditioned media was determined using a BioRad detergent compatible protein assay kit, as recommended by the manufacturer.

To undergo fluorometric assay, the conditioned media of approximately 1 µg total protein and the fluorogenic peptide substrate were incubated for 60 minutes at room temperature. By doing this, the enzymatic action of the plasmin on the substrate released a fluorophore (7-amido-4-trifluoromethylcoumarin). The fluorescent signal was then measured using a spectrophotometer.

The fibrinogenolytic assay was carried out with 10 µg of human fibrinogen being incubated in 10 mM tris (hydroxymethyl) aminomethane HCl in the presence of human plasmin (1U) or granulosa cell-conditioned media (1µg total protein) at 37°C for 2 hours. Human plasmin plus fibrinogen were incubated with and without the plasmin inhibitor D-VAL-Phe-Lys-CMK (chloromethyl ketone dihydrochloride; 1µM) in order to assess the specificity of plasmin cleavage of fibrinogen. The reaction was stopped using a denaturing loading buffer containing 2.4 M urea. Thereafter, the reaction mixture was boiled and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fibrinogenolytic products were then visualized by staining the gel with Coomassie blue.

Calcineurin inhibitors level

In paper V, 250µl of whole blood were drawn from the rat aorta at euthanasia. Tacrolimus (TAC) levels were measured by chemiluminescent immunoassay (CMIA). Cyclosporine-A (CyA) levels were measured by enzyme immunochemistry using a CyA-specific assay, according to the manufacturer's instruction.

Quantitative real-time polymerase chain reaction (RT-PCR)

RT-PCR was used in papers I, II, IV, and V, Trizol Reagent Invitrogen was used to isolate the total ribonucleic acid (RNA) from granulosa, theca, granulosa lutein, and HGL5 cells, according to the manufacturer's protocol. Using Taq-Man methodologies, the messenger ribonucleic acid (mRNA) expression levels for the desired proteins were analyzed by RT-PCR. Mx3000P QPCR system was used to perform the polymerase chain reaction (PCR) reactions in papers I and II, and the ABI Prism 7000 Sequence Detection

was used in papers IV and V. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative amount of mRNA in each sample and then normalized.

Microscopic assessment

Ovulation rate assessment and ovarian preservation

In paper V, the ovulation rate was assessed 20 hours after the human chorionic gonadotropin (hCG) injection. After dissection, the left ovary was kept in RNA later and stored immediately at -20°C . The right ovary was kept in 4% formaldehyde. The ampullary regions of the fallopian tubes were then opened to release oocytes. The oocytes were incubated with hyaluronidase for ten minutes over a glass microscope slide in order to be scattered. Then, they were covered with a coverslip and counted under a Nomarski interference microscope.

White blood cells subpopulation in peripheral blood

In paper V, the rat aorta was punctured at euthanasia to obtain whole blood. The blood was spread onto a glass slide and air dried. It was then fixed by immersion in Romanowsky stock solution for 5 minutes and rinsed in distilled water and air dried again. The cells were then identified according to their specific characteristics by microscopic assessment.

Immunohistochemistry

Immunohistochemistry was used in papers I and V. Intact follicles were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned for immunohistochemistry. Sections were deparaffinized, dehydrated, rinsed in sterile water, and then treated to block endogenous peroxidase activity. Sections were then incubated with antibodies for the desired proteins studied, washed, and incubated with a secondary antibody. The sections were treated with a conjugated streptavidin alkaline phosphatase to amplify the reaction signal and visualized by the Vulcan Fast Red Chromogen Kit, counterstained with hematoxylin and Permout Mounting Media in paper I and x400, Leica DM4000B in paper V.

Statistical Analysis

PAPER I

Kruskal-Wallis test and post hoc comparisons were performed using Dunn's multiple comparison test or Tukey's; p-value of <0.05 was considered statistically significant.

Student's t-test was used for additional analysis.

PAPER II

Bartlett's χ^2 test was used to check for heterogeneity of variance and, thereafter, Bonferroni post hoc test and Bonferroni's multiple comparison tests were performed.

PAPER III

Values between pre-ovulatory and ovulatory phases were analyzed using unpaired student's t-test, and a p-value of <0.05 was considered statistically significant.

PAPER IV

Kruskal-Wallis and Mann-Whitney tests were performed to test for significant differences between groups. P-value of <0.05 was considered statistically significant.

PAPER V

Kolmogrov-Smirnoff test was used to test for normal distribution. Student t-test or Mann-Whitney test were used to compare between groups. P-value <0.05 was considered statistically significant.

RESULTS AND COMMENTS

The results of Papers I-V are summarized and commented upon in this section

Results and comments for Paper I

The human ovulatory process is a complex biological course of action that involves several mediator systems, which, in a partly repetitive fashion, causes the rupture of the follicle with the release of a fertilizable oocyte. The major structural strength of the follicle is given by the extracellular matrix (ECM), which is distributed in its outer layers. The two basement membranes, underlying the theca interna and the ovarian surface epithelium, are made up of collagen type IV and laminin. In the theca layer of the follicle, collagen type III is the predominant ECM component in addition to collagen type I in the external theca layer. Collagen type I and III are also the major ECM components in the parts of the ovarian stroma and tunica albuginea that are between the preovulatory follicle and the exterior.

All these collagen-rich structures must be breached, as the follicle ruptures and allows an oocyte to pass through from its antrum, through the layers of granulosa cells and theca cells, and onto the surface of the ovary.

There exists a large amount of data regarding the specific proteinases that are active in the breakdown of the wall of the preovulatory follicle in animal species, mostly rodents. However, the literature on this subject is limited regarding human ovulation. There is human data from cell-culture experiments available, but these studies have all utilized human granulosa-lutein cells obtained from in vitro fertilization (IVF) cycles. These cells come from an artificial hyperstimulated cycle, and it is questionable whether the physiology of these cells will mimic the normal situation.

The present study is one of the first to examine proteinase expression in the human ovulatory follicle of the natural menstrual cycle. Moreover, the study focused on the expression and regulation of the proteinase matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs) in human ovulation. Expressions before and during different intervals of ovulation were compared, and comparisons were also made between the two major cell compartments of the follicle: the theca compartment and the granulosa compartment. Expression was evaluated via a quantitative reverse transcription polymerase chain reaction (RT-PCR).

Results

During the early ovulatory phase, 12 to 18 hours after the triggering of ovulation, the expression of several proteinases increased in both the granulosa cells and theca cells. Thus, MMP19, MMP 1, ADAMTS1, and ADAMTS9 were all increased in the granulosa cells during early ovulatory phase, as compared to preovulatory phase. In the theca cells, only MMP 19 was significantly increased in its expression. During the late ovulatory phase (18 to 34 hours after the triggering of ovulation), there was still an increase in the expression of all four proteinases in the granulosa cell compartment. In the theca cell compartment, only MMP19 was still elevated, as compared to the preovulatory phase. During the postovulatory phase, only the theca cells were available for analysis, with results indicated that MMP19 showed a prolonged elevation. There were no changes seen in the expression of MMP and MMP13. In summary, the results reveal the rapid and early induction of MMP19 in both the theca and granulosa cells and that these elevated expression levels are sustained throughout the ovulatory process. There was also the transient increased expression of MMP1, ADAMTS1, and ADAMTS9 during the early and late ovulatory phase, which was especially evident in the granulosa cell compartment. The results of messenger ribonucleic acid (mRNA) expression were mimicked by immunohistochemistry showing the localization of the proteins during ovulation. Two cell culture systems were used to examine the expression of the four different proteinases. Granulosa-lutein cells were pre-cultured for 6 to 7 days to regain their responsiveness to human chorionic gonadotropin (hCG) after the receptor desensitization occurring after the hCG challenge in vivo, around 36 hours before oocyte pick-up. These cells responded in vitro to hCG via the expression of MMP1 and ADAMT 1, but no changes were observed for MMP19 or ADAMTS9. Virally transformed granulosa cells, in order for an easily performed cell-culture with a large number of passages, have been used in research regarding ovarian physiology. In this study, we used 5HGL cells that are granulosa-like. Since the expression of the luteinizing hormone (LH) receptor might have been compromised, the cells were challenged with the direct stimulation of the LH-operative second messenger systems via forskolin and phorbol-12-myristate-13-acetate (PMA). Increased expressions of MMP1, ADAMTS1, and ADAMTS9 were observed but were of a lower magnitude than in the granulosa-lutein cells.

Comments

This study is one of the first to examine proteinase expression in the dominant preovulatory/ovulatory follicle of the natural menstrual cycle of women. The expression patterns of these four MMPs/ADAMTS found in the human are like those seen in previous studies on animal species, including detailed studies on

rat, mouse, and rhesus macaque. Thus, the results highlight that the function of these proteinases in ovulation is well preserved in mammalian ovulation.

Studies on several different species have indicated that MMP1 is a key regulator in the breakdown of the follicle wall, and the results of the present study verified these observations. Expression of MMP1 in the granulosa cells increased more than 200-fold during the early ovulatory phase compared to the preovulatory phase and then declined during the late ovulatory phase. Expression in the granulosa cells was manifold higher than that in theca cells. These results indicate that this early mRNA expression with presumed protein expression verified by the immunohistochemistry experiments is important in the events of follicular rupture rather than in the tissue remodelling of the transition into a corpus luteum, which would reach its peak activity around and after follicular rupture.

The other MMP that showed alterations in expression levels was MMP19. The expression of MMP19 increased in both the granulosa and theca cells, and, in comparison to MMP1, this high expression was more prolonged. Thus, increased expression was observed in the theca cells throughout ovulation and also during the postovulatory phase. This time course of this expression suggests that this MMP is important in the breakdown of ECM to accomplish follicular rupture and also in tissue remodelling during luteinisation.

We evaluated the expression of two different ADAMTS in the human follicle during ovulation. This is the first study to explore these ADAMTS in human ovulation, with the assumption that ADAMTS also are important in human ovulation, as has also been shown before in rodent species. We found a pronounced increase in the expression of ADAMTS1 and ADAMTS9 in the granulosa cells, during both the early and late ovulatory phases, but no changes were seen in expression in the theca cells. The results strongly support the fact that the granulosa cell-derived production of these ADAMTS is important for ovulation to progress normally.

In the present study, we used two different cell culture systems to examine if the *in vivo* situation could be mimicked *in vitro*. The experiments represent a start to the evaluation of systems that later could be used for studies on the regulation of expression and production of ovulatory mediators, such as proteinases. The results showed that the granulosa-lutein cells allowed to reverse to a LH-responsive state which is promising as a future tool for studying the intracellular regulation of ovulatory mediators. The virally transformed granulosa cells, albeit much easier to culture, were more questionable in this regard, since the expression showed fairly large differences compared to the *in vivo* situation.

Results and comments for Paper II

During ovulation, there is a continued breakdown of the extracellular matrix (ECM) of the exterior wall of the preovulatory follicle so an oocyte can be released. Studies on several different species have shown that three families of proteases are likely to be instrumental in this process, which includes matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs), and the plasmin/PA families of proteases, which would then be instrumental in the breakdown of different collagens, laminin, and other ECM components in the two basement membranes (collagen type IV and laminin), the theca externa (mainly collagen type III), the external stroma of the ovary, and in the tunica albuginea (mainly collagen type I and III) that covers the top of the follicle that is on its way toward ovulation. The activation and regulation of these proteases are regulated by different inhibitors, and the delicate balance between these two variables is essential for site-directed ECM breakdown at the top of the follicle so that other parts of the follicle can be spared and the tissue integrity toward the basal part of the follicle maintained. This is most likely important in the smooth transition and remodelling of a ruptured follicle into a corpus luteum. One important protease inhibitor is tissue factor pathway inhibitor 2 (TFPI2), which belongs to the Kunitz family of serine protease inhibitors. The present study set out to determine the expression of TFPI2 in human and rat follicles during ovulation as well as to increase understanding of the regulation of this serine protease inhibitor in follicle cells during ovulation in humans.

Expressions before and during different intervals of ovulation were compared in the human menstrual cycle and in a human chorionic gonadotropin (hCG)-gonadotropin primed model of the immature rat. Cell culture experiments with interfering ribonucleic acid (RNA) were used, and assays for the quantification of messenger ribonucleic acid (mRNA), plasmin activity, cell viability, and steroid levels were applied.

Results

It was observed that the ovulatory trigger hCG induced the expression of TFPI2 in both cell compartments (granulosa and theca) of the ovulatory human follicle as well as in the entire ovulatory rat ovary, which mainly contains of large ovulating follicles due to their hyperstimulation by gonadotropin priming, which was induced during the prepubertal stage. In humans, a massive (around 2000-fold) induction of TFPI2 was seen in the granulosa cells during the early ovulatory phase, and, at this stage, the increase in theca cells was around 35-fold. In both cell compartments, TFPI2 expression was maintained over the late ovulatory phase and declined after ovulation. In the rat, with an ovulatory time span of around 12 hours (h), the

increase in TFPI2 expression 8 h after hCG was just below 200-fold and, at 24 h post hCG, the levels had declined to those observed before the triggering of ovulation. The regulation of TFPI2 was studied in rat granulosa cells obtained before the ovulatory trigger. In these experiments, hCG triggered TFPI2 expression and directed the activation of protein kinase A (PKA) and protein kinase C (PKC) via forskolin, Phorbol-12-myristate-13-acetate (PMA) also induced TFPI2 expressions. The signalling pathways that are operative in gonadotropin-mediated TFPI2 expression were explored using pharmacological agents that inhibit the second messengers PKA and PKC as well as down-stream mitogen-activated protein kinase (MAPK) signalling. The results indicated that PKA, mitogen-activated protein kinase kinase 1/3 (MEK1/3), and p38MAPK are important in the mediation of the signal to achieve TFPI2 production in granulosa cells. Additional experiments concerning the regulation of TFPI2 expression indicated the roles of the epidermal growth factor (EGF) receptor and the protease-activated receptor 1 (PAR1) but not of signals mediated by progesterone or prostaglandins. The role of TFPI2 in the regulation of plasmin activity was observed in the cell culture experiments, utilizing small interfering RNA (siRNA) against TFPI2. To establish a broader picture of the downstream function of TFPI2, a TFPI2-knockdown approach with siRNA and combination microarray analysis was used. Several genes were found to be down-regulated, and the results were confirmed by a real time polymerase chain reaction (RT-PCR), such as for amphiregulin (AREG) and interleukin 6 (IL6).

Comments

The study made use of a combination of experiments on both the human and the rat, which is the species that is most well-studied in terms of ovarian physiology, especially the regulation of ovulation. We report, for the first time, a marked increase in the expression of TFPI2 in the theca and granulosa cells of the ovulatory human follicle during the natural menstrual cycle as well as in the rat ovary. Moreover, we also examined the mechanisms involved in TFPI2 expression and found that PKA, EGFR, MEK1/3, p38MAPK, and PAR-1 are important in signalling. Further studies will be able to highlight the exact mechanisms involved in this regulation.

The cellular knock-down experiments clearly showed that TFPI2 influences a great number of pathways that's implicated in ovulation and that play roles both in proteolytic degradation, vascular changes, and the expansion of the cumulus granulosa cells around the oocyte. Thus, TFPI2 plays a central role in ovulation, although its regulation is not mediated by progesterone-progesterone receptor (PR) interaction, which is regarded as the main pathway for ovulation regulation. The reasons could be that TFPI2 will affect the

proteolytic processing of the growth factors, thereby affecting the gene expression of several mediators with growth-factor-dependent expression.

In conclusion, the data supports the fundamental role of TFPI2 in remodelling ECM during ovulation as well as in the regulation of the expression of para-/autocrine ovulatory mediators.

Results and comments for Paper III

Most studies within the ovulation field have investigated the roles of specific molecules involved in ovulation using *in vivo* experiments, *in vitro* perfusion experiments, cell cultures, or a molecular biology analysis of tissues/samples. The molecular biology approach has, in general, been used to analyse messenger ribonucleic acid (mRNA) expression via a quantitative real time polymerase chain reaction (RT-PCR) to acquire data on the regulation of certain genes, signalling pathways, or down-stream events after the perturbation of specific pathways. Studies on this aspect has used isolated granulosa and theca cells of species with larger follicles (e.g., human, monkey, bovine) as well as whole ovarian cell extracts from rodents with follicle sizes that do not allow for the separation of these cell compartments. To get closest to the roles of any mediator, it is of value to focus on protein levels. This can be applied by using a western blot technique for protein extracts from cells/tissues; however, drawbacks include the fact that these kind of experiments require a substantial amount of cells/tissue, since their sensitivity to quantitate proteins, in relation to a microarray and RT-PCR, in addition to their amplifying techniques for quantitating mRNA, is so much lower. Immunohistochemistry is a classical technique to detect proteins in tissues, but this methodology does not allow for the accurate quantification of protein levels.

In large species, including primates, it is possible to obtain follicular fluid from larger follicles. The fluid-filled antrum develops from the early antral stage of the follicle and will then expand during later stages of folliculogenesis. It constitutes a major part of the microenvironment for the oocyte, cumulus cells, and the non-mural granulosa cells of the follicle wall. Via the modern proteomic approach, which is based on mass spectrometry, it is possible to identify and quantify many proteins in a small volume of a liquid sample. Prior to the present study, there existed some reports on the proteomic analysis of follicular fluid from *in vitro* fertilization (IVF) cycles in addition to comparisons at earlier stages. However, since the follicular fluid that is analysed is from an artificial hyperstimulated cycle, there will probably be deviations in protein regulation between these IVF cycles and ovulation during the human natural menstrual cycle.

The present study aims to examine the full protein content of human follicular fluid during natural ovulation. We used modern mass spectrometry with isobaric tags for relative and absolute quantification. This was applied to follicular fluid from the preovulatory phase and ovulatory phase.

Results

We identified over 500 proteins in the follicular fluid of the periovulatory interval, and 115 of these proteins were found in all the samples. Differences in abundance between the preovulatory phase and ovulatory phase were observed for 25 proteins, with most of these proteins being up-regulated during ovulation. The up-regulated proteins belonged to the biological processes of inflammation, coagulation, lipid metabolism, complement activation, and antioxidation. The five proteins that were down-regulated during ovulation were enzymes or involved in lipid metabolism or iron transport.

Comments

This study is the first to analyse the proteomic profile of human follicular fluid in a near state of physiological ovulation. Its difference from the physiologic state is that ovulation was triggered by human chorionic gonadotropin (hCG) rather than by the natural, mid-cyclic peak of luteinizing hormone (LH). In this unique biological material from the follicular fluid of the human natural menstrual cycle, over 500 proteins were identified. Notably, proteins that showed changes in the level of follicular fluid during ovulation may play functional roles in this process. Out of the 20 upregulated proteins, several had previously been identified as ovulatory mediators or having the potential as such. Thus, it is well described that ovulation has many similarities to inflammation, and the present study also identified several of these proteins being upregulated. Some new biological pathways, such as coagulation and complement activation, were also indicated by the results of the present study as being operative in ovulation. Furthermore, *in vitro* studies combined with *in vivo* experiments of animals may highlight the exact mechanisms behind the action for these mediators. It is known that the complement system plays a role in the innate immune system, and it may be that this component of immune regulation is essential in the biologically important process of ovulation.

A limitation of this study was the low number of individual samples, and it could be that this is not representative of a larger population or that other significant differences would be observed if the population was larger. However, it was cumbersome to acquire this biological material, with over 50 women being screened for possible admittance into the study; thus, we do not predict that a similar larger study will be conducted in the near future, and we should therefore use the information on proteins produced by the present study

and combine this data with results from other studies examining mRNA expression, samples of IVF populations, and studies on non-human primate species.

In conclusion, this study has, for the first time, examined the proteome profile of human follicular fluid during ovulation. Data on certain proteins may be essential in other scientific studies and to ultimately increase understanding of the human ovulatory process, which may provide clinically valuable information concerning anovulation, luteinized unruptured follicle syndrome, and the development of female contraceptives that could directly target the ovary.

Results and comments Paper IV

The number of potential ovulatory mediators is continuously expanding due to results from microarrays and proteomic studies that has identified several new products that are upregulated during ovulation in terms of messenger ribonucleic acid (mRNA) or protein levels. The studies have also exemplified new potential pathways that may be operative. Moreover, we had indications from microarray data that osteoprotegerin (OPG) and the receptor activator of nuclear factor kappa B ligand (RANKL) mRNA were present in human follicles during ovulation and that there was a variation in their expressions during the ovulatory interval. These proteins have never been linked to ovulation, but rather associated to bone physiology, specifically to the osteoblast. The usual role of OPG is to be a decoy receptor for RANKL, which will thereby decrease the binding of RANKL to the proper receptor activator of the nuclear factor kappa B (RANK). The molecular triad of OPG-RANKL-RANK has later been linked to physiological events in endocrine organs, such as the thyroid gland, as well as in muscular organs (e.g., heart) and immune organs (e.g., spleen). A connection to the ovary is that OPG is induced by oestradiol and chemokines, which are major ovary-derived substances emitted before and during ovulation, respectively.

Moreover, there is a link between alterations in the molecular triad of OPG-RANKL-RANK and atherosclerosis, which is an inflammatory type of disease. Thus, since ovulation is parallel to inflammation in many mediator systems, this OPG-RANKL-RANK system may play a functional role in human ovulation.

Results

The study used synthesized complementary deoxyribonucleic acid (cDNA) from extracted mRNA obtained from separated granulosa and theca cells, which were obtained during four distinct phases of ovulation of the natural menstrual cycle. In human granulosa cells, the mRNA levels of OPG showed a late increase during ovulation, with low levels during early ovulation and a 2000-fold increase over the preovulatory stage and late ovulatory phase, corresponding to 18 to 34 hours (h) after human chorionic gonadotropin (hCG). The increased expression of OPG persisted during the period of luteinisation, with a 1000-fold increase during the postovulatory stage. The time-pattern of OPG expression in the theca cells was similar but of a lower magnitude.

The expression of RANKL showed an early onset with significantly already increased levels in both the granulosa and theca cells during the early ovulatory stage. The magnitude of increase was around 10-fold higher in the granulosa cells compared to the theca cells.

We also examined whether there would be variations in the serum levels of OPG and RANKL during the different reproductive phases. Minor variations were seen during the menstrual cycles, and the serum levels of OPG and RANKL were stable during breastfeeding and after menopause, with substantially lower levels in postmenopausal women than their fertile counterparts.

Comments

This study is the first study on any species to examine the expression of OPG and RANKL in the ovary. The marked increase of both mediators with slightly different expression profiles in time suggests that they both play roles in ovulation. The early expression of RANKL, prior to the increase in OPG, would suggest the major activity of RANKL during the first early and midovulatory events, considering that there will be some time for the mRNA to be transcribed into the protein as well as for the release of the protein from intracellular sources.

Functional studies need to be performed on the expression and roles of OPG and RANKL in the follicle during ovulation. The preferred research setting for such experiments should be in the rat, considering the large extent of accumulated knowledge on ovulation in this species. Experiments could be performed with well-established *in vitro* perfusion techniques, in which pharmacological blockers or neutralizing antibodies could be administered with subsequent add-backs as positive controls. This system takes in account the near physiological situation of intact tissue architecture and communication between cells, since it is preserved.

A limitation of this study was that further quantification on the protein level was not performed, which was due to a lack in biological material with extracted proteins.

Another way for detecting protein is immunohistochemistry, in which antibodies against specific proteins are applied on tissue sections. The present study, still in manuscript form, would benefit from this method of protein detection for OPG and RANKL, which will also allow for the examination of the distribution within the tissues. We are also aware of the limited number of samples per observation point during ovulation as well as the small number of participants in the three different groups of women that were examined for blood levels.

In conclusion, this study has, for the first time, demonstrated the expression of two new potential mediators of ovulations. It is quite likely that the OPG-RANKL-RANK system is operative in tissue remodelling in conjunction with ovulation, which is in line with the role of the system in bone tissue remodelling. Future studies should be performed to examine the functional aspects of this system within the ovary.

Results and comments Paper V

It is becoming more common for women on immunosuppressive medication following an organ transplantation to attempt to become pregnant. Most young women that have undergone organ transplantation and later will attempt pregnancy are those with kidney grafts, but there is also increasing numbers of young patients that have undergone transplantation of either the heart, lung, or liver. Uterus transplantation is a new modality of organ transplantation, with the target group so far only being women of fertile age. The basis of modern immunosuppression is the use of calcineurin inhibitors, with cyclosporine-A being the standard with an increasing global use in the last decade, in addition to the alternative calcineurin inhibitor tacrolimus. Any of these immunosuppressive agents are used in 95% of all solid organ transplant recipients. Several reproductive functions of both males and females are driven by immune cells, which work in close cooperation with the somatic cells of a specific organ. Thus, ovulation has many similarities with the traditional local inflammatory reaction, and several subtypes of leukocytes have been shown to play active roles in this process. There exist limited data on the effect of calcineurin inhibitors on reproductive processes such as ovulation. Additionally, animal studies have suggested the negative effects of calcineurin inhibitors on implantation; however, studies on their effects on ovulation are currently lacking. The aim of the present study was to examine the effect of the two calcineurin inhibitors, cyclosporine-A and tacrolimus, on ovulation with a well- characterized, gonadotropin-primed, immature rat model used as

the research tool. The immunosuppressive agents were administered at a constant rate by mini-osmotic pumps to achieve stable blood concentrations that would be similar to the human situation. A sham-operated group with saline-containing mini-osmotic pumps was used as the control group.

Results

The levels of cyclosporine-A and tacrolimus in the respective groups were within the therapeutic levels, which did not alter the peripheral count of any leukocyte examined. There was a clear decrease in the ovulation rate of the group treated with cyclosporine-A, but a similar ovulation rate was seen in the rats treated with tacrolimus and the controls. There were indications that both of the calcineurin inhibitors affected the ovulatory influx of neutrophils, since the expression of myeloperoxidase, which is a neutrophil-derived enzyme, decreased in both these groups as compared to controls. However, the other neutrophil-specific marker that was used, neutrophil-expressed elastase, did not significantly change, although its tendency to decrease was observed in the tacrolimus group. We used two well-known ovulatory mediators and measured the expression levels of the three groups. There was a decreasing tendency for the transcriptional regulator runt-related transcription factor 2 (RUNX2) in the group treated by tacrolimus. The distribution and density of the macrophages and T-cells were evaluated via immunohistochemistry examining newly formed corpora lutea. No difference in the densities of these subsets of leukocytes were observed for the newly formed corpora lutea.

Comments

This study is, to our knowledge, the first to examine the effect of calcineurin inhibitors on ovulation in any species. This is surprising since cyclosporine has been used in clinical transplantation for more than four decades and that organ transplantation among the fertile age group is becoming more common. Today, throughout the world, around 20% of all patients with solid organ transplants are of fertile age. Moreover, currently, tacrolimus, which comes in a once daily dosage, has been increasingly used, since some studies have indicated a somewhat better organ survival effects as compared to cyclosporine. The applied gonadotropin-primed animal model is well characterized in terms of the events of ovulation. The ovulation rate was only depressed after the administration of cyclosporine-A, and, after tacrolimus treatment, a normal ovulation rate was observed. This is reassuring, since tacrolimus is gaining more of the market share in solid organ transplantation. We measured several possible effectors on ovulation. Specifically, there was a decrease in the myeloperoxidase expression. This would indicate the lower density of the neutrophils or that the neutrophils expressed less of this enzyme.

Further studies should be conducted to examine the effects on the ovulation rate as well as ovulatory mediators with wider dose ranges for the calcineurin inhibitor. Another limitation of the study is that we studied the effects on a rodent species with the aim to extrapolate these results to the human situation. Even though most aspects of ovarian physiology show many similarities between humans and rats, there are large differences in the lengths and changes of the ovarian cycle, a rat has an ovulatory interval that, in length, is less than half that of a human. Thus, at a later stage, it would be preferable to expand these studies to also include a large mono-ovulatory species, such as a non-human primate. Another aspect is that it is likely that a large proportion of the transplanted women of fertile age will need assisted reproduction with in vitro fertilization (IVF) to become pregnant, which is also true for those with uterus transplantations in which the oviducts are excluded in the graft. Any disturbance in ovulation by the immune system would then be of less importance, but other aspects, such as risk of ovarian hyperstimulation syndrome, implantation, early placentation, and pregnancy, would have to be studied.

In conclusion, the study, for the first time, showed that a calcineurin inhibitor exerts an inhibitory function on ovulation. The result may have implications for female patients with solid organ transplants aiming to become pregnant. Early examinations conducted to ascertain the occurrence of ovulation should then take place.

DISCUSSION

The focus of this thesis is intra-ovarian mechanisms of ovulation, with special emphasis concerning the human ovulatory process. Data from this thesis may become essential in future studies and lead ultimately to detailed knowledge concerning the human ovulatory process and how disturbances can occur in this. This may provide us with clinical valuable information concerning anovulation, luteinized unruptured follicle syndrome, ovarian hyperstimulation syndrome (OHSS) as well as in the development of new strategies for female contraception, with direct action on the ovary.

The studies described in this thesis outline a typical sequence of ovulatory changes. Ovulation is initiated by the luteinizing hormone (LH) surge, with the first responders being granulosa and theca cells. Both these cell types, express LH receptors, although at higher density in granulosa cells. Both these cell types produce cytokines, chemokines, prostaglandins, steroids, and other paracrine inflammatory mediators. These mediators activate resident immune cells and attract additional leukocytes. Proteases, which are produced by somatic ovarian cells and leukocytes, weaken the basement membrane between the granulosa cell compartment and the theca cell compartment. These proteases and the structural changes facilitate neoangiogenesis with invasion of vascular endothelial cells and additional immune cells. Traditional inflammatory mediators, in concert with follicle-specific stimuli, trigger cumulus expansion and the detachment of the cumulus oocyte complex from the basal granulosa cells. At the follicle apex, there is vascular constriction, detachment of cells, and extensive extracellular matrix (ECM) remodelling. The remainder of the follicle undergoes angiogenesis, functional differentiation of granulosa and theca cells, tissue remodeling, and contraction. Ultimately, these functional and structural changes culminate in both rupture when the tensile strength of the follicle can no longer hold back the fluid pressure inside the antrum.

A main focus of the thesis is inflammation and the role of ECM degradation and regulation. As outlined in the Papers and the introduction several ECM components, mostly collagens, give the support and structure to the follicle.

With use of new CLARITY technique it is evident how complex the ECM is arranged, in and around the follicle as well as for the vasculature of the ovary (Feng et al., 2017). The degradation of the ECM is a delicately balanced process and, with synthesis, activation, and regulation of the matrix metalloproteinases (MMPs), plasminogen activators (PAs), and a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS), as discussed in Paper I and II being essential. The ECM homeostasis, with all these components, is important in the ovary as well as in other organs Birkedal-Hansen et al., 1993).

In this regard it should also be mentioned that osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL), as examined in Paper IV, are important for ECM turnover and remodelling in bone tissue (Costa Fernandes et al., 2019). In Paper IV any further functional studies were not performed but the very high ovarian expression levels of these two components of this ligand-decoy receptor system suggests an active role in the tissue remodeling of ovulation. This is further discussed in Paper IV.

Much literature is supporting and promoting a paramount role for MMPs in ovulation. As also described in the Introduction there is a morphological fragmentation of the collagenous matrix at the follicular apex (Bjersing & Cajander, 1971, Fukumoto et al., 1981). Moreover, biochemical methods found decreased collagen content in the follicle after the LH surge and this was most pronounced at the top of the follicle. It is likely that part of this degradation of the follicular ECM occur via the action of a cascade of proteolytic events such as MMPs (Paper I), plasmin (Paper II) and other inflammatory-related proteins (Paper III). Importantly, results of those papers were merely descriptive and naturally a functional role could not be elucidated in a safe study situation in the human. However, corroboration for a role of the MMPs system in follicular rupture is from animal studies where ovulation was inhibited by blocking MMP activity with chemical MMP inhibitors or blocking antibodies towards MMPs in the rat (Brännström et al., 1988), hamster (Gottsch et al., 2002), sheep (Ichikawa et al., 1983) and rhesus macaque (Peluffo et al., 2011). Naturally, the non-human primate species of the macaque is very close to the human concerning the reproductive anatomy and physiology, so results from this species could be extrapolated to the human. Moreover, as shown in Paper I, the expression profiles of MMPs in the human mimics that in the rhesus macaque although with some differences in specific MMPs as outlined below. Thus, studies from The Oregon group of Stouffer aspirated granulosa cells from the dominant follicle prior to human chorionic gonadotropin (hCG) administration (0 h) or 12, 24, or 36 h after hCG to examine messenger ribonucleic acid (mRNA) expression of MMP1, MMP2, MMP3, MMP7, MMP9, tissue inhibitor of metalloproteinase (TIMP)1, and TIMP2 (Chaffin & Stouffer, 1999). At the 12 h examination point 12 h after hCG, there was an increase in MMP1, MMP2, MMP7, TIMP1, and TIMP2. However, MMP9 mRNA did not increase until immediately prior to ovulation (Chaffin & Stouffer, 1999). In Paper I, we examined also the expression of MMP1 in granulosa cells but additionally also in theca cells. Although the interval from hCG to follicular rupture is similar (36-38 h) in the rhesus macaque and the human, we were only able to examine two time points (early ovulatory phase; late ovulatory phase). Moreover, the samples from the studies of this thesis (Papers I-IV) were not as exact on the hour in relation to the study by Stouffer (Chaffin & Stouffer, 1999).

Although the experimental design was somewhat similar, we could not schedule our sterilization surgeries on demand, out of logistic reason at the hospital but also from a patient and doctor perspective. Nevertheless, the human material we have acquired is to our knowledge unique since it is the first study where granulosa cells and theca cells have been obtained from the human natural menstrual cycle. In comparison, studies from the Copenhagen group, that also have looked at human ovulation, has used granulosa cells obtained before and after hCG in hyperstimulated in vitro fertilization (IVF) cycles (Wissing et al., 2014), and it is known from several comparative studies that the levels of mediators can differ markedly between these two settings of patients.

Progesterone is an early key mediator in ovulation, where progesterone binding will cause further downstream signaling. In the study by Stouffer and coworkers one group of macaques were given an inhibitor of progesterone synthesis and another group received this treatment and add back of the non-metabolizable progestin R5020 (Chaffin & Stouffer, 1999). Granulosa cells were examined at 12 and 36 h after hCG. The blockage of progesterone synthesis, and presumably progesterone receptor (PGR) activation, decreased mRNA levels for MMP1, MMP2, MMP7, TIMP1, and TIMP2, with demonstrated reversal for MMP1 and TIMP1 by the non-metabolizable progestin R5020 (Chaffin & Stouffer, 1999). This finding further points towards the importance of MMP1 in ovulation in primates, including the human. In Paper I, we found a very pronounced increase of MMP1 expression in granulosa cells during the early ovulatory phase and this increase in mRNA levels was 200-fold as compared to the preovulatory stage. A follow-up study from the same group also included other factors related to ADAMTS and PA systems, in addition to MMPs (Chaffin & Stouffer, 1999). There was an increase at 12 h for some MMPs (MMP1, MMP10, and MMP19) and ADAMTS (ADAMTS4, ADAMTS9, and ADAMTS15), as well as PLAU. After follicular rupture the mRNA levels MMP1, MMP10, MMP19, ADAMTS1, ADAMTS4, ADAMTS9, and urokinase-type plasminogen activator (PLAU) were elevated again, suggesting roles for some of them in ovulation and some of them in structural luteinization.

It is more difficult to conduct these types of experiments in the human. In an attempt to get this precious material in order to get insight into human ovulation our group at Sahlgrenska methodically collected the dominant follicle before, during and after ovulation. In this clinical trial, where around 50 women were initially participating the tissue was extracted from women operated laparoscopically for tubal sterilization. In this clinical study, women were monitored by serial transvaginal ultrasound (TVU) for around two cycles before surgery to determine if accurate follicular development occurred. During the menstrual cycle for the laparoscopic sterilization, ultrasound was

performed every 1–2 days to enable the surgery to be planned at the accurate stage of the menstrual cycle. The study population was randomly divided into four groups (pre-ovulatory phase, early ovulatory phase, late ovulatory phase and post-ovulatory phase) to distinguish between the different ovulatory stages. The ovulation trigger was hCG and the surgery was conducted in different phases, where the preovulatory phase was defined as the stage when the dominant follicle was >14 mm and <17.5 mm prior to hCG administration. The early ovulatory phase was defined as 12 to 18 hours after hCG, the late ovulatory phase was 18 to 24 hours after hCG and the postovulatory phase was 44 to 77 h after hCG. At the time of surgery, the whole dominant follicle was excised, as schematically shown in Fig. 8

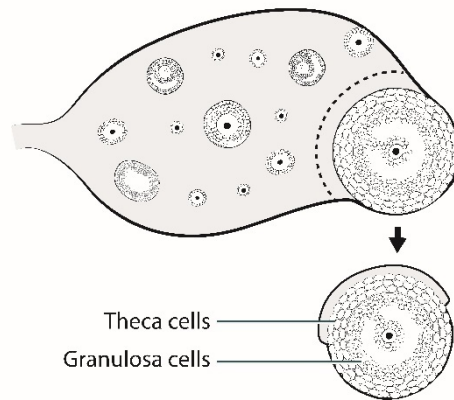


Fig. 8. Type of ovarian excision to obtain material from periovulatory follicle.

The material has been used for studies on extracellular matrix (Lind et al., 2006), the gelatinases (Lind et al., 2006), the stromelysins (McCord et al., 2012) and other MMPs and ADAMTS (Rosewell et al., 2015), as discussed below.

Dissection of the dominant human follicle into the granulosa and theca cell compartments has provided additional data into ovulatory changes in the MMP system. In one study, the changes in the stromelysins (MMP3, MMP10, and MMP11) was examined across the periovulatory period (McCord et al., 2012). Expression of MMP10 mRNA was low in human granulosa and theca cells from follicles collected at the preovulatory phase. After ovulation trigger, there was a marked increase in the levels of MMP10 mRNA and the elevation remained at the late ovulatory phase. In theca cells, MMP10 mRNA levels returned to preovulatory levels by the postovulatory period. In contrast, levels of mRNA for MMP11 decreased to less than half of the preovulatory follicles, after ovulation triggering.

In Paper I, the expression of the major MMPs and ADAMTS were examined in the granulosa and theca cell compartments across the human periovulatory period. The ovulation trigger hCG elevated levels of mRNA for MMP1, MMP19, ADAMTS1, and ADAMTS9 in the granulosa cell compartment. There were differences in the temporal mRNA expression pattern of the diverse MMP and ADAMTS in granulosa cells that varied slightly among the proteinases with MMP1, ADAMTS1, and ADAMTS9 mRNA increasing during the early ovulatory period whereas MMP19 mRNA was increased during the late ovulatory period. For the other collagenases, MMP8 and MMP13 mRNA expression was extremely low and did not change throughout the periovulatory period in either granulosa or theca cells. In theca cells, MMP19 was the only proteinase that was induced by hCG, with mRNA expression increasing during the late ovulatory period and remaining elevated during the postovulatory period. Determining the localization of these proteinases revealed an overall general pattern with increased staining intensity present in the granulosa and theca cell layers after hCG administration. This immunohistochemistry result correlated with the observations of the mRNA expression.

Follicular fluid can be used as one body fluid to detect changes in the ovary and especially in the closely situated granulosa cells, this was used in Paper III. Previously, MMP2 and MMP9 activity were detected in follicular fluid by gelatin zymography (D'Ascenzo et al., 2004, Nikolettos et al., 2003). Interestingly, in IVF follicular fluid the levels of gelatinase seemed to be higher for agonist cycles than for antagonist cycles of drug administration (Bilen et al., 2014). One study compared the protein levels of MMP in the follicle between normally ovulating women and of patients undergoing ovarian hyperstimulation for IVF (D'Ascenzo et al., 2004). Lower MMP levels were found in follicular fluid of IVF patients compared with those of normally ovulating women, but the inverse relationship was seen for TIMP1.

The PA system is also involved in ovulation and that was studied to some extent in Paper II. There are several studies proposing a role for this system in ovulation. Thus, in the rat, ovulation is preceded by a transient and cell specific expression of tissue-type plasminogen activator (PLAT) resulting in proteolytic activity localized to the surface of the ovary overlaying the ovulatory follicle prior to ovulation (Liu et al., 2013). In the rhesus macaque, exogenous hCG administration showed that granulosa cells are the major source of PLAT production, as reflected by changes in mRNA and proteolytic activity to facilitate the breakdown of the follicular wall to permit oocyte release (Liu et al., 2004). Studies in the human reported presence of low levels of PLAT mRNA and a lack of PLAU mRNA in IVF granulosa-lutein cells by Northern blot analysis in human granulosa cells collected from preovulatory follicles at the time of IVF (Jones et al., 1989, Weimer et al., 1984). In Paper

II, the regulation of plasmin activity by tissue factor pathway inhibitor 2 (TFPI2) was studied in rat granulosa cells. The granulosa cells were stimulated by forskolin and phorbol 12-myristate 13-acetate (PMA). Interfering RNA to knockdown TFPI2 showed increased plasmin activity, demonstrating the interplay between these two systems.

In the present thesis, several aspects of ovulation and its relationship to inflammation has been explored. In Paper I dramatic changes in certain proteases were seen at ovulation in the human. Importantly, this increase in expression levels of proteases showed a temporal profile suggestive of participation in follicular rupture, rather than in later events. In Paper II it was demonstrated that the protease inhibitor TFPI2, which has a role in controlling inflammation, is also regulated by an ovulatory trigger. Several inflammatory mediators were regulated downstream of TFPI2. Inflammatory mediators were also seen in higher follicular fluid levels in women at ovulation than before as shown in Paper III. Also, the OPG-RANKL system, as studied in Paper IV, has a role in inflammatory diseases such as arthritis and atherosclerosis. In Paper V of the present study, suppression of the immune system/inflammation by cyclosporine A decreased ovulation rate. Collectively, the results of this thesis further emphasize the similarities between the ovulation reaction and the inflammatory reaction.

There may exist some clinical states with relevance to the results of the thesis. One is luteinized unruptured follicle (LUF) syndrome, which is defined as absence of follicle rupture and without release of an oocyte. This happens in the presence of a seemingly normal follicular growth in the follicular phase, normal endocrinology, and a normal span of the luteal phase. The most reliable diagnostic tool for LUF is repeated examinations with TVU, showing a growing follicle, normal follicle before ovulation and then no follicular collapse/ corpus luteum (Marik et al., 1978). The formation of LUF may be linked to dysregulation of the ovulation-associated inflammatory changes. Thus, the general cyclooxygenase inhibitor indomethacin change the vasculature of the preovulatory sheep follicles (Murdoch et al., 1989) in a similar manner as observed in LUF in women. Another, indication of a link between LUF and inflammation is in women previously diagnosed with LUF syndrome, an injection of the cytokine colony-stimulating factor just before hCG reduced the occurrence of LUF to 4% of the cycles as compared to 19% in those women not receiving the cytokine (Shibata et al., 2016).

The findings of the present study may also have implications for diagnosis and treatment of ovarian hyperstimulation syndrome (OHSS). This is a pathophysiological state with massive inflammation and with signs of overexpression of several ovulatory mediators (Nastri et al., 2010). Several of these have been covered in the present thesis.

The fact that ovulation is inflammation-like in its mediation is also suggested by the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on ovulation. In a first study on this aspect the effect of two different non-selective prostaglandin synthase inhibitors (indomethacin or azapropazone) were compared regarding ovulation rate (Killick & Elstein, 1987). The incidence of LUF (10% in control cycles) in azapropazone or indomethacin cycles were 50% and 100%, respectively. Normal steroid levels, also concerning progesterone were found in LUF cycles. A selective peroxidase/cyclooxygenase prostaglandin-endoperoxide synthase 2 (PTGS2) inhibitor was used in a study from Sahlgrenska, and the agent rofecoxib was given from midfollicular phase (Pall et al., 2001) even if ovulation was not inhibited, it was delayed by more than one day in almost 70% of the women.

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