Université de Montréal

THE INVOLVEMENT OF NITRIC OXIDE IN BOVINE FOLLICULAR DEVELOPMENT AND OVULATION

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Thèse présentée à la Faculté de médecine vétérinaire
en vue de l'obtention du grade de
philosophiae doctor (Ph.D.)
en sciences vétérinaires
option reproduction

Août, 2013

Résumé

Comprendre les événements paracriniens qui régulent la fertilité chez la vache est nécessaire non seulement en raison de l'importance agricole de cette espèce, mais aussi pour son utilisation potentielle comme modèle chez l'humain. L'oxyde nitrique (NO), un gaz de radicaux libres, a été impliqué dans la croissance folliculaire et l'ovulation chez les rongeurs et d'autres espèces, mais chez la vache c'est une énigme fascinante : le NO est produit par les cellules de la granulosa bovine et est régulé par la FSH, mais la présence et le profil d'expression des enzymes responsables de la synthèse de NO (NOS) dans les cellules de la granulosa tout au long de la croissance folliculaire ne sont pas claires. Les objectifs de cette thèse sont: (1) élucider le mécanisme de contrôle des NOS et les conséquences de la production d'oxyde nitrique pour le fonctionnement des cellules de la granulosa au cours du développement folliculaire chez la vache et (2) déterminer la régulation des NOS pendant la cascade ovulatoire induite par LH chez les cellules de la granulosa bovine et si l'activité des NOS pour l'expression des gènes critiques dans la cascade ovulatoire chez cette espèce. Les résultats sont séparés en 2 articles. Dans le premier article, la régulation de NOS2 dans les cellules de la granulosa bovine a été explorée. L'abondance des ARNm codant pour NOS2 a été stimulée par la FSH et l'IGF1 en augmentant l'estradiol, et un blocage de l'action de l'estradiol a conséquemment réduit les niveaux d'ARNm codant pour NOS2. De plus, l'inhibition de l'activité des NOS a augmenté l'apoptose dans les cellules de la granulosa in vitro. Dans le second article, il a été démontré que le pic de LH induit une activation des NOS dans les cellules de la granulosa, et que l'activité de NOS induit la production de NO, ce qui est essentiel pour l'expression des gènes critiques dans la cascade ovulatoire induite par LH comme EREG/AREG/PTGS2. Ensemble, les résultats présentés dans ces 2 articles suggèrent que les niveaux physiologiques d'activité des NOS peuvent contribuer à la croissance et la survie des cellules de la granulosa et indiquent également que NO peut être essentiel pour l'ovulation chez les bovins.

Mots-clés: oxyde nitrique, NOS, ovaire, follicule, cellules de la granulosa, apoptose, ovulation, vache

Abstract

Understanding the paracrine events that regulate fertility in the cow is necessary not only because of the agricultural importance of this species, but also its potential use as a model for humans. Nitric oxide (NO), a free-radical gas, has been implicated in follicular growth and ovulation in rodents and other species, but the cow is an intriguing enigma: NO is produced by bovine granulosa cells and is regulated by FSH, but the presence and the expression pattern in granulosa cells of the enzymes responsible for NO synthesis (NOS) throughout follicular growth are unclear. The objectives of the present thesis were (1) to elucidate the mechanism of control of NOS and the consequences of nitric oxide production for granulosa cell function during follicle development in cattle; and (2) to determine the regulation of NOS during the LH-induced ovulatory cascade in bovine granulosa cells and whether NOS activity is critical for the ovulatory cascade in this species. The results are separated in 2 articles. In the first article, the regulation of NOS2 in bovine granulosa cells was explored. Abundance of mRNA encoding NOS2 was stimulated by FSH and IGF1 through increased estradiol, and a blockade of estradiol action consequently lowered NOS2 mRNA levels. Further, inhibition of NOS activity increased apoptosis in granulosa cells in vitro. In the second article, it was demonstrated that the LH surge induces NOS activation in granulosa cells, and that NOS activity induces the production of NO, which is essential for EREG/AREG/PTGS2 expression, critical genes in the LH-induced ovulatory cascade. Together, the results presented in these 2 articles suggest that physiological levels of NOS activity may contribute to growth and survival of granulosa cells, and also indicate that NO may be essential for ovulation in cattle.

Key words: nitric oxide, NOS, ovary, follicle, granulosa cells, apoptosis, ovulation, cow

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List of abbreviations

3β-HSD: 3β-hydroxysteroid dehydrogenase

17β-HSD: 17β-hydroxysteroid dehydrogenase

A4: Androstenedione

ADAM: A Disintegrin and Metalloproteinase

AREG: Amphiregulin

AKT: Protein Kinase B

Bax: Bcl-2–associated X protein

Bcl-2: B-cell lymphoma 2

BH4: Tetrahydrobiopterin

BP: Base Pair

BTC: Betacellulin

cAMP: Cyclic Adenosine Monophosphate

CAMKII: Calcium/calmodulin-dependant Kinase II

cGMP: Guanosine 3'5'-Monophosphate

CL: Corpus luteum

COC: Cumulus-Oocyte Complexes

CYP11A1: Cytochrome P450 Cholesterol Side-Chain Cleavage, Family 11, Subfamily A, Polypeptide 1

CYP17: cytochrome P450 17α-hydroxylase

CYP19A1: Cytochrome P450, Family 19, Subfamily A, Polypeptide 1

D: dominant follicle

DNA: Deoxyribonucleic Acid

eCG: Equine chorionic gonadotropin

eNOS: Endothelial Nitric Oxide Synthase

E2: Estradiol

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

EGR1: Early Growth Response 1

EREG: Epiregulin

ERK: Extracellular-Signal-Regulated Kinase

FAD: flavin adenine dinucleotide

Fas: Apoptosis Antigen 1

FasL: Apoptosis Antigen 1 Ligand

FGF2: Fibroblast Growth Factor 2

FMN: Flavin Mononucleotide

FSH: Follicle-Stimulating Hormone

FSHR: Follicle-Stimulating Hormone Receptor

GnRH: Gonadotropin-Releasing Hormone

GPCR: G protein-coupled receptor

GVBD: germinal vesicle breakdown

H2AFZ: H2A histone family, member Z

HAS2: Hyaluronan Synthase 2

hCG : Human Chorionic Gonadotropin

HDL: High-density Lipoprotein

HSD17B1: Hydroxysteroid (17-beta) dehydrogenase 1

HSD3B2: Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid deltaisomerase 2

IGF1: Insulin-like Growth Factor-1

iNOS: Inducible Nitric Oxide Synthase

LDL: Low-density Lipoprotein

LH: Luteinizing Hormone

LHR: Luteinizing Hormone Receptor

LPS: Lipopolysaccharide

MAPK: mitogen-activated protein kinase

mRNA: Messenger RNA

NADPH: nicotinamide adenine dinucleotide phosphate reduced form

ND: non-dominant follicle

NOS: Nitric Oxide Synthase

NOS1: Neuronal Nitric Oxide Synthase

NOS2: Inducible Nitric Oxide Synthase

NOS3: Endothelial Nitric Oxide Synthase

NOx: Nitrogen Oxide Species

P4: Progesterone

PG: Prostaglandin

PGE2: Prostaglandin E2

PGF2α: Prostaglandin F2α

PI3K: Phosphatidylinositol 3-Kinase

PTGS2: Prostaglandin-Endoperoxide Synthase 2

PTX3: Pentraxin related gene

RAS: Rat Sarcoma

RNA: Ribonucleic Acid

RT-PCR: Reverse Transcription-Polymerase Chain Reaction

SDS: Sodium Dodecyl Sulfate

sGC: Soluble Guanylyl Cyclase

PKA: Protein Kinase A

PKG: Protein Kinase G

PMSG: pregnant mare's serum gonadotropin

PKC: Protein Kinase C

SNAP: S-nitroso-N-acetylpenicillamine

SNP: Sodium Nitroprusside

StAR: Steroidogenic Acute Regulatory protein

TGF-β: Transforming Growth Factor Beta

TSG-6: Tumor Necrosis Factor-Inducible Gene 6 Protein

ZP: Zona Pellucida

Acknowledgements

I want to express my deeply-felt thanks to Dr. Christopher Price for everything. Thank you for being more than an excellent supervisor, but also a great person. It was an honor to be your Ph.D. student.

I want to give special thanks to my special lab mates Hilda Guerrero, Atefeh Abedini and Fatiha Sahmi. Thanks for your collaboration and advices. Thanks for your friendship and for making the lab an excellent place to be.

My deepest gratitude to my collaborator and friend Dr. Valério Portela. He not only gave me the pleasure to know Dr. Price, but also directly and indirectly participated in my formation as a Ph.D. student.

I want to thank Alysson Macedo and Gustavo Zemke, for all the help during their training in our lab.

I am happy to acknowledge all the CRRA employees who helped me during this period. I want to thank Mira Dobias-Goff, Vickie Roussel and Jacinthe Therrien for their technical support and their kindness. My sincere gratitude to Eliane Auger, Geneviève Provost, Julie Blouin and Micheline Sicotte for helping me to solve all bureaucratic obstacles. Thank you ladies!

I want to thank all CRRA students who shared their experiences, knowledge and friendship during all these years.

I want to thank the Fonds québécois de la recherche sur la nature et les technologies (FQRNT) for the scholarship and the Natural Sciences and Engineering Research Council (NSERC) for the financial support for the experiments.

I would like to thank my mother Regina, my father Jurandir, my brother Alexandre and my sisters Cristina and Clarissa, for all the encouragement and emotional support sent from thousands kilometer from here. You are always on my mind. I love you guys!

I want to express my deeply-felt thanks to my wife Lucilene Bernardi, my best friend and love. Thank you for always being there for me. Thank you for sharing this amazing experience with me.

I would like to thank God for these blessed years of my Ph.D.

Introduction

Ovarian follicles are the functional units of the ovary. Each follicle contains normally one oocyte, the female reproductive germ cell. The other cells that surround the oocyte to compose a mature ovarian follicle are somatic cells, and include cumulus and mural granulosa cells, and the cells of the theca layer [1]. The formation, development and maturation of an oocyte is defined as oogenesis, while the process that involves the proliferation and differentiation of somatic cells, and consequently, the maturation of the whole ovarian follicle is refered to as folliculogenesis. Both oogenesis and folliculogenesis are linked in an intimate and mutually dependent relationship [2, 3].

During the course of folliculogenesis, oocytes first acquire meiotic competence and then gradually acquire developmental competence, a biochemical and molecular state that allows a mature oocyte to undergo normal fertilization, support normal preimplantation embryo development and subsequent healthy growth of the implanted embryo to term. The support for the acquisition of oocyte competence is maybe the most important function of the follicle, with granulosa cells exerting an essential role [4, 5]. For this reason, it is crucial that granulosa cells are healthy and working properly. To guarantee the good functioning of granulosa cells, many endocrine factors such as gonadotropins, paracrine growth factors and intracrine modifiers of cell function modulate their development and function. Some, such as FSH and LH, have well defined roles in granulosa cells function, but the roles of others are less well defined. This is the case for nitric oxide (NO), a short-lived gas produced by the action of the enzyme nitric oxide synthase (NOS). This free-radical is produced in the ovary and has been implicated in different ovarian processes of several species. Although many studies have determined that NO modulates processes like steroidogenesis, follicular growth, oocyte maturation and ovulation [6-8], many questions about the regulation of the NO generation system as well the physiological effects of NO in granulosa cells still need to be answered.

This thesis contains results from studies using cell models carefully selected to represent granulosa cells at different stages of development in cattle. The regulation of NOS mRNA levels in granulosa cells by natural ligands, including gonadotropins, steroids and growth factors in conditions that mimic follicle growth and differentiation, as well in conditions that simulate the periovulatory period, have been determined. The roles exerted by NO on granulosa cells during these two distinct physiological moments have also been described. These findings may provide new and clinically relevant information on the physiological role of a highly potent free radical gas in the ovary. The data obtained will advance significantly our understanding of follicle development and ovulation and should lead to better clinical approaches to infertility.

Chapter 1:

Literature review

1. Ovarian follicular development and growth

Follicular development and growth can be driven by different regulators and involve complex interactions between the three main cell types within the follicle: theca cells, granulosa cells and the oocyte. The systemic endocrine regulation of folliculogenesis is related not only to the pituitary gonadotropins FSH and LH, but various locally produced hormones and growth factors. The oocyte has been confirmed as a major regulator of preantral and early antral follicular growth. On the other hand, late steps of antral follicle development and growth involve gonadotropins and growth factors, specially the insulin-like growth factor (IGF) system [3, 9].

The following sections will focus mainly on basic aspects of ovarian follicular development and growth in cattle.

1.1 Ovarian follicles

In ruminants, ovarian follicular formation is completed during fetal life. In cattle, follicular growth is initiated before the last primordial follicles are formed and then continues throughout fetal, neonatal and adult life [10]. Ovarian follicles can be classified as primordial, primary, secondary and tertiary or antral follicles (Figure 1). Some authors divide antral follicles in early or small antral follicles and late or large antral follicles. They not only present differences in their morphology, but also in their responsiveness to different regulators [3, 11].

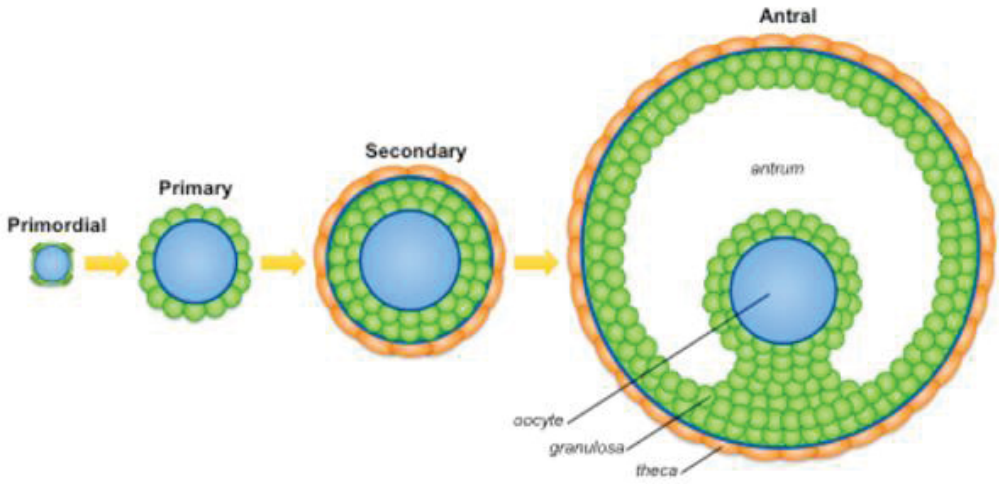

Figure 1. Ovarian follicle classification (http://www.bme.umich.edu/labs/shikanov/).

1.1.1 Primordial follicles

In cattle, after day 90 of fetal life, the first follicles separate themselves by producing a basement membrane, forming the primordial follicles which are the largest population of follicles in the ovary. Each primordial follicle contains a small non-growing oocyte and a layer of non-dividing flattened pre-granulosa cells encapsulated by the follicular basal lamina. The ovary has a reservoir of primordial follicles that is depleted as follicles gradually and regularly leave this resting pool and initiate growth [11]. In these follicles, the oocyte and granulosa cells have receptors for some growth factors, but not LH or FSH. The primordial follicles, however, do not require gonadotropins for their survival and continued development [12]. Many of the proteins expressed in primordial follicles are associated with cell maintenance and preparation for growth. A primordial follicle expresses hundreds of genes that fulfill housekeeping and signalling functions, cytoskeletal events, DNA repair, mRNA processing, ribosomal function, protein synthesis and ubiquitination. The delay between the appearance of the first primordial and the first primary follicles is relatively long, at 50 days in cattle [13, 14].

1.1.2 Primary follicles

The first activated primary follicles do not appear in bovine fetal ovaries until Day 140 of pregnancy. Once follicles have left the pool of primordial follicles they undergo gonadotropin-independent growth, meaning that FSH and LH are not essential for their growth. These small pre-antral follicles present continuous growth that is mainly controlled by factors secreted by the oocyte [12]. The transformation of the flattened pre-granulosa cells of the primordial follicle into a single layer of cuboidal granulosa cell marks the transition to primary follicle [13]. As a follicle grows to the primary stage, the granulosa cells not only change shape but also divide and increase in number and the oocyte enlarges. The primary follicle is also characterized by the development of the zona pellucida (ZP), that was absent in primordial follicles. Several hundred genes not found in primordial follicles are activated during this stage of growth, including those related with synthesis of the ZP, as well some involved in mitochondrial function, cell signalling and communication [11].

1.1.3 Secondary follicles

The secondary follicles are a group of large preantral follicles. They gain multiple layers of granulosa cells, from two to six layers around the oocyte. They also present a well delimited zona pellucida and a theca interna. The secondary follicles are considered gonadotropin-responsive because these follicles present not only FSH-responsive granulosa cells but are also characterized by the development of LH-responsive theca interna. The acquisition of the enzymes required for thecal androgen production is essentially complete before antrum formation [14, 15].

1.1.4 Antral follicle formation

The antrum is a fluid-filled cavity that is formed in the follicles under the influence of FSH. A follicle with an antrum is named tertiary or antral follicle (Figure 2). As antral follicles form, the granulosa cells differentiate into two anatomically and functionally distinct lineages; the mural granulosa cells that line the wall of the follicle and that have principally a steroidogenic role; and the cumulus cells, that form an intimate life-support association with the oocyte [16, 17].

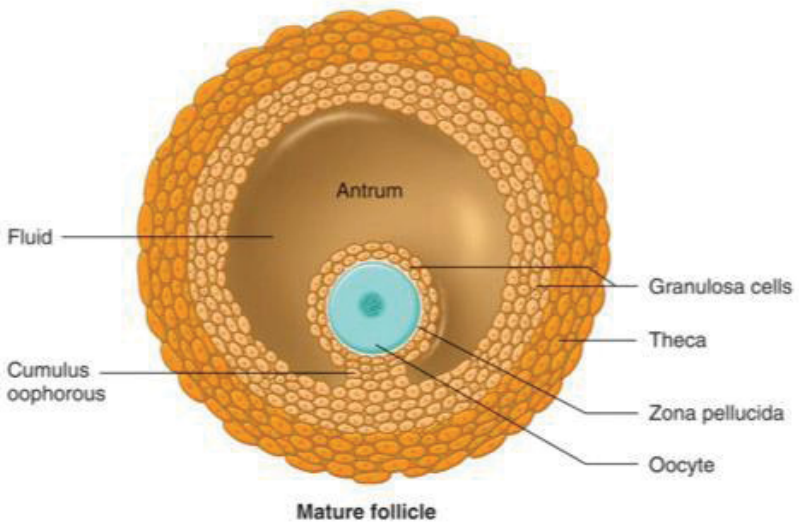

Figure 2. Antral follicle structure (http://www.studyblue.com/notes/note/n/week-7-female-reproductive-system-parysek/deck/5613015).

As follicle development progresses, follicles gradually become more and more reliant on gonadotropins, first as gonadotropin-responsive follicles and then as gonadotropin-dependent follicles [3].

Follicular growth is highly related to the secretion of steroids, especially estrogens. They are necessary for granulosa cell proliferation, growth of the oocyte and acquisition of LH receptors [18, 19].

1.2. Follicle steroidogenesis

One of the most important functions of the follicle is the production of steroids. Follicular steroidogenesis in ruminants, as in other species, starts usually with cholesterol and ends with the formation of several steroid metabolites [20]. This involves both theca and granulosa cells (Figure 3). Basically, cholesterol is imported into the cell through internalization of blood-borne lipoproteins. Within the cell, cholesterol is maintained within lipid droplets as cholesterol esters. The enzyme cholesterol ester hydrolase converts the cholesterol esters to free cholesterol. Within the cytoplasm the free cholesterol is mobilized to the mitochondria, and then internalized. This internalization of cholesterol by the mitochondria is the rate-limiting step for the general steroidogenic pathway, and is mediated by steroidogenic acute regulatory protein (StAR). Once inside the mitochondria, cholesterol is converted to pregnenolone by the enzyme cytochrome P450 cholesterol side-chain cleavage (CYP11A1 or P450scc). Pregnenolone can then be converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD or HSD3B2), or to 17α-hydroxypregnenolone by the enzyme cytochrome P450 17α- hydroxylase (CYP17 or P45017-OH). In ruminant luteal and granulosa cells, the enzyme CYP17 is not expressed, and so steroidogenesis goes through to progesterone; this progesterone is not metabolised further, and is secreted. In theca cells, however, there is abundant CYP17 activity, and so pregnenolone is converted to 17α-hydroxypregnenolone. This 17α-hydroxypregnenolone then undergoes sequential conversion to androstenedione by CYP17 and 3β-HSD activities. Ruminant theca cells convert limited amounts of androstenedione to testosterone with the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD or HSD17B1), and both androstenedione and testosterone are secreted. A good portion of these secreted

androgens are absorbed by the neighbouring granulosa cells and are further converted to estrogens. Ruminant granulosa cells prefer to metabolize androstenedione to estrone by the enzyme cytochrome P450 aromatase (CYP19A1), and then the estrone is metabolized to estradiol by 17β-HSD. Alternatively, testosterone can be metabolised to estradiol by CYP19A1 [21, 22].

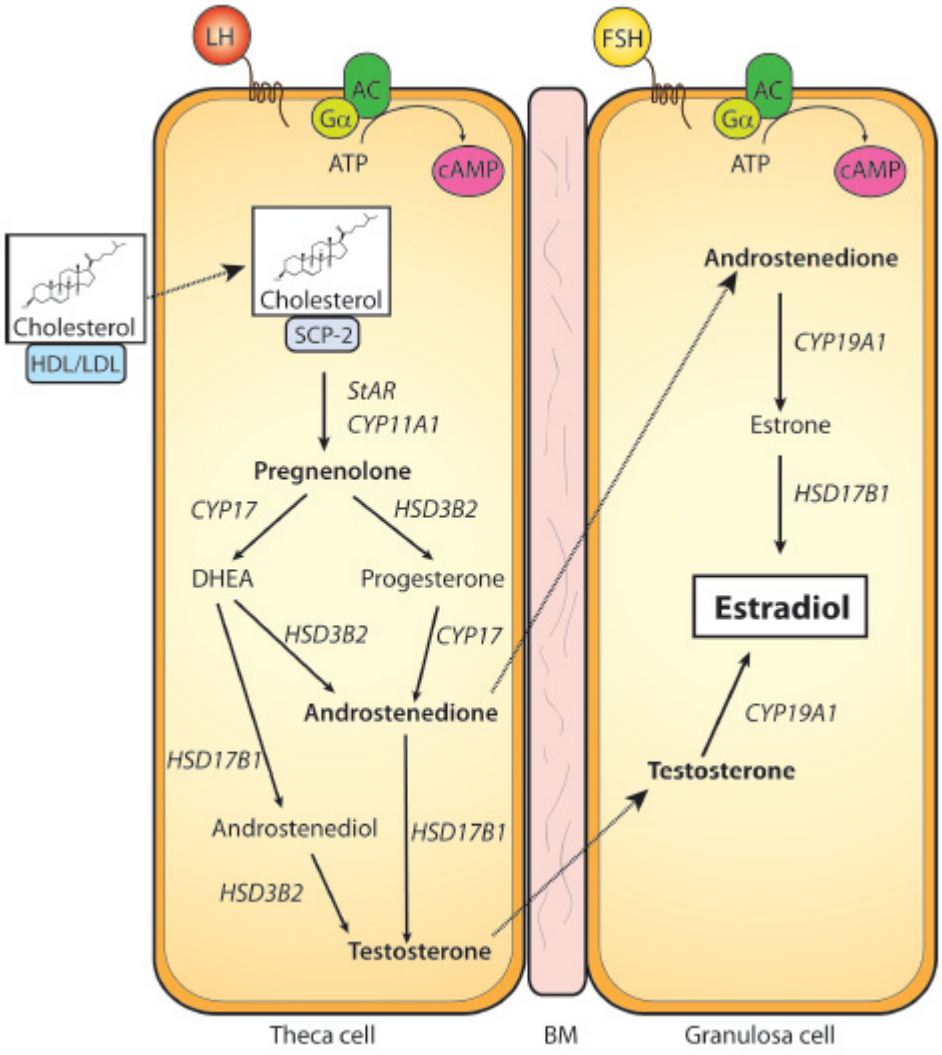

Figure 3. Major steroidogenic pathways in the follicle (Taken from [22]).

1.2.1 Roles of gonadotropins and insulin-like growth factor-1 (IGF1) in steroidogenesis

The production of both estradiol and progesterone is regulated within the follicle throughout follicle growth. Summarizing the steroidogenic pathway described above, in ruminants, granulosa cells convert theca-derived androgens to estrogens with the enzyme CYP19A1 and may convert androstenedione to testosterone and/or estrone to estradiol with 17β-HSD. These and other steroidogenic enzymes are under the regulation of gonadotropins and growth factors.

Theca and luteal cells express LH receptors and the steroidogenic enzymes present in these cells are normally up-regulated by LH. Consequently, LH induces androgen secretion from theca cells and stimulates progesterone secretion from luteal cells. In granulosa cells of smaller follicles, the only gonadatropin receptor expressed is FSHR; and FSH regulates both estradiol and progesterone secretion. In cattle as well as other species, FSH acts mainly through a cAMP pathway and can be considered one of the primary stimulators of granulosa CYP19A1 expression, but also regulates the expression of CYP11A1 [23, 24]. In larger follicles, LHR is also expressed in granulosa cells and LH modulates mainly progesterone secretion [25, 26].

A number of growth factors have also been shown to alter steroid production. A growth factor critical to follicular growth is IGF1. It stimulates estradiol and progesterone secretion in bovine follicles. IGF1 acts through PI3K and PKC pathways to increase expression of CYP19A1 [24, 27] and other steroidogenic enzymes in bovine granulosa cells and to stimulate progesterone and androstenedione secretion in theca cells [28-30].

1.3 Follicular dynamics in the cow

The cow, like women and mares, is a mono-ovulatory species, and generally ovulates one follicle per cycle. As a non seasonal polyestrus species, the cow continually has estrous cycles all year around. The entire estrous cycle averages 21 days

and studies using ultrasonic imaging to monitor follicle populations in different size categories or to monitor individually identified follicles have convincingly documented that follicular growth occurs in a wave-like fashion and that the majority of estrous cycles in cattle are comprised of two or three such waves. Two-wave cycles are consistently shorter (19–21 days) than three wave cycles (22–23 days) [31, 32].

In gonadotropin-responsive and gonadotropin-dependent follicles, inadequate support from gonadotropins leads to their regression. It is the developing dependence on gonadotropins that transforms folliculogenesis from a linear process in the preantral and early antral stages of development into a wave-like process during the terminal stages of folliculogenesis, as gonadotropin-dependent follicles grow and regress in a regular sequential pattern of waves [3].

1.3.1 Follicle wave emergence

During the antral growth stage, the most advanced follicles in the pool of gonadotropin-responsive follicles are those that emerge concomitantly with the increases in FSH to form what is commonly referred to as the cohort of gonadotropin-dependent follicles. In a more classic concept, follicular wave emergence is defined as the sudden growth of a group of small follicles that are initially detected by ultrasonography at a diameter of 3–5 mm. In cattle, in both two- and three-wave estrous cycles, the emergence of the first follicular wave occurs consistently on the day of ovulation [33]. Until recently, reference to a follicular wave in cattle was limited to follicles larger than 4 mm, based simply on the limit of resolution of existing ultrasound equipment. The accessibility to new ultrasound scanners capable of resolving structures as small as 1 mm permitted some authors to determine that, 1 or 2 days before conventionally defined wave emergence, 1–3 mm follicles have already developed in a wave-like manner in association with a FSH surge in plasma. During this phase, the follicles grow at an approximately similar rate and each follicle has the capacity for future dominance and no follicle exerts dominance over its cohort [34, 35].

1.3.2 Follicle selection and diameter deviation

The follicles emerging in the same wave present a similar growth rate for approximately 2 days, after which one follicle is selected for further growth. In cattle, as in other monovular species, this process is known as follicle selection [36, 37]. Selection of the dominant follicle is associated with decreasing blood FSH concentrations during the first 3 days of the wave. The nadir in FSH is reached 4 days after wave emergence, and concentrations remain low for the next 2–3 days. One of the reasons by which the selected follicle may continue its growth is related to the IGF system. IGF1 increases the sensitivity of small follicles (around 5 mm in cattle) to gonadotropins and simulates their transition from the gonadotropin-responsive to the gonadotropin-dependent stages [38].

The moment when the selected follicle continues its growth, while the remaining follicles cease growing, is known as diameter deviation [39]. At the beginning of deviation, the largest follicle in cattle is about 8.5 mm and second largest follicle is about 7.2 mm [40]. Although there is no significant difference in size, intrafollicular biochemical events ensure future dominance of the selected follicle. The intrafollicular factors responsible for these biochemical changes include those related to the IGF system, steroids, inhibin-A/activin-A peptides, gonadotropin receptors and angiogenic factors [40-42] . However, IGF1 and its associated system, estradiol secretion and the presence of LH receptors have been temporally and/or functionally well implicated with follicle deviation and may be useful markers. In cattle, it was shown that the concentrations of free IGF1 remains constant or increase in the largest follicle before the equivalent period at the beginning of deviation [43], which is also marked by an increase in estradiol levels. IGF1 not only induces estradiol secretion in granulosa cells, but also stimulates granulosa cell proliferation and synergizes with gonadotropins to promote differentiation of granulosa cells [29, 44]. All these data support the concept that the IGF-system via IGF1 is an initiator of the beginning of follicle deviation and therefore a good marker for selection.

1.3.3 Follicle dominance

Follicle dominance is defined as the emergence of one follicle as significantly larger than the rest of the cohort, and that is morphologically a functionally dominant [9]. Probably the most important characteristic of the dominant follicle is its greater capacity for estradiol production. After the wave emergence, estradiol content in the follicular fluid of the growing dominant follicle increases at least 20-fold by the day of selection [35, 37]. In cattle, follicular-fluid concentrations of estradiol begin to increase differentially in the largest versus second largest shortly before or at the expected beginning of deviation. This dominant follicle secretes sufficient estradiol and inhibin to suppress FSH, which as a consequence, promotes atresia in the remaining gonadotropin-dependent follicles and preventing the emergence of a new cohort of gonadotropin-responsive follicles [39, 45, 46].

The increased estradiol secretory capacity of the dominant follicle is because it is molecularly distinct from the others even before the beginning of deviation. Estradiol synthesis is dependent upon gonadotropic stimulation of both androgen synthesis in theca cells and its aromatization to estradiol in granulosa cells. In cattle, the selected follicle presents higher expression of the gene for CYP19A1 near the beginning of deviation. Levels of mRNA for CYP17 in theca cells and for CYP19A1 in granulosa cells are higher in early dominant follicles than in recruited follicles, whereas mRNA for CYP11A1 is higher in granulosa, but not theca cells [47-49]. This explains the increased potential for estradiol production by the selected dominant follicle in comparison to the subordinate follicles.

Studies performed to determine changes that occur in granulosa cells when the most successful follicle of the cohort becomes dominant show that the majority of the transcripts up-regulated in granulosa cells of the dominant follicle are encoded by genes that regulate not only estradiol synthesis, but also cell proliferation and survival, signalling, organ development and extracellular tissue remodelling [50, 51].

1.3.4 Ovulatory follicles

In cattle, about three days after emergence, one or a few follicles achieve potentially ovulatory status. The dominant follicle shifts its gonadotropin dependence from FSH to LH during the FSH nadir, and is able to continue to grow while the subordinates regress. In bovine, LH receptor (LHR) mRNA in granulosa cells is detected in follicles greater than 8 mm, but not in follicles smaller than 8.0 mm or in subordinate follicles [26, 52].

The ovulation occurs, however, if the preovulatory follicle grows in the correct endocrine milieu that involves appropriate progesterone and estradiol levels and LH pulse frequency (Figure 4). Pulse frequency and amplitude of LH are influenced by circulating concentrations of both progesterone and estradiol. High levels of progesterone produced by a functional corpus luteum (CL) during diestrus or pregnancy suppress LH pulse frequency. The non-ovulatory wave is marked by the presence of a CL, and consequently, high levels of progesterone. In these conditions LH pulse frequency is suppressed and the gonadotropin-dependent dominant follicle undergoes atresia, secreting less estradiol and inhibin so that FSH concentration can increase and start a new wave [36, 53]. On the other hand, the dominant follicle present at the onset of luteolysis becomes the ovulatory follicle. The plasma progesterone concentrations decrease and the LH pulse frequency increases, permitting the dominant follicle grow larger and remain dominant. Increasing estradiol concentrations with decreasing progesterone after luteolysis increase LH pulse frequency, culminating in a large prevulatory LH surge and ovulation [54].

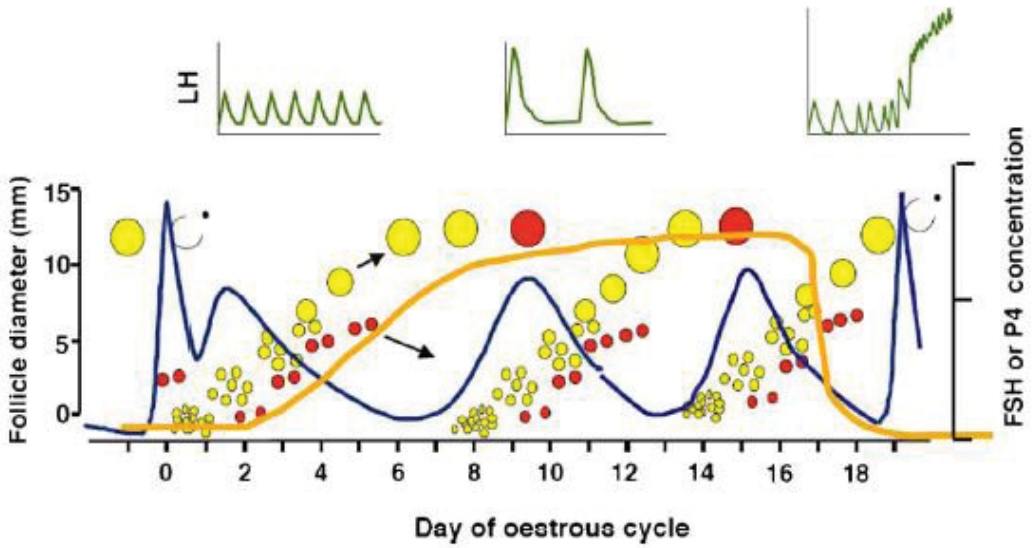

Figure 4. Ovarian follicular dynamics during the estrous cycle. Schematic of the pattern of secretion of FSH (blue line), LH (green lines), and P4 (orange line); and the pattern of growth of ovarian follicles during the estrous cycle in cattle (Taken from [55]).

1.4 Follicle atresia

The bovine ovary contains hundreds of thousands of follicles at birth, but very few follicles successfully ovulate and more than 99.9% undergo atresia, the process of degeneration of ovarian follicles. Atresia happens at various stages of follicular development [56], but the collective evidence suggests that the rate of follicular atresia is very low during the preantral stages of growth while the transition to an antral follicle is accompanied by a significant increase in the rate of atresia, indicating that it is a physiological challenge for the follicle to form an antrum and to maintain the granulosa–oocyte syncytium. It has been estimated that the incidence of atresia in bovine follicles is greatest after antrum formation, just before the final stages of follicular development [3, 57].

Both subordinate and dominant follicles may stop their growth and regress through the atretic process under different circumstances. General morphological signs

of atresia include decrease of follicle wall thickness characterized by the reduction of granulosa cells layer thickness, which becomes loose and disorganized. An advanced stage of atresia is characterized by follicle cell degeneration, initially in the granulosa cell layer. The death of granulosa cells leads to almost total destruction of the granulosa cells layer lining the inner follicular wall with the consequent destruction of follicular structure [57, 58]. All these morphological changes in an atretic follicle are preceded and/or accompanied by molecular and biochemical changes that include a marked decrease in concentrations of estradiol in follicular fluid and reduced expression of mRNA encoding FSHR, several steroidogenic enzymes and many survival genes [48, 59, 60].

1.4.1 Apoptosis in ovarian follicles

Apoptosis is a process of cell self-destruction and is an event associated with the initiation and progression of ovarian follicular atresia in all vertebrate species [56, 61]. Cell death is mediated through caspase activity. Caspases are cysteine proteases that cleave their substrate proteins specifically at an aspartate residue. They are constitutively expressed in an inactive proenzyme form and are activated after cleavage at specific aspartate residues. The activation of execution caspases, such as caspase-3, 8 and 9, indicates the point of no return in the apoptotic pathway. These proteins either directly or indirectly cleave a broad array of proteins necessary for cell survival, such as those involved in DNA maintenance and repair and organization of intermediate filaments. During the apoptosis, the nucleus breaks into several fragments, then the cell breaks up into several membrane bound smooth-surfaced apoptotic bodies [58].

Apoptosis of granulosa cells is an early feature of atresia in bovine follicles [58, 62, 63]. A number of mechanisms have been proposed to induce apoptosis and activation of caspases in granulosa cells. These include binding of specific ligands to their respective receptors, such as tumor necrosis factor-alpha [61], inhibition of cell–cell contact [64], presence or absence of specific growth factors [65], and altered levels

of hormones such as estrogens and androgens [66]. Some studies demonstrate that a high concentration of progesterone may play an important role in initiating the regression of non-ovulatory dominant follicles during the bovine estrous cycle [58]. In addition, follicular apoptosis may be induced by oxidative stress [67].

A particular and interesting trigger for apoptosis in granulosa cells is Fas antigen [68]. Fas is a cell surface receptor that induces apoptosis when bound by Fas ligand (FasL). The Fas system has been shown to mediate bovine granulosa cell apoptosis. In this species, granulosa and theca cells are susceptible to FasL-induced apoptosis to varying degrees. The expression of Fas mRNA and responsiveness of granulosa cells to FasL-induced apoptosis is higher in atretic subordinate follicles compared with healthy dominant follicles [65, 69]. The fact that both mRNA and protein for Fas and its ligand are high in follicles undergoing atresia, indicate that the Fas pathway is involved in the initiation and/or progression of apoptosis [69, 70]. Activation of the Fas pathway leads to cleavage and activation of caspases. Furthermore, cell death is inhibited by reagents that prevent binding of FasL to Fas, providing evidence that apoptosis is mediated, at least partially, by binding of endogenous Fas and FasL on granulosa cells [71].

1.4.2 Anti-apoptotic mechanisms in granulosa cells

Follicle cells are thought to initiate apoptosis in the presence of cytotoxic signals or in the absence of necessary survival signals [61]. Gonadotropins and growth factors have been reported to play critical roles in preventing apoptosis in granulosa cells of antral follicles [72]. The process of apoptosis in follicles is associated with decreased levels of FSHR and LHR mRNAs, and consequently, a consistent decreased response of granulosa cells to gonadotropins [59]. FSH binding to its receptor promotes ovarian follicle survival and growth not only by stimulating proliferation, but also inhibiting apoptosis by up-regulating the expression of intracellular anti-apoptotic proteins such as X-linked inhibitor of apoptosis protein [73, 74]. IGF1 also stimulates bovine granulosa

cell proliferation and survival [75]. In addition, IGF1 inhibits FasL-induced apoptosis of bovine granulosa cells [65].

The effects of FSH and IGF1 on follicle cell survival, however, are also related to their ability to stimulate estrogen synthesis. During follicular development, both FSH and IGF1 stimulate estrogen production in vivo and in vitro. Some studies have implicated estrogen as an inhibitor of apoptosis [66]. In cattle, follicles that are selected for continued growth and development to the ovulatory stage have increased capacity to secrete estradiol relative to follicles destined to undergo atresia [36]. The occurrence of apoptosis in individual atretic follicles is correlated with decreased levels of CYP19A1 mRNA and intrafollicular estrogen levels. One of the mechanisms used by estradiol to protect bovine granulosa cells from FasL-induced apoptosis in vitro is related to its effect on progression through the cell cycle [19].

2. Ovulation

Ovulation is the rupture of the follicle wall and release of the oocyte-cumulus complex. The ovulatory process depends on a coordinated activity of gonadotrophins, steroid hormones and mediators involved in an inflammatory reaction, such as prostaglandins. Some of the most significant changes that occur during the periovulatory period include meiotic maturation of the oocyte, follicular rupture and ovulation; and the shift in follicular steroidogenesis from androgen/estradiol to progesterone as the primary steroid product secreted by granulosa cells [76].

The following section will describe briefly the main aspects related to the preovulatory cascade in the cow and also in other species, especially rodents due to the large body of literature in mice and rats.

2.1 The preovulatory cascade

The main trigger of the preovulatory cascade is LH, which activates a cascade of signaling events that are propagated throughout the ovarian preovulatory follicle to promote ovulation of a mature egg. Although LH directly stimulates theca and granulosa cells, its effects on cumulus cells and oocytes are probably indirect, as both cell types express few or no LH receptors and fail to respond when directly stimulated by LH [77]. In minutes to hours post-LH, several genes are rapidly and transiently up-regulated, causing the required physiological and phenotypic changes in the follicular cells that culminate in ovulation and luteinization.

2.1.1 LH signaling pathways activation

LH activates a number of cellular signaling cascades within the preovulatory granulosa cell. The LHR, a classical G-protein-coupled receptor (GPCR), activates adenylate cyclase, resulting in a large intracellular cAMP increase that activates the cAMP-dependent serine kinase protein kinase A (PKA) [78]. Although LH rapidly induces in a PKA-dependent manner the expression of several genes in the preovulatory follicle, other important pathways are activated by LH for the induction of essential genes for ovulation (Figure 5), including extracellular regulated kinase (ERK1/2 or MAPK3/1), phosphoinositide 3-kinase/AKT (PI3K/AKT) and mitogen-activated protein kinase 14 (MAPK14 or p38) signaling pathways [77, 79, 80].

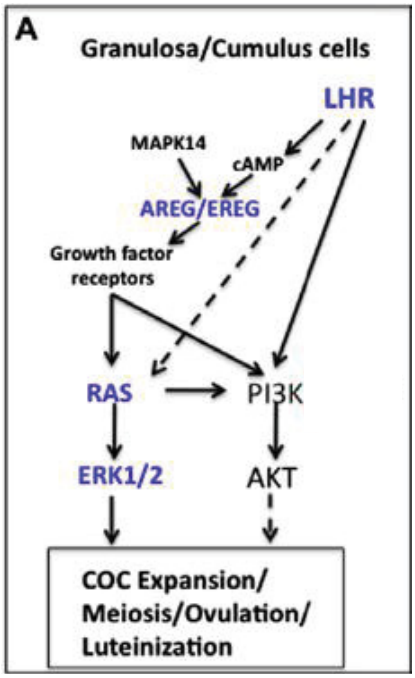

Figure 5. Important signaling cascades in ovulation (Modified from illustration on [81]).

Studies with porcine cumulus cells demonstrated that inhibition of MAPK14 or PKA activity resulted in significant inhibition of MAPK3/1 phosphorylation [82], suggesting that these pathways may converge on MAPK3/1. Moreover, mice in which ERK1 and ERK2 have been disrupted in granulosa cells exhibit normal follicle growth, but in response to LH, the COCs fail to expand, oocytes fail to re-enter meiosis, and follicles fail to either ovulate or luteinize [83]. The ERK1/2 pathway seems to be essential to LH effects during the preovulatory period.

2.1.2 ADAMs

ADAMs are type I transmembrane proteins with both metalloproteinase and disintegrin domains [84]. ADAMs are implicated in cell–cell and cell–matrix

interactions and shedding of membrane-bound precursors such as EGF family ligands [85, 86]. In terms of the preovulatory cascade, LH induces ADAMs expression/activity which in turn cleave and release the pre-formed EGF-like growth factors from the surface of mural granulosa cells [87, 88]. Increased expression of EGF family ligands accompanies the cascade of events resembling an inflammatory and/or tissue remodeling process during the preovulatory period, and shedding and action of such autocrine and paracrine signals is critical for LH actions [79, 89, 90].

2.1.3. EGF-like growth factors

The EGF-like factors include amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC). These factors were identified as genes rapidly induced by LH in granulosa cells and by EGF in cumulus cells as illustrated in the previous figures above [91]. Studies with mice in vivo demonstrate that AREG and EREG mRNA levels are increased within 1 h after hCG injection [87, 92-94]. In cattle, a recent in vitro study indicates that LH increased EREG mRNA levels within 1 h but did not alter AREG mRNA levels in mural granulosa cells until 6 h after challenge [95]. Once released, AREG/EREG then act in a paracrine manner to stimulate the EGF receptor of cumulus cells [89, 96]. EGF receptor activation results in AREG/EREG expression in the cumulus and increased prostaglandin-endoperoxide synthase 2 (PTGS2) expression. This same loop occurs in mural granulosa cells with EREG/AREG acting in autocrine manner to intensify the cascade [95].

Disruption of the EGF ligand/receptor signaling pathway in mice compromises ovulation, indicating that activation of this pathway is essential for LH-induced ovulation to occur [97]. The EGF-like factors bind their cognate receptors present on granulosa cells and cumulus cells, activate RAS, a small GTPase involved in transmitting signals, and induce expression of downstream target genes, including not only PTGS2 but also hyaluronan synthase 2, TNF-α– induced protein 6 and several other genes, each of which is a target of ERK1/2 in cultured cells [90, 98]. Thus,

ERK1/2 controls a master switch that mediates the global reprogramming of granulosa cells downstream of EGF-like–factor activation of the EGF receptor pathway. Several transcriptional regulators are known to affect ovulation and appear to help mediate the effects initiated by ERK1/2 (Figure 6).

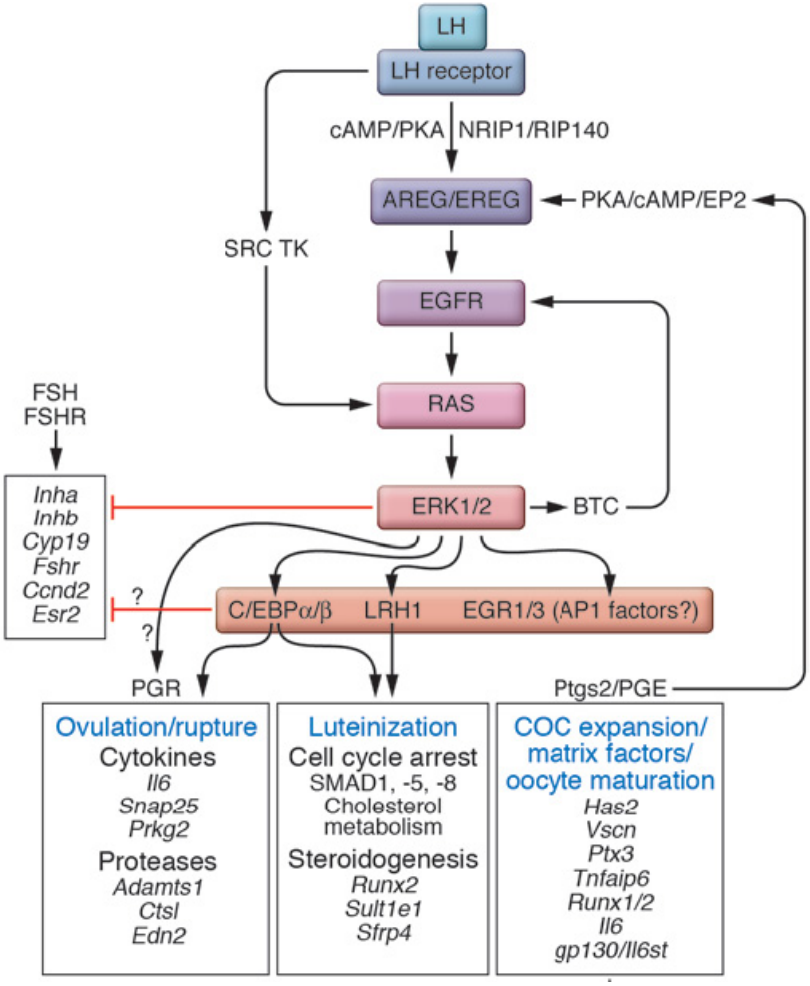

Figure 6. LH-induced RAS/ERK1/2 signaling pathway. Activation of ERK1/2 is essential to turn off the FSH regulated gene expression program that controls genes essential for preovulatory follicle growth and differentiation. Key transcription factors that are activated by ERK1/2 phosphorylation and affect ovulation and luteinisation (Taken from [90]).

2.1.4. Prostaglandins

A key element in the cascade that culminates in the rupture of the follicle wall is the synthesis of prostaglandins (PGs) by granulosa cells. Prostaglandins are produced from arachidonic acid by a LH-inducible enzyme, prostaglandin-endoperoxide synthase 2 (PTGS2; [99, 100]. In cattle, concentrations of prostaglandin E2 (PGE2) and prostaglandin F2alpha (PGF2α) in the follicular fluid remain low until late in the periovulatory period, increasing dramatically in the hours preceding ovulation [101]. In addition, studies in vitro using bovine granulosa cells collected from preovulatory follicles and challenged with LH show that PTGS2 mRNA levels significantly increase by 6 hours [95] or later [101]. In vivo studies with this species indicate that PTGS2 mRNA abundance increases around 12 hours post-hCG/GnRH challenge and suggest that PGs secretion do not commence until late in the periovulatory period, between 18 and 24 h after GnRH [102-104].

The essential participation of PGs to the ovulation has been demonstrated in different studies. PTGS2-null mice failed to ovulate [105] and the use of a broad-spectrum prostaglandin inhibitor (indomethacin) blocked ovulation in mice, rats, rabbits, sheep, pigs and primates [77]. In cattle, the intrafollicular injection of indomethacin [106] or a PTGS2-selective inhibitor also inhibited ovulation [107]. PGs promote a highly localized inflammatory response in the follicle prior to ovulation and act, at least in part, by stimulating the expression of proteases, including plasminogen activators, in the follicle wall to promote follicular rupture [108]. Prostaglandins have been also involved in a wide array of ovulatory and luteal events, including cumulus expansion, oocyte maturation, angiogenesis and progesterone production [109, 110].

2.1.5 Cumulus expansion and oocyte maturation

As a consequence of the ovulatory surge of LH, cumulus cells respond with a specific pattern of gene induction that leads the cumulus cells to produce a hyaluronan-

rich matrix that surrounds the oocyte prior to ovulation. This process, known as cumulus expansion or mucification, depends on a specific cascade of intracellular signals and extracellular matrix gene expression within the cumuls-oocyte complex (COC), and exerts a key role in the ovulatory process [111-113]. The set of genes expressed in periovulatory cumulus cells are critical for normal rates of ovulation and fertility. Studies of knockout mice demonstrated that EREG-null mice and PTGS2-null mice both exhibit defective cumulus expansion, reduced ovulation rate and infertility [97, 105]. Genes encoding COC matrix components are also induced in cumulus cells by FSH or EGF in conjunction with oocyte signals, including HAS-2, TSG-6 and pentraxin-3 [112-115].

Cumulus cell expansion and resumption of meiosis with germinal vesicle breakdown (GVBD) are major events in oocyte maturation. In mammalian follicles, primary oocytes enter meiosis but are arrested at the diplotene stage of prophase I. The oocytes stay in this dormant state for months and years until the preovulatory stage. In response to the preovulatory LH surge, the germinal vesicle of the oocytes in preovulatory follicles undergoes GVBD, which is then followed by chromatin condensation and the formation of meiotic spindles while the oocyte progresses through the maturation process. The transition from metaphase I (MI) to metaphase II (MII) is accompanied by extrusion of the first polar body. The maturing oocyte is the site of phosphorylation events that activate or deactivate the proteins involved in progression of the cell cycle [116]. Several kinases, including members of the mitogen-activated protein kinase (MAPK) family, are activated by a kinase pathway during this period [117].

Signals produced by cumulus cells appear to influence oocyte maturation [17]. The PTGS2-derived PGs produced by cumulus cells appear to constitute critical mediators not only of cumulus expansion, as described above, but also for oocyte maturation. PGE2 is the main PTGS2-related prostaglandin produced by cumulus cells both in vivo and in vitro and acts directly on the oocyte to activate the proteins involved

in meiosis progression. [118-120]. PGE2 acts as a direct enhancer of oocyte MAPK activity during the maturation process [110].

2.2. Follicule rupture

The follicle wall rupture that characterizes ovulation is one of the most important processes in female reproduction. The ovulation can be considered similar to an inflammatory process that is characterized by vascular changes and proteolytic degradation of the follicle wall [121-123].

As with all the other intrafollicular processes during the preovulatory period, follicle rupture is also linked to PGs action. In cattle, the inhibition of PGs synthesis by the intrafollicular administration of indomethacin blocks the LH surge-induced key mediators of extracellular matrix remodeling, consequently blocking ovulation [108]. PGE2 presents a key role in ovulation as a mediator of proteolytic degradation of the follicle wall. PGE2 alone or in combination with LH increased fibrinolytic activity in the medium of cultures of rat granulosa cells, whereas the PG synthesis inhibitor indomethacin blocked gonadotropin-induced fibrinolysis. Tissue-type PA (tPA) and urokinase-type PA (uPA) are serine proteases that convert plasminogen into the active proteolytic enzyme plasmin and studies demonstrate that PGs, especially PGE2, regulates PA-mediated proteolysis [124].

2.3 Corpus luteum

The LH surge causes ovulation and rapidly initiates a program of terminal differentiation of the ovulated follicle into a transient endocrine gland, the corpus luteum (CL) through a process termed luteinization. This essential process of luteal development is, as described previously, marked by the remodeling of extracellular matrix and by the differentiation and proliferation of cells derived from the

postovulatory follicle, such as granulosa, theca, and vascular endothelial cells [125]. In cattle, the CL rapidly develops within 2–3 days after ovulation, which is accompanied by active angiogenesis and vascularization from the preovulatory follicle [126].

2.3.1. Corpus luteum functions

The most important function of the CL is the production of progesterone, which is required for achievement and maintenance of pregnancy. In cattle, after CL formation, plasma progesterone concentrations progressively increase. Plasma progesterone concentrations peak between 10 and 14 days post-ovulation. The CL also produces many vasoactive factors such as nitric oxide [126], endothelin-1 [127], angiotensin II [128] and PGF2α [129]. In the cow, these factors are involved in the regulation of CL blood flow and progesterone secretion. If pregnancy does not occur successfully in this species, the CL is only functional for 17–18 days and it will regress in a process called luteolysis [126, 130].

2.3.2. Luteolysis

If there is no maternal recognition of pregnancy, PGF2α released from the endometrium of the nonpregnant cow induces luteolysis, characterized by hypoxic cell death resulting from hyalinization of blood vessels. In ruminants, it is well known that pulsatile PGF2α release from the uterus on days 17–18 of the estrous cycle is essential to induce regression of the CL [131]. Luteolytic PGF2α induces a drastic decrease in progesterone secretion from the CL as well as CL volume and blood flow in the non-pregnant cow [132, 133].

3. Nitric oxide system

For many years, nitric oxide (NO) was considered an atmospheric pollutant, formed as a product of nitrogen burn from industrial and automobile exhaust fumes. By 1987, it was confirmed that this labile molecule could be synthesized within cells of live organisms [134, 135]. In the following years many studies were published indicating that NO was a mediator of a variety of biological functions. In 1992, NO was named **"Molecule of the Year"** by Daniel E. Koshland, Editor for Science. In 1998, the researchers who discovered NO as a signal molecule in the cardiovascular system, Robert F. Furchgott, Louis J. Ignarro and Ferid Murad, were awarded with **"The Nobel Prize in Physiology or Medicine"** [136]. The signal transmission by a gas that could be produced with in a cell, penetrate through membranes and regulate the function of another cell, represented a new principle for signalling in biological systems.

3.1. Nitric oxide biosynthesis

Within the cell, NO is generated as a co-product of the enzymatic reaction that converts the amino acid L-arginine into the amino acid L-citrulline. The enzymes responsible for this reaction are called nitric oxide synthase (NOS). The amount of NO produced by each cell type depends not only on which NOS is present, but also the intensity of its activity in response to different stimulis. The proper amount of substrates and co-factors are also critical for NO synthesis. Availability of L-arginine is essential for NO generation because it is known that L-arginine is the only physiological nitrogen donor for NOS-catalyzed reactions [137-140].

3.1.1 Nitric oxide synthases

The different NOS enzymes are named according to the tissues from which the original cDNA and protein were isolated. The neuronal (nNOS) and endothelial (eNOS) enzymes, were first found in brain and vascular endothelial cells, respectively [141]. The inducible enzyme (iNOS) was first detected in macrophages, and expressed in response to inflammatory cytokines and lipopolysaccharides [137]. Some authors also consider a mitochondrial (mNOS) enzyme [142, 143], however, this is controversial and it is believed that the mNOS could be another NOS translocated to the mitochondria [144]. In this thesis, only information related to the neuronal, inducible and endothelial NOS will be reviewed. The current gene symbols for nNOS, iNOS and eNOS are NOS1, NOS2 and NOS3, respectively [145].

Molecular cloning has shown that different bovine NOS share around 60% homology in this species [146]. The mammalian NOS proteins show a very high level of conservation [146]. The first NOS to be purified and cloned was the rat NOS1, which is constitutively expressed at high levels in the brain [147]. The bovine NOS1 gene located in the chromossome 17 and consists of 25 exons and 24 introns. The transcript presents a length of 3975 bps and a protein of 1325 residues (Ensembl: ENSBTAG00000002023). In cattle, NOS1 can be detected in heart, kidney, intestin, spleen, brain, liver, uterus and testis [148].

The inducible NOS, NOS2, was first isolated from activated murine macrophages and characterized by a subunit of molecular mass of approximately 130-135 kDa [149, 150]. As the name suggests, the NOS2 is not generally expressed in unstimulated cells, although exceptions to this rule of course exist. Bovine NOS2 shows a high degree of similarity to NOS2 from other species, and also shares a common protein domain structure. In cattle, a 3471 bp transcript and a protein of 1156 amino acids have been identified [146]. The bovine NOS2 gene is located in chromosome 19 and contains 26 exons and 25 introns (Ensembl: ENSBTAT00000009062.5). This

enzyme is induced in a wide range of cell types and tissues. In bovine tissues, NOS2 can be detected in heart, kidney, intestin, spleen, brain, liver, uterus and testis [148].

The endothelial NOS gene, NOS3, is highly conserved among species [158] and is a membrane-bound protein of 135 kDa synthesized by endothelial cells and other cell types. In bovine tissues, NOS3 can be detected in heart, kidney, intestine, spleen, brain, liver, uterus and testis [148]. The NOS3 gene plus 2.9 kilobases of 5'-flanking sequence has been isolated and characterized in cattle. The gene (Ensembl: ENSBTAG00000017680) is located in chromosome 4 and spans 20 kilobases and contains 26 exons and 25 introns.

3.1.1.1 Transcriptional regulation of NOS

Analysis of the bovine NOS2 promoter, sequenced from a Holstein animal (GenBank: AF333248), identified a TATA box 30 bp upstream of the bovine transcription start and binding sites for the several transcription factors [151], including AP-1, IRF-1, Ets-1 and NF-kB [152, 153]. Increased expression of NOS2 can be correlated with a number of pathological situations and several studies indicate that NOS2 expression can be induced by immunostimulatory cytokines, oxidative stress and bacterial products [154]. On the other hand, there are many reports that show expression of NOS2 during normal physiology in response to signals that are noninflammatory or nonimmunologic. The induction of NOS2 in cattle has been shown following stimulation of cells with viruses, bacteria, LPS and cytokines [155-157], but also induced by hormones and different factors.

The 5'-flanking region of NOS3 lacks a typical TATA box but contains numerous putative transcription factor binding sites. These include consensus sequences for an AP-1 site, an NF-1 site, a tumor necrosis factor responsive element, two sterol regulatory elements, 3 acute-phase response element, two sterol regulatory elements, 3 acute-phase response elements, 6 GATA motifs, 16 CACCC boxes, 5 Sp1 sites, 15

estrogen half-palindromic motifs, and 9 fluid shear stress-responsive elements [158]. Although NOS3 is considered a constitutively expressed gene, it is known that NOS3 may be transcriptionally regulated [159].

3.1.1.2 NOS protein structure

NOS proteins possess a bi-domain structure, consisting of two identical monomers, which are functionally divided into two major domains: a C-terminal reductase domain and an N-terminal oxygenase domain [160]. The reductase domain has binding sites for calmodulin, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The oxygenase domain has binding sites for the cofactors heme and tetrahydrobiopterin (BH4 or H4B) and the substrate L-arginine [161, 162] (Figure 7).

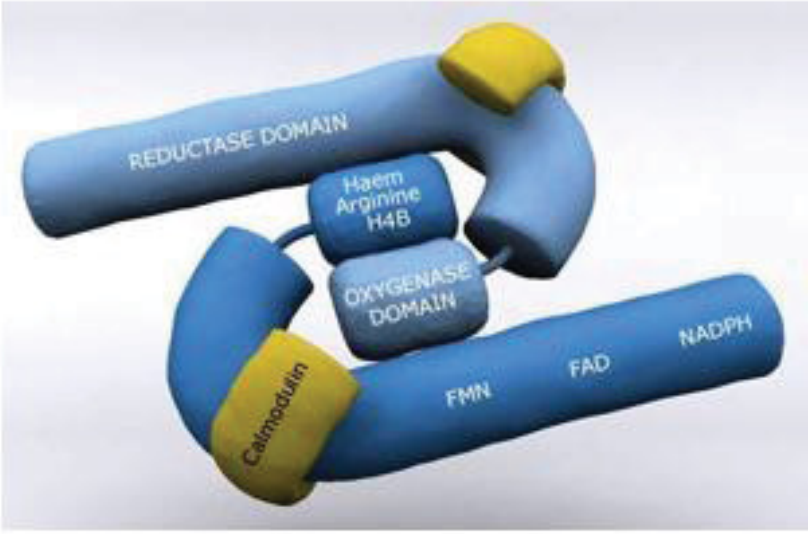

Figure 7. The general structure of the NOS enzymes (http://www.reading.ac.uk/nitricoxide/intro/no/synthesis.htm)

3.1.2 Enzymatic reaction

As mentioned previously, each NOS functions as a dimer consisting of two identical monomers. The heme is critical to the enzymatic reaction because it participates in dimerization, as NOS exists as monomers in its absence. Monomers of all the enzymes are unable to bind to BH4 or L-arginine [150, 163]. Each enzyme acts as a dimeric protein in catalysing the NADPH-dependant electron oxidation of L-arginine. Briefly, the reductase domain transfers electrons from NADPH along the flavins and calmodulin to the catalytic heme centre in the N-terminal portion of the protein [161]. L-arginine is then hydroxylated by NOS to form N-hydroxy-L-arginine (NHA) as an intermediate, which is subsequently oxidized to yield L-citrulline in addition to NO (Figure 8), in a 1:1 stoichiometry [160].

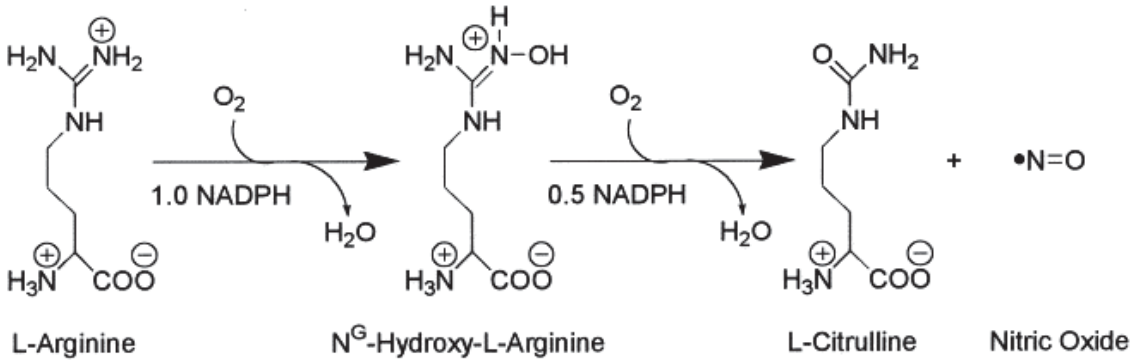

Figure 8. Nitric oxide synthesis from L-arginine (Taken from [164]).

3.1.3 NOS activation

An important molecule related to NOS activation is calcium. Increase in intracellular calcium triggers a cascade of events leading to NOS activation and NO synthesis. Intracellular calcium binds to calmodulin to form a calcium–calmodulin

complex and to regulate the binding of calmodulin to the 'latch domain', which permits electron transfer from NADPH via flavin groups within the reductase domain to a heme-containing active site, thereby facilitating the conversion of oxygen and L-arginine to L-citrulline and NO [165, 166].

The NOS1 and NOS3 enzymes are functionally similar and neither contain bound calmodulin. In the presence of calcium, however, when the high affinity association between calcium and calmodulin refered to above occurs, it results in the activation of the enzyme. For this reason NOS1 and NOS3 are commonly classified as calcium/calmodulin-dependent [162, 167], although NOS3 can also be activated in a calcium-independent manner [168]. These two NOS catalyse NO production within seconds in response to diverse stimuli and produce small quantities (at nM) of NO [150, 169]. On the other hand, NOS2 contains calmodulin so tightly bound that it is considered to be a subunit rather than a cofactor [170]. This synthase has the shortest sequence and binds calmodulin at all physiological concentrations of calcium and unlike the other two enzymes it is not regulated by calcium, therefore NOS2 activity is regarded as calcium-independent [141, 171]. There are only a few intracellular mechanisms that regulate NOS2 activity, which is generally considered to be at the transcriptional level. The NOS2 protein levels can be acutely induced [154] and this enzyme is characterised by release of large quantities (at μM) of NO even hours after exposure to inducing agents [172, 173].

Apart from calcium, several other factors can regulate NOS activity, especially NOS3 activation. NOS3 can be activated by certain stimuli without a sustained increase in calcium being necessary [169]. At the post-translational level, NOS3 activity is highly regulated by substrate and cofactor availability as well as endogenous inhibitors, lipid modification, direct protein-protein interactions, phosphorylation, O-linked glycosylation, and S-nitrosylation. The NOS3 signalosome is perhaps the best characterized of the three NOS isoforms since it has been clear for a few years that the association with calmodulin and caveolin has profound effects on the intracellular localization and activity of NOS3 [162, 169, 174]). This enzyme can be phosphorylated

on serine, threonine, and tyrosine residues, with numerous putative phosphorylation sites (Figure 9).

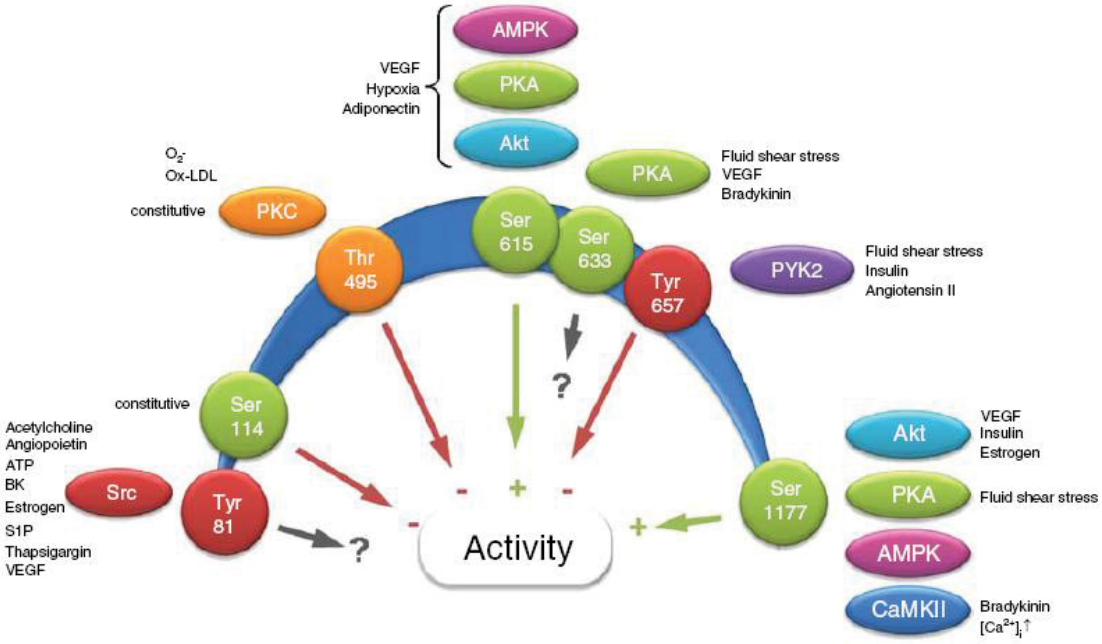

Figure 9. The regulation of NOS3 by phosphorylation. Schematic depiction of confirmed NOS3 phosphorylation sites, and their influence on enzyme activity (*green arrows* activation, *red arrows* inhibition, *black arrow* no direct effect on enzyme activity). The numbers refer to the human sequence (Taken from [169].

3.2 Nitric oxide chemistry

Nitric oxide is a simple, diatomic and non polar molecule. It is a colourless gas at room temperature and pressure. This inorganic free radical is also endogenously produced as a gas with a very short half-life from milliseconds to few minutes [141, 175]. Although NO has a very short half-life, due to its high solubility, NO can freely diffuse through biological membranes. Its chemistry and redox state nevertheless facilitate its interaction with various biomolecules to regulate different intracellular and

intercellular events. In this section a few illustrative examples of the reactivity of NO will be presented.

3.2.1 NO redox species and its interactions

Nitric oxide either diffuses directly to its target or it is converted to different derivates. One of the most unique and important chemical features of NO is that it is a paramagnetic species. Unlike other carbon, oxygen or nitrogen-centred radicals, NO does not have the tendency to dimerize at standard temperature and pressure, so it is capable of forming high-affinity-nitroso complexes with a variety of metal complexes [176]. In a general view, NO can be converted to a variety of nitrogen oxide species (NOx); to an organonitrosyl (E-NO) compound, where E is a sulfur-, nitrogen-, or carbon-containing moiety or to a metal-nitrosyl (M-NO) complex [175]. Some of these species are better suited for delivery of NO and others for longer-term storage.

From a biological point of view, some important reactions of NO are those with oxygen in its various redox forms. Nitric oxide gas reacts with O_2 to form nitric dioxide gas (NO_2), which dimerizes to dinitrogen tetroxide (N_2O_4). The N_2O_4 dismutates spontaneously in water and buffers at pH 7.4 to yield the stable end products nitrite (NO_2^-) and nitrate (NO_3^-). Estimation of NO_2^-, NO_3^- in aqueous biological samples is used to provide indirect means of estimating endogenous NO production [141, 177, 178]. Other important nitrogen oxide is peroxynitrite (ONOO-), that is formed in vivo by the diffusion-limited reaction between NO and superoxide [179]. This anion is highly oxidizing and can even effect tyrosine nitration, resulting in a variety of pathophysiological effects ranging from inflammation to cancer [180].

3.3 Nitric oxide biology

The NO synthesized within the cells freely diffuses through the membranes and acts as an intracellular and extracellular biological messenger, interacting with a variety of biomolecules such as enzymes, cytokines, membrane receptors, transcription factors and DNA to modulate several physiological and pathological processes in mammals and other living organisms [181]. The roles exerted by NO, however, may vary according to its concentration, when and where it is produced, and whether NO acts directly or via some of its redox species as cited above [175, 182].The effects and the mechanism of action of NO are strictly dependent on its concentration as well as on the presence of metals, proteins and low-molecular-weight thiols in a given cell. For this reason NO may exert dual effects on the same process in the same cell.

3.3.1 Mechanisms of action of NO

Nitric oxide has the capacity to modulate the activity of proteins through reversible reactions with available functional groups, notably with iron and thiols [175, 176]. NO can directly react with heme proteins such as cytochrome P450 [183-185], cyclooxygenase [186, 187] and guanylyl cyclase [182, 188]. This last one was one of the first targets identified for NO in biology. Soluble guanylyl cyclase (sGC) is a heterodimeric enzyme consisting of α- and β- subunits and a prosthetic heme group with ferrous iron [189]. It has been proposed that unique binding interactions of NO with the heme iron in guanylate cyclase allows the liberation of the transaxial ligand, histidine, which leads to enzyme activation that catalyzes the conversion of GTP into guanosine 3’5’-monophosphate (cGMP)[188]. The result is an increase in cGMP that represents an important intracellular second messenger that mediates many key biological actions of NO. The cGMP exerts its physiological actions through cGMP-dependent protein kinase (PKG), cGMP-regulated phosphodiesterases (PDE2, PDE3) and cGMP-gated cation channels, among which PKG might be the primary mediator. Importantly, the cGMP

signal is compartmentalized within a cell so that specific targeted proteins can be regulated by the same "generic" cGMP to exert differential physiological effects.

Although initially the physiological effects of NO were thought to be predominantly related to the activation of guanylate cyclase and thus to enhanced production of cGMP, the influence of NO in normal cellular function as well as in a wide range of pathophysiological conditions has been linked to S-nitrosylation [190]. In this process, NO modifies protein functions via covalent attachment of a NO group to reduced thiol (Cys) groups of free amino acids, peptides and proteins to form a S-nitrosothiol or S-nitrosoprotein [191]. S-nitrosylation seems to be one of the principal post-translational protein modifications that play a role in cell signaling, including phosphorylation, acetylation and ubiquintylation [192].

3.3.2 Roles of nitric oxide in general physiology

The NOS enzymes have been found in a variety of cell types, including neurons, gastric and bronchial epithelium, skeletal muscle, macrophages, cardiomyocytes, hepatocytes, chondrocytes and many others tissues and cells types of living organisms. The production of NO is, therefore, almost ubiquitous and NO seems to be associated with a wide range of functions. The known roles of NO in biology continue to grow and now range from neuroprotection and the immune response to protein regulation and chemotherapeutic resistance [193-195], vasodilation, maintaining the endothelial cell barrier, inhibition of platelet aggregation and neutrophil adhesion to endothelial cells [196], reduction of smooth-muscle cell proliferation and migration [197] and the control of apoptosis [198].

3.3.3 Roles of nitric oxide in general physiopathology

NO exerts important roles in carcinogenesis and tumor progression. It has been demonstrated that NO may be both pro- and anti-tumorigenic, depending on the concentration and the tumor microenvironment in question [199]. This free radical is involved in immune system responses and pathogenesis of several disorders. NO may act as a critical agent of host defense, but also as a central mediator of different pathogenesis. As mentioned previously some of the NO actions must depend on what sort of cell, which NOS is involved and NO interactions. High levels of NO inhibit a wide array of microorganisms, but can also potentially damage the host, contributing to the pathology [200]. Generally, the NOS normally associated with many pathologies is the inducible enzyme, NOS2. The production of NO by NOS2 is higher than that from other NOS enzymes, so concentration can easily increase up to cytotoxic levels. The antimicrobial effect may be also consequence of the formation of reactive nitrogen intermediates. Nitric oxide can react with other radicals, resulting in the formation of peroxynitrite, a potent oxidant effective in inducing cytotoxicity. These molecules induce oxidative and nitrative stress to kill intracellular microorganisms as part of the innate immune response [201].

As mentioned in a previous section, NO may modulate, at different levels, the activity of cyclooxygenases, precursor enzymes of PGs synthesis [186, 202]. Although the basal release of NO and PGs exerts a protective role in many pathophysiological conditions, NO and PGs released simultaneously in large amounts may be detrimental for cell survival. The overt production of NO and PGs is now known to occur in tissues affected by the inflammatory processes of rheumatic diseases, chronic degenerative disorders, central neurodegenerative processes associated with brain ischemia, as well as in neuroinflammatory diseases [187].

3.4 Nitric oxide functions in the ovary

NO has been also implicated in the regulation of mammalian reproduction. Specifically in the female, it has been demonstrated that NO is generated by ovarian cells and within the ovarian vasculature. The involvement of NO in the modulation of ovarian function is documented by several studies which indicate that NO exerts critical roles in the regulation of steroidogenesis, follicle development and survival, ovulation, oocyte maturation, cumulus expansion and corpus luteum function and lifespan [7, 8, 203].

3.4.1 Nitric oxide and steroidogenesis

The involvement of NO in steroid secretion by the ovaries has been evaluated in many studies through the use of exogenous NO donors and/or NOS inhibitors. Several studies indicate a negative correlation between NO and steroidogenesis. The negative effect on steroid production by NO has been demonstrated in different species and in different conditions. NO appears to inhibit steroidogenesis in human granulosa–luteal cells [204], rat granulosa cells [205] and porcine granulosa cells [206, 207]. The action of NO is, in part, attributable to the down-regulation of CYP19 gene transcription, but NO may also directly inhibit aromatase activity probably by binding to the CYP19 heme portion and then altering the enzyme activity [208, 209]. In addition, some authors suggest that the negative effect of NO on both basal and gonadotropin stimulated estradiol production may be, at least in part, exerted through an inhibition of androstenedione secretion [210]. Another mechanism by which NO may regulate steroidogenesis in granulosa cells is through cGMP. The cGMP pathway was indicated as one of the mechanisms used by NO to inhibit steroidogenesis in cultured granulosa cells from mice [205]. It has been suggested that cGMP increases phosphodiesterase 2 (PDE2) activity which in turn increases cAMP hydrolysis, the FSH intracellular second messenger. In swine, while some studies indicate that the effect of NO on steroidogenesis seem to be induced, at least in part, via a cGMP-dependent pathway

[207], others suggest it is not [211]. Studies with bovine granulosa cells also show that NO donors reduce estradiol and progesterone secretion, but the use of cGMP analogs did not efficiently inhibit steroidogenesis [212]. Interestingly, a more recent study with bovine granulosa cells indicates that high concentrations of the nitric oxide donor sodium nitroprusside (SNP) inhibited both progesterone and estradiol synthesis by cGMP-independent pathways, while low SNP concentrations stimulated E2 synthesis through the activation of soluble nitric oxide-sensitive guanylyl cyclase [213].

Some reports demonstrate the existence of a positive correlation between NO and steroid secretion. Follicular NOx (NO metabolites: nitrite plus nitrate) levels and estradiol concentration increased concomitantly in porcine ovarian follicles [214]. A similar relationship between NOx and estradiol concentrations is found in human ovarian follicles [215]. The increase of nitrite and nitrate levels in the serum of postmenopausal women subjected to E2 replacement therapy substantiates the positive effect of estrogens on NO production [216]. Estradiol has been reported to induce NOS3 in cultured endothelial cells [217] and it has been shown that estrogen induced vasodilation and increased blood flow is mediated via NO generation [218]. Additionally, ovarian perfusion with a NOS inhibitor in rats causes decreased E2 synthesis, suggesting that appropriate NO concentrations may positively regulate E2 synthesis [219].

3.4.2 Nitric oxide production and follicle development and growth

An involvement of NO production in follicle growth has been indicated in some species. In women, NO levels change during follicular growth and a positive correlation between follicular NOx concentrations and follicular size was reported [215, 216]. NO seems to be necessary for follicle development in mice too. Reduced growth rate and persistent basement membranes were associated with in vitro disruption of NO [220]. Some authors suggest that NO may influence follicle development by mediating the effects of gonadotropins on the blood-follicle barrier, thus influencing its permeability to

different substances [221], but other evidence indicates that NO acts at a molecular level to regulate follicle development and growth. A growth promoting effect of NO is supported by the observation that NO increased, while NOS blockers reduced the expression of EGF receptors in rat granulosa cells [222]. However, treatment of bovine granulosa cells from different size follicles with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) did not influence granulosa cell proliferation [223].

3.4.3 Nitric oxide and apoptosis in granulosa cells

A further mechanism through which NO may be involved in the control of follicular development is its effects on apoptosis [224]. Although NO exerts pro-apoptotic properties in many cell types [198, 225], a protective effect of NO has been observed in rat granulosa cells from immature [226, 227] and preovulatory [228] follicles. It is reported that NO inhibits FasL-induced apoptosis in rat granulosa cells by suppressing the activation of caspases [229]. Moreover, some reports show that NO donors decreased the expression of genes as Bax, a pro-apoptotic member of the Bcl-2 protein family [228]. In human granulosa cells, different data have been reported and do not clearly define NO involvement in the regulation of apoptosis [230]. On the other hand, high NO levels have been shown to reduce apoptosis in bovine granulosa cells [223]. In this same species, the addition of SNAP also decreases significantly the number of apoptotic nuclei in blastocysts [231]. This confirms that NO may exert a cell protective function, as suggested in a study with bovine embryos [232].

3.4.4 Nitric oxide and the ovulatory process

Studies performed with different species and using different approaches, provide strong evidence that NO also participates in the ovulatory process. Although one study in rats suggested that a preovulatory decrease in NO concentrations is a prerequisite for the ovarian response to LH and successful ovulation [233], most of the reports indicate

that NO is an essential mediator of ovulation. In rats, the administration of NOS inhibitors, intraperitoneally or via the ovarian bursa, inhibits ovulation, an effect reversed by a NO donor [234]. Similar results have been reported in hCG-treated rabbits [235, 236]. Studies conducted on NOS knockout mouse models provide further support for the role of NO in ovulation. NOS3 deficiency in the mouse has been shown to be associated with a significant reduction in ovulatory potential after hCG treatment [237-239]

It has been suggested that NO might contribute to follicle rupture by increasing intrafollicular pressure [240], either by increasing the vascular flow and the transudation of fluid to the follicular antrum or by stimulating the contractile elements of the ovarian follicle. But the most relevant mechanism by which NO stimulates the ovulatory process probably involves the production of prostaglandins, which contribute to enhancing the inflammatory process in the periovulatory period. In different cell types and tissues, it has been shown that NO regulates PTGS2 at the level of transcription, translation and enzyme activity [187]. PTGS2 is activated by NO at lower concentrations via binding to the heme moiety and so augmenting enzymatic activity [186, 241], whereas higher concentrations of NO may inhibit both synthesis and activity of PTGS2 [242]. A crosstalk between NO and PG biosynthetic pathway has been reported in the ovary. Blocking intraovarian NO production by NOS inhibitors diminished the production of PGE2 and PGF2α in response to hCG injections, while intrabursal injection of NO donor stimulated prostaglandin synthesis in rabbits [235] and rats [243]. A stimulatory effect of a NO donor on PGF2α production by large bovine follicles has been also reported [244].

3.4.5 Nitric oxide and oocyte maturation

Nitric oxide synthesis seems to be also important for oocyte maturation. NOS3 knockout mice exhibited a reduced number of oocytes in metaphase II of meiosis; a high percentage of oocytes remained in metaphase I or were atypical compared to controls

[237]. The effects of NO on oocyte maturation have also been evaluated by the use of NO donors. However, the effect of NO generated by an exogenous donor on mouse oocyte maturation can be stimulatory or inhibitory depending on its concentration. NO inhibited [237] or stimulated [245-249] nuclear maturation in mice. Besides NO donors, several drugs that inhibit NO synthesis are being used in studies on ovarian physiology, and they show that manipulating amounts of NO during in vitro culture also affects oocyte maturation in cattle [250]. One of these studies indicated that the accumulation of cGMP was probably not responsible for the effects of NO on meiosis [251]. NO affects the in vitro maturation of bovine COC, modulating the viability of cumulus cells and of oocyte, the progression of meiosis after GVBD, the migration of cortical granules, and cleavage and blastocyst development [252]. Another study with bovine ovaries suggests that a defective NOS3/NO system is related to a reduced follicle vasculature and may affect oocyte quality, thus inducing a premature decline of fertility [231].

3.4.6 Nitric oxide and corpus luteum formation and luteolysis

Nitric oxide is also involved in the regulation of corpus luteum formation, function and lifespan. Different studies indicate, however, that action of NO may depend on the stage of CL development. In rats, a positive effect of NO has been suggested in the midstage CL. NO stimulated both glutathione, a major antioxidant, and progesterone production, thus favoring the maintenance of the CL [253]. It has been suggested that NO is also possibly involved in the control of luteal vascularization [254]. Together with PGE, NO seems to act through its effects on vasculature and proteolytic processes [255]. Other findings indicate that NOS2 mediated NO secretion stimulated PGE synthesis, which is effective in increasing progesterone production [256]. Prostaglandin E has been demonstrated to enhance basal progesterone secretion also in newly formed CL from pseudopregnant rabbits [257]. In mares, it is suggested that NO may play a role in CL growth during early luteal development, when vascular development is more intense [258].

On the other hand, NO has been suggested to be an important mediator of luteolysis in several species. NO produced by the rat ovaries during the last 2 days of CL development increased PGF2α production in the ovary and diminished serum progesterone concentrations leading to CL involution [253, 259]. In cattle, NO stimulated the synthesis of PGF2α, which in turn increased NOS activity, thus activating a positive feedback mechanism between PGF2α and NO to ensure luteal regression and, consequently, progesterone production decrease [260, 261]. The same has been suggested in humans [262] and rabbits [257].

3.5 NOS expression in the ovary

The expression and activity of NOS vary in the ovary and may depend on stimulus, cell type and animal species involved [7]. Although NOS3 was initially, with NOS1, considered as constitutively expressed, it is has been established that their expression can be regulated at the transcriptional level under various conditions [263]. In contrast, the expression of NOS2 is considered inducible, mainly in response to immune stimulus, as inflammatory cytokines [137]. But other non-inflamatory stimulus may influence on NOS2 expression, as it will be described in this section.

3.5.1 Nitric oxide synthases identified in the ovary

In most studies in NOS expression in reproductive tissues, NOS2 and NOS3 are the enzymes commonly detected in follicles, while NOS1 is normally poorly or not detected. In rats, NOS3 was detected in oocytes, granulosa cells of immature and gonadotropin-stimulated ovaries [264] and in blood vessels [265]. NOS2 was also detected in rat granulosa cells [226, 265] and a study demonstrated that both NOS2 and NOS3 are expressed in rat stroma, thecal and luteal cells [266]. In mice, both NOS3 and NOS2 were localized in oocyte, theca and granulosa cells [220, 248, 267]. In humans, NOS3 expression was reported in granulosa-luteal cells [204]. In porcine follicles,

NOS3 was localized in oocytes, cumulus cells, and granulosa cells and NOS2 was mainly localized in the oocytes, granulosa and theca cells [268-270].

In cattle, the situation is not clear. While some studies demonstrated that NOS3 protein was not detected in granulosa cells of growing follicles from cattle [271], and mRNA encoding NOS2 was not detected in cultured bovine granulosa cells [272], other studies were published indicating that NOS were identified in ovarian cells [231, 273, 274]. According to these authors, both NOS3 and NOS2 were detected in oocytes, granulosa and theca cells and other compartments of the ovary, including corpus luteum, corpus albicans, surface epithelium and stroma.

3.5.2 NOS expression during follicle development

The expression of NOS2 and NOS3 is regulated by gonadotropins and other factors in the ovarian cells of different species, suggesting that both participate in the ovarian functions. In immature rats, follicular development induced by pregnant mare's serum gonadotropin (PMSG) is associated with an increase in NOS3, while NOS2 expression remains relatively constant [264, 265]. On the other hand, some authors observed a decrease in NOS2 mRNA levels induced by PMSG in granulosa cells from immature rat follicles and suggest that NO may act as a cytostatic factor [226]. In cultured rat granulosa cells, FSH does not induce NOS2 mRNA, but interleukins induce activation of NOS2 [226, 275].

In porcine granulosa cells of antral follicles, the endothelial NOS was expressed only in the presence of FSH [207, 269]. Studies of pig oocytes from small follicles (1–3 mm) showed that amounts of NOS3 protein were constant after culture [268], while amounts of NOS2 protein decreased [214], suggesting a differential gene expression during follicular development. In sheep, it was reported that NOS3 mRNA and protein expression changes throughout follicular growth and atresia, and the pattern for NOS3 protein expression follows the pattern of vascular development during folliculogenesis

[276]. In cows, NOS3 protein was detected in granulosa cells during all the stages of folliculogenesis [274].

3.5.3 NOS expression during peri-ovulatory period

In rats, stimulation with hCG induces an increase of both NOS2 and NOS3 [264, 266]. In pigs, low levels of NOS3 were detected in oocytes and granulosa cells at the early stage of follicle development in comparison to NOS3 levels in both cell types in preovulatory follicles [277]. In cattle, NOS3 and NOS2 transcripts were detected in oocytes, however, relative abundance of these transcripts decreased after in vitro maturation [274]. Other studies made similar observations and suggested that the reduction in NOS3 transcript could be involved in the reduction in NO necessary for germinal vesicle breakdown [273, 278].

3.5.4 NOS expression during CL formation and luteolysis

The expression as well activity of NOS enzymes may change from CL formation up to its regression. In rats, NOS2 expression decreased with CL aging [279]. NOS2 expression also declined with CL aging in the rabbit [280, 281] and sheep [254]. In the human CL, NOS3 is the most abundant NOS with highest values during the late luteal phase, but immunoreactive NOS2 did not show well defined phase-specific changes [262]. In bovine CL, it was possible to detect NOS2 and NOS3 in endothelial and luteal cells. The levels of NOS3 and NOS2 were increased from the early to late luteal phase of the estrous cycle and then decreased in regressed luteal phase in the cow [282].

Chapter 2:

Hypotheses and objectives

Hypotheses and objectives

The pattern of NOS enzyme expression in the follicle of ruminants is not clear. In cattle, the expression of NOS genes in granulosa cells is controversial, but it has been demonstrated that FSH stimulates NO production in cultured bovine granulosa cells and that the inhibition of endogenous NOS activity decreased bovine cumulus cell viability and affected oocyte maturation. These findings indicate the existence of endogenous NOS activity in the granulosa layer of this species.

Studies indicate that nitric oxide may present anti-apoptotic properties. In rodents, nitric oxide suppressed follicle apoptosis, decreasing both mRNA and protein levels of pro-apoptotic genes. Considering that during follicle growth in cattle, one follicle is selected for further growth and the other follicles in the cohort regress and undergo atresia through apoptosis our first hypothesis is that NOS are expressed in bovine granulosa cells under regulation by gonadotrophic hormones, and its activity may be related to granulosa cell survival.

Nitric oxide has been also correlated with ovulation. In studies performed with different polyovulatory species, inhibition of NO production blocks induced ovulation. It is suggested that the most relevant mechanism by which NO stimulates the ovulatory process probably involves the production of prostaglandins. Therefore, our second hypothesis is that NOS expression and activity is critical for expression of genes essential for the ovulatory cascade in bovine granulosa cells.

To test the hypotheses mentioned above we established the following objectives for the present thesis:

1. To elucidate some of the mechanisms controlling NOS expression and the consequences of nitric oxide production for granulosa cell function during follicular development in bovine.

2. To determine the regulation of NOS expression during the LH-induced ovulatory cascade in bovine granulosa cells, and to assess whether NOS activity is critical for the expression of genes involved in the ovulatory cascade in this species.

Chapter 3:

Articles

Article 1.

Regulation of inducible nitric oxide synthase expression in bovine ovarian granulosa cells.

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Article published in **Molecular and Cellular Endocrinology 335 (2011) 189-194**

Abstract

Nitric oxide (NO) is a potential regulator of ovarian follicle growth, and ovarian granulosa cells reportedly generate NO in response to gonadotrophins, suggesting that the regulated form of nitric oxide synthase (iNOS) is present. The objectives of the present study were to gain insight into the expression and role of iNOS in the follicle. Messenger RNA encoding iNOS was detected in granulosa cells, and abundance was higher in growing dominant follicles compared to subordinate follicles ($P<0.01$). FSH ($P<0.05$) and IGF1 ($P<0.01$) stimulated oestradiol secretion and iNOS mRNA abundance in granulosa cells in vitro, whereas FGF2 ($P<0.05$) and EGF ($P<0.01$) decreased oestradiol secretion and iNOS expression. The addition of an anti-oestrogen prevented FSH-induced iNOS mRNA accumulation. Inhibition of endogenous NO production did not affect steroidogenesis in granulosa cells, but increased FasL mRNA abundance, caspase-3 activation and the incidence of apoptotic cell death ($P<0.05$). These results demonstrate that iNOS is expressed in ruminant granulosa cells and is regulated by gonadotrophins and oestradiol. Physiological levels of NO may contribute to the survival of granulosa cells.

Key words: ovary, nitric oxide, follicle, steroidogenesis, apoptosis

Introduction

In monovulatory species, follicle growth occurs in waves, and a cohort of follicles arises in each wave under the influence of a transient rise in circulating FSH concentrations. As FSH concentrations decline, only one follicle will be 'selected' for continued growth; all the others will become atretic. The continued growth of the dominant follicle has been linked to increased IGF1 bioavailability and oestradiol production (Beg et al., 2002; Fortune et al., 2004). A variety of paracrine signalling molecules has also been implicated in the regulation of follicle growth (Buratini & Price, 2011; Webb et al, 2007), and there is a growing awareness of potential roles for intracrine effectors such as nitric oxide.

Nitric oxide (NO) is a free radical gas produced by the conversion of L-arginine to L-citrulline by a family of nitric oxide synthases (NOS). Nitric oxide is generated in most organ systems, including the major reproductive tissues (Rosselli et al., 1998). The predominant NOS enzymes present in reproductive tissues are endothelial (eNOS, also known as NOS3) and inducible NOS (iNOS, also known as NOS2); the expression of eNOS is commonly constitutive whereas that of iNOS is considered to be regulated. The importance of NO in fertility has been demonstrated with studies of knockout mice, in which loss of either eNOS or iNOS disrupted oestrous cyclicity (Jablonka-Shariff et al., 1999). The viability of double eNOS/iNOS knockout mice is severely compromised (Tranguch and Huet-Hudson, 2003).

A number of studies suggest that NO affects ovarian function. High concentrations of NO in follicular fluid have been associated with reduced oestradiol secretion and

lower oocyte quality in women undergoing in vitro fertilization (Lee et al., 2004; Vignini et al., 2008). Similarly, the addition of NO donors to follicular granulosa cells in vitro inhibited steroidogenesis in humans, pigs, rats and cattle (Basini et al., 1998; Masuda et al., 1997; Snyder et al., 1996; Van Voorhis et al., 1994). Some evidence also indicates that NO inhibits the expression of genes responsible for triggering the apoptotic cascade, at least in rats (Chen et al., 2005; Yoon et al., 2002).

The pattern of NOS enzyme expression has been described in the follicle in polyovulatory species. In rats, iNOS mRNA was located to granulosa cells of immature but not antral follicles (Matsumi et al., 1998; Van Voorhis et al., 1995), whereas eNOS mRNA was detected in granulosa cells of gonadotrophin-stimulated antral follicles (Jablonka-Shariff and Olson, 1997). Similarly in pigs, eNOS but not iNOS mRNA has been measured in granulosa cells from antral follicles (Grasselli et al., 2001; Takesue et al., 2001), although iNOS protein was detected in granulosa cells in one immunohistochemistry study (Kim et al., 2005) but not in another (Tao et al., 2004).

In contrast, the situation in monovulatory species is not clear. Endothelial NOS protein was not detected in granulosa cells of growing follicles from sheep and cattle (Grazul-Bilska et al., 2006; Grazul-Bilska et al., 2007), and mRNA encoding iNOS was not detected in cultured bovine granulosa cells (Herath et al., 2007). These data are puzzling as FSH stimulated NO production in cultured bovine granulosa cells (Basini and Tamanini, 2000), and inhibition of endogenous iNOS activity with aminoguanidine decreased bovine cumulus cell viability (Matta et al., 2009). These latter findings argue

for the existence of endogenous, inducible NOS activity in the granulosa layer of monovulatory species.

We hypothesise therefore that iNOS is expressed in bovine granulosa cells, and is under regulation by gonadotrophic hormones. The objectives of the present study were to determine the hormonal regulation of iNOS expression in granulosa cells in vitro, to investigate the physiological role of NO in granulosa cells in vitro by inhibiting endogenous iNOS activity, and to extend these studies to an in-vivo model of follicle growth and atresia.

Materials and methods

In vitro studies

To study the regulation of iNOS expression and role of nitric oxide on granulosa cell function, we cultured granulosa cells in serum-free medium as described (Gutiérrez et al., 1997) with slight modifications. Materials were obtained from Invitrogen Life Technologies (Burlington ON, Canada) unless otherwise stated. Briefly, bovine ovaries were collected from adult cows at abattoir, and were transported to the laboratory in

PBS containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). Follicles between 2 and 5 mm diameter were dissected and granulosa cells were collected by rinsing the follicle wall with Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12). The granulosa cells were washed twice by centrifugation at 980 *g* for 20 min each, and either frozen in Trizol for RNA extraction or suspended in DMEM/F12

containing Hepes (15 mM), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1 %; Sigma-Aldrich, Oakville ON, Canada), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1.1 mM), androstenedione (A4; 10^{-7} M at start of culture, and 10^{-6} M at each medium change) and insulin (10 ng/ml). The number of cells was counted with a haemocytometer and the viable cells were assessed by the dye exclusion method using

0.4 % Trypan Blue. Cells were seeded into 24-well tissue culture plates (Sarstedt Inc; Montréal QC, Canada) at a density of $1x10^6$ viable cells per well in 1 ml medium. Cultures were maintained at 37 °C in 5 % CO_2 in air for 6 days, with 700 μl medium being replaced every 2 days. Depending on experiment, cells were treated with insulin like growth factor 1 (IGF1; LongR3 analogue), fibroblast growth factor-2 (FGF2), ICI 182780, aminoguanidine (all from Sigma-Aldrich), epidermal growth factor (EGF; R&D Systems, Minneapolis, MN) and/or bovine FSH (lot AFP-5332B; Dr Parlow, NIDDK). All hormonal treatments were started on day 2 of culture. Medium samples were collected on day 6 and stored at −20 °C until steroid assay, and cells were collected in Trizol and stored at −80 °C until RNA extraction. All series of cultures were performed on at least three different pools of cells collected on different occasions.

In vivo study

To determine if iNOS expression differs between growing and regressing follicles in vivo, follicles were collected from cycling cows at defined stages of follicle growth. The experimental animals were obtained from a herd of Angus cattle on a farm in the State of Rio Grande do Sul, Brazil. The experimental procedures were approved by the Federal University of Santa Maria Animal Care and Use Committee. Ovulation and subsequent emergence of the first follicular wave of the cycle were induced by an injection of prostaglandin-F2α as described (Evans and Fortune, 1997). The animals were slaughtered at a local abattoir during the first follicle wave around the expected time of follicle deviation, and the ovaries were transported to the laboratory.

The two largest follicles from each pair of ovaries were dissected and their diameter was measured. Cows with a follicle greater than 10 mm were likely post-deviation and were not used. The follicular fluid was aspirated, centrifuged and frozen for steroid assay. The antral cavity was repeatedly flushed with saline solution and granulosa cells recovered by centrifugation at 1200 g for 1 min and pooled with the follicular fluid pellet. The samples were collected into Trizol and the total RNA was extracted immediately according to the manufacturer's protocol. A total of 12 follicles from six cows were used. The dominant follicle in each animal was identified by follicular fluid oestradiol concentration and evaluation of mRNA encoding cytochrome P450 aromatase (CYP19A).

Real-time RT-PCR

For both in vivo and in vitro samples, gene expression was assessed by relative real-time RT-PCR. Total RNA (1 µg for cultured samples and 0.2 µg for in vivo samples) was first treated with 1U DNase (Promega, Madison, WI USA) at 37 °C for 5 min to digest any contaminating DNA. The RNA was reverse transcribed in the presence of 1 mM oligo(dT) primer and 4 U Omniscript Rtase (Qiagen, Mississauga, ON Canada), 0.25 mM dideoxy-nucleotide triphosphate (dNTP) mix, and 19.33 U RNase Inhibitor (GE Healthcare, Baie-d'Urfé, QC Canada) in a volume of 20 µl at 37 °C for 1 h. The reaction was terminated by incubation at 93 °C for 3 min. Real-time PCR was conducted in an ABI Prism 7300 instrument in 25 µl reaction volume containing 12.5 µl of 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 9.5 µl of water, 1 µl of each sample cDNA and bovine-specific primers for amplifying histone H2AFZ (Portela et al., 2010), CYP19A (sense primer 2a, antisense 3b) (Hamel et al., 2005), iNOS (Tesfaye et al., 2006), and FasL (sense: 5'-AGCCAAAGGCATAC -3', antisense: 5'-TGCCTGTTAAATGA-3'). A common thermal cycling parameter (3 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C) was used to amplify each transcript. Identity of the iNOS amplicon was confirmed by sequencing; there was no significant homology with bovine eNOS or nNOS sequences. Melting curve analyses were routinely performed to verify product identity. Samples were run in duplicate and were expressed relative to H2AFZ as housekeeping gene. Data were normalised to a calibrator sample (a mix of cDNA

samples from cultured granulosa cells) using the ΔΔCt method with correction for amplification efficiency (Pfaffl, 2001).

Apoptosis assessment

Apoptosis in cultured cells was measured by flow cytometry and immunofluorescence for cleaved caspase-3. Flow cytometry was performed essentially as described (Blondin et al., 1996). After culture, cells were recovered by scraping the plate with a rubber spatula. The cells were washed 3 times in ice-cold PBS by centrifugation and resuspending the cells, then fixed overnight in 70 % ethanol before staining with propidium iodide (50 mg/mL in PBS with 0.1 % Triton X and 20 mg/mL RNase A). A minimum of 25,000 propidium iodide stained cells/sample were sorted on a FACSVantage SE (BD Biosciences, Oakville ON, Canada) and analysed with Cell Quest Pro software (BD Biosciences). The number of cells in the ‘‘sub-G1’’ peak was quantified and represented the number of apoptotic cells. Proportions of apoptotic cells were transformed to arcsines before statistical analysis.

For immunofluorescence, cultured cells were fixed in 4 % paraformaldehyde for 20 min, washed in 2 % Triton-X and and then in 0.05 % Tween, blocked in 5 % BSA, and incubated with cleaved caspase-3 antibody (1:150; Cell Signaling Technology, Danvers MA). After the primary antibody, the cells were washed in PBS and then incubated with Cy3-conjugated second antibody (Jackson ImmunoResearch, West Grove, PA) and counterstained with DAPI. Cells were examined under an Olympus

FV1000 laser-scanning confocal microscope with multi-argon laser light source. Digital images were captured and mean fluorescence intensity in each field for Cy3 and DAPI were quantified with ImageJ software (NIH). Results are expressed relative to DAPI.

Steroid assay

Oestradiol was measured in follicular fluid and conditioned medium in duplicate by RIA as described previously (Bélanger et al., 1990), without solvent extraction. Intra- and inter-assay coefficients of variation were 6% and 9%, respectively. The sensitivity of this assay was 10 pg/tube, equivalent to 0.3 ng/µg protein. Steroid concentrations in culture medium were corrected for cell number by expressing per unit mass of cell protein. Cells were lysed with 100 µl 1 N NaOH for 2 h and neutralised with 100 µl 1N HCl, and total cell protein was measured by the Bradford protein assay (Bio-Rad, Mississauga, ON, Canada).

Statistical analysis

The in-vivo data were analysed by ANOVA with follicle group as a main effect. For the in-vitro data, doses of hormones and growth factors were used as the main effects and culture replicate was included in the model as a random effect in the F-test. Data were transformed to logarithms when not normally distributed (Shapiro–Wilk test). Differences between means were tested with the Tukey–Kramer HSD test. All analyses were performed with JMP software (SAS Institute, Cary, NC). Data are presented as means ± SEM.

Results

iNOS mRNA abundance is under hormonal regulation

We first investigated the regulation of iNOS mRNA abundance in granulosa cells using a non-luteinising culture model. In this model, FSH and IGF1 stimulate cell proliferation and oestradiol secretion (Cao et al., 2006; Gutiérrez et al., 1997). Cells were cultured in the presence of graded doses of FSH or IGF1 for the last 4 days of culture. FSH stimulated oestradiol secretion and iNOS mRNA abundance in a dose-dependent manner with an approximate 4-fold increase in iNOS mRNA levels ($P<0.05$, Fig. 1A). Similar effects were noticed for IGF1 ($P<0.01$, Fig. 1B).

In a second series of cultures, cells were cultured in the presence of IGF1 (10 ng/ml) with or without EGF (10 ng/ml), or with FSH (1 ng/ml) with or without FGF2 (10 ng/ml). These growth factors were used at doses previously demonstrated to inhibit oestradiol secretion in this cell model (Cao et al., 2006), and they significantly decreased oestradiol secretion and iNOS mRNA levels in the present study ($P<0.05$, Fig. 1C, D).

Oestradiol regulation of iNOS mRNA levels

Increases in iNOS mRNA levels were generally accompanied by increased oestradiol secretion, therefore we determined if oestradiol had a direct effect on iNOS mRNA. First we cultured cells with graded doses of FSH with the aromatase substrate, A4, or with the non-aromatisable androgen, dihydrotestosterone (DHT; 1.0μM). In the

presence of A4, FSH stimulated oestradiol secretion and iNOS mRNA levels, but in the presence of DHT, FSH failed to stimulate oestradiol secretion (not shown) or iNOS mRNA (Fig 2A). We then cultured FSH- or IGF1-stimulated cells with the antioestrogen ICI 182,780 (10μM), which resulted in a marked decrease of iNOS mRNA abundance ($P<0.05$, Fig 2B). Conversely, culture with oestradiol (in the absence of FSH to minimise endogenous oestradiol secretion) significantly increased iNOS mRNA abundance (Fig 2C).

Endogenous iNOS activity inhibits apoptosis

To explore the role of NO, granulosa cells were cultured with IGF1 (10 ng/ml) with or without the selective iNOS inhibitor, aminoguanidine (1 mM). Addition of aminoguanidine had no consistent effect on steroid secretion (not shown) or CYP19 mRNA abundance, but increased the proportion of apoptotic cells, cleaved caspase-3 protein and abundance of mRNA encoding the pro-apoptotic factor Fas ligand ($P<0.05$, Fig 3). Aminoguanidine also increased the proportion of apoptotic cells cultured with FSH (not shown).

iNOS mRNA is regulated during establishment of the dominant follicle in vivo

The above studies in vitro suggest that iNOS mRNA levels are upregulated by IGF and that endogenous iNOS activity may contribute to granulosa cell survival. In cattle, the dominant follicle is characterised by a healthy layer of granulosa cells growing under the influence of IGF, therefore we determined if iNOS mRNA

abundance differs between growing and non-growing follicles using a well-defined in vivo model. There was no significant difference in mean diameters between dominant and non-dominant follicles of the first follicle wave ($P>0.05$, Fig. 4A), however the status of the dominant follicle was confirmed by higher oestradiol concentrations ($P<0.01$, Fig. 4B) and by higher levels of granulosa cell CYP19A mRNA ($P<0.05$, Fig. 4C) compared to the non-dominant follicle. Abundance of iNOS mRNA in granulosa cells was higher in dominant follicles than in subordinate follicles ($P<0.01$, Fig. 4D).

Discussion

The physiology of nitric oxide in the bovine follicle was, until now, an enigma: nitric oxide is generated by granulosa cells (Basini et al., 1998) and regulated by FSH (Basini and Tamanini, 2000), and yet neither eNOS nor iNOS had been reported in this cell type (Grazul-Bilska et al., 2007; Herath et al., 2007). A recent study identified both proteins in granulosa cells by immunohistochemistry, but also in theca, stroma, surface epithelium and in corpora lutea and albicantia (Pires et al., 2009). In the present report, we describe the expression of iNOS in bovine granulosa cells in vivo and after serum-free culture, and its regulation by FSH and IGF1. These data explain the ability of granulosa cells to generate NO, and offer insights into its physiological role.

This is the first study to describe the presence of iNOS mRNA in ruminant granulosa cells. In contrast, Herath and colleagues could not dectect iNOS mRNA by PCR (Herath et al., 2007). The difference between studies is difficult to explain, as both

employed a similar serum-free culture system, although the primers for PCR were different. In the present study, iNOS mRNA was consistently detected in granulosa cells over the course of numerous cultures, and was also detected in granulosa cells of growing first-wave follicles. These data are consistent with the generation of NO in cultured cells (Basini and Tamanini, 2000).

Abundance of mRNA encoding iNOS was regulated. FSH and IGF1 both increased iNOS mRNA abundance, and to our knowledge, no other similar studies have been reported. In rats, injection of eCG induced follicle development and NOS activity in whole-ovary extracts (Faletti et al., 1999), likely owing to an increase in thecal eNOS expression (Jablonka-Shariff and Olson, 1997). In the present study, EGF and FGF2 inhibited iNOS mRNA abundance in granulosa cells, which is consistent with the effect of EGF on iNOS in cultured rat granulosa cells (Matsumi et al., 2000). A common observation in these experiments was that treatments that increased oestradiol secretion (FSH, IGF1) also increased iNOS mRNA abundance, whereas those that decreased oestradiol secretion (FGF2, EGF) decreased iNOS mRNA levels.

To gain insight into the nature of the relationship between oestradiol and iNOS, we evaluated whether oestradiol directly alters iNOS mRNA levels. In the absence of an aromatisable substrate, FSH was unable to stimulate iNOS mRNA abundance, and blockade of oestradiol action caused marked down-regulation of iNOS mRNA levels in FSH- and IGF1-stimulated cells. Further, the addition of oestradiol directly stimulated iNOS mRNA accummulation. These data suggest that the effects of gonadotrophins and growth factors on iNOS mRNA are mediated at least in part by oestradiol. This is in

agreement with studies demonstrating regulation of eNOS/iNOS by oestradiol in uterine/oviductal tissue in humans, sheep and cattle (Guang et al., 2005; Lapointe et al., 2006; Zhang et al., 1999).

The function of NOS in the follicle remains to be clarified. In cultures of porcine (Masuda et al., 1997), bovine (Basini et al., 1998) and human (Van Voorhis et al., 1994) granulosa cells addition of NO donors inhibited steroidogenesis, leading to the general conclusion that NO is a negative regulator of follicle function. However, these data should be interpreted with caution, as NO donors have been shown to be cytotoxic (Faes et al., 2009; Viana et al., 2007). In the present study, we inhibited endogenous NO production with the selective iNOS inhibitor, aminoguanidine (Misko et al., 1993). We reasoned that reduced intracellular NO generation would increase follicle function, but aminoguanidine did not enhance oestradiol secretion or CYP19A mRNA levels, possibly because oestradiol secretion is already raised in these IGF1-stimulated granulosa cells (Glister et al., 2001; Gutiérrez et al., 1997; Silva and Price, 2000). It has also been suggested that NO inhibits apoptosis of granulosa cells (Basini et al., 1998; Matsumi et al., 2000; Yoon et al., 2002). One of the critical mediators of apoptosis in follicles is the Fas/FasL system, and NO inhibited FasL-induced apoptosis in rat granulosa cells (Chen et al., 2005). Consistent with these studies, we showed that inhibition of endogenous iNOS activity increased FasL mRNA abundance and increased the incidence of caspase-mediated apoptosis. Oestradiol promotes granulosa cell development by inhibiting FasL-induced apoptosis (Quirk et al., 2006), and the results

of the present study suggest that one mechanism of oestradiol action may be through the generation of NO.

These findings can be placed in the context of follicle growth. In monovulatory species, follicle growth occurs in waves, and a cohort of follicles arises in each wave. All but one of these follicles will become atretic, and the continued growth of the dominant follicle has been linked to increased IGF1 bioavailability and oestradiol production (Beg et al., 2002). This in turn would increase NO production and decrease the susceptibility of the cells to FasL mediated apoptosis. This scenario is supported in the present study by the higher levels of iNOS mRNA in the dominant, oestrogenic first-wave follicle compared with subordinate non-oestrogenic follicles. Collectively, these data are consistent with a role for physiological levels of NO in the inhibition of apoptosis in granulosa cells.

In summary, the present results demonstrate the expression and regulation of iNOS in bovine granulosa cells. Abundance of mRNA encoding iNOS is stimulated by FSH and IGF1 through increased oestradiol, and a blockade of oestradiol action consequently lowers iNOS mRNA levels. The present data suggest that endogenous NO production protects granulosa cells from apoptosis and consequently inhibits follicle atresia.

Acknowledgements

We are grateful to Dr A.F. Parlow and National Hormone & Peptide Program, NIDDK for bovine FSH, Dr K. Bertolin (Université de Montréal) for assistance with immunofluorescence, and Luiz Francisco Piccini Borges and the staff of Fazenda São Francisco for care of the animals. This work was supported by NSERC and CNPq (Brazil).

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Figures

Figure 1.

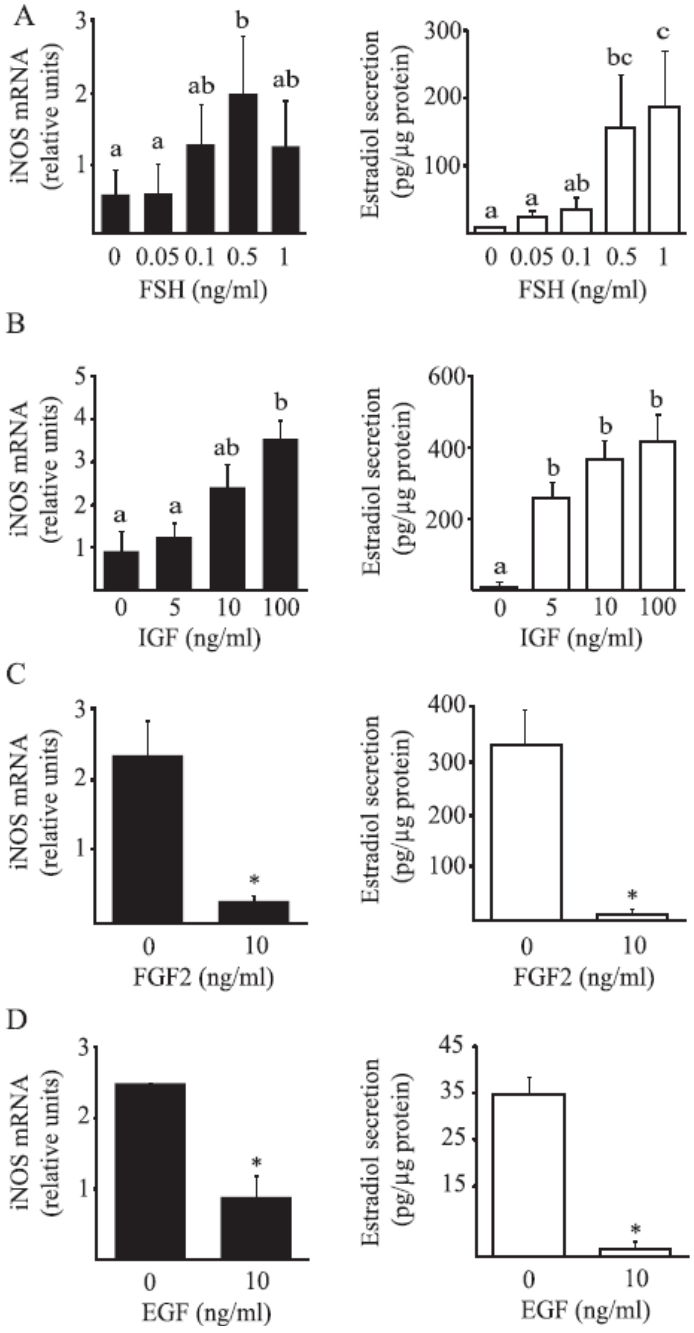

Fig 1. Effect of hormones and growth factors on abundance of iNOS mRNA and oestradiol secretion from granulosa cells in vitro. Cells were cultured for 6 days under non-luteinising conditions (see Materials and Methods for details), and treated with FSH (A), IGF1 (B), FSH with and without FGF2 (C) or IGF1 with or without EGF (D). Data are means ± SEM of three independent cultures. Bars with different letters are significantly different ($P<0.05$)

Figure 2.

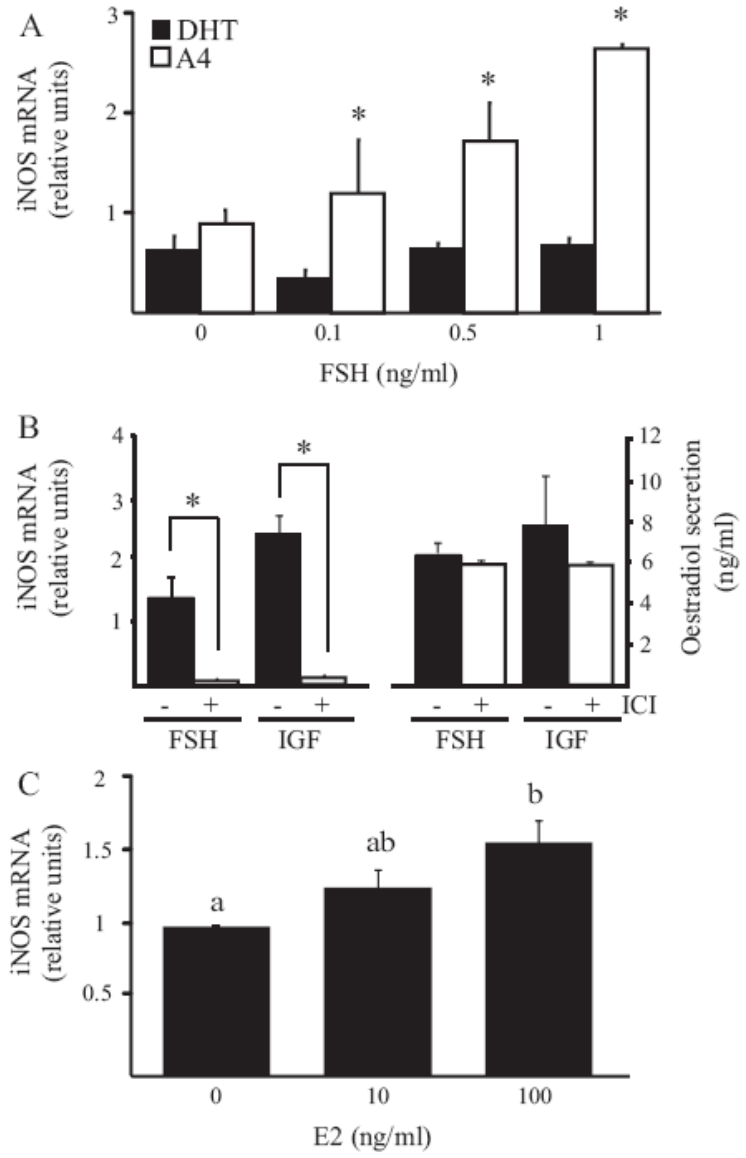
A
DHT
A4
iNOS mRNA (relative units)
FSH (ng/ml)
0
0.1
0.5
1
B
iNOS mRNA (relative units)
Oestradiol secretion (ng/ml)
ICI
FSH
IGF
FSH
IGF
C
iNOS mRNA (relative units)
a
ab
b
E2 (ng/ml)
0
10
100

Fig 2. iNOS mRNA abundance is stimulated by oestradiol. **A)** Granulosa cells were cultured for 6 days under non-luteinising conditions with graded doses of FSH and either an aromatisable (A4) or non-aromatisable androgen (DHT). **B)** Granulosa cells were cultured in the presence of IGF1 (10 ng/ml) alone or with the antioestrogen, ICI 182,780 (10 μM); and FSH (1 ng/ml) alone or in the presence of ICI 182,780. **C)** Cells were cultured in the presence of the indicated doses of oestradiol (without FSH). Data are means ± SEM of three independent cultures. Asterisks denote differences between treatments ($P<0.05$), and bars with different letters are significantly different ($P<0.05$).

Figure 3.

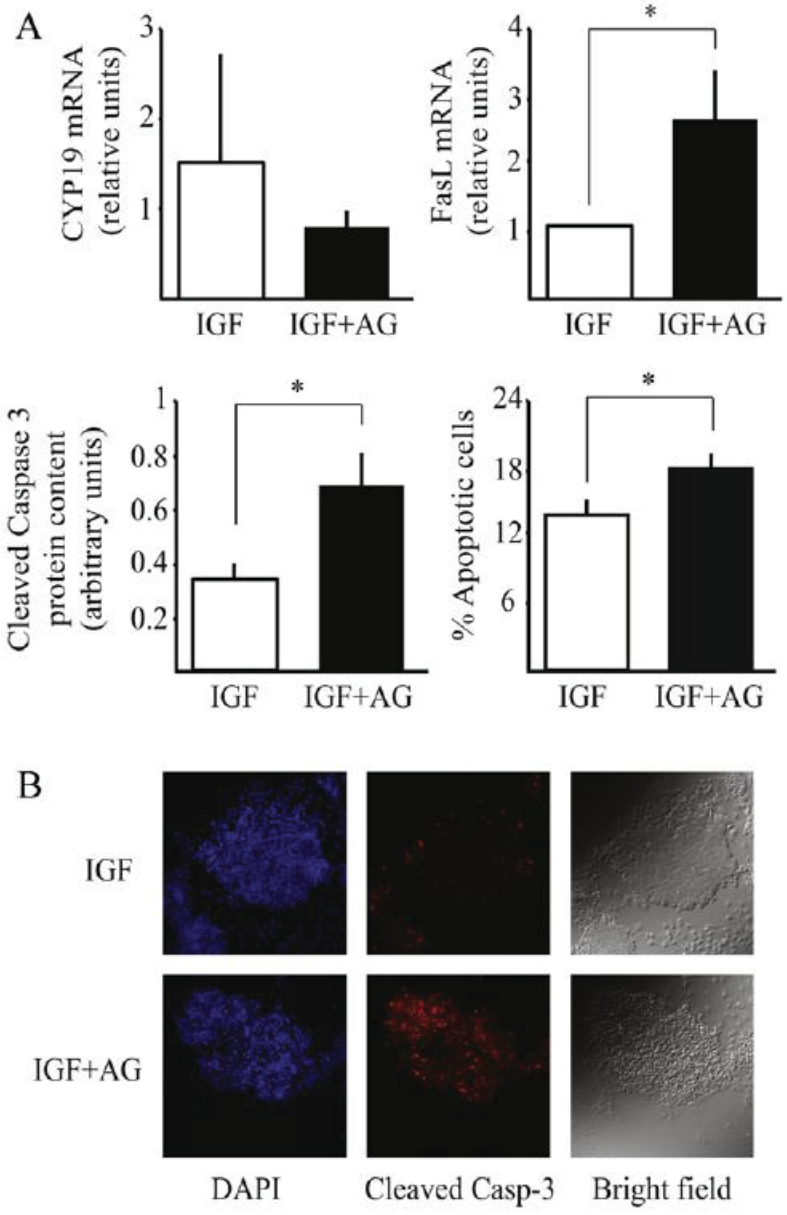
A
CYP19 mRNA (relative units)
3
2
1
IGF
IGF+AG
FasL mRNA (relative units)
4
3
2
1
*
IGF
IGF+AG
Cleaved Caspase 3 protein content (arbitrary units)
1
0.8
0.6
0.4
0.2
*
IGF
IGF+AG
% Apoptotic cells
24
18
12
6
*
IGF
IGF+AG
B
IGF
IGF+AG
DAPI
Cleaved Casp-3
Bright field

Fig 3. Effects of an iNOS inhibitor on IGF1-stimulated granulosa cells in vitro. **(A)** Endogenous iNOS activity was inhibited with aminoguanidine (AG) and the abundance of mRNA encoding Fas ligand (FasL), levels of cleaved caspase-3 protein and the proportion of apoptotic cells were measured. Data are means ± SEM of three independent cultures. Asterisks denote differences between treatments ($P<0.05$). **(B)** Representative culture showing nuclear staining (DAPI), cleaved caspase-3 fluorescence and brightfield images of the same clump of granulosa cells treated with IGF1 or IGF1 plus AG.

Figure 4.

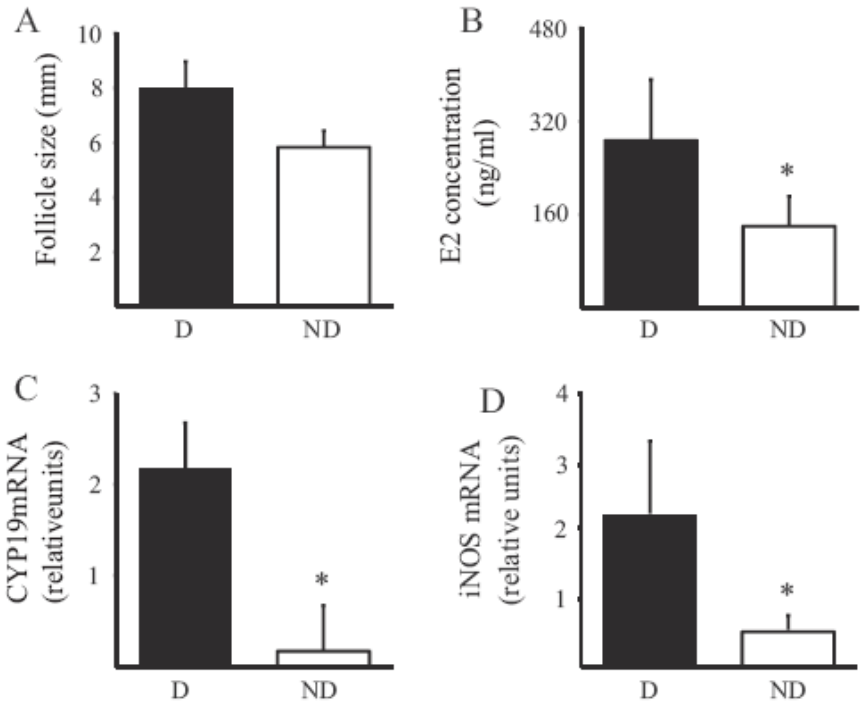
A
Follicle size (mm)
10
8
6
4
2
D
ND
B
E2 concentration (ng/ml)
480
320
160
*
D
ND
C
CYP19mRNA (relativeunits)
3
2
1
*
D
ND
D
iNOS mRNA (relative units)
4
3
2
1
*
D
ND

Fig 4. Follicle size, oestradiol (E2) concentration and abundance of mRNA encoding aromatase (CYP19) and iNOS in six pairs of early dominant (D) and non-dominant (ND) follicles. The two largest follicles were collected from cows around the time of deviation in the first wave of the oestrous cycle. Data are means ± SEM of six cows. Asterisks denote differences between dominant and non-dominant follicles ($P<0.05$).

Article 2.

Nitric oxide synthase activity is critical for the LH-induced ovulatory cascade in bovine granulosa cells

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This manuscript will be submitted to **Free Radicals Biology and Medecine.**

Abstract

In rabbits and rodents, nitric oxide (NO) is generally considered to be critical for ovulation. In monovulatory species, however, the importance of NO has not been determined. The objectives of the present study were (1) to determine if nitric oxide synthase (NOS) enzymes are regulated by LH and (2) to determine if endogenous NO is critical for expression of genes essential for the ovulatory cascade in bovine granulosa cells (GC). We employed a short-term GC culture system in which epiregulin (EREG), amphireguln (AREG) and prostaglandin-endoperoxide synthase 2 (PTGS2) mRNA levels are acutely upregulated by LH. Time- and dose-response experiments demonstrated that LH had a significant stimulatory effect on endothelial NOS (NOS3) mRNA abundance but in a prostaglandin-dependent manner. NO production was stimulated by LH before a detectable increase in NOS3 mRNA levels was observed. Pretreatment of cells with the NOS inhibitor, L-NAME, blocked the effect of LH on the epidermal growth factor (EGF)-like ligands EREG and AREG, as well as PTGS2 mRNA abundance and protein levels. Similarly, EGF treatment increased mRNA encoding EREG, AREG and the early response gene EGR1, and this was inhibited by pretreatment with L-NAME. Interestingly, pretreatment with L-NAME had no effect on either ERK1/2 or AKT activation. Taken together, these results suggest that endogenous NOS activity is critical for LH-induced ovulatory cascade in granulosa cells of a monotocous species and acts downstream of EGF receptor activation but upstream of the EGF-like ligands.

Key words: ovary, nitric oxide, follicle, prostaglandins, ovulation

Introduction

Nitric oxide (NO) is a free radical gas generated within the cell by the conversion of L-arginine to L-citrulline by a family of nitric oxide synthases (NOS). Nitric oxide signaling and NOS activity modulate cellular functions in a number of organ systems, including the reproductive system, and particularly in the ovary. The predominant NOS enzymes present in female reproductive tissues are endothelial (eNOS, also known as NOS3) and inducible NOS (iNOS, also known as NOS2). A number of studies describe NOS activity in granulosa and theca cells of the ovarian follicle of several species and indicate that NO affects ovarian steroidogenesis, follicle development, apoptosis and oocyte maturation as well the ovulatory process [1].

The involvement of NO in ovulation has been reported in different species. NO production is stimulated by hCG in follicular cells of rodents [2], horses [3] and rabbits [4]. The largest preovulatory follicle in the chicken also contains higher concentration of NO than smaller follicles [5]. In addition, it has been demonstrated that NOS inhibitors block hCG-induced ovulation in rats [6-8] and rabbits [4]. The importance of NO for ovulation in monovulatory species has not been established. Some studies in women undergoing in vitro fertilization report a positive correlation between NO levels in follicular fluid and size of preovulatory follicles [9]. In ruminants, although NO has been considered critical to oocyte maturation in cows [10-12], the role of NOS/NO system in ovulation has not been elucidated.

The ovulatory process is initiated by the surge of LH that leads to a cascade of events that culminates in the rupture of the follicle wall and release of the oocyte. A key element of this cascade is the secretion of prostaglandins (PG) by granulosa cells [13].

Prostaglandins are synthesized by a LH-inducible enzyme, prostaglandin-endoperoxide synthase 2 (PTGS2, also known as COX2) [14]. It has been suggested that NO induces the ovulatory process through regulation of PG secretion. Inhibition of NO production reduced PG secretion and the administration of NO donors stimulated PG synthesis [2, 4] at least in part by altering PTGS2 mRNA levels [15].

In the last decade, it has been demonstrated that the LH-induced cascade leading to PTGS2 expression involves the EGF-like factors epiregulin (EREG) and amphiregulin (AREG). Briefly, LH induces the release of EREG/AREG from the surface of mural granulosa cells, which then act in an autocrine and paracrine manner to stimulate the EGF receptor of mural and cumulus cells. EGF receptor activation results in EREG/AREG expression and induction of PTGS2 expression [16, 17]. It is not known if NO affects EGF-like factor expression.

To better understand the involvement of NO in ovulation in monovulatory species, the objectives of the present study were (1) to determine if nitric oxide synthase (NOS) enzymes are regulated by LH and EGF, and (2) to determine if endogenous NO is critical for expression of genes essential for the ovulatory cascade in bovine granulosa cells.

Materials and methods

Cell culture

The granulosa cell culture was as previously described [18] in which EREG, AREG and PTGS2 mRNA levels are acutely upregulated by LH. The reagents were obtained from Invitrogen except where otherwise stated. Briefly, bovine ovaries were

collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir and were transported to the laboratory in PBS at 35°C containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (1 μg/ml). Five to eight ovaries that each contained a single large follicle (>10 mm diameter) were selected for each replicate. Cells were collected from the large follicle by aspiration, pooled and were washed twice by centrifugation at 219 x g for 20 min each. Cell viability was estimated with 0.4% Trypan Blue Stain. Cells were seeded into 24-well tissue culture plates (Sarstedt) at a density of 1 x 10^6 viable cells per well in 1 ml DMEM-F12 supplemented with sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1 %; Sigma-Aldrich), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1.1 mM), androstenedione (10^{-7} M), FSH (1 ng/ml), insulin (10 ng/ml) and 2% fetal calf serum (Hyclone). Cultures were maintained at 37°C in 5% CO2 for 24 h. Medium was then replaced with serum-free DMEM-F12 with antibiotics for 18 h, at which point treatments were added for the times shown in Results.

Cells were stimulated with bovine LH (AFP11743B; NIDDK), epidermal growth factor (EGF; R&D Systems) or prostaglandin E2 (PGE2; Sigma-Aldrich) at the doses and time periods described in *Results*, after which cells were recovered for analysis. In some experiments cells were treated 2 h before LH/EGF challenge with the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma-Aldrich) or the prostaglandin synthesis inhibitor indomethacin (INDO; Sigma-Aldrich).

Nucleic acid extraction & RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions and quantified by absorbance at 260 nm. Total RNA (0.2

μg) was first treated with 1 U of DNase (Promega) at 37℃ for 5 min to digest any contaminating DNA, followed by 65℃ for 5 min for DNase inactivation. The RNA was reverse transcribed in the presence of 1 mM oligo(dT) primer and 4 U of Omniscript RTase (Omniscript RT Kit; Qiagen), 0.25 mM dideoxy-nucleotide triphosphate (dNTP) mix, and 19.33 U of RNase Inhibitor (GE Healthcare) in a volume of 20 μl at 37℃ for 1 h. The reaction was terminated by incubation at 93℃ for 5 min.

Real-time PCR was performed in an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems). Bovine-specific primer sequences were histone H2AFZ, PTGS2, EREG, AREG and STAR all in [18], NOS3 [19] and NOS2 [20]. Common thermal cycling parameters (3 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C) were used to amplify each transcript. Melting curve analyses were performed to verify product identity. Samples were run in duplicate, and were expressed relative to histone H2AFZ as housekeeping gene. Data were normalized to a calibrator sample using the Pfaffl ΔΔCt method with correction for amplification efficiency [21].

Western blotting

After challenge with PGE2 (10 μM), LH (400 ng/ml) or EGF (5 ng/ml), with or without the NOS inhibitor L-NAME (100 μM), cells were harvested at different time points as described in the *Results*. For protein extraction, cells were washed with cold PBS and lysed in 100 μl/well cold RIPA buffer (25 mM Tris-HCL pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, and protease and phosphatase inhibitor cocktails). The homogenate was centrifuged at

6000 g for 5 min at 4°C. The resulting supernatant was retained and stored at -20°C. Protein concentrations were determined by BCA protein assay (Pierce).

Samples were resolved on 12% SDS-polyacrylamide gels (15 μg total protein per lane) and electrophoretically transferred onto PVDF membrane in a Bio-Rad wet Blot Transfer Cell apparatus (transfer buffer : 39 mM glycine, 48 mM Tris-base, 1% SDS, 20% methanol, pH 8.3). After transfer, the membranes were blocked in TTBS (10 mM Tris-HCL, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h then incubated overnight with the primary antibodies (anti-ERK, #9102, 1:1000; anti-phospho-ERK (Thr202/Tyr204), #9101, 1:1000; anti-AKT, #9272, 1:1000; anti-phospho-AKT (Ser473), #9271, 1:1000; all from Cell Signaling Technology) and anti-PTSG2 (#160112, 1:1000; Cayman Chemical) diluted in TTBS at 4°C. After washing three times with TTBS, membranes were incubated for 2 h at room temperature with 1:20 000 anti-rabbit (GE Healthcare) or with 1:200 000 anti-mouse (Calbiochem) horseradish peroxidase-conjugated immunoglobulin G diluted in TTBS. After five washes in TTBS, protein bands were revealed by chemiluminescence (ECL; Millipore) and autoradiography. Semiquantitative analysis was performed with NIH Image J software.

Real time NO measurement

Intracellular NO production was assessed with the fluorescent NO-sensitive dye 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) essentially as described [22]. Cells were cultured as described previously, and pretreated with DAF-FM DA (10 μM) 2 h before the addition of LH (400 ng/ml) or EGF (5 ng/ml). Cells were examined under an Olympus FV1000 laser-scanning confocal microscope at time

0 and 180 min. Digital images were captured from the same field and fluorescence intensity (ImageJ software) was assessed.

Statistical Analysis

All experiments were performed with three or four independent replicates, with each replicate using ovaries collected at different times. Replicates were usually performed at intervals of one week. Data that did not follow a normal distribution (Shapiro-Wilk test) were transformed to logarithms. Analysis of data was performed with JMP software (SAS Institute) with treatment and/or time as main effect and culture replicate (where appropriate) as a random variable in the F-test. Differences between means were tested with the Tukey-Kramer HSD test. Data are presented as means ± SEM.

Results

The first series of experiments was performed to determine the regulation of NOS mRNA and activity by LH in bovine granulosa cells. A time-course experiment demonstrated that LH had a significant stimulatory effect on NOS3 and NOS2 mRNA abundance at 12 h post-challenge ($P<0.05$, Fig. 1A, B). Abundance of NOS2 mRNA was considered to be low owing to the high mean threshold Ct values for this mRNA (37-39) and this gene was not investigated further. The effect of dose of LH on abundance of mRNA encoding NOS3 and genes involved in the ovulatory cascade was then tested at the 12 h time point; LH significantly increased NOS3, EREG and AREG

mRNA levels ($P<0.05$, Fig. 2A). Further cultures demonstrated that EGF also stimulated NOS3, EREG and AREG mRNA levels at 6 h post-challenge ($P<0.05$, Fig. 2B).

As the peak for NOS3 mRNA abundance occurred only at 12 h after LH challenge, a relatively late time point in the LH-induced preovulatory cascade and that corresponds to the initial increase in prostaglandin secretion in vivo in cattle, we then tested whether prostaglandin regulates NOS3 expression. Addition of PGE2 stimulated NOS3 mRNA abundance ($P<0.05$, Fig. 3A). Moreover, when cells were pretreated with indomethacin, a non-selective PTGS inhibitor, EGF was not capable of increasing NOS3 mRNA levels ($P<0.05$; Fig. 3B).

The effect of LH and EGF on NO production was then assessed with the NO-sensitive dye, DAF-FM DA. The results indicated that both LH and EGF increased NO production 3 h after challenge ($p<0.05$; Fig. 4).

A second series of experiments was then performed to assess whether NOS activity is critical for the expression of important genes involved in the ovulatory cascade. Challenge of cells for 6 h with LH increased EREG, AREG, PTGS2 and STAR mRNA levels, and pretreatment with the NOS inhibitor, L-NAME, effectively blocked the effect of LH on EREG, AREG and PTGS2, but not STAR mRNA abundance ($P<0.05$; Fig. 5A). Pretreatment with L-NAME also blocked the LH-induced increase in PTGS2 protein levels ($P<0.05$; Fig. 5B). L-NAME alone had no effect on basal gene expression.

To further explore if NO is necessary for EGFR-dependent signaling, cells were pretreated with a single dose of L-NAME and challenged with EGF for 1, 2, 4 and 8 h. EGF alone increased mRNA encoding EREG and early growth regulatory factor-1 (EGR1) within 1 h, and this was inhibited by pretreatment with L-NAME. EGF alone

also increased abundance of mRNA encoding AREG and the nuclear orphan receptors NR5A1 and NR5A2 after 8 h, and L-NAME inhibited the effect of EGF ($P<0.05$; Fig. 6).

To determine the site of action of NO, the effect of inhibition of NOS activity on ERK1/2 and AKT activation was measured by Western blotting. EGF stimulated ERK1/2 and AKT phosphorylation (Ser473 and Thr202/Tyr204, respectively), and pretreatment with L-NAME had no effect on either ERK1/2 or AKT activation ($P>0.05$; Fig. 7).

Discussion

In rabbits and rodents, nitric oxide is generally considered to be necessary for ovulation, and acts in part through increased prostaglandin secretion. In monovulatory species, however, the importance of nitric oxide has not been determined. In the present study, we employed an in-vitro model of preovulatory events in cattle to provide the first evidence that nitric oxide is necessary for the preovulatory cascade in a monotocous species, that this likely involves very early generation of nitric oxide not requiring transcriptional activation of NOS enzyme genes, and that the locus of action of nitric oxide is the EGF-like factors EREG and AREG.

In the present study we detected an increase of both NOS3 and NOS2 mRNA levels in granulosa cells after treatment with LH. Nevertheless, NOS2 was detected at very low levels and was not investigated further. The presence of NOS2 and NOS3 and their regulation by gonadotropins has been examined in the rat ovary by several authors, but the contribution of each NOS to ovulation is still not clear. One of these studies

found that NOS2 protein was barely detectable during follicle development and the ovulatory process [23]; other reported maximal levels of NOS2 mRNA in unstimulated ovaries and its reduction after hCG injection [24]. An increase of both NOS2 and NOS3 after hCG stimulation has been reported [25, 26]. On the other hand, a more recent study demonstrated the presence of only NOS3 protein levels in the ovary of rats 10 h after hCG treatment [27]. Moreover, studies with NOS2-and NOS3-knockout mice indicated that disruption of NOS2 had no effect on ovulation rate in superovulated prepubertal females, but NOS3 deficiency had a significant negative effect [28, 29]. Although little is known about NOS regulation in GC during the preovulatory period in ruminants, recently our group published a study determining the regulation of NOS2 in bovine GC during follicular development, suggesting that NOS2 expression is estradiol-dependent [20]. The decline in circulating estradiol levels during the periovulatory period [30] could explain the NOS2 low mRNA abundance in the preovulatory model used in the present study.

Our results demonstrate that LH stimulated NOS3 mRNA levels around 12 h post-challenge, which is a fairly late event in the preovulatory cascade. This increase coincides with LH-dependent PTGS2 mRNA expression in this culture system [18] and to increased PG secretion in vivo after the LH surge [31]. This suggests that NOS3 mRNA may be regulated by PG, and that any role that NO plays in the induction of ovulation is not likely due to transcriptional activity of the NOS3 gene, which we confirmed by direct stimulation of NOS3 mRNA levels with PG and by the ability of the PTGS2 inhibitor indomethacin to abrogate the effect of EGF on NOS3 mRNA levels. These results are supported by reports showing that PGE2 induces NOS3 expression in

cerebral microvessel endothelial cells in pigs [32] and that PGF2α induces NOS3 expression in bovine corpus luteum [33].

It has been also reported that NOS activity increases in the period that precedes ovulation in other species. The intracellular NO measurement performed in our study indicated that NO production was increased in GC in the first 3 hours after LH/EGF challenge. These results support the hypothesis that NOS3 may be the most important NOS acting in our preovulatory model: while NO generation from NOS2 depends almost exclusively on the transcriptional increase of this enzyme, NO generation can rise as a consequence of NOS3 activation even before a significant increase of its expression levels. In hepatocytes of mice it was shown that EGF-induced NOS3 activation is dependent on the EGFR/PI3K/AKT signaling [34]. In human epidermal keratinocyte cell line, HB-EGF-induced NOS3 activation depends on both ERK1/2 and PI3K/AKT pathways [35]. However, whether LH activates NOS3 in a manner dependent or independent of EGFR signaling in our cell model is still unknown. Interestingly, NOS3 can be also activated by calcium increase [36], and in swine GC it was demonstrated that LH induces a rapid and biphasic rise in calcium [37].

The NO generated from NOS activation appears to be critical in the preovulatory period. Although a recent study suggests that NO inhibits oocyte maturation and ovulation in rats [8], it has been demonstrated that knockout of the NOS3 gene severely impairs both oocyte maturation and ovulation in mice [28, 29, 38] and that NOS inhibitors suppress hCG-induced ovulation in rats [6, 7] and rabbits [4]. Some of these studies also used NO-generating drugs (NO donors) to stimulate or restore ovulation; however, these data should be interpreted with caution as NO donors have been shown to be cytotoxic [39, 40]. Some authors suggest that the positive relationship between NO

and ovulation is related to a crosstalk between NO and PG secretion. Inhibition of NO production reduced ovarian prostaglandins synthesis in rats and rabbits [2, 4]. It was shown in different cells types that NO may stimulate PTGS2 mRNA levels and PTGS2 activity directly. NO interacts with various pathwayswhich can influence PTGS2 expression such as the B-catenin/TCF, cAMP/PKA/CREB and JNK/Jun/ATF2 signaling cascades [15]. NO also activates PTGS2 by binding directly to its heme group [41]. Our results confirm that inhibition of NOS activity reduces mRNA and protein levels of PTGS2 in preovulatory granulosa cells of cattle.

LH induces PTGS2 expression in GC by stimulating the release of the EGF-like ligands, EREG and AREG. These activate the EGF receptor and its downstream signaling that includes a positive feedback on EREG and AREG expression [42]. A previous study from our group using the same cell culture system demonstrated that LH increased EREG mRNA levels within 1 h and AREG mRNA levels 6 h after challenge [18]. Our present results clearly indicate that inhibition of NOS activity effectively blocked the effect of LH on EREG, AREG and PTGS2 mRNA abundance after 6 hours. Similarly, inhibition of NOS activity effectively reduced the ability of EGF to induce EREG and AREG expression. To our knowledge, this is the first report that indicates the expression of EGF-like factors is NO-dependent.

The EGF receptor (EGFR) activation is essential to the signaling cascade that leads to PTGS2 expression, which entails activation of the ERK1/2 and AKT kinase cascades [43, 44]. We demonstrated in this study that L-NAME affected EREG/AREG/PTGS2 expression but did not affect ERK1/2 or AKT activation. Inhibition of NOS activity affected expression of the early-response gene EGR1 that, like EREG and AREG, is ERK1/2-dependent [45]. Although not conclusive, these

results suggest that NO must be critical downstream of ERK1/2 signaling but upstream of EGR1 and the EGF-like ligands. Different transcriptional regulators are known toaffect ovulation and appear to help mediate the effects initiated by ERK1/2. Mice lacking nuclear receptor–interacting protein 1 (Nrip1; also known as RIP140) exhibit impaired ovulation and reduced expression of EREG, AREG, and other ovulation-related genes [46]. RIP140 could be a potential target of NO in this preovulatory system.

Taken together, these results suggest that the LH surge induces granulosa cells to activate NOS; NOS activity induces production of NO, which is essential for EREG/AREG/PTGS2 expression. In conclusion, NOS activity is critical for LH-induced ovulatory cascade in granulosa cells and NO may be essential for ovulation in cattle.

Acknowledgements

We are grateful to Dr A.F. Parlow and National Hormone & Peptide Program, NIDDK for bovine FSH and LH. This work was supported by NSERC and FQRNT (Canada).

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Figures

Figure 1.

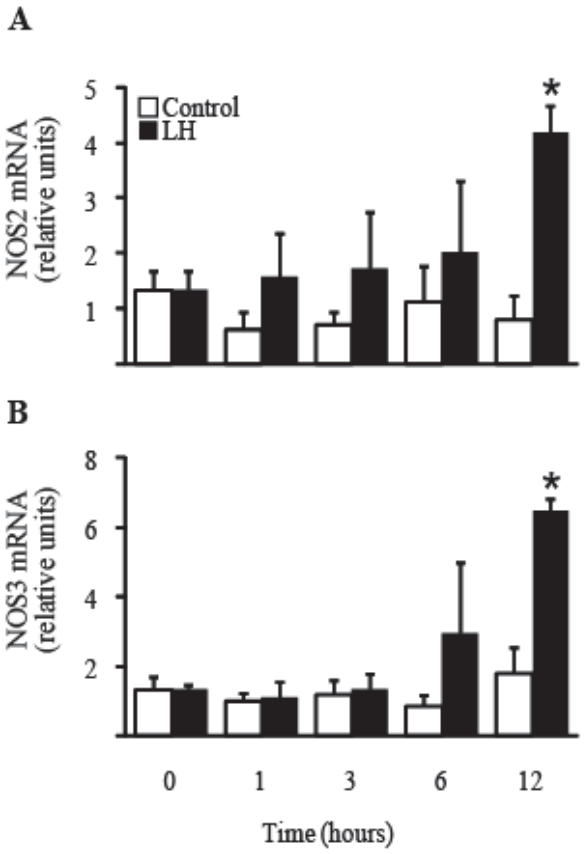

Fig. 1. LH induces NOS mRNA abundance in a time-dependent manner. Bovine granulosa cells from large follicles (≥10 mm diameter) were cultured with serum for 24 h, and then in serum-free medium for a further 18 h, before adding LH (400 ng/ml) for the times given. Messenger RNA abundance was measured by real-time PCR. Data represent the mean ± SEM for three independent replicate cultures. LH-stimulated data are expressed relative to the control data at each time point. An asterisk (*) denotes significant increases over control ($P < 0.05$).

Figure 2.

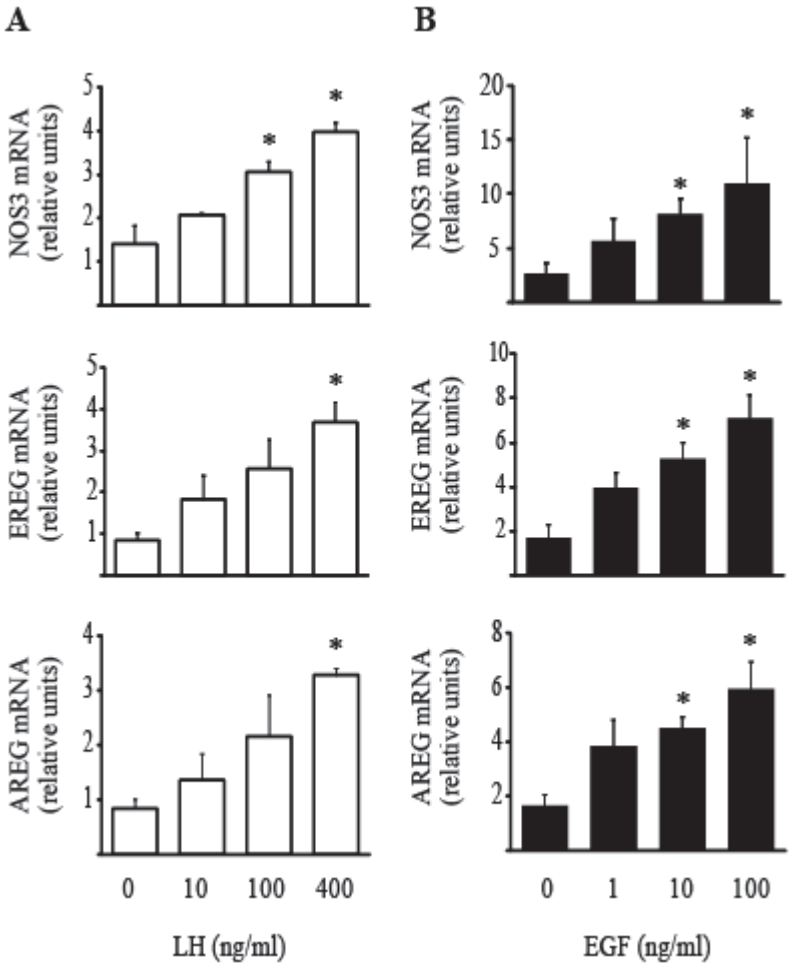
A
B
NOS3 mRNA (relative units)
EREG mRNA (relative units)
AREG mRNA (relative units)
0
10
100
400
LH (ng/ml)
0
1
10
100
EGF (ng/ml)

Fig 2. NOS3 mRNA levels are regulated in a dose-dependent manner by LH and EGF. Cells were cultured as described in the legend for Figure 1 and were treated for 12 h with the indicated doses of LH **(A)** and for 6 h with the indicated doses of EGF **(B)**. Messenger RNA abundance was measured by real-time PCR. Data represent the mean ± SEM for three independent replicate cultures. Asterisks (*) denote differences between treatments ($P<0.05$).

Figure 3.

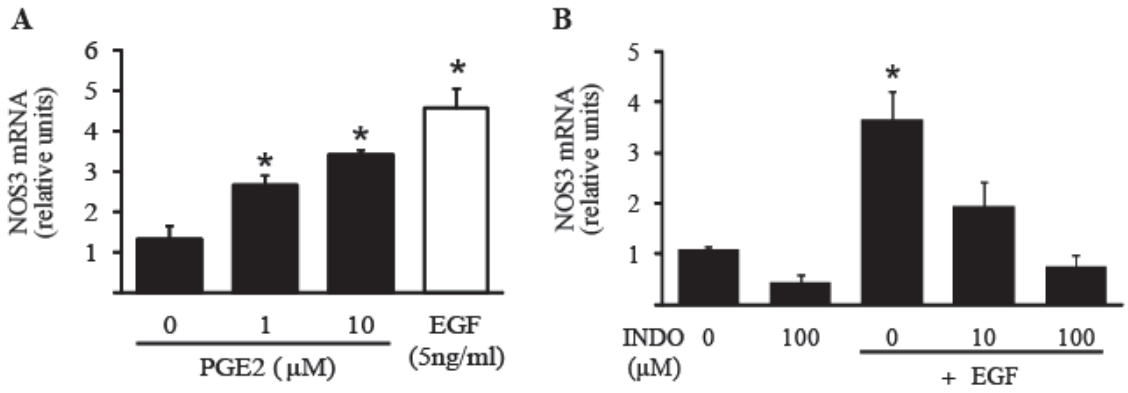
A
NOS3 mRNA (relative units)
6
5
4
3
2
1
*
*
*
0
1
10
EGF (5ng/ml)
PGE2 (μM)
B
NOS3 mRNA (relative units)
5
4
3
2
1
*
INDO (μM)
0
100
0
10
100
+ EGF

Fig. 3. Prostaglandins regulate NOS3 mRNA levels. Bovine granulosa cells were cultured as described in Figure 1. **(A)** Effect of treatment for 6 h with the indicated doses of PGE2 and EGF. **(B)** Effect of pretreatment with indomethacin, a nonselective PTGS inhibitor on NOS3 mRNA abundance 6 h after challenge with EGF. Messenger RNA abundance was measured by real-time PCR. Data represent the mean ± SEM for three independent replicate cultures. Asterisks (*) denote differences between treatments ($P<0.05$).

Figure 4.

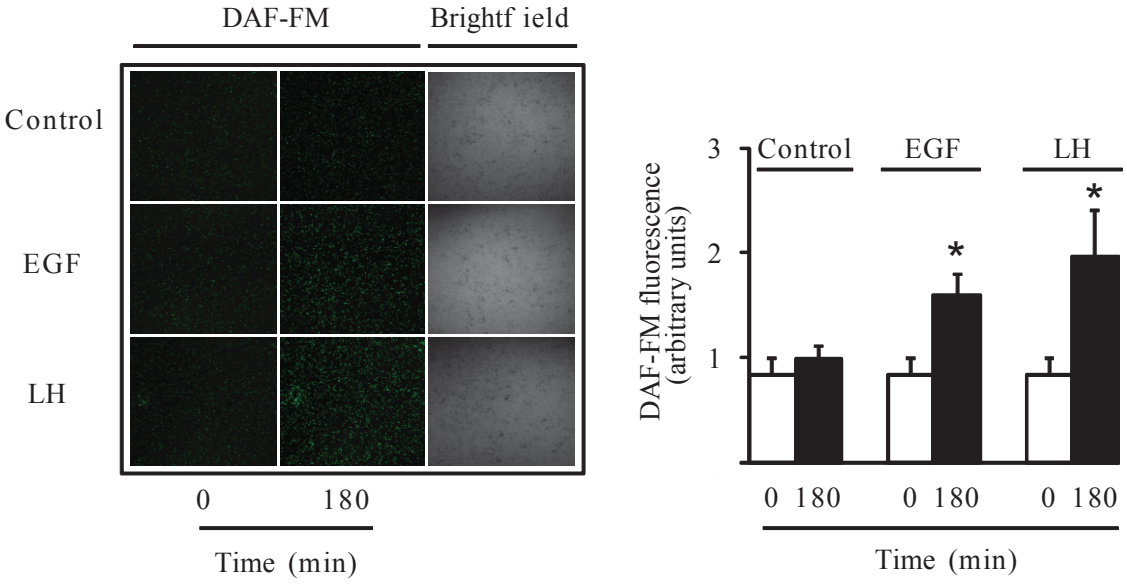
DAF-FM
Brightf ield
Control
EGF
LH
0
180
Time (min)
DAF-FM fluorescence (arbitrary units)
3
2
1
Control
EGF
LH
*
*
0 180
0 180
0 180
Time (min)

Fig. 4. LH and EGF increase nitric oxide production. Intracellular NO production was assessed with the fluorescent NO-sensitive dye DAF-FM DA. Cells were cultured as described previously, and pretreated with DAF-FM DA (10μM) 2 h before the addition of LH (400 ng/ml) or EGF (5 ng/ml). Cells were examined under laser-scanning confocal microscope at time 0 and 180 min. Digital images were captured from the same field and fluorescence intensity was assessed. Data represent the mean ± SEM for three independent replicate cultures. An asterisk (*) denotes significant increases over control ($P < 0.05$).

Figure 5.

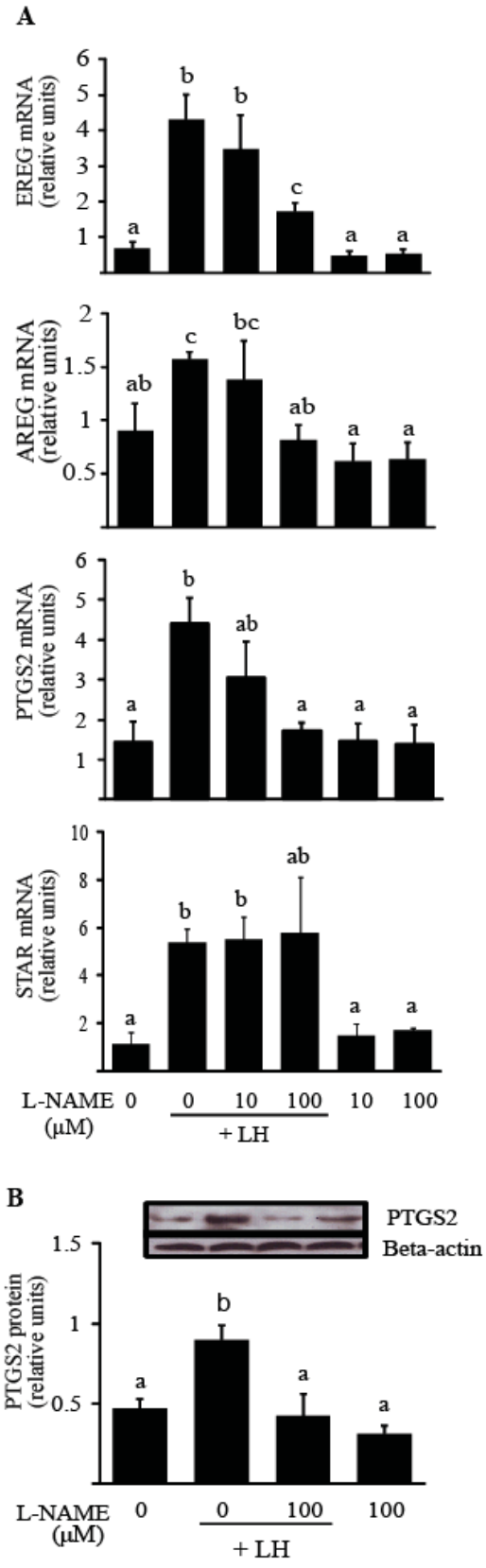
A
EREG mRNA (relative units)
AREG mRNA (relative units)
PTGS2 mRNA (relative units)
STAR mRNA (relative units)
L-NAME (μM) 0 0 10 100 10 100
+ LH
B
PTGS2
Beta-actin
PTGS2 protein (relative units)
L-NAME (μM) 0 0 100 100
+ LH

Fig. 5. Effect of NOS inhibitor on LH-induced preovulatory genes. Bovine granulosa cells were cultured as described in Figure 1. Cell were treated with different doses of L-NAME, a NOS inhibitor, on abundance of mRNA encoding genes involved in the ovulatory cascade at 6 h after LH challenge **(A)** and on PTGS2 protein levels at 12 h after LH (400 ng/ml) challenge **(B)**. Protein secretion was measured by western blot and a blot of one independent culture is shown with the samples in the same order as in the graph. Messenger RNA abundance was measured by real-time PCR. Data represent the mean ± SEM for three independent replicate cultures. Bars with the same letters are not significantly different ($P > 0.05$).

Figure 6.

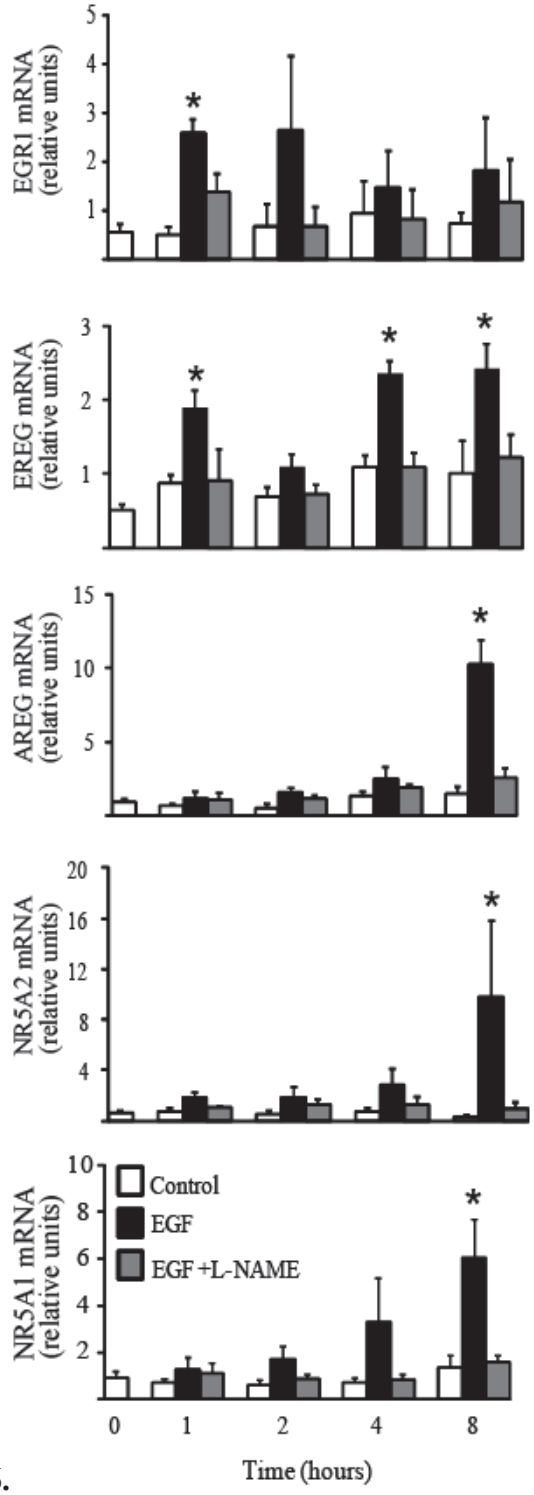

Figure 6.

Fig. 6. Effect of NOS inhibitor on EGF-induced preovulatory genes. Bovine granulosa cells were cultured as described in Figure 1 and were pretreated with L-NAME (100 μM), a NOS inhibitor, before EGF (5 ng/ml) challenge. Messenger RNA abundance was measured by real-time PCR. Data represent the mean ± SEM for three independent replicate cultures. An asterisk (*) denotes significant increases over control at each time point ($P < 0.05$).

Figure 7.

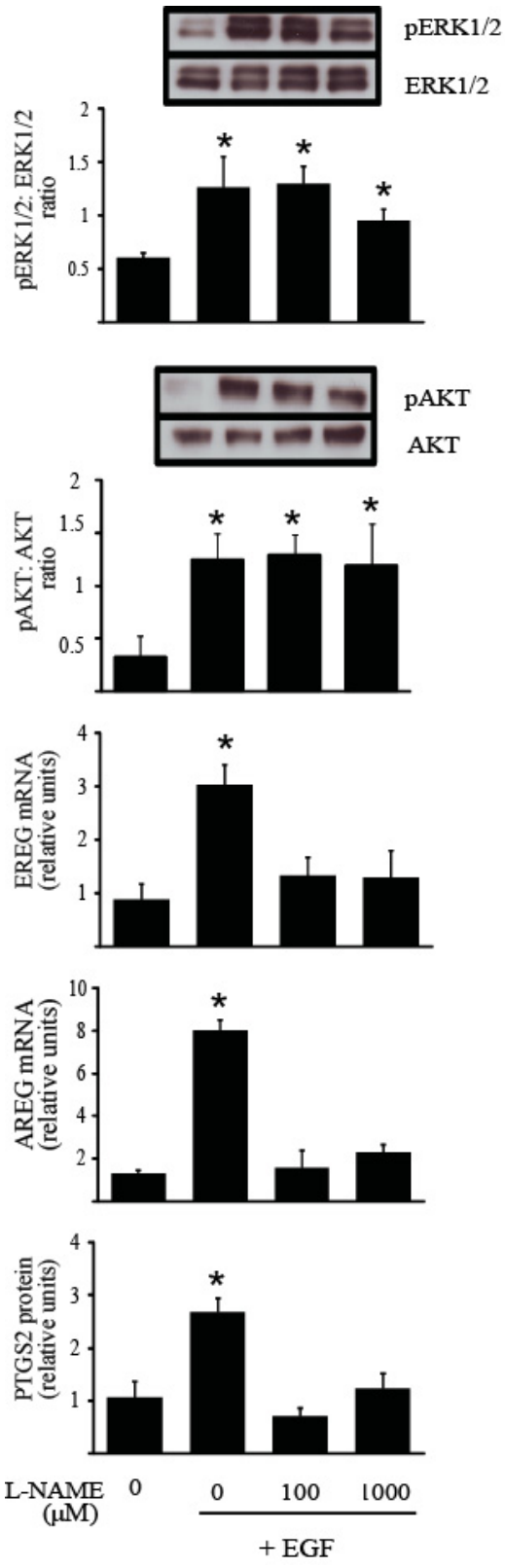
pERK1/2
ERK1/2
pERK1/2: ERK1/2 ratio
2
1.5
1
0.5
*
*
*
pAKT
AKT
pAKT: AKT ratio
2
1.5
1
0.5
*
*
*
EREG mRNA (relative units)
4
3
2
1
*
AREG mRNA (relative units)
10
8
6
4
2
*
PTGS2 protein (relative units)
4
3
2
1
*
L-NAME (μM)
0
0
100
1000
+ EGF

Fig. 7. Nitric oxide is not essential for EGF-induced ERK1/2 and AKT phosphorylation. Bovine granulosa cells were cultured as described in Figure 1. In the presence of different doses of L-NAME, EGF was able to induce ERK1/2 and AKT phosphorylation. Samples were collected 15 min after EGF challenge to measure phosphorylation by western blot, and the blot of one independent culture is shown with the samples in the same order as in the graph. Cells from the same pools were pretreated with different doses of L-NAME and challenged for 6 h with EGF to confirm L-NAME effects on abundance of mRNA encoding genes involved in the ovulatory cascade in granulosa cells. Data represent the mean ± SEM for three independent replicate cultures. An asterisk (*) denotes significant increases over control ($P < 0.05$).

Chapter 4:

General discussion

General discussion

Disorders of ovarian function are major causes of infertility, subfertility and cancer. A better comprehension of the regulation of granulosa cell function is likely to be critical in resolving the cause of several ovarian disorders, as well overcoming ovarian follicle ageing and improving the success rate of assisted reproductive technologies. Models for granulosa cells culture are of considerable interest, especially animal models. Ruminants, particularly cattle, offer great potential, not only because of the agricultural importance of this species, but also its potential use as a model for humans. Many aspects of follicle growth in cows are similar to those in humans. Both species are predominantly mono-ovulatory, and sizes of follicles at different stages of development are similar [283]. The dynamics of follicle wave emergence appear to be similar in cows and women [33, 284], and reproductive ageing in cattle and women share many features [285].

Events associated with the normal progression of proliferation and differentiation of granulosa cells are critical for growth of the follicle, health of the egg and the process of ovulation. Granulosa cell health and death are regulated by endocrine factors such as gonadotropins, paracrine growth factors and intracrine modifiers of cell function. The free radical gases comprise a group of intracrine agents that have been linked to ovarian function. One of these is nitric oxide (NO). This short-lived gas is produced by the action of the enzymes nitric oxide synthase (NOS), and has several effects on cell function.

The regulation of NOS and the contribution of NO for the follicle growth and the ovulatory processes in cattle were until now unclear. Together, the novel results reported in this thesis not only indicate that bovine granulosa cells express NOS2 and NOS3, but also demonstrate a difference in the pattern of expression/activity of these enzymes throughout follicle growth in the cow. The NO generated by the activity of each of these enzymes seems to exert important functions at least in two distinct physiological

moments. During the establishment of follicle dominance, NO seems to be a critical anti-apoptotic agent produced by granulosa cells to avoid atresia. On the other hand, in the preovulatory period, NO seems to act as an essential mediator of the ovulatory cascade.

The above mentioned results were obtained using different cell models and in vivo samples carefully selected to represent granulosa cells at different stages of development. In the first article of this thesis, the objectives were to gain insight into the expression and role of NOS2 during follicle growth in cattle. For this, granulosa cells from follicles between 2-6 mm were cultured in serum-free conditions. In this nonluteinizing model, the cells retain their typical follicular (estrogenic) phenotype and responsiveness to FSH and IGF1, but not LH [23, 28, 286]. Herein, this model will be described as the differentiation model. Preliminary results indicated that mRNA levels for both NOS2 and NOS3 were detected in granulosa cells cultured in this model. However, for the first article we decided to focus only on NOS2, because it is known to be inducible in a large number of cell types, whereas the others are generally expressed constitutively [263]. Another relevant criteria for this choice was the fact that in a cell that expresses more than one NOS, NOS2 presents a higher contribution to NO generation [169, 172, 173]. While NOS3 is commonly associated with basal production of NO (nM), NOS2 activity may generate NO at more elevated levels (μM).

For the second article of this thesis the objectives were to determine the regulation pattern of NOS during the preovulatory period and to determine if endogenous NO is essential for expression of genes critical for the ovulatory cascade in bovine granulosa cells. A second in vitro bovine model was then employed, in which cells from large follicles (>10mm) are conditioned with FSH then challenged with LH or EGF; both induce the expression of genes involved in ovulation, including EREG, AREG, PTGS2 and others [95]. As this model can simulate in vitro the LH-induced ovulatory cascade in bovine granulosa cells, it will be described here as the periovulatory model. The first series of experiments using this second model also demonstrated the expression of both NOS2 and NOS3. But surprisingly, NOS2 levels were lower than expected. A time-

course experiment demonstrated that LH had a significant stimulatory effect on NOS3 and NOS2 mRNA abundance at 12 h post-challenge (figure 1; chapter 3; article 2). Abundance of NOS2 mRNA was considered to be low owing to the high mean threshold Ct values for this mRNA (37-39 cycles). The high mRNA abundance for NOS2 detected in all the experiments performed with the differentiation model was not verified in the periovulatory model. These results suggest that NOS expression pattern changes throughout the follicle growth in cattle. In sheep, it was demonstrated that NOS3 protein and mRNA expression changes substantially during follicular growth. NOS3 mRNA levels increased in granulosa cells at 12 and 24 h after hCG treatment [276]. In swine it was demonstrated that NOS3 levels increase in late stages of follicle development and the authors suggested that these changes might be related to the gonadotropins surge and necessary to the ovulation in this species [277]. In rats, NOS2 and NOS3 are also differentially regulated during follicular development [264].

In the first article, the differentiation model employed was used to mimic in vitro the conditions of a growing follicle. In this model, FSH and IGF1 stimulate cell proliferation and estradiol secretion [28, 287]. When we challenged granulosa cells with graded doses of FSH and IGF1, there was stimulation on estradiol secretion and NOS2 mRNA abundance (Figure 1; chapter 3; article 1). To complement these results, we determined if NOS2 mRNA abundance would differ between growing and non-growing follicles using a well-defined in vivo model described in the article 1. The results indicated that NOS2 abundance was higher in growing dominant follicles compared to subordinate follicles. The status of the dominant follicle was confirmed by higher estradiol concentrations and CYP19 mRNA abundance (figure 4; chapter 3; article 1). As in both in vivo and in vitro models NOS2 expression was positively correlated with estradiol levels, we then decided to determine if estradiol had a direct effect on NOS2 mRNA. In fact, the addition of an anti-estrogen prevented FSH- and IGF1-induced NOS2 mRNA accumulation. In addition, estradiol alone significantly increased NOS2 mRNA abundance (figure 2; chapter 3; article 1). This data clearly show that NOS2 expression in bovine granulosa cells is, at least in part, estradiol-dependent. This could

explain the lower NOS2 mRNA levels detected in granulosa cells used in the periovulatory model. In this model, CYP19 levels decrease in a time-dependent manner and, consequently estradiol secretion decrease with time (data not shown). This is supported by the fact that circulating estradiol levels decline during the peri-ovulatory period in vivo [288]. So, the NOS2 increase observed at 12 h post LH-challenge in our periovulatory model was probably stimulated in a different manner than the estradiol-dependent stimulation verified in the differentiation model.

The different pattern of NOS2 and NOS3 gene expression detected in both articles 1 and 2, suggests that the amount of NO required by the follicle during its growth in cattle may change. Although in the first article NOS3 regulation was not studied, it was clearly demonstrated by different experiments that NOS2 mRNA levels increased in healthy growing follicles. In cattle, the dominant follicle is characterised by a healthy layer of granulosa cells growing under the influence of IGF [289]. Our studies using the differentiation model indicated that NOS2 mRNA abundance is stimulated in a dose-dependent manner by IGF1 (figure 1; chapter 3; article 1). To confirm our in vitro findings, we demonstrated with in vivo samples that NOS2 abundance was higher in growing dominant follicles compared to subordinate follicles (figure 4; chapter 3; article 1). Supported by the literature of NO as an anti-apoptotic agent in several cells types, including granulosa cells [227, 229], we decided to determine if NOS2 expression/activity was one of the mechanisms used by dominant growing follicles to avoid atresia. As we hypothesized, endogenous NOS2 activity inhibited apoptosis in bovine granulosa cells. Using the differentiation model, granulosa cells were cultured with IGF1 with or without the selective NOS2 inhibitor, aminoguanidine. Addition of aminoguanidine increased the abundance of mRNA encoding the pro-apoptotic factor Fas ligand, cleaved caspase-3 protein and, consequently, the proportion of apoptotic cells (figure 3; chapter 3; article 1).

Overcoming atresia by a dominant follicle is a critical process and is dependent of the balance between death and survival factors. Among the survival factors is estradiol, the hallmark of follicle health. Estradiol is a well known anti-apoptotic factor

that acts in different manners to inhibit apoptosis [19]. The results from our first article suggest that one of the mechanisms by which estradiol combats apoptosis in granulosa cells and guarantees follicle dominance is through NO generation. The fact that during the dominant follicle growth NOS2 is more highly expressed than in granulosa cells from preovulatory follicles, suggests that the amount of NO necessary to inhibit apoptosis is larger than the amount necessary to mediate the ovulatory cascade.

In terms of NOS gene expression regulation, results obtained in the article 2, using the periovulatory model, demonstrate that NOS3 is transcriptionally regulated by LH. Although the time-course experiment with LH suggested that the mRNA increase in NOS3 was a relatively late event in the preovulatory cascade, the experiments using the NOS activity inhibitor L-NAME indicated that NO generated in the first hours after LH challenge was essential for the expression of important preovulatory genes like EREG and AREG (figure 5; chapter 3; article 2). These results demonstrate that NO generated at lower levels by basally expressed NOS3 enzymes is essential for LH-induced signaling cascade. A similar effect could be observed when cells were challenged with EGF with or without L-NAME. Although we did not study the mechanisms of activation of NOS3 in the first hours after LH or EGF treatments, our results indicated that NO production increased in bovine granulosa cells in the first 3 hours after challenge (Figure 4; chapter 3; article 1). These results suggest that NOS3 is post-translationally regulated in our periovulatory model and that NO produced by this initial regulation is critical for the early events of the ovulatory cascade. Although not verified in our studies, it is known that phosphorylation of NOS3 at specific residues causes the enzyme to produce higher levels of NO [169].

In support of the importance of NOS3 activity, the blockage of NOS3 with L-NAME resulted in the inhibition of PTGS2 mRNA levels at 6 h and PTGS2 protein levels at 12 h after LH challenge (figure 5; chapter 3; article 2). Interestingly, PGs alone stimulated NOS3 mRNA abundance (figure 3; chapter 3; article 2), indicating the existence of a positive feedback loop between NO and PGs; and potentially explaining the late increase in NOS3 mRNA levels observed only at 12 h post-LH. The biological

actions of prostaglandins have been attributed to their interaction with cell surface G protein–coupled receptors, FP for PGF2α and EP2, EP3, and EP4 for PGE2. Both EP2 and EP4 receptors typically couple to Gs to increase dramatically and immediately cAMP levels in the cytoplasm [32]. In bovine, the LH/FSH surge also modulates the levels of mRNA for PGs receptors. In granulosa cells, mRNA levels for EP2 increase post-GnRH and remain elevated. In contrast, early in the periovulatory period, while EP4 mRNA levels increase transiently, EP3 mRNA levels decrease transiently [76]. In swine cerebral microvascular endothelium, it has been reported that PGE2 increase NOS3 expression via EP3 receptors. More importantly, in addition to plasma membrane EP3 receptors, in the same cells mentioned above it has been also demonstrated the presence of functional perinuclear EP3 receptors for PGE2. The stimulation of the perinuclear EP3 receptor can induce the expression of NOS3, a process that depends on nuclear envelope K_{Ca} channels, protein kinases, and NF-ĸB [32].

Together the results presented in the 2 articles presented in this thesis confirm our hypothesis that different amounts of NO may be required at different times during follicle development in cattle. It is possible to suggest a "switch" between NOS2 and NOS3. During the establishment of follicle dominance, the high concentration of NO produced from NOS2 expression/activity seems to be crucial, but in the periovulatory period, especially in the early events of preovulatory cascade, lower levels of NO generated from NOS3 activity may play a very important role. Although we do not have absolute values for NO produced in each cell model evaluated, the literature indicates different amounts produced by the activity of each NOS, with NOS2 responsable for the highest production [169, 172].

In terms of mechanisms of action, in both article 1 and 2 we decided to use only NOS inhibitors (aminoguanidine and L-NAME, respectively). These are competitive inhibitors and produce a time-dependent inactivation of NO generation [220]. It means that in terms of NO action on granulosa cells, our results are simply based in the evaluation of the consequences of reduction/inhibition of NO generation. Although useful experiments would require also the addition of NO directly to our models, as NO

is a free radical gas, it is difficult to reproduce physiological intracellular concentrations of NO. Many authors employ exogenous NO donors in their experiments. Although useful, NO donors must be used with extreme caution because some of them easily reach cytotoxic levels [213, 252]. Moreover, NO may exert different roles according to concentration. A good example comes from studies of the effects of NO in cancer, showing that NO either promotes or inhibits tumorigenesis. These conflicting findings have been resolved, in part, by the levels of NO donors used such that low levels promote tumor growth and high levels inhibit tumor growth [193]. To reproduce a similar amount of NO produced endogenously by granulosa cells in our differentiation and periovulatory models would require precise measurement of NO concentrations and a detailed search for the more appropriated NO donor and the optimization of its use.

Many of the physiological functions of NO seem to be mediated by cGMP [290]. So, an interesting alternative to test NO effects on granulosa cells in our models could be the use of cGMP analogs and/or drugs that inhibit cGMP generation in response to endogenous NO. However, studies with bovine granulosa cells and COCs indicated that some of the effects exerted by NO (NO donors) on granulosa cell steroidogenesis, as well oocyte maturation can be cGMP-independent [212, 251]. As NO could be acting by more than one mechanism and interacting with different molecules in each of our models we preferred to use only NOS inhibitors and correlate our results with NOS expression/activity patterns and NO generation profile.

The information about NOS regulation and NO function presented in this thesis may be extremely useful for the development or improvement of drugs or techniques to control or improve the efficiency of NO signaling. Pharmacological therapies target increased NO bioavailability by influencing the activity of endogenous NOS. This can be achieved by oral tetrahydrobiopterin (BH4) supplementation for example [291]. Besides the supplementation with co-factors crucial for the NOS enzymatic reaction, supplementation with the substrate L-aginine as a therapeutic approach may also represent an interesting option. A study with poor responder women concluded that oral L-arginine supplementation may improve ovarian response, endometrial receptivity and

pregnancy rate [292]. Interestingly, prolonged treatment with N-acetylcysteine and L-arginine restores gonadal function in patients with polycystic ovary syndrome [293].

NO levels can also be raised by using NO-releasing donor compounds. Several studies have demonstrate that dietary supplementation with NO donors induce benefits in exercise performance, for example [294]. NO donors represent useful means of systemic NO delivery and have been used for many years as effective therapies for many disorders [295]. However, as mentioned previously, the use of exogenous NO donors must be done with extreme caution and optimisation of the delivery compound is an essential step required to avoid systemic toxicity and side effects [296]. Nanotechnology has benefited a number of biomedical areas including drug delivery. The use of nanostructured materials, like nanoparticles, as drug delivery systems has begun to impact medicine due to beneficial size-dependent physical and chemical properties [297]. Nitric oxide-releasing nanoparticles have presented promising results in the acceleration of wound healing [298, 299], antimicrobial efficacy [300] and on the inhibition of ovarian cancer cell growth [301].

Other existent technique to manipulate NO system involves gene therapy. Research has concentrated on comparing the effect of gene delivery of NOS enzymes in healthy and diseased animal models. In intimal hyperplasia, restenosis, vascular tone and ischemia-reperfusion injury, for example, most results demonstrate therapeutic benefits following vascular gene delivery of all NOS in pre-clinical models of cardiovascular disease [302]. The focus of gene therapy has been to deliver the NOS gene directly to the site of injury resulting in a local increase of NO generation. This approach avoids the problem with systemic NO toxicity. The direct delivery of NOS genes to the follicle, especially granulosa cells, could be a tool to solve problems with follicle growth and anovulation.

Although in this thesis we did not the mechanism used by NO to inhibit apoptosis in the growing dominant follicle or mediate the ovulatory cascade in cattle, we clearly identified genes and proteins affected by NO reduction/absence. These data

indicate the importance of NO for the follicle development and growth; and reveal novel direct or indirect NO targets in granulosa cells. An emerging pathway for NO action is S-nitrosylation. This process is the direct modification of proteins by adding a NO moiety to the amino acid cysteine [190, 192]. The impact of S-nitrosylation in healthy and diseased ovaries is unknown. Future studies involving the identification of proteins S-nitrosylated during the follicle dominance establishement and ovulation may permit the selection of potential targets and/or mechanism to be manipulated with the objective of improving fertility efficiency or even combat ovarian disorders in cattle and women.

Final conclusions

In conclusion, the studies reported in this thesis not only confirm the presence of an active nitric oxide system in bovine granulosa cells, but also demonstrate that nitric oxide synthase expression and activity are regulated by gonadotropins, estradiol and growth factors throughout follicle development and growth in cattle. Physiological levels of nitric oxide contribute to the survival of granulosa cells, exerting an important role in the establishment of follicle dominance. Moreover, NOS activity seems to be critical for the LH-induced ovulatory cascade in granulosa cells and NO may be essential for ovulation in cattle. We provide novel insight regarding the role of NO upstream of PTGS2. To our knowledge, this is the first study indicating an interaction between NO and EGF-like factors during the preovulatory period.

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