Glucocorticoid production and action in bovine luteinized granulosa cells

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ウシ黄体化顆粒膜細胞における糖質コルチコイド産生と作用

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要約

黄体化顆粒層細胞（LGC）において progesterone（P4）と cortisol，および黄体化の指標である pentraxin 3（*PTX3*），regulator of G protein signaling 2（*RGS2*）と vanin 2（*VNN2*）の発現はともに上昇するが，それらの関係性についてはよく分かっていない。そこで本研究では P4 と cortisol が互いの生産に及ぼす影響と，これらのステロイドが上記の遺伝子発現に与える影響をウシ LGC の培養系を用いて調べた。Chapter 3 ではウシ顆粒層細胞の黄体化に伴う機能的，形態的変化と cortisol 生産機序について調べた。直径 2-5 mm の卵胞から吸引採取した顆粒層細胞を 10%ウシ胎児血清を含む DMEM/F-12培養液で14日まで培養した。P4生産とそれに関与する steroidogenic acute regulatory protein （*STAR*），cholesterol side-chain cleavage enzyme （*CYP11A1*），および 3β-hydroxysteroid dehydrogenase type 1 （*HSD3B1*）の発現は培養開始後 10 日まで増加し，その後高いレベルで推移した。P4 から cortisol への変換を仲介する 2 つの酵素のうち 21-hydroxylase （*CYP21A2*）の発現は低く，4 日以降にさらに低下した。Cortisol 生産の最終段階を担う 11β-hydroxylase（*CYP11B1*）の発現は認められなかった。また培養期間中に高い P4 の生産が認められたにも関わらず，P4 からの cortisol 生産は認められなかった。一方で cortisone から cortisol への変換を仲介する 11β-hydroxysteroid dehydrogenase type 1 （*HSD11B1*）の発現は 2 日目に顕著に増加した後，8 日目にかけて減少し，その後はほぼ一定に保たれた。Cortisol 生産は HSD11B1 の基質である cortisone を培地中に加えた時のみに認められた。LGC で副腎皮質において cortisol 合成を刺激する主要な因子である ACTH と angiotensin II（Ang II）の受容体の発現が認められたことから，これらの因子が LGC の cortisol 生産に与える影響を 6 日目の LGC を用いて調べた。ACTH，もしくは Ang II による 24 時間の処置は LGC の P4 生産を抑制したが，cortisol 生産に関与する *CYP21A2*，*CYP11B1*，H*SD11B1* のいずれの発現にも影響を与えなかった。Chapter 4 では P4 が cortisol の生

産および黄体化関連因子発現に与える影響を調べた。6日目，もしくは12日目のLGCをtrilostane（P4合成阻害剤），nomegestrol acetate（NA，合成プロジェスタージェン），もしくはP4の存在下で24時間培養した。TrilostaneによりP4生産 阻害された結果 *HSD11B1*，*HSD3B1*，*PTX3*，*RGS2*，および *VNN2* の発現と cortisol の生産の増加が認められた 一方，*STAR* 発現の低下が認められた。NA もしくは P4 の同時添加は容量依存的に trilostane の効果を低減させ，*HSD11B1*，*HSD3B1*，*PTX3*，*RGS2*，および *VNN2* の発現と cortisol の生産を低下させ，*STAR* と *HSD11B2* の発現を増加させた。Chapter 5 では cortisol が P4 の生産および黄体化関連因子発現に与える影響を調べた。cortisol は容量依存的に *HSD11B1*，*HSD3B1*，*PTX3*， *RGS2*，および *VNN2* の発現を増加させ，*STAR* と *HSD11B2* の発現を低下させたが，P4 合成には影響を与えなかった。以上のことから，1）ウシ LGC は局所で生産される P4 を基質とした cortisol 生産は行えないが，副腎皮質由来の cortisone を基質とした *HSD11B1* による cortisol 生産を行っていること，2）プロジェスタージェンは *HSD11B1* の発現を抑えることで局所的な cortisol の生産を抑制すること，3）プロジェスタージェンと cortisol は *STAR*，*HSD3B1*，*HSD11B1*，*HSD11B2*, *PTX3*，*RGS2*，および *VNN2* の発現を異なる方向に調節することが明らかになった。このことは黄体化の過程で P4 と cortisol が共に顆粒層細胞で生産され、自己分泌的に黄体機能の調節を行っていることを示唆するものである。

Abstract

Progesterone (P4) and cortisol production and expression of luteinizing markers: pentraxin 3 (*PTX3*), regulators of G-protein signaling 2 (*RGS2*), and vanin 2 (*VNN2*), increase in luteinized granulosa cells (LGCs), but their relationship is not well established. Therefore, this study investigated the effects of P4 and cortisol on each other's production and their effects on the expression of the above genes in cultured bovine LGCs. In chapter 3, the functional and morphological changes associated with luteinization of granulosa cells (GCs) and the mechanism of cortisol production were investigated. GCs were collected from follicles of 2–5 mm diameter and cultured in DMEM/F-12 supplemented with 10% fetal calf serum (FCS) for up to 14 Days. P4 production and expression of steroidogenic acute regulatory protein (*STAR*), cholesterol side-chain cleavage enzyme (*CYP11A1*), and 3β-hydroxysteroid dehydrogenase type 1 (*HSD3B1*) rapidly increased until Day 10 and stayed high thereafter. The expression of 21-hydroxylase (*CYP21A2),* one of the two enzymes required in the conversion of P4 into cortisol, was low and decreased further after 4 Days, whereas the expression of 11β-hydroxylase (*CYP11B1*), which is responsible for the final stage of cortisol production, was not detectable. Moreover, cortisol production from P4 was not detected, although high P4 production was observed during the culture period. On the other hand, the expression of 11β-hydroxysteroid dehydrogenase type 1 (*HSD11B1*), which converts cortisone to cortisol, dramatically increased on Day 2, decreased until Day 8, and stayed relatively constant thereafter. Cortisol production was only observed when cortisone, a substrate for HSD11B1, was added to the culture medium. Because the LGCs expressed receptors for adrenocorticotrophin hormone (ACTH) and angiotensin II (Ang II), which are major factors that stimulate cortisol in the adrenal cortex, the effects of these factors on cortisol production by the LGCs were investigated on Day 6 of culture. A 24-h treatment with ACTH or Ang II downregulated P4 production by the LGCs but did not affect the expression of *CYP21A2*, *CYP11B1*, or *HSD11B1*, and cortisol production was undetected. In Chapter 4, the effects of P4 on cortisol production and the expression of factors related to luteinization were investigated. LGCs were treated with trilostane (a P4 synthesis inhibitor), nomegestrol acetate (NA, a synthetic progestogen), or P4 for 24 h on Days 6 and 12 of the culture. Trilostane suppressed P4 production and *STAR* expression while elevating *HSD11B1* and *HSD3B1, PTX3, RGS2*, and *VNN2* expression and cortisol production. Concomitant treatments with NA or P4 dose-dependently decreased cortisol production, *HSD11B1*, *HSD3B1*, *PTX3, RGS2*, and *VNN2* expression but

elevated *STAR* and *HSD11B2* expression in both Day 6 and Day 12 LGCs. In Chapter 5, the effect of cortisol on P4 production and the expression of factors related to luteinization were examined. Cortisol increased *HSD11B1*, *HSD3B1*, *PTX3*, *RGS2*, and *VNN2* expression while decreasing *STAR* and *HSD11B2* without influencing P4 production. Altogether, these results indicate that: 1) bovine LGCs cannot produce cortisol using the locally produced P4 as a substrate but produce cortisol via the HSD11B1 pathway using the adrenal-sourced cortisone as a substrate; 2) progestogens suppress cortisol production by regulating *HSD11B1* expression; and 3) progestogens and cortisol differentially regulate *STAR*, *HSD3B1*, *HSD11B1*, *HSD11B2*, *PTX3, RGS2*, and *VNN2* expression in bovine LGCs. This suggests that both P4 and cortisol are produced by GCs during luteinization and that they regulate luteal function in an autocrine manner.

CHAPTER 1. General Introduction

1.1. Introduction

In livestock farming, reproduction is an essential aspect of evaluating efficiency. Reproductive efficiency has a major influence on the economics of livestock farming. This, therefore, highlights the importance of establishing and maintaining a pregnancy and successful parturition.

The corpus luteum, a transient endocrine organ in the ovary, produces the hormone progesterone (P4), which is essential for preparing the uterus for pregnancy establishment and maintenance in mammals. Therefore, it is very important to maintain an adequate supply of P4 by maintaining a functioning CL to meet these demands. An inadequate supply of P4 has been associated with inadequate or abnormal luteal function, which substantially has a negative impact on fertility. P4 also plays an important role in regulating the estrous cycle. Put together, this makes it very important to understand the factors that influence CL formation and function for intervention purposes, such as assisted reproduction.

In preovulatory follicles, a cascade of complex biochemical and morphological events takes place, leading to ovulation and CL formation. Several studies have been conducted to understand the complex series of events and the diverse signaling pathways in follicular cells during this period. However, due to the complex nature of events, they are not fully understood. One aspect of these events that is not fully understood is the role of the elevated cortisol level as well as its interaction with the concurrently elevated progesterone (P4) level during this period.

1.1.1. Events in preovulatory follicles

1.1.1.1. Progesterone production and action

During the follicular phase of the estrous cycle, when circulating P4 levels are low, the preovulatory follicle produces high levels of estradiol (E2), which stimulates gonadotropin-releasing hormone (GnRH) pulse frequency in the hypothalamus via positive feedback. This causes an increase in luteinizing hormone (LH) secretion, resulting in the LH surge [1]. Pre-ovulatory LH pulses are essential for the final stage of follicle maturation, ovulation, and are thought to stimulate and prepare steroidogenic cells, granulosa cells (GCs) and theca cells (TCs), in the follicle for luteinization [2]. The ensuing LH surge activates the processes of ovulation,

oocyte meiosis, cumulus cell oocyte complex expansion, and luteinization. The subsequent decrease in LH level is linked to a significant decrease in the expression of the E2-producing enzyme aromatase and E2 production by GCs. This leads to increased P4 production, which coincides with luteinization and follicular rupture [3]. To produce P4, cholesterol is initially moved by the steroid acute regulatory (STAR) protein into the mitochondria, where the cytochrome P450 side-chain cleavage (CYP11A1) enzyme in the inner membrane converts it into pregnenolone. The 3-hydroxysteroid dehydrogenase (HSD3B) in the mitochondria and endoplasmic reticulum then converts pregnenolone into P4 [4]. Locally produced P4 plays a crucial role in reproductive function. It is involved in regulating the estrus cycle, ovulation, implantation, and pregnancy [5–8]. In GCs, P4 has been shown to inhibit proliferation, leading to inhibited follicular development [9–11]. Studies have also shown that P4 enhances its own secretion and prevents apoptosis in luteal cells [12–16] and GCs [9,13,17–20]. Research has also shown that P4 mediates its actions through two P4 nuclear receptors, PRA and PRB [21]. Because P4 is involved in these important physiological processes, it is an important factor in reproductive function.

1.1.1.2. Luteinization and its markers

Luteinization is a term that refers to the morphological and biochemical changes in the TCs and GCs in the preovulatory follicle in response to LH stimulation. This differentiation process of the steroidogenic cells shifts their primary steroid production from E2 to P4 during the transition from preovulatory follicle to CL. As a result of several studies in vitro and in vivo, luteinization has been characterized by significant alterations in tissue structure, extracellular matrix adhesion proteins, angiogenesis, intracellular signaling, hormone production, and the regulation of genes and the cell cycle [22–25]. Generally, luteinization is characterized by a remarkable elevation in P4 production and expression of the renowned steroidogenic proteins involved in its production: STAR, CYP11A1, and HSD3B [26–31]. Therefore, the increase in the expression of these proteins and P4 production is a hallmark of luteinization. Recently, genomic studies in GCs showed that the regulators of G-protein signaling 2 (*RGS2*), pentraxin 3 (*PTX3*), and vanin 2 (*VNN2*) were among the many other genes upregulated during the LH surge. Therefore, these genes have recently been referred to as "luteinizing markers" [32–35].

Pentraxin 3 (PTX3) is a prototype long pentraxin that belongs to a group of pentraxin proteins that have displayed significant evolutionary conservation and are expressed in various types of cells. A wide variety of cell types,

especially mononuclear phagocytes, dendritic cells, fibroblasts, and endothelial cells, swiftly produce and release PTX3 in response to basic inflammatory cues such as those from tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and those involving the Toll-like receptor [TLR] [36–38]. PTX3 expression has been shown to provide protection and defense against neurodegeneration, myocardial damage, adverse effects of stress, and infections from fungal, bacterial, and viral pathogens [38–40]. PTX3 is expressed in the female reproductive tissues, including those of the ovary and uterus, and has been shown to play an important role in ovarian function [33,35,41–43].

Regulators of G-protein signaling (RGS) are a set of proteins that regulate G protein-coupled receptor (GPCR) signaling. Several investigations have demonstrated that some RGS proteins are expressed substantially and constitutively in resting cells. On the contrary, RGS2 is generally modestly expressed but is significantly upregulated when stimulated by various stimuli and in various types of cells [44–48]. The elevated expression of RGS2 has been associated with the modulation of several biological processes, such as immunological responses, bone formation, and cardiovascular function [49].

Vanins are significantly homologous proteins that are encoded by the VNN1, VNN2, and VNN3 orthologs and have a comparable genomic structure [50,51]. These proteins are well-known for their pantetheinase activity in mammalian tissues. In this activity, pantetheine is hydrolyzed to produce pantothenic acid, which is commonly known as vitamin B5 or pantothenate, and cysteamine. Pantothenic acid functions to protect against cellular oxidative stress by increasing glutathione levels [52] and as a substrate for the biosynthesis of coenzyme A. Coenzyme A serves as a cofactor in the biosynthesis of fatty acids and the oxidation of pyruvate to power the citric acid cycle [53], among several other biological functions. The fatty acids are very essential for steroidogenesis in steroid-producing organs. Cysteamine also provides significant protection against oxidative stress and type 1 diabetes in islet cells [54,55].

PTX3, RGS2, and VNN2 are also expressed in other ovarian cells and tissues, such as cumulus cells, TCs, and the CL, and they have been associated with fertility functions in female reproductive studies [38,56–59]. In the cumulus oocyte complex, PTX3 plays a role in cumulus expansion and is therefore important for the successful release of the matured oocyte during ovulation. PTX3 knockout mice experienced infertility as a result of defective ovulation and oocyte fertilization associated with defective cumulus expansion [38,56,57]. Studies

have also shown that PTX3 mediates the pro-survival actions of interferon tau in luteal endothelial cells and luteinized GCs (LGCs) [60], and its expression is higher in CL during pregnancy than during regression [42]. On the other hand, RGS2 knockdown in early mouse embryo development resulted in abnormal embryo development or embryo arrest at 2 or 4 cells [58]. The downregulation of VNN2 expression in GCs from cows with severe negative energy balance at 60 Days postpartum was associated with suboptimal fertility [59].

The expression of *PTX3*, *RGS2* and *VNN2* has only recently attracted attention in luteinization studies [32–35]. therefore, their function and regulation in the luteinization process are not well understood. They may play important roles in the inflammatory process of ovulation as is the case in inflammatory responses of extraovarian cells [36–40,60–72]. Additionally, although the two C_{21} steroids, P4 and cortisol, are present during the luteinizing process which starts during the periovulatory period [73–76], their influence on the expression of *PTX3*, *RGS2* and *VNN2* in LGCs is unknown.

1.1.1.3. Glucocorticoids in preovulatory follicles

Cortisol (corticosterone in rodents) is the prime glucocorticoid hormone produced by the adrenal glands in the adrenal cortex. To produce cortisol, the 21-hydroxylase (CYP21A2) converts P4 to 11-deoxycortisol (11-DOC), a weak but active glucocorticoid. The 11-DOC is thereafter converted to cortisol (corticosterone in rodents) through a cascade of reactions mediated by 11β-hydroxylase-1 (CYP11B1) [77,78]. Cortisol secretion follows a diurnal pattern that is regulated by the hypothalamic-pituitary-adrenal (HPA) axis. The corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) of the hypothalamus stimulate the corticotropes of the anterior pituitary to secrete adrenocorticotrophin hormone (ACTH). In turn, ACTH stimulates the zona fasciculata of the adrenal cortex to produce glucocorticoids. Elevation of the circulating cortisol levels is prompted by stress or physiological signals, but these levels still follow the diurnal pattern. The diurnal release pattern is a result of cortisol's negative feedback on the hypothalamus and anterior pituitary, which suppresses the additional release of CRH and ACTH, respectively, and balances the HPA feedforward [79,80].

Although glucocorticoids are classically known to be produced in the adrenal glands, there is a growing body of evidence suggesting extra adrenal production of glucocorticoids in various organs, including the ovaries. It was previously thought that the ovary does not express the necessary enzymes for the de novo production of corticosteroids. However, studies in the recent past have suggested otherwise. Studies have shown that follicular

cortisol levels in cattle and humans are higher than those in their serum during the periovulatory period [73,74]. In humans and macaques, GCs primed with FSH and treated with hCG expressed *CYP21A2* [81,82] and produced 11-DOC. CYP11B1 is expressed in the pig ovary [83], and it is LH/hCG inducible in the murine ovary [84]. In cattle, the TL and GC from small/large and healthy/atretic follicles have been shown to express CYP21A2, but barely express CYP11B1. That study also showed that both *CYP21A2* and *CYP11B1* were more apparently expressed in the mid-luteal CL [85]. Conversely, other researchers have demonstrated that the follicular wall of cattle ovaries can produce cortisol via the de novo pathway [86]. If this pathway is functional in bovine GCs, then cortisol production is bound to increase with the increased P4 production that comes with luteinization, which may explain increased cortisol levels in the luteinizing follicles during the periovulatory period.

In glucocorticoid target tissues like adipose tissue, the brain, liver, and lung, cortisol can also be generated from the circulating adrenal-sourced inert cortisone in a reaction mediated by a bidirectional enzyme called 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) [87]. On the other hand, its isoform, the type 2 (HSD11B2) enzyme, is a unidirectional enzyme that converts cortisol to cortisone. As a result, the two HSD11B isoforms regulate glucocorticoids' local levels and activities. The expression and functionality of the glucocorticoid metabolizing enzymes HSD11B1 and HSD11B2 in the ovary have been described by many researchers. They have been shown to be expressed in many ovarian cell types, including the oocyte, cumulus cells, GCs, TCs, luteinized GCs, corpus luteum, and ovarian surface epithelium. Ovarian expression of these two enzymes has been shown to be developmentally regulated. The GCs, in particular, express HSD11B2 before exposure to the LH surge or ovulatory dose of LH/hCG, but express HSD11B1 during luteinization. The increased expression of HSD11B1 in the GCs of the luteinizing follicles corresponds with the increased follicular cortisol levels. This indicates that the increased HSD11B1 expression during the periovulatory period leads to increased HSD11B1-mediated cortisol production from the circulating adrenal-sourced cortisone and thus increased cortisol levels. Similarly, HSD11B1-mediated cortisol production increases during the early to mid-luteal stage of the CL [88,89].

In the target tissues/cells, glucocorticoids act via the glucocorticoid receptor (NR3C1) to regulate various physiological processes such as protein and carbohydrate metabolism, immune and stress responses, skeletal

growth, cardiovascular function, reproduction, and cognition. NR3C1 is expressed in follicular cells, CL, and other ovarian tissues [90]. Therefore, the increased cortisol levels suggest roles for cortisol during the periovulatory period and the ensuing CL formation process. However, the role of cortisol during these events is not fully understood. Cortisol is thought to play anti-inflammatory roles during the ovulatory process and wound healing after ovulation because it is induced and increased by LH/hCG, along with other biochemical reactions like cytokine production, prostaglandin synthesis, histamine release, and proteolysis [91,92]. Therefore, cortisol may protect LGCs in the ovulatory follicles from the adverse effects of the inflammatory process of ovulation. Cortisol may also promote luteinization, as it has been shown to increase P4 production by GCs. Cortisol may also be important during the events that follow ovulation, as glucocorticoids have also been shown to suppress apoptosis induced by cytokines and other stimulants in rat and human GCs [93–95] and cattle CL [89].

Put together, the evidence above suggests cortisol and P4 interaction in LGCs, as there seems to be the presence of the two metabolic pathways for cortisol production in LGCs and the possibility of P4's influence on these processes. The above evidence also indicates that the LGCs are exposed to circulating and possibly locally synthesized glucocorticoids that may act in both an endocrine and an autocrine manner to regulate their physiology. Therefore, the studies in this dissertation endeavored to explore these possibilities by investigating the effects of P4 and cortisol on each other's production and their effects on the expression of luteinizing markers (*PTX3*, *RGS2*, and *VNN2*) in *in vitro* cultured bovine LGCs. For this purpose, the following were examined: cortisol and P4 production in Chapter 3; P4's influence on cortisol production and luteinizing markers in Chapter 4; and the effects of cortisol on P4 production and luteinizing markers in Chapter 5.

STAR

Cholesterol shuttle

Cholesterol

| **CYP11A1**

Pregnenolone

| **HSD3B**

Progesterone

| **CYP17A1**

17α-Hydroxyl progesterone

| **CYP21A2**

11-Deoxycortisol

| **CYP11B1**
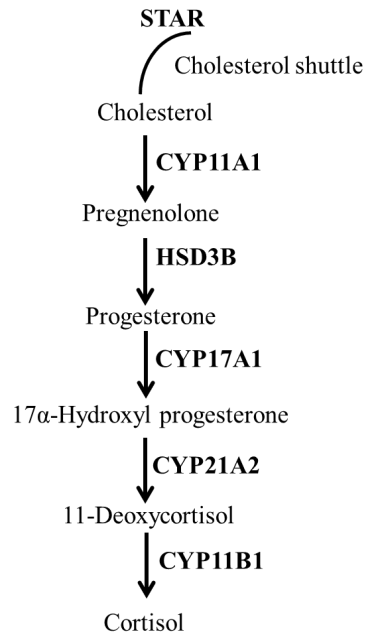
Cortisol

Figure 1.1. The biosynthesis pathway for the conversion of cholesterol to progesterone and subsequently cortisol in the adrenal glands.

CHAPTER 2. General Materials and Methods

All chemicals and hormones used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.1. Granulosa cell preparation

Bovine ovaries were harvested from a local slaughterhouse and brought to the laboratory in saline containing penicillin (200 U/mL; Meiji Seika Pharma Co. Ltd., Tokyo, Japan) and streptomycin (100 µg/mL; Meiji Seika Pharma). Using a syringe fitted with an 18-gauge needle, follicles ranging in diameter from 2 to 5 mm were aspirated. Under a microscope, only clear sheets of GCs were carefully collected from the aspirates and pooled. The pooled cells were centrifuged for pelleting, and the supernatant was removed. The cells were then subjected to three series of washing in PBS and centrifuging at 1800 rpm for 10 min. The pelleted cells were dispersed using Accumax (Innovative Cell Technologies, San Diego, CA, USA) and filtered through a 200 µm strainer (PluriSelect, Leipzig, Germany), washed in Dulbecco's Phosphate-Buffered Saline (DPBS), and pelleted. The red blood cells in the pellet were then lysed with 200 µL of lysing buffer (Hybri-Max), and the remaining GCs were washed with DPBS by filling the tube up to 15 mL and then pelleted again. The pellet of GCs was resuspended in culture media, and the viable cells were counted using the trypan blue exclusion technique, in which the dead cells take up the dye whereas the live cells remain unstained. Here, 10 µL of cells were diluted 1:1 with 10 µL of 0.4% (w/v) trypan blue solution and left to sit for 3 minutes at room temperature. After that, the number of live cells was counted on a hemocytometer under a phase contrast microscope.

2.2. Granulosa cell culture

GCs were cultured using the method outlined by Shimizu et al. (2009), with some modifications [27]. Briefly, 10^5 cells per well were cultured in 4 or 24 multi-well plates (Nunc; Thermo Fisher Scientific, Roskilde, NY, USA) containing 500 µl of DMEM/F-2 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% [v/v] FCS (Fetal Clone III; Thermo Fisher Scientific, Waltham, MA, USA), 0.1% [w/v] bovine serum albumin (BSA), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml sodium selenium. The GCs were cultured at 37 °C with 5% CO_2 in humidified air. The culture media were changed every 24 h.

2.3. Experimental treatments

Experiment details are presented in the respective chapters. Nevertheless, GCs and spent media were collected at the end of each experiment. At the collection, the GCs were counted, washed, and lysed by vortexing in 200 µL of TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), followed by a spin down, and kept at -20 °C until RNA extraction. Similarly, the spent media were kept at -20 °C until the steroid hormone assay.

2.4. Steroid hormone assay

P4 and cortisol in the spent media were measured using commercial enzyme immunoassay (EIA) kits for P4 and cortisol, respectively (Cayman Chemical Company, Ann Arbor, MI, USA). To assess blank values, cultures without GCs were set up for each experiment, and the blank values were subtracted from the assay results. The P4 assay had a range of 7.8 to 1,000 pg/mL with a detection limit of approximately 10 pg/mL. The cross-reactivities of the P4 EIA antiserum (Item No. 482602) were pregnenolone (61%), 17-hydroxyprogesterone (0.5%), and cortisol (<0.001%). The cortisol assay had a range of 6.6 to 4,000 pg/mL with a sensitivity of approximately 35 pg/mL. The cross-reactivities of the cortisol EIA antiserum (Item No. 400362) were cortisone (0.13%), pregnenolone (<0.01%), and P4 (<0.01%). The intra- and inter-assay coefficients of variation (CVs) for cortisol and P4 were less than 10% and less than 15%, respectively.

2.5. Gene Expression analysis

2.5.1. Total RNA extraction

Total RNA was extracted from GCs using TRIzol Reagent according to the manufacturer's instructions. The GC lysates in 200 µL of TRIzol were spun down. A total of 40 µL of 100% chloroform was then added to each tube and shaken vigorously for 30 sec. The samples were incubated at room temperature for 3 min and then centrifuged at 11500 rpm for 15 minutes at 4 °C to obtain 3 separate phases of the extracts: the colorless upper aqueous phase containing RNA, a cloudy interphase containing DNA, and a red organic phase containing protein. 100 µL of the upper colorless supernatant was removed into a new RNase-free tube with 2 µL of diluted Dr GenTLE (Takara Bio Inc., Otsu, Shiga, Japan), a carrier for DNA/RNA at precipitation. To precipitate the RNA, 100 µL of isopropanol was added to the tubes, mixed, incubated for 10 min at room temperature, and then

centrifuged at 11500 rpm at 4 °C for 15 min to obtain a pellet. The supernatant was discarded, and the RNA pellet was washed with 500 μL of 75% ethanol and then centrifuged at 11500 rpm at 4 °C for 10 min.

2.5.2. Complementary DNA (cDNA) synthesis

To avoid false positive PCR results, contaminating genomic DNA (gDNA) was removed prior to cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, after completely removing ethanol, the RNA pellet was resuspended with a mixture of 1μL gDNA wipeout and 6 μL THE RNA storage solution (Thermo Fisher Scientific). The samples were transferred to 200 μL tubes, incubated at 42 °C for 2 min, and then placed on ice. A 3 μL reverse transcription reaction mixture of 0.5 μL reverse transcriptase, 2 μL reverse transcription buffer (Mg2+ and dNTPs), and reverse transcription primer mix (Oligo-dTs and random primers) was then added to each tube, spun down, and incubated at 42 °C for 30 minutes and then 95 °C for 3 min.

2.5.3. Primer design

Based on the reported bovine sequences (Table 1), primer pairs targeting functional gene transcripts were designed using the Primer-BLAST tool of the National Center for Biotechnological Information (NCBI) [96]. To ensure efficient amplification, products larger than 250 bps were avoided. Two sets of primers were used to quantify progesterone receptor (PR) isoforms. Primers for isoform B (*PRB*) were chosen from the N-terminus region, which is unique to PRB. Additional primers were made to amplify isoforms A and B (*PRA/B*). As previously reported, the isoform A (PRA) expression level was calculated by subtracting the PRB value from the PRA/B value [97].

2.5.4. Quantitative PCR (qPCR)

Quantitative PCRs were performed using FastStart Essential DNA Green Master (Roche Diagnostics GmbH, Mannheim, Germany) and LightCycler Nano (Roche, Basel, Switzerland). The amplification program consisted of an initial activation at 95 °C for 10 min, followed by 45 cycles of these amplification steps: denaturation at 94 °C for 10 sec, annealing at 60 °C for 10 sec, and extension at 72 °C for 15 sec. The authenticity of the PCR products was validated by monitoring the melting curve. Amplification of all genes in this study was carried out in a PCR reaction volume of 10 μL comprising 0.8 μL PCR grade H_2O, 5 μL Green Master 2x conc., 0.1 μL each of forward and reverse primers, and a 4 μL cDNA sample. A positive control (corpus luteum for *STAR*,

CYP11A1, *HSD3B1, PTX3*, *RGS2*, and *VNN2*; GCs from the preovulatory follicle for CYP19A1; adrenal gland for *CYP21A2*, *CYP11B1*, *MC1R*, *MC3R*, *AGTR1*, and *AGTR2*; liver for *HSD11B1* and *NR3C1*; kidney for *HSD11B2*) and a negative control (dH2O) were included in each PCR run. The intra- and inter-assay CVs for all quantifications were less than 10% and less than 15%, respectively. The results were normalized to the geometric means of three stably expressed reference genes, ribosomal protein L4 (*RPL4*), ribosomal protein L15 (*RPL15*), and TATA-box binding protein (*TBP*), as mentioned elsewhere [98].

2.5.5. Statistical analysis

All statistical analyses were conducted using the R statistical software [33]. The normality of the data was tested using the Bartlett test, and the data were transformed to base-10 logarithms after failing the test. The data were analyzed using ANOVA, followed by Tukey's multiple comparison test. The results were expressed as mean ± SEM, and differences between the groups were considered significant at $P < 0.05$.

Table 1.1. List of primers

Gene (bp)		Sequence (5'-3')	GenBank No.	Position[a]
RPL4	F	ACTCCGAGCACCACGCAAGA	NM001014894.1	945-964
(116)	R	TGGTGTTCCTGCGCATGGTCT	NM001014894.1	1060-1040
RPL15	F	GCGGCAGCCATCAGGGTGAG	NM001077866.1	17-36
(90)	R	AGGAAGCGCATCACGTCCGA	NM001077866.1	106-87
TBP	F	GCCTTGTGCTTACCCACCAACAGTTC	NM001075742.1	1133-1158
(200)	R	TGTCTTCCTGAAACCCTTCAGAATAGGG	NM001075742.1	1332-1305
STAR	F	CAGCAGAAGGGTGTCATCAGAG	NM_174189.3	767-788
(149)	R	AGGACCTGGTTGATGATGGTCT	NM_174189.3	915-894
CYP11A1	F	CCCTGAAAGTGACTTGGTTCTTCA	NM_176644.2	1209-1232
(118)	R	GTCAAACTTGTCCGGACTGGAG	NM_176644.2	1326-1305
HSD3B1	F	CCTTGTACACTTGTGCCCTGAG	NM_174343.3	640-661
(118)	R	AACTTGCAGTGATTGGTCAGGA	NM_174343.3	757-736
CYP19A1	F	TTGCAAAGCATCCCCAGGTT	U18447.1	1117-1136
(151)	R	AGGTCCACAACGGGCTGGTA	U18447.1	1267-1248
CYP21A2	F	CCTGGAGCTGTTCGTGGTG	NM_174639.1	1442-1460
(122)	R	GCTGGACCTTGAGGTTGACA	NM_174639.1	1563-1544
CYP11B1	F	CCATCGAAGCCAGCACCTTA	NM_174638.3	592-611
(60)	R	CTGGGCACAAACATGAGCTG	NM_174638.3	730-711
PRA/B	F	AGGGCAATGGAAGGACAGCATA	NM_001205356.1	1717-1738
(139)	R	ACTTTCGGCCTCCAAGAACCAT	NM_001205356.1	1855-1834
PRB	F	ATCTTCCCTCGGTCCTGCCA	NM_001205356.1	178-197
(98)	R	GCTTCCCCTCCGGAATACGC	NM_001205356.1	275-256
NR3C1	F	ACGGCTATTCAAGCCCTGG	NM_001206634.1	1561-1579
(89)	R	GCAGAGTTTGGGAGGTGGTC	NM_001206634.1	1649-1630
HSD11B1	F	AAGCAGACCAACGGGAGCATT	NM_001123032.1	532-552
(111)	R	GGAGAAGAACCCATCCAGAGCA	NM_001123032.1	642-621
HSD11B2	F	CGAGCACTTGAATGGGCAGTT	NM_174642.2	1033-1053
(123)	R	CCTGGGTAATAGCGGCGGAGT	NM_174642.2	1155-1135
PTX3	F	TGGTCGCTGATGCTGTGATT	NM_001076259.2	861-880
(107)	R	GGCCACCGAGTCACCATTTA	NM_001076259.2	967-948
RGS2	F	TCCCAGCGGGAGAGAGATAA	NM_001075596.1	11-30
(158)	R	AGCTCAAACGGCTCTTCCAA	NM_001075596.1	168-149
VNN2	F	ACAGTGTCGCTCTTTGGGAG	NM_001163920.1	1425-1444
(85)	R	TCAGGTACGCTGTTGCTGAG	NM_001163920.1	1509-1490
MC1R	F	CAGGGGAGCCATGAGTTGAG	NM_174108.2	220-239
(115)	R	CGTGCAGTTAAGGGAACCCA	NM_174108.2	334-315
MC3R	F	CTGAGGTCTTCTTGGCCCTG	FJ433881.2	467-486
(154)	R	TTGGACATGCCCTGATGGAC	FJ433881.2	620-601
AGTR1	F	CCGAGTGAGAGCGGACTGAT	NM_174233.2	268-287
(88)	R	AGACCTTCTGGAATGGCTTCA	NM_174233.2	355-335
AGTR2	F	GCTTGTCTGTCCTCATTGCC	AJ277986.1	233-252
(99)	R	CTTCTCAGGTGGGAAAGCCA	AJ277986.1	331-312

[a]Nucleotide position in the reported sequence.

RPL4: ribosomal protein L4, *RPL15*: ribosomal protein L15, *TBP*: TATA-box binding protein, *STAR*: steroidogenic acute regulatory protein, *CYP11A1*: cholesterol side-chain cleavage enzyme, *HSD3B1*: 3β-hydroxysteroid dehydrogenase type 1, *CYP19A1*: Aromatase, *CYP21A2*: 21-hydroxylase, *CYP11B1:* 11β -hydroxylase, *PRA/B*: progesterone receptor isoform A/B, *PRB*: progesterone receptor isoform B, *NR3C1*: glucocorticoid receptor, *HSD11B1*: 11β-hydroxysteroid dehydrogenase type 1, *HSD11B2*: 11β-hydroxysteroid dehydrogenase type 2. *PTX3*: pentraxin 3, *RGS2*: regulators of G-protein signaling 2, *VNN2:* vanin 2, *MC1R*: melanocortin receptors 1, *MC3R*: melanocortin receptors 3, *AGTR1*: angiotensin receptors 1, *AGTR2*: angiotensin receptors 2.

CHAPTER 3. Progesterone and Cortisol production

3.1. Introduction

In peri-ovulatory follicles, the LH surge induces the luteinization of GCs and switches the primary steroid production from estrogen to P4 [2,99]. During this period, follicular levels of glucocorticoids are also upregulated [73,74,81]. The concurrent upregulation of P4 production with cortisol levels during this period raises the possibility of de novo cortisol production from P4. While HSD11B1-mediated cortisol production in the ovary is generally accepted by many researchers, the de novo production of corticosteroids in the ovary still remains a debate among researchers.

Several studies have shown the upregulation of HSD11B1-mediated cortisol production in ovulatory follicles and cultured LGCs [73–76]. The increased HSD11B1-mediated cortisol production is also observed during the early to mid-luteal stages of the CL [88,89]. Similarly, recent studies in various species suggested the possibility of CYP21A2 and CYP11B1-mediated cortisol production from P4 in the ovary; LGCs from humans and macaques express CYP21A2 [81,82], and those from murine and pig ovaries express CYP11B1 [83,84]. Amweg et al. (2017) demonstrated that the bovine follicular wall can produce cortisol using this pathway [86]. The bovine CL has also been shown to express both CYP21A2 and CYP11B1 in the mid-luteal stage [85]. If this pathway is active in bovine GCs, then the cells can produce cortisol from P4, and the production may increase with the sharp rise in P4 supply brought on by luteinization. Collectively, these findings suggest that LGCs may produce cortisol via two distinct metabolic pathways.

The studies in this chapter, therefore, used a simple culture system to investigate: 1) the functional and morphological changes associated with luteinization of GCs; and 2) the production of cortisol via the HSD11B1 and CYP21A2/CYP11B1 pathways in bovine LGCs *in vitro*.

3.2. Material and Methods

Details of the collection, culture, and processing of GCs are tabulated in Section 2.2 of Chapter 2.

3.2.1. Experiment 1: Changes in progesterone and cortisol production, and related gene expression in bovine granulosa cells undergoing luteinization *in vitro*

Bovine GCs were cultured for up to 14 Days, and the spent media and cells were collected every two Days to monitor the occurrence of luteinization and the expression of related genes. The production of P4 and expression of related genes and "luteinizing markers," *VNN2*, *PTX3*, and *RGS2*, were investigated in cultured GCs every two Days over a 14-Day culture period. During this period, the de novo production of cortisol and the expression of related genes were also investigated.

3.2.2. Experiment 2: Effect of angiotensin II and adrenocorticotrophin hormone on cortisol production and expression of related genes in bovine luteinized granulosa cells

To investigate stimulated cortisol production via the CYP21A2/CYP11B1 pathway, LGCs were treated with/without doses of Ang II (0.1, 1 µM) and ACTH (0.1, 1 µM) on Day 12 of culture, when P4 production was highest (see results of experiment 1). The production of progesterone and cortisol and the expression of *CYP21A2*, *CYP11B1*, *HSD11B1*, *NR3C1*, angiotensin receptors 1 and 2 (*AGTR1* and *AGTR2*) and melanocortin receptors 1 and 3 (*MC1R* and *MC3R*) were investigated.

3.2.3. Experiment 3: HSD11B1-mediated cortisol production, and expression of related genes in bovine luteinized granulosa cells

HSD11B1-mediated cortisol production was investigated in cultured GCs. On Day 6, the LGCs were treated with/without 100 nM of cortisone, a substrate for HSD11B1-mediated cortisol production, for 24 h. The production of cortisol and expression of *HSD11B1, HSD11B2*, and *NR3C1* were investigated in cultured LGCs. Here, data was analyzed using the Welch two-sample t-test, expressed as mean ± SEM, and significant differences from the control expressed as *$P<0.05$, **$P<0.01$, ***$P<0.001$.

3.3. Results

3.3.1. Changes in progesterone and cortisol production, and related gene expression in bovine granulosa cells undergoing luteinization *in vitro*

The number of GCs and their P4 production continuously increased until Day 10 (Figs. 2.1A, B), while acquiring morphological traits typical of luteinized GCs, as previously described [26,27] (Figs. 2.4A-G). The expression

of steroidogenic acute regulatory protein (*STAR*), cholesterol side-chain cleavage enzyme (*CYP11A1*), 3β-hydroxysteroid dehydrogenase type 1 (*HSD3B1*), and *PRA* increased in the first half of the culture period (Days 2–8), reached a plateau, and remained high in the second half (Days 10–14) (Figs. 2.1C-F). The expression of *PRB* steadily decreased (Fig. 2.1G) throughout the 14-Day culture, resulting in a rapid decrease in the ratio of *PRB* to *PRA* (Figs. 2G, H). A significant level of *CYP19A1* expression was observed on Day 0, which was equivalent to around 7% of the level found in GCs from E2-rich ovulatory follicles. After a steep decline on Day 2, the expression eventually declined to less than 1/100 of the initial value (Fig. 2.2A). *VNN2* and *RGS2* expression sharply declined on Day 2 until Day 4, and then increased steadily during the rest of the culture period (Figs. 2.2B, C). The expression of *PTX3* dramatically increased on Day 2, sharply decreased on Day 4, and continued to decrease for the rest of the 14-Day culture period (Fig. 2.2D).

The 14-Day culture period can be divided into two phases: the proliferating phase (Days 2–8), in which the GCs increased in number and P4 production, and the confluent phase (Days 10–14), in which the cell number and P4 production reached a plateau and remained high.

During the 14-Day culture period, no de novo production of cortisol was detected. CYP21A2 expression was low on Days 0 and 2 and further decreased thereafter (Fig. 2.3A), while CYP11B1 was not detected. The HSD11B1 expression, on the other hand, dramatically increased on Day 2, declined on Days 4 and 6, and remained relatively unaltered thereafter (Fig. 1.3B). The expression of 11β-Hydroxysteroid dehydrogenase type 2 (HSD11B2) and glucocorticoid receptor (NR3C1) paralleled P4 production (Figs. 2.3C, D).

3.3.2. Effect of angiotensin II and adrenocorticotrophin hormone on cortisol production and expression of related genes in bovine luteinized granulosa cells

Treatment with doses of ACTH significantly suppressed P4 production while increasing MC1R and MC3R. A similar trend was observed in Ang II-treated GCs. Both Ang II and ACTH did not influence the expression of *CYP21A2*, *CYP11B1*, or *HSD11B1* in GCs. ACTH tended to increase the expression of *NR3C1* (Figs. 2.5A-H). In all the GCs, no *de novo* cortisol production was detected.

3.3.3. HSD11B1-mediated cortisol production, and expression of related genes in bovine luteinized granulosa cells

Cortisol production and gene expression were compared between the GCs treated with or without cortisone. Although there was no significant difference in P4 production or the expression of *HSD11B1*, *HSD11B2*, or *NR3C1*, cortisol production was significantly higher in GCs treated with cortisone (Figs. 2.6A-E).

3.4. Discussion

In ovulatory follicles, luteinization of GCs results in the shift of their primary steroid production from estrogen to P4. During this period, HSD11B1-mediated cortisol production from cortisone [3–6] and possibly CYP21A2 and CYP11B1-mediated cortisol production from P4 [23–28] by ovulatory follicles also increases. Although cortisol production by GCs via the HSD11B1 pathway is comparably well documented, its production via CYP21A2/CYP11B1 is still not clear. In this study, it was found that LGCs could not synthesize cortisol via the CYP21A2/CYP11B1 pathway but were capable of HSD11B1-mediated cortisol production.

The increase in P4 synthesis, the expression of *STAR*, *CYP11A1*, and *HSD3B1*, the changes in phenotype, and the decrease in CYP19A1 expression by the GCs during the 14-Day culture indicated the luteinization of the GCs, as was previously reported [26–31]. On this basis, the cultured GCs in this and the next chapters were referred to as "LGCs". The LGCs barely expressed *CYP21A2* and did not express *CYP11B1*, the genes for the enzymes that are crucial for the de novo production of cortisol from P4. Moreover, although P4 increased during the 14-Day culture period, cortisol production was not detected. On the other hand, the LGCs expressed appreciable levels of *HSD11B1* and could produce cortisol from the supplemented cortisone.

In the adrenal glands, cortisol production via the CYP21A2/CYP11B1 pathway is known to be stimulated by ACTH [77,78], but stimulation by Ang II has also been demonstrated [100]. In this study, the LGCs expressed receptors for ACTH, the *MC1R* and *MC3R*, and, for Ang II, the *AGTR1* and *AGTR2*, thereby suggesting their actions on the LGCs. Even though both ACTH and Ang II downregulated P4 production by the LGCs, the hormones did not influence their expression of *CYP21A2*, *CYP11B1*, *HSD11B1*, or *NR3C1*. Additionally, cortisol production was undetected in both ACTH- and Ang II-treated and non-treated LGCs. However, ACTH appeared to enhance its effect on P4 production by upregulating the expression of its receptors in the LGCs. Put together, these results suggest that bovine LGCs are unable to produce cortisol from P4 but are capable of

converting the adrenal-sourced cortisone to cortisol. Similarly, in humans and macaques, luteinized GCs expressed *CYP21A2*, but *CYP11B1* was undetected in those studies [81,82]. On the contrary, Amweg et al. (2017) showed the expression of CYP11B1 in GCs and TCs from healthy bovine follicles at various growth stages, as well as the production of cortisol from P4 by the follicular wall [86]. However, in that study, the shown CYP11B1 expression by GCs may have included very low expression, as evidenced by the large error bars, which indicated a standard deviation greater than the group mean values.

In response to gonadotropins, the expression of the "luteinizing markers" *VNN2*, *RGS2*, and *PTX3* increases in ovulatory follicles [32,35,56,101–105]. In this study, despite the fact that by Day 2 of culture the GCs had begun to transform morphologically, an indication of the onset of luteinization, the expression of *RGS2* and *VNN2* GCs had sharply decreased on that Day and remained low until Day 4, contrary to the events *in vivo* [102,103]. This suggests that gonadotropins may be necessary for the upregulation of these genes during the early stages of luteinization. However, more research is needed to fully understand this. On the other hand, the expression of *PTX3* in LGCs dramatically increased on Day 2 and then steadily decreased thereafter, thereby mimicking the findings *in vivo*, where the expression was upregulated in ovulatory follicles but downregulated in the CL of the estrous cycle [42]. Altogether, this study shows that bovine LGCs express *VNN2*, *PTX3*, and *RGS2* and that their expression levels vary with luteinization.

In this study, the *PRB/PRA* ratio consistently decreased throughout the 14-Day culture, suggesting a decrease in sensitivity to P4 in advanced LGCs.

In summary, studies in this chapter have demonstrated: 1) the luteinization of bovine GCs using a simple *in vitro* model; 2) bovine LGCs primarily produce cortisol from adrenal-derived cortisone through HSD11B1 and not via the CYP21A2/CYP11B1 pathway.
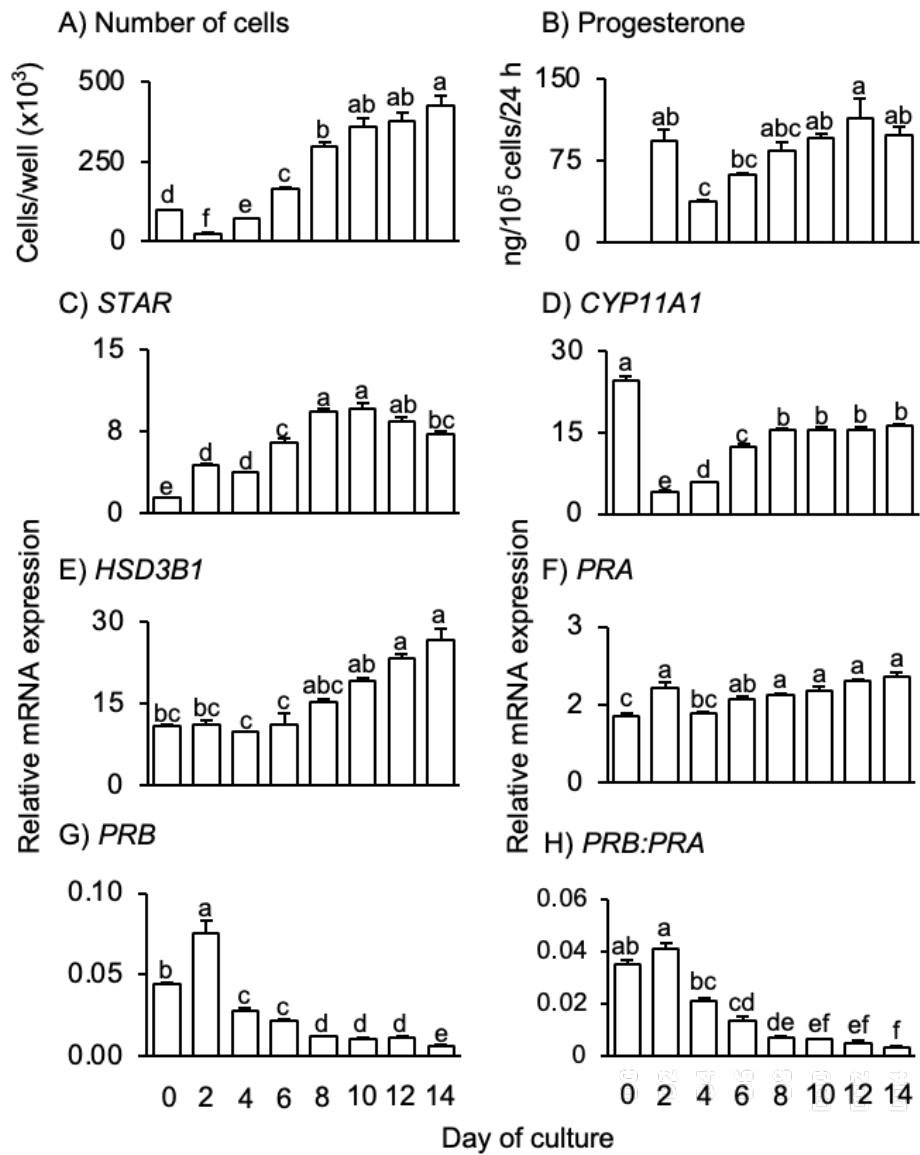
Figure 3.1. Changes in cell number (A), progesterone production (B), and expression of *STAR* (C), *CYP11A1* (D), *HSD3B1* (E), *PRA* (F), *PRB* (G), and the ratio of *PRB* to *PRA* (H) by the bovine granulosa cells cultured for 14 days. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
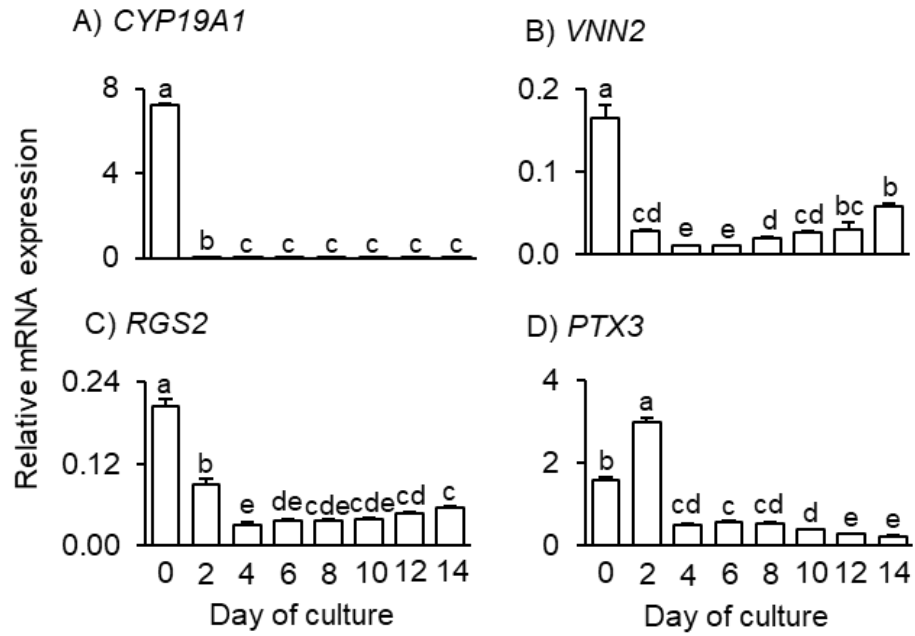
Figure 3.2. Changes in the expression of *CYP19A1* (A), *VNN2* (B), *RGS2* (C) and *PTX3* (D) by the bovine granulosa cells cultured for 14 days. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
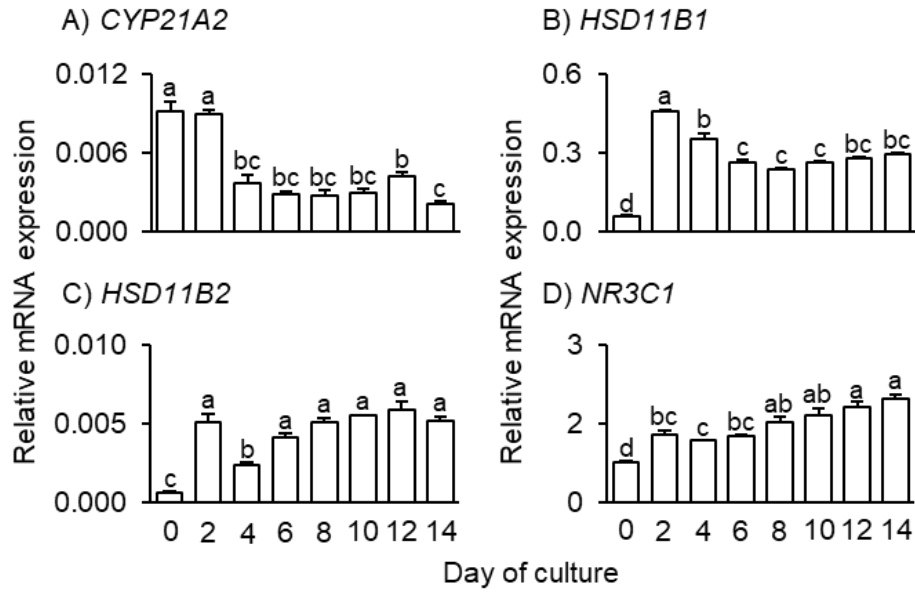
Figure 3.3. Changes in the expression of *CYP21A2* (A), *HSD11B1 (*B), *HSD11B2* (C) and *NR3C1* (D) by the bovine granulosa cells cultured for 14 days. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
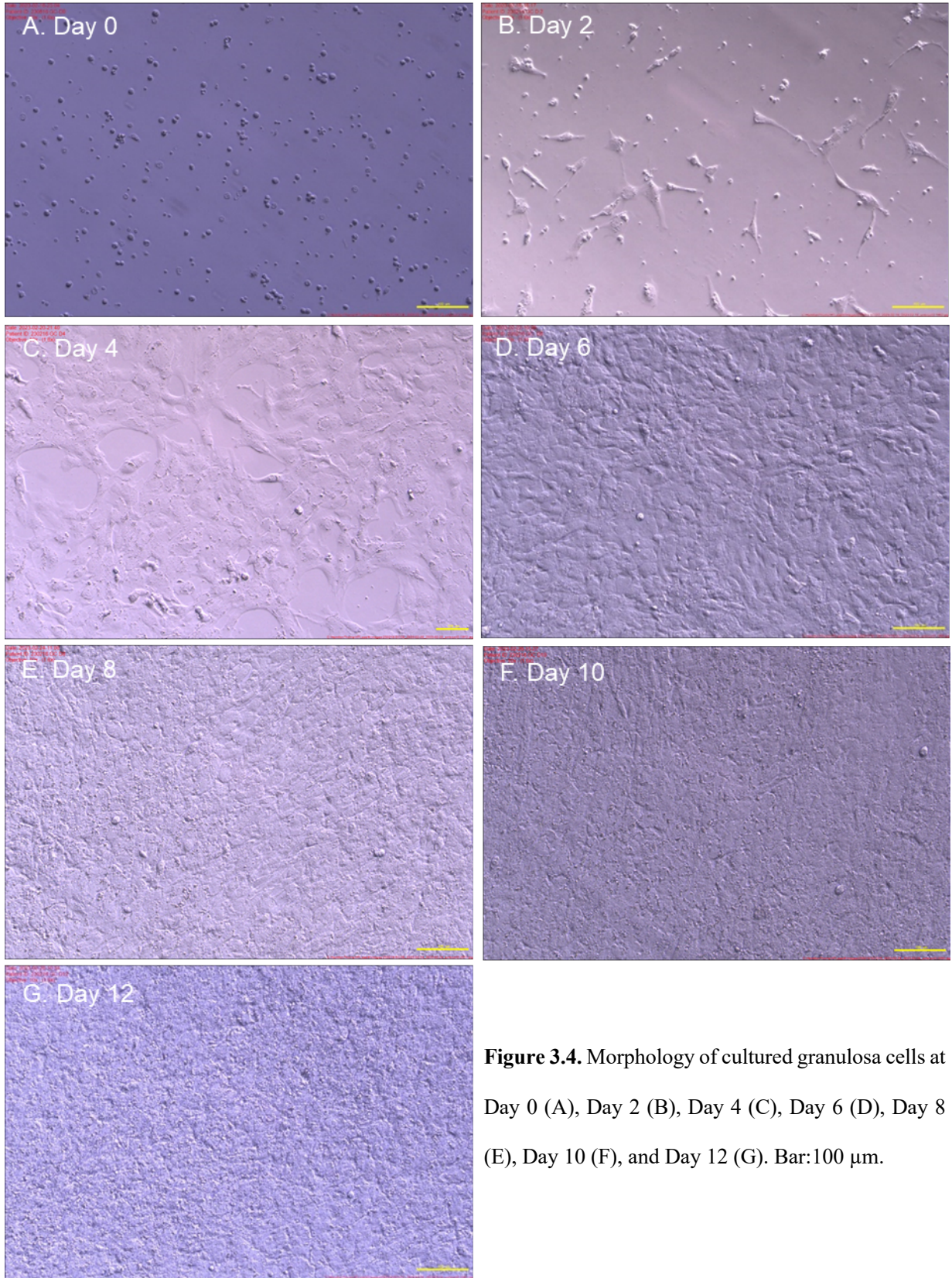
Figure 3.4. Morphology of cultured granulosa cells at Day 0 (A), Day 2 (B), Day 4 (C), Day 6 (D), Day 8 (E), Day 10 (F), and Day 12 (G). Bar:100 μm.
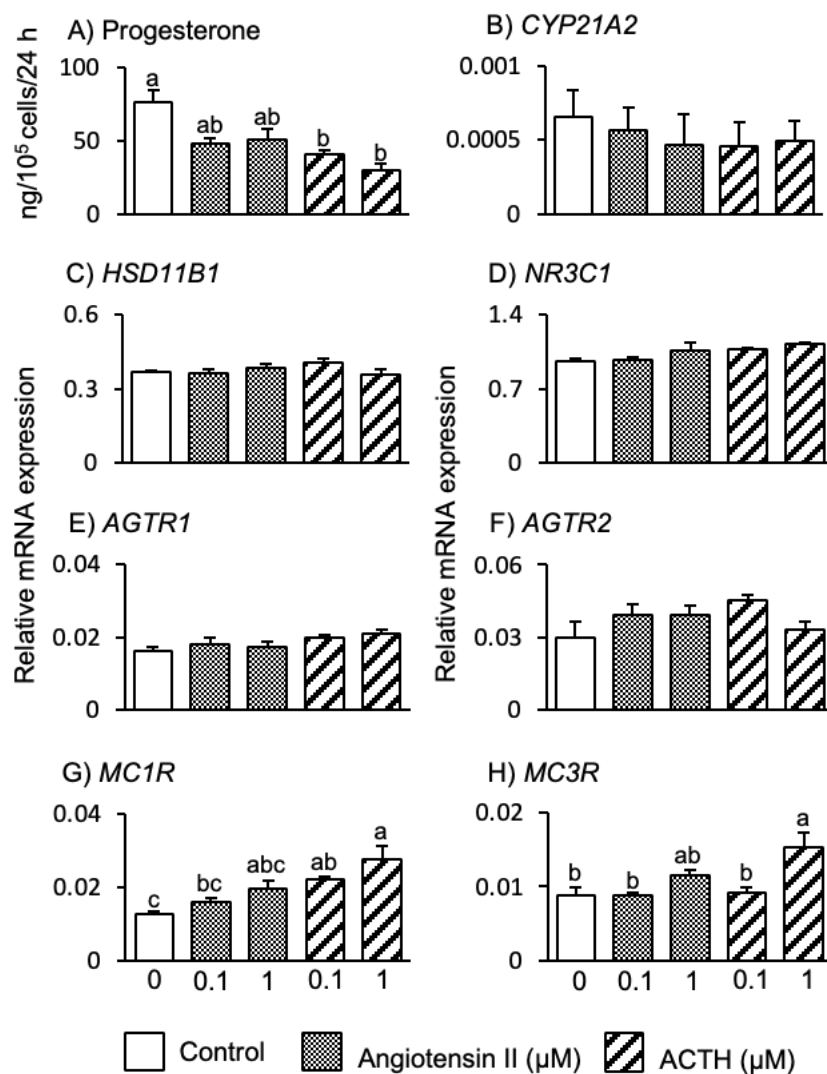
Figure 3.5. Effects of angiotensin II and adrenocorticotrophin hormone (ACTH) on progesterone production (A), and expression of *CYP21A2* (B), *HSD11B1* (C), *NR3C1* (D), *AGTR1* (E), *AGTR2* (F), *MC1R* (G) and *MC3R* (H) expression by luteinized bovine granulosa cells. The cells were treated with/without angiotensin II (0.1, 1 μM) and with/without ACTH (0.1, 1 μM) for 24 h on Day 12 of culture. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
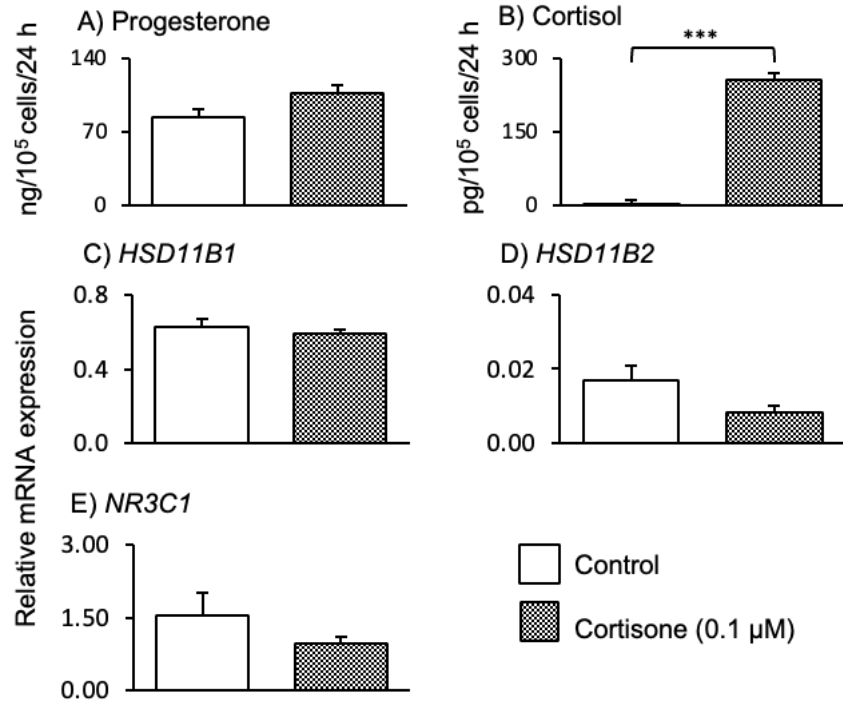
Figure 3.6. Production of progesterone, cortisol and expression of *HSD11B1*(A), *HSD11B2* (B) and *NR3C1* (C) by luteinized bovine granulosa cells (LGCs). Day 6 LGCs were treated with/without 0.1 µM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). *P<0.05, **P<0.01, ***P<0.001: Significantly different from control (Welch two-sample t-test).

CHAPTER 4: Regulation of HSD11B1-mediated cortisol production

4.1. Introduction

P4 production is concurrently elevated with HSD11B1-mediated cortisol production by GCs in luteinizing follicles [73–76]. Similarly, both P4 and HSD11B1-mediated cortisol production increase during the early and mid-luteal stages of the CL [88,89]. Studies have shown the regulation of *HSD11B1* expression in GCs such as LH and cytokines [106,107]. However, although P4 is the main regulatory factor produced by LGCs, to our knowledge, its influence on the glucocorticoid metabolism has not been reported.

In the ovaries, the locally produced P4 acts through the classic nuclear receptor (PR) to play various important roles. In periovulatory follicles, P4 inhibits the apoptosis of GCs [20,108–110] and plays crucial roles in ovulation and luteinization [5,6]. Studies have also shown that P4 regulates ovarian steroidogenesis, as it has been shown to regulate its production by regulating the gene expression of steroidogenic proteins. In GCs and the CL, P4 acts through PR to stimulate its own production and play luteo-protective roles [16,111–115]. Despite numerous studies on P4's roles in the ovary, its effect on cortisol, a co-produced steroid in luteal cells and CL, is unknown. P4 may stimulate cortisol production, as is the case in cumulus cells, where P4 regulates the expression of *HSD11B1* and the HSD11B1-mediated conversion of cortisone to cortisol [116].

In the previous chapter, it was established that the LGCs express *HSD11B1* and are capable of HSD11B1-mediated cortisol production. However, the expression pattern of the HSD11B1 suggested somewhat P4's influence as the gene expression increased when luteinization had set in, decreased when P4 concentration increased in the medium, and remained stable after Day 6 of culture when P4 levels had reached a plateau. The results suggested that P4 may downregulate *HSD11B1* expression in bovine LGCs.

PTX3, *RGS2*, and *VNN2* are expressed in the ovary and are upregulated in the periovulatory follicles, and thus have recently been referred to as "luteinizing markers" [35,56,117,118]. Ovarian studies have shown that *PTX3*, *RGS2*, and *VNN2* are regulated by gonadotropins in GCs [35,56,102,103,117–119]. While P4 concentration is also upregulated during the periovulatory period, its effect on these markers is unknown.

In this chapter, therefore, the above hypothesis was tested, and the effect of progestogens on HSD11B1-mediated cortisol production and expression of luteinization markers was investigated.

4.2. Material and Methods

Details of the collection, culture, and processing of GCs are tabulated in Section 2.2 of Chapter 2.

4.2.1. Experiment 1: Effect of forskolin on progesterone and cortisol production and gene expression in bovine luteinized granulosa cells

Forskolin, a diterpene that stimulates P4 production in ovarian cells, including GCs [120–122], was used to investigate the effect of endogenous P4 on HSD11B1-mediated cortisol production. Here, GCs were treated with/without 10 µM forskolin and with/without 0.1 µM cortisone, a substrate for HSD11B1, for 24 h on Day 6. A similar experiment was conducted with 10 µM forskolin and with/without 10 µM RU 486 (PR and NR3C1 antagonist) in the presence of 0.1 µM cortisone. The effect of forskolin on the expression of *HSD11B1*, *HSD11B2*, *NR3C1*, *STAR*, *CYP11A1*, and *HSD3B1* was also investigated.

4.2.2. Experiment 2: Effects of progestogens on cortisol production and gene expression in bovine luteinized granulosa cells

To investigate the effect of progestogens on HSD11B1-mediated cortisol production, GCs were cultured with/without 10 µM trilostane (3β-hydroxysteroid dehydrogenase inhibitor) and doses of a synthetic progestogen, nomegestrol acetate (NA: 0.01, 0.1, 1, or 10 µM), in the presence of 0.1 µM cortisone, a substrate for HSD11B1, for 24 h on Days 6 and 12. The experiment was repeated using P4 (0.003, 0.03, 0.3, or 3 µM) instead of NA on Days 6 and 12. A similar experiment was also conducted with/without 10 µM trilostane and NA (0.01, 0.1, 1, or 10 µM) on Day 6, but without cortisone to eliminate the effect of cortisol. In these experiments, NA was used instead of P4 to facilitate the simultaneous assessment of the effect of progestogens on cortisol and P4 production. Endogenous P4 synthesis could be measured in the presence of the P4 agonist because NA does not cross-react with the P4 enzyme immunoassay (EIA) antiserum. The effect of progestogens on the expression of *HSD11B1*, *HSD11B2*, *NR3C1*, *STAR*, *CYP11A1*, *HSD3B1*, *PTX3, RGS2*, and *VNN2* was also investigated.

4.3. Results

4.3.1. Effect of forskolin on progesterone and cortisol production and gene expression in bovine luteinized granulosa cells

Treatment with forskolin increased the production of P4, the expression of *CYP11A1*, *HSD3B1*, *PRA*, *PRB*, *HSD11B1*, and *NR3C1*, and the ratio of *PRB* to *PRA* in LGCs (Figs. 3.1A, D-G, I, K). However, it suppressed *HSD11B2* expression in LGCs (Fig. 3.1J). Cortisol production was significantly higher in GCs treated with cortisone but tended to be lower when LGCs were concomitantly treated with cortisone and forskolin (Fig. 3.1B). To determine the influence of endogenous P4 on cortisol production, LGCs were treated with/without forskolin and RU486 in the presence of cortisone. Treatment with forskolin and/or RU486 downregulated cortisol production (Fig. 3.2A). Forskolin elevated *HSD11B1*, *NR3C1*, *CYP11A1*, *HSD3B1*, *PRA*, and *PRB* expression and the ratio of *PRB* to *PRA* while decreasing *HSD11B2* expression (Figs. 3.2B, D, F-I, C), and RU 486 reversed these effects on *HSD11B1* and *HSD3B1* expression.

4.3.2. Effects of progestogen on cortisol production and gene expression in bovine luteinized granulosa cells

Treatment with trilostane suppressed P4 production to less than the assay's detection limit. Trilostane upregulated cortisol production and *HSD11B1* and *HSD3B1* expression (Figs. 3.3A, B, G) but suppressed *HSD11B2* and *STAR* expression in Day 6 LGCs (Figs. 3.3C, E). Treatment with NA dose-dependently reversed these effects. Similar results were observed for cortisol production and the expression of these genes in Day 12 LGCs (Fig. 3.5). NA tended to, and significantly upregulate *CYP11A1* in Day 6 and Day 12 LGCs, respectively (Figs. 3.3F, 3.5F). Treatment with NA dose-dependently downregulated *PTX3*, *RGS2*, and *VNN2* expression in both Day 6 and 12 LGCs (Figs. 3.4 and 3.6). When P4 was used instead of NA, similar results were obtained for both Day 6 and 12 LGCs (Figs. 3.7-3.10). The expressions of *PRA* and *PRB* and the ratio of *PRB* to *PRA* were not affected by progestogens. The ratio of *PRB* to *PRA* decreased by about half in the Day 12 LGCs compared to the Day 6 LGCs for both NA and P4 experiments (Figs. 3.3, 3.5, 3.7, 3.9).

In these experiments, spent media contained up to seven ng/mL (approx. 20 nM) of cortisol at the end of the 24 h culture. To eliminate the possible effect of cortisol, the experiment was repeated with trilostane and NA but without cortisone on Day 6. In the absence of cortisone, trilostane upregulated *HSD11B1* and *HSD3B1*

expression but suppressed *STAR* expression, whereas NA reversed this effect in Day 6 LGCs (Figs. 3.11A, D, F). Lower doses of NA elevated the trilostane-suppressed *PTX3* expression. NA dose-dependently decreased *RGS2* and *VNN2* expression (Fig. 3.12).

4.4. Discussion

Cortisol production via the HSD11B1 pathway increases simultaneously with P4 production by GCs in luteinizing follicles. However, the role of P4, the main regulatory factor produced by LGCs, in glucocorticoid metabolism has not been reported. This chapter demonstrates that progestogens suppress the expression of *HSD11B1* and the conversion of cortisone to cortisol.

In this study, treatment with forskolin on Day 6 of culture increased P4 production and the expression of *HSD3B1*, *CYP11A1*, and *HSD11B1* while decreasing cortisol production by LGCs. HSD11B1 is a bidirectional enzyme with both reductase (which regenerates cortisol from cortisone) and dehydrogenase (which inactivates cortisol into cortisone) activities. Therefore, the results in this study suggest that forskolin may have, directly or indirectly, via increased P4 production, increased the dehydrogenase activity of HSD11B1, which may have decreased cortisol production. Similarly, in rats, forskolin increased the expression of HSD11B1 by GCs from preovulatory follicles. In that study, however, HSD11B1-mediated cortisol production was shown [106].

On the other hand, treatment with RU486 alone or in combination with forskolin decreased both cortisol production and the expression of *HSD11B1* in bovine LGCs in this study. RU486 has a high affinity for both PR and NR3C1, making it an antagonist for both P4 and cortisol actions. Therefore, the results also suggest that cortisol may also regulate its production in bovine LGCs. Put together, the effect of the increased endogenous P4 on HSD11B1-mediated cortisol production was unclear, as treatment with forskolin and RU486 downregulated cortisol production.

When local P4 synthesis was suppressed in both Day 6 and Day 12 LGCs, *HSD11B1* expression and cortisol production increased, whereas concomitant treatment with NA or P4 reversed these effects. These results clearly indicate that locally produced P4 is capable of suppressing HSD11B1-mediated cortisol production in an autocrine manner in the bovine LGCs. The dose-dependent experiments also indicate that high levels of NA or P4 are necessary to suppress cortisol production. In bovine CL, *HSD11B1* expression increased at the developing stage but decreased at the highest P4-producing stage (mid-luteal phase) [89,123]. In the previous chapter, the

expression of *HSD11B1* increased sharply on Day 2 and then decreased gradually afterward as P4 production increased. Taken together, these results suggest that HSD11B1-mediated cortisol production is somewhat suppressed in CL, or LGCs producing high levels of P4.

Progestogens may also suppress cortisol production by increasing *HSD11B2* expression. Although not as consistent as *HSD11B1*, treatment with NA or P4 increased or tended to increase the expression of *HSD11B2* in the present study. If this is the case, P4 suppresses cortisol production by regulating both activating and inactivating enzymes. Nevertheless, the expression level of *HSD11B2* was much lower than that of *HSD11B1*, implying that P4 regulates cortisol production mainly through regulating *HSD11B1* expression in LGCs.

In this study, progestogens regulated the expression of *PTX3*, *RGS2*, and *VNN2* in bovine LGCs in the absence and presence of locally produced cortisol. The expression of *RGS2* and *VNN2* when local production of P4 was suppressed using trilostane indicated P4's suppressive effects on these markers in the absence and presence of locally produced cortisol. This was evident when the progestogens dose-dependently reversed these effects. On the other hand, treatment with trilostane in the absence of locally produced cortisol downregulated *PTX3* expression, and this effect was reversed by lower doses of NA. In the presence of locally produced cortisol, treatment with trilostane upregulated *PTX3* expression, but concomitant treatment with progestogen dose-dependently reversed this effect. This therefore suggests that progestogens situationally regulate *PTX3* expression in LGCs. *PTX3*, *RGS2*, and *VNN2* are upregulated in the periovulatory follicles, regulated by gonadotropins in GCs, and have recently been referred to as "luteinizing markers" [35,56,102,103,117–119]. Considering that their expression is concurrently upregulated with P4 production, the results in the present suggest that their upregulation is predominantly in response to the LH surge and/or other unknown factors and not to P4. However, further studies are necessary to elucidate this. Put together, these results suggest that P4 also regulates the expression of *PTX3*, *RGS2*, and *VNN2* in bovine LGCs.

P4 regulates gene expression by activating nuclear PR. The *PR* expressed in GCs is encoded by two isoforms: *PRA* and *PRB* [20]. Although both PRA and PRB work as ligand-activated transcription factors, their functional activities may differ [124,125]. PRB activates P4 target genes, whereas PRA predominantly represses PRB activity. This implies that the relative levels of PRA and PRB in a given cell determine the cellular response to P4. The *PRB* to *PRA* ratio was reduced by about half in the Day 12 LGCs compared to the Day 6 LGCs. This

may explain why the expression of *HSD11B1* stayed relatively high after Day 8 in the face of high P4 levels (Fig. 3.3B). Additionally, because forskolin increased P4 levels, *HSD11B1* expression, and the *PRB* to *PRA* ratio while decreasing cortisol production, the results suggest that the forskolin-induced P4 may have upregulated the dehydrogenase activities of HSD11B1 by acting through PRB. Nonetheless, further studies are necessary to elucidate this further.

The results in this study also suggest that progestogens may downregulate P4 production, as they tended to downregulate the expression of *HSD3B1* in bovine LGCs. On the contrary, in rat and human GCs exposed to gonadotropin, P4 has been shown to upregulate its production [113,114]. Because gonadotropins were added to the culture medium in both the rat and human studies, the differences in observations could be attributed to species differences and/or differences in culture conditions.

In conclusion, studies in this chapter demonstrated that progestogens: 1) suppress cortisol production by suppressing *HSD11B1* expression and perhaps by increasing *HSD11B2*; 2) may suppress P4 production by suppressing *HSD3B1* expression; and 3) P4 suppresses *PTX3*, *RGS2*, and *VNN2* expression in bovine LGCs.
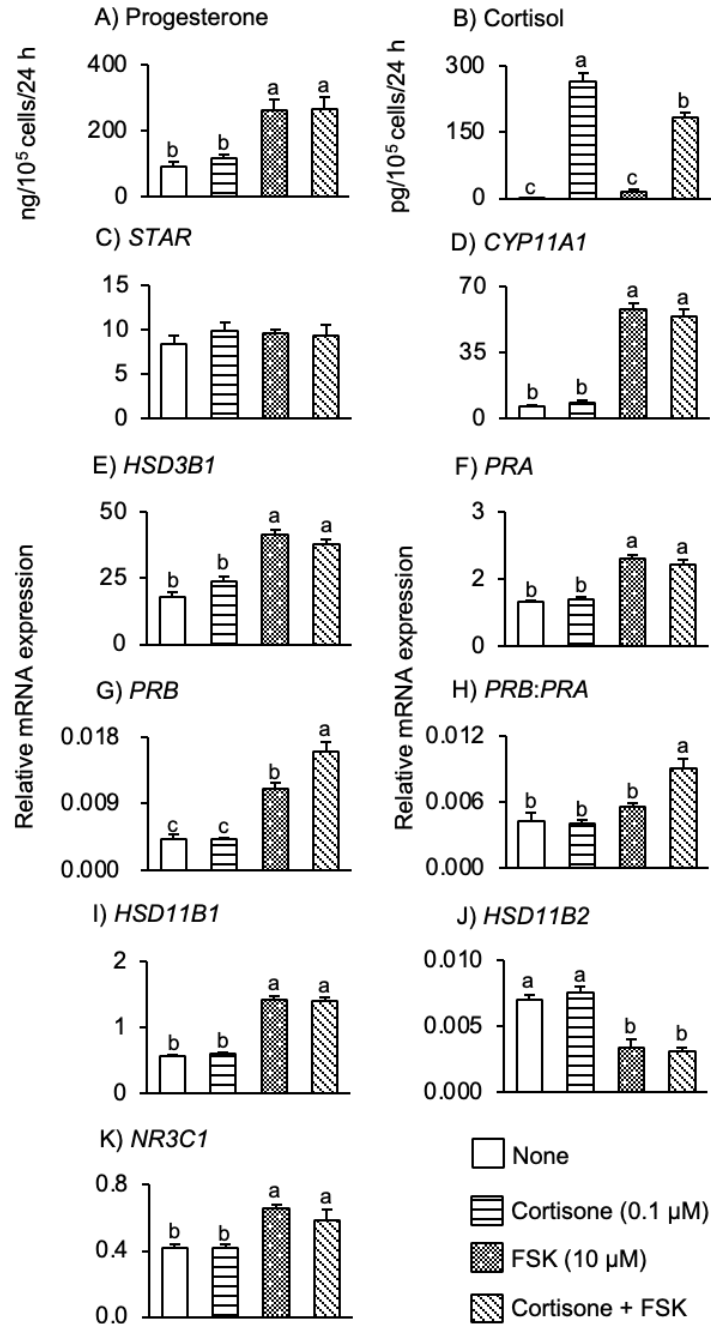
Figure 4.1. Effects of forskolin (FSK) on production of progesterone (A) and cortisol (B), and expression of *STAR* (C), *CY11A1* (D), *HSD3B1* (E), *PRA* (F), *PRB* (G), *HSD11B1* (H), *HSD11B2* and *NR3C1* (K), and the ratio of *PRB* to *PRA* (H) in luteinized bovine granulosa cells. The cells were treated with/without 0.1μM cortisone and with/without 10 μM FSK for 24 h on Day 6 of culture. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
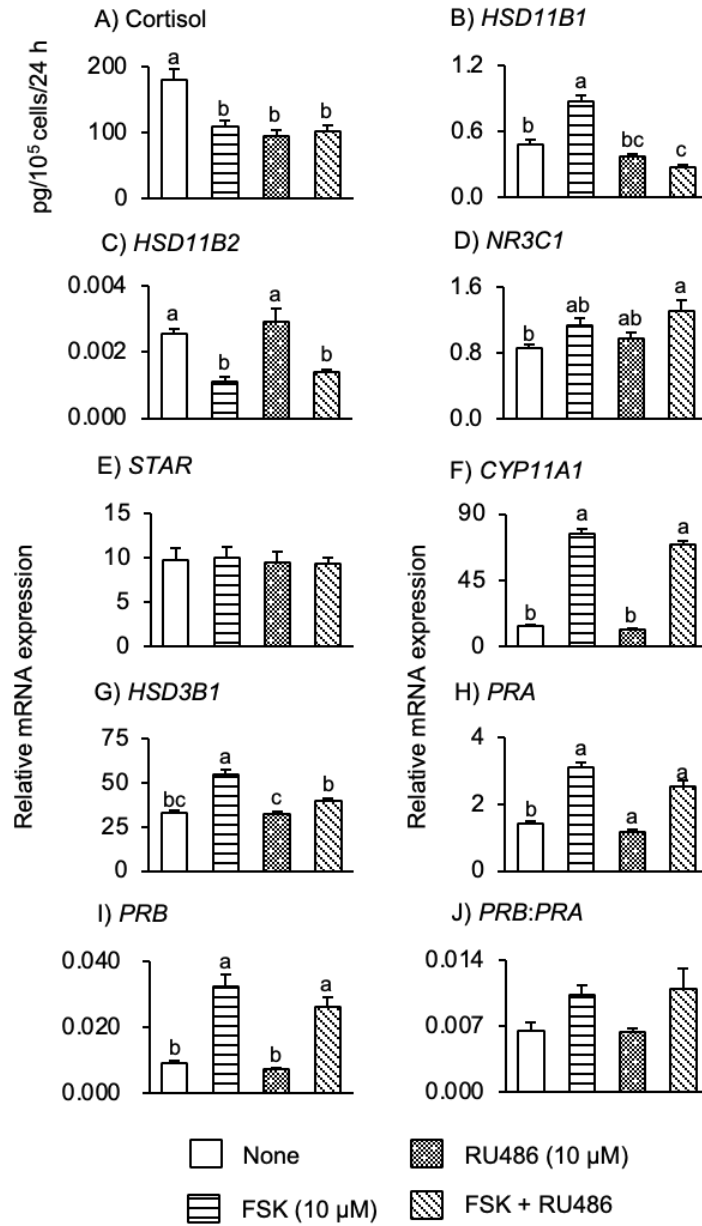
Figure 4.2. Effects of forskolin (FSK) induced progesterone on cortisol production (A), and expression of *HSD11B1* (B), *HSD11B2* (C), *NR3C1* (D), *STAR* (E), *CY11A1* (F), *HSD3B1* (G), *PRA* (H) and *PRB* (I), and the ratio of *PRB* to *PRA* (J) in luteinized bovine granulosa cells. The cells were treated with/without 10 μM FSK and with/without 10 μM RU486 in the presence of 0.1 μM cortisone for 24 h on Day 6 of culture. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
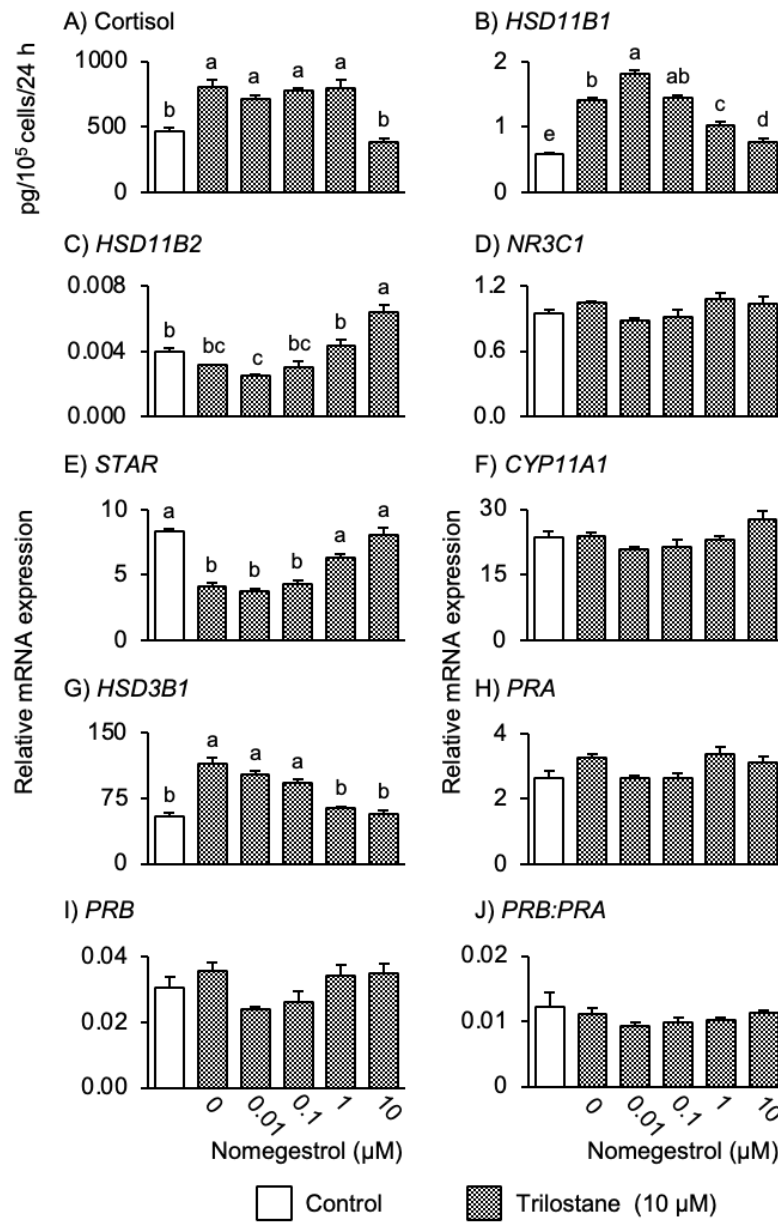
Figure 4.3. Effects of nomegestrol acetate on cortisol production (A), and expression of *HSD11B1* (B), *HSD11B2* (C), *NR3C1* (D), *STAR* (E), *CYP11A1* (F), *HSD3B1*(G), *PRA* (H), *PRB* (I), and the ratio of *PRB* to *PRA* (J) in cultured bovine luteinized granulosa cells (LGCs). Day 6 bovine LGCs were treated with/without trilostane (10 μM) and with/without nomegestrol acetate (0, 0.01, 0.1, 1, or 10 μM) in the presence of 0.1 μM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
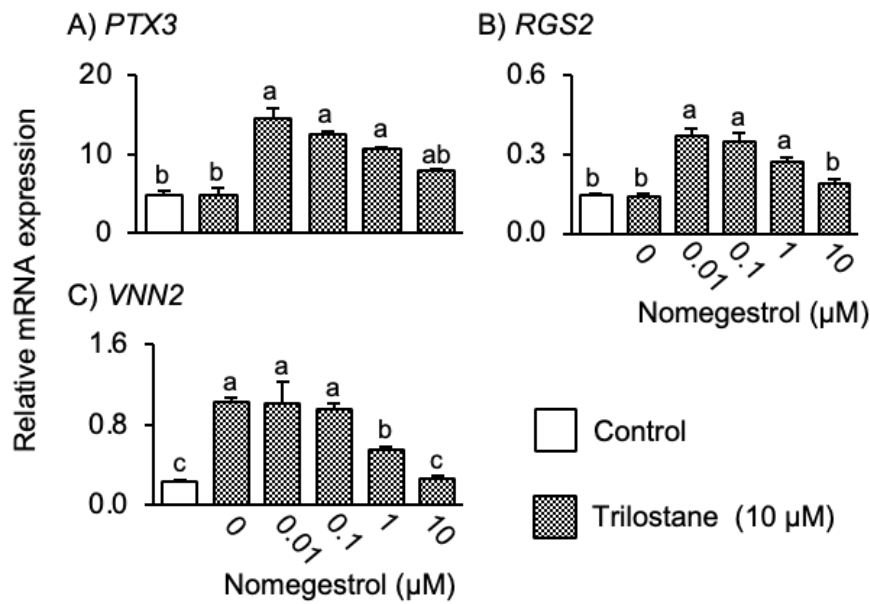
Figure 4.4. Effects of nomegestrol acetate on expression of *PTX3* (A), *RGS2* (B), and *VNN2* (C) in cultured bovine luteinized granulosa cells (LGCs). Day 6 bovine LGCs were treated with/without trilostane (10 µM) and with/without nomegestrol acetate (0, 0.01, 0.1, 1, or 10 µM) in the presence of 0.1 µM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).

Figure 4.5. Effects of nomegestrol acetate on cortisol production (A), and expression of *HSD11B1* (B), *HSD11B2* (C), *NR3C1* (D), *STAR* (E), *CYP11A1* (F), *HSD3B1*(G), *PRA* (H), *PRB* (I), and the ratio of *PRB* to *PRA* (J) in cultured bovine luteinized granulosa cells (LGCs). Day 12 bovine LGCs were treated with/without trilostane (10 μM) and with/without nomegestrol acetate (0, 0.01, 0.1, 1, or 10 μM) in the presence of 0.1 μM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).

Figure 4.6. Effects of nomegestrol acetate on expression of *PTX3* (A), *RGS2* (B), and *VNN2* (C) in cultured bovine luteinized granulosa cells (LGCs). Day 12 bovine LGCs were treated with/without trilostane (10 μM) and with/without nomegestrol acetate (0, 0.01, 0.1, 1, or 10 μM) in the presence of 0.1 μM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
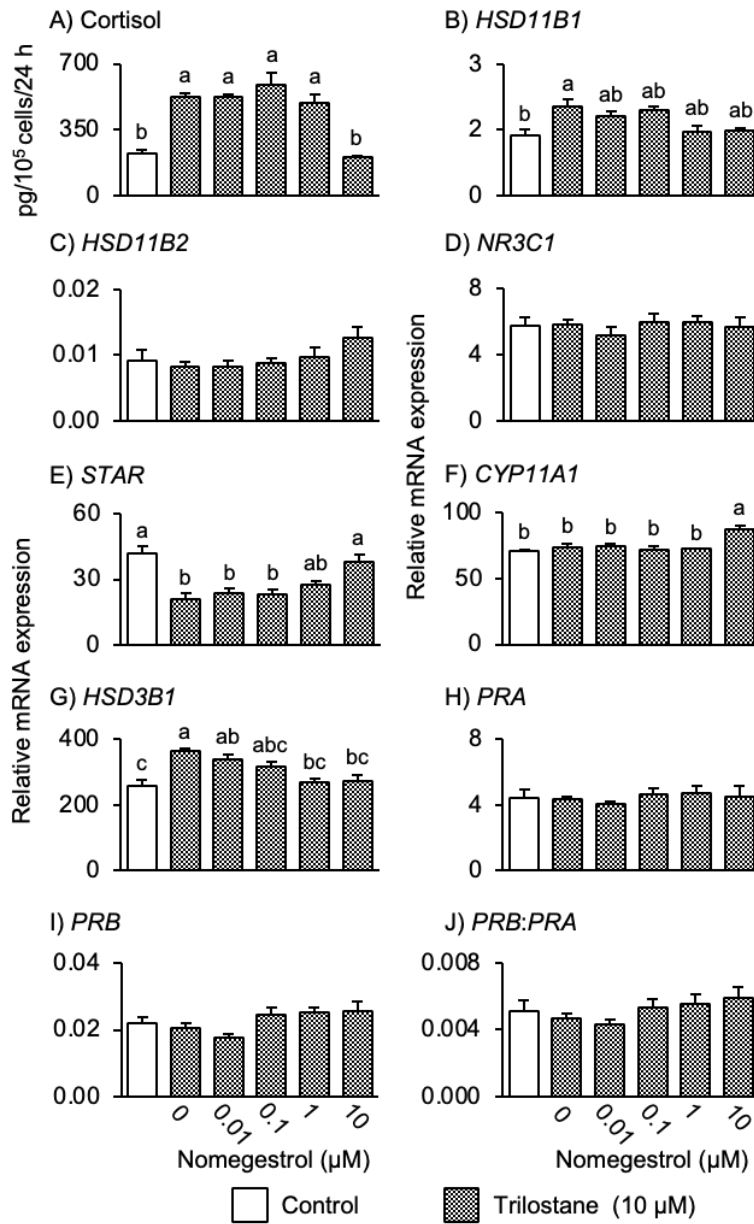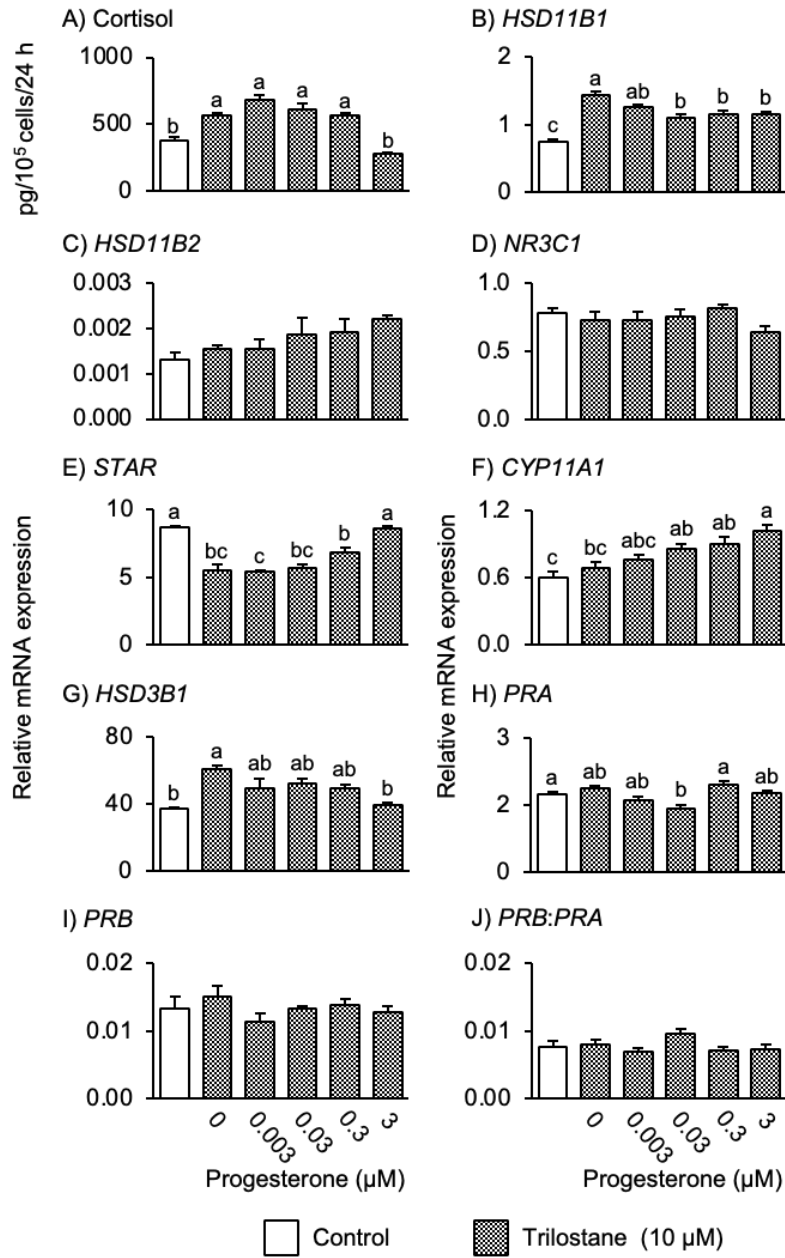
Figure 4.7. Effects of progesterone on cortisol production (A) and expression of *HSD11B1* (B), *HSD11B2* (C), *NR3C1* (D), *STAR* (E), *CYP11A1* (F), *HSD3B1*(G), *PRA* (H), *PRB* (I), and the ratio of *PRB* to *PRA* (J) in cultured bovine luteinized granulosa cells (LGCs). Day 6 bovine LGCs were treated with/without trilostane (10 μM) and with/without progesterone (0, 0.003, 0.03, 0.3, or 3 μM) in the presence of 0.1 μM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test)

Figure 4.8. Effects of progesterone on expression of *PTX3* (A), *RGS2* (B), and *VNN2* (C) in cultured bovine luteinized granulosa cells (LGCs). Day 6 bovine LGCs were treated with/without trilostane (10 μM) and with/without progesterone (0, 0.003, 0.03, 0.3, or 3 μM) in the presence of 0.1 μM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
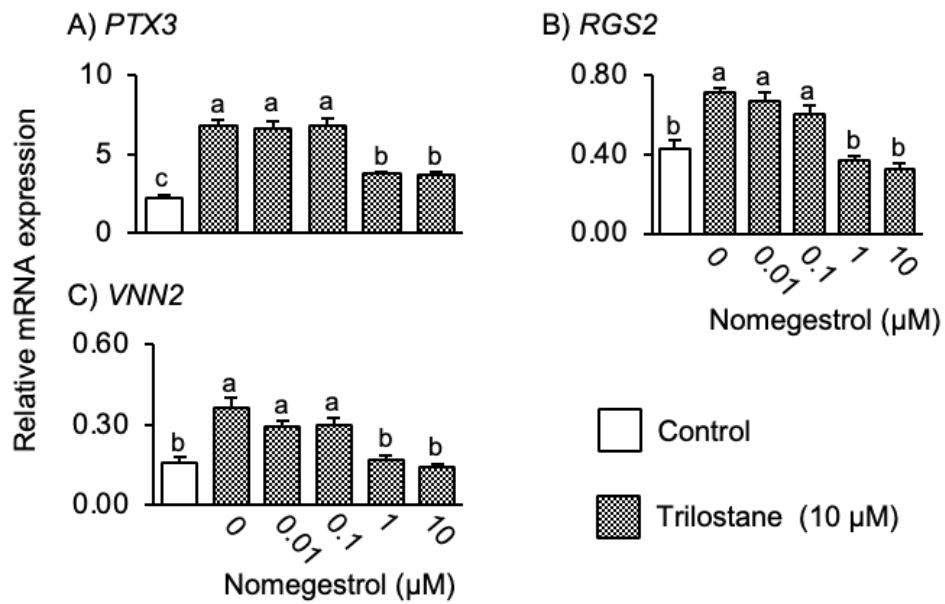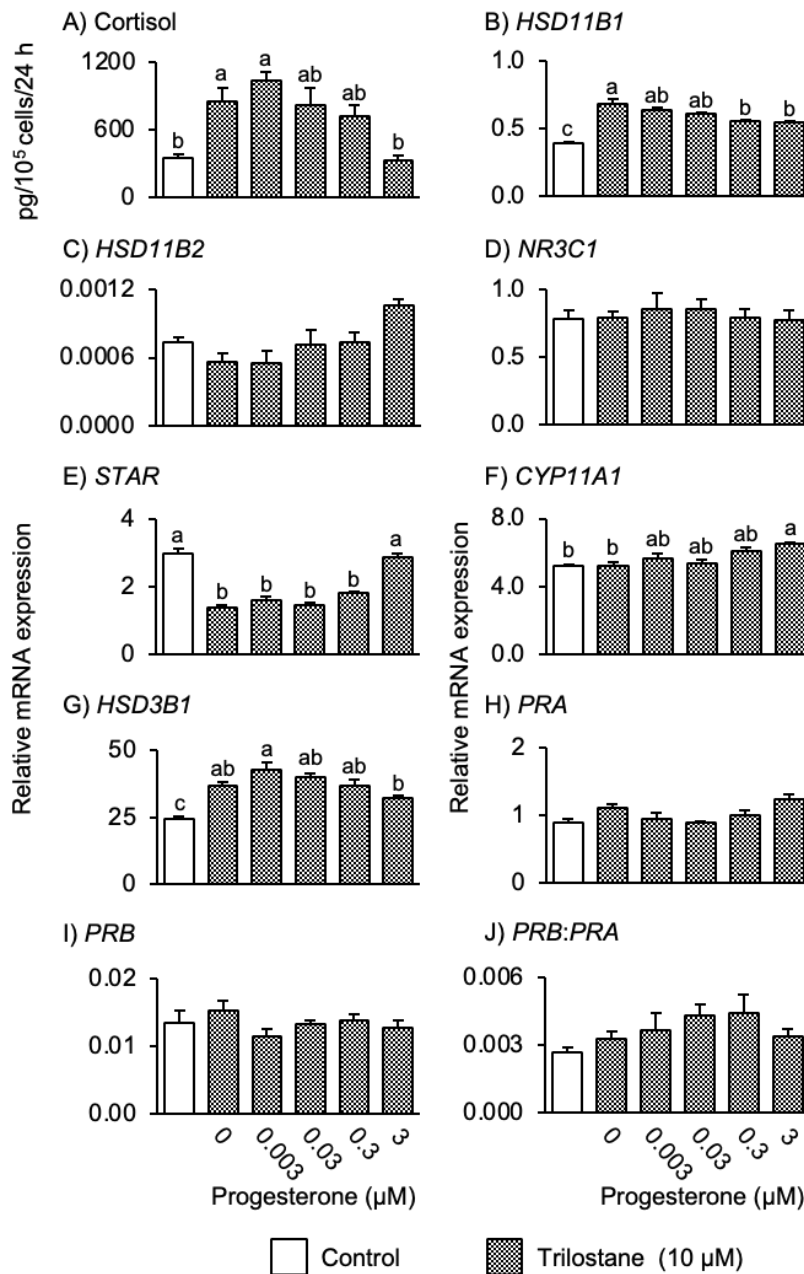
Figure 4.9. Effects of progesterone on cortisol production (A) and expression of *HSD11B1* (B), *HSD11B2* (C), *NR3C1* (D), *STAR* (E), *CYP11A1* (F), *HSD3B1*(G), *PRA* (H), *PRB* (I), and the ratio of *PRB* to *PRA* (J) in cultured bovine luteinized granulosa cells (LGCs). Day 12 bovine LGCs were treated with/without trilostane (10 μM) and with/without progesterone (0, 0.003, 0.03, 0.3, or 3 μM) in the presence of 0.1 μM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
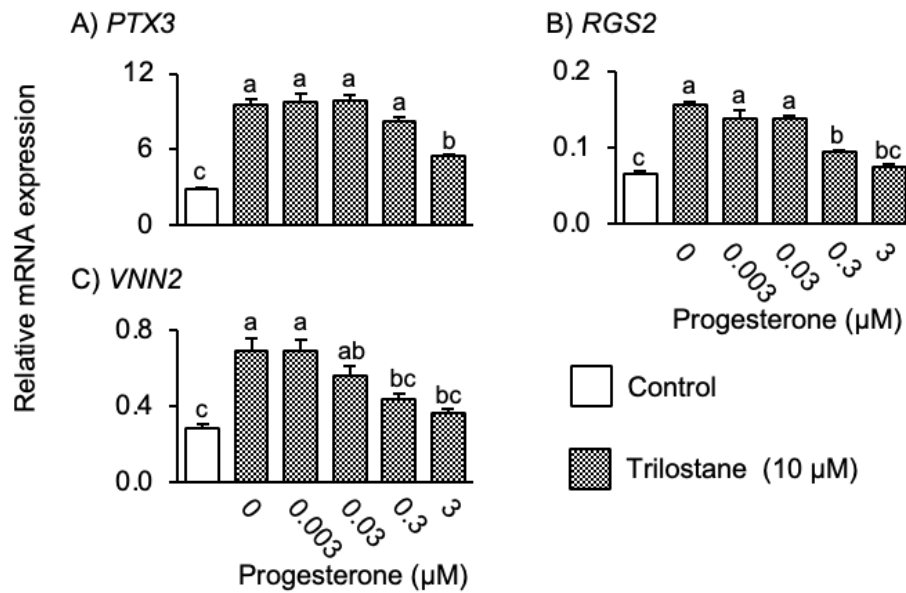
Figure 4.10. Effects of progesterone on expression of *PTX3* (A), *RGS2* (B) and *VNN2* (C) in cultured bovine luteinized granulosa cells (LGCs). Day 12 bovine LGCs were treated with/without trilostane (10 μM) and with/without progesterone (0, 0.003, 0.03, 0.3, or 3 μM) in the presence of 0.1 μM cortisone for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
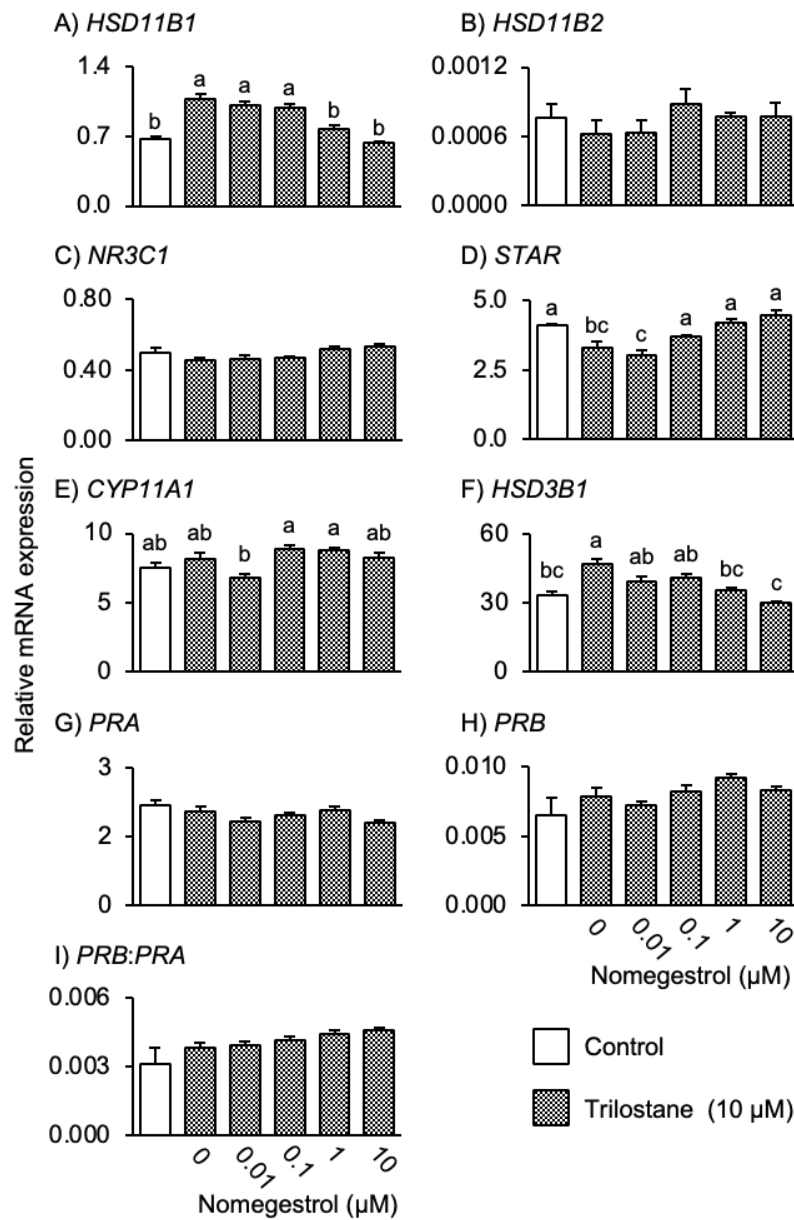
Figure 4.11. Effects of nomegestrol acetate on the expression of *HSD11B1* (A), *HSD11B2* (B), *NR3C1* (C), *STAR* (D), *CYP11A1* (E), *HSD3B1* (F), *PRA* (G), *PRB* (H), and the ratio of *PRB* to *PRA* (I) in cultured bovine luteinized granulosa cells (LGCs) in the absence of cortisol. Day 6 bovine LGCs were treated with/without trilostane (10 μM) and with/without nomegestrol acetate (0, 0.01, 0.1, 1, or 10 μM) for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey multiple comparison test).
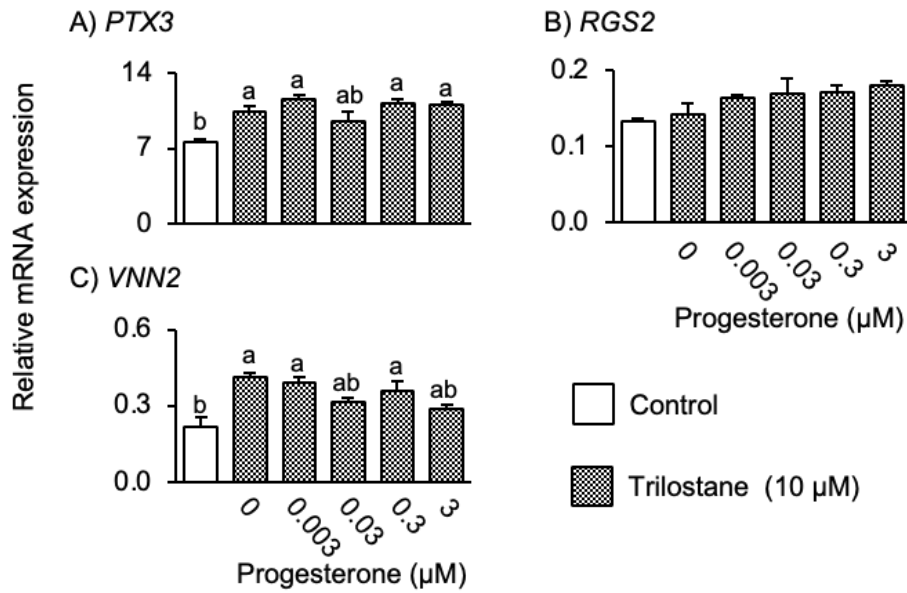
Figure 4.12. Effects of nomegestrol acetate on expression of *PTX3* (A), *RGS2* (B) and *VNN2* (C) in cultured bovine luteinized granulosa cells (LGCs) in the absence of cortisol. Day 6 bovine LGCs were treated with/without trilostane (10 µM) and with/without nomegestrol acetate (0, 0.01, 0.1, 1, or 10 µM) for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey multiple comparison test).
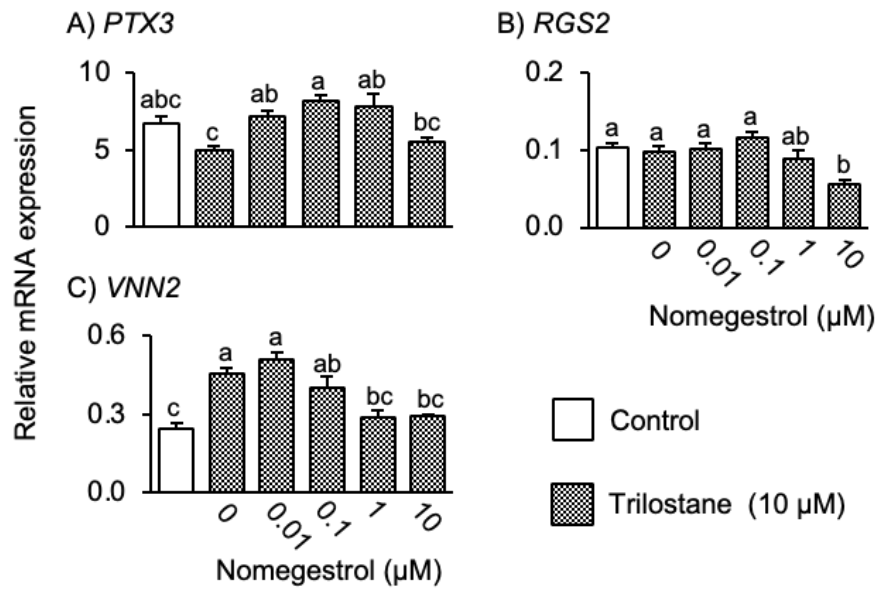
CHAPTER 5. Effects of Cortisol on Luteinization

5.1. Introduction

Glucocorticoids constantly regulate ovarian function throughout reproductive development. These steroids directly regulate ovarian function by acting through their receptor, NR3C1, which is expressed in various ovarian cell types, including GCs. During the periovulatory period, cortisol, the prime glucocorticoid in mammalian species (corticosterone in rodents), is elevated in preovulatory follicles [73–76]. However, the physiological importance of cortisol in the peri-ovulatory period is not fully understood. Cortisol may protect GCs from the adverse effects of inflammation associated with ovulation [76,90,92,126]. Additionally, cortisol has been shown to play a role in maintaining luteal function during the early and mid-luteal stages of CL [89]. However, the role of cortisol during the luteinization process is not fully understood. Cortisol may also promote luteinization, as glucocorticoids have been shown to increase P4 production in cultured GCs from various species, including cattle [127–134] by modulating the expression of the classical luteinization markers: *STAR*, *CYP11A1*, and *HSD3B1*.

Recently, genomic studies in GCs showed that the genes of three proteins, PTX3, RGS2, and VNN2, were also among the many other genes upregulated during the LH surge and have been recently referred to as "luteinizing markers." In various tissues and/or cells, these three proteins have been associated with inflammatory responses [36–40,60–72]. Even though cortisol is well known for its roles in inflammatory responses and is concurrently elevated with the gene expression of the three proteins, its influence on the expression of the genes in GCs or LGCs is unknown. Cortisol may protect GCs and LGCs by regulating the expression of *PTX3*, *RGS2*, and *VNN2*. In the previous chapters, it was shown that the bovine LGCs in the present studies express appreciable levels of the glucocorticoid receptor, *NR3C1*, and thus suggest the action of glucocorticoids on these cells. In this chapter, therefore, the effect of cortisol on the production of P4 and the expression of luteinizing markers (*PTX3*, *RGS2* and *VNN2*) in the bovine LGCs was investigated.

5.2. Material and Methods

Details of the collection, culture, and processing of GCs are tabulated in Section 2.2 of Chapter 2.

5.2.1. Experiment 1: Dose effects of cortisol on progesterone production and gene expression in bovine luteinized granulosa cells

The effects of cortisol on P4 production and gene expression were investigated by treating GCs with/without cortisol (0.01, 0.1, or 1 µM) and with/without 10 µM RU486 (PR and NR3C1 antagonist) for 24 h on Day 6 and Day 12. Here, P4 production and the expression of *STAR*, *CYP11A1*, *HSD3B1*, *PR*s, *PTX3*, *RGS2*, *VNN2*, *HSD11Bs*, and *NR3C1* in bovine LGCs were investigated.

5.2.2. Experiment 2: Dose effects of cortisol on gene expression in bovine luteinized granulosa cells in the absence of progesterone

The effects of cortisol on gene expression were investigated by treating LGCs with/without cortisol (0.01, 0.1, or 1 µM) in the presence of 10 µM trilostane to eliminate the effect of P4 on Day 6 and Day 12. The expression of *STAR*, *CYP11A1*, *HSD3B1*, *PR*s, *PTX3*, *RGS2*, *VNN2*, *HSD11Bs* and *NR3C1* in bovine LGCs was investigated.

5.2.3. Experiment 3: Cortisol and progestogen interaction in bovine luteinized granulosa cells

To investigate the interaction between cortisol and progestogens, LGCs were treated with/without 1 µM cortisol and with/without 10 µM NA in the presence of 10 µM trilostane on Day 6 for 24 h. The expression of *STAR*, *CYP11A1*, *HSD3B1*, *PR*s, *PTX3*, *RGS2*, *VNN2*, *HSD11Bs* and *NR3C1* in bovine LGCs was investigated.

5.3. Results

5.3.1. Dose effects of cortisol on progesterone production and gene expression in bovine luteinized granulosa cells

Cortisol dose-dependently suppressed *STAR* and *PRB* expression (Figs. 4.1B, F) and significantly or tended to upregulate *CYP11A1* and *HSD3B1* expression (Figs. 4.1C, D) in Day 6 LGCs without affecting P4 production (Figs. 4.1A, G). RU486 reversed these effects. Treatment with cortisol dose-dependently increased the expression of *PTX3*, *RGS2*, *VNN2*, and *HSD11B1*, but tended to decrease that of *HSD11B2* (Figs. 4.2A-E). These effects were reversed by RU486.

In Day 12 LGCs, cortisol dose-dependently suppressed expression of *STAR* but upregulated HSD*3B1* expression (Figs. 4.3B, D). Treatment with cortisol tended to downregulate the expression of *PRB* and the ratio of *PRB* to *PRA* but did not affect the expression of *CYP11A1*, *PRA*, or P4 production (Figs. 4.3F, C, E, A). Cortisol dose-dependently upregulated the expression of *PTX3*, *RGS2*, *VNN2*, and *HSD11B1*, while downregulating *HSD11B2* (Figs. 4.4A-E). The effects of cortisol on gene expression were reversed by RU486. In both Day 6 and Day 12 LGCs, cortisol did not affect *NR3C1* expression (Figs. 4.2F, 4.4F).

5.3.2. Dose effects of cortisol on gene expression in bovine luteinized granulosa cells in the absence of progesterone

At the end of experiment 1, the spent media contained up to 1.8 mg/mL P4 (approx. 6 mM). To eliminate the possible effect of P4 on the expression of genes, experiment 1 was repeated with trilostane on Days 6 and 12.

In the absence of P4, cortisol dose-dependently suppressed *STAR* and upregulated *CYP11A1* and *HSD3B1* expression in the Day 6 LGCs (Figs. 4.5A-C). Cortisol tended to decrease the expression of *PRB* but did not influence *PRA* expression or the *PRB* to *PRA* ratio (Figs. 4.5D-F). In a dose-dependent manner, cortisol increased the expression of *PTX3*, *RGS2*, *VNN2*, and *HSD11B1* and tended to decrease *HSD11B2* without influencing *NR3C1* expression in the Day 6 LGCs (Figs. 4.6A-F).

In Day 12 LGCs, cortisol dose-dependently suppressed *STAR* and *PRA* expression but upregulated *HSD3B1* without affecting *CYP11A1* and *PRB* expression or the *PRB* to *PRA* ratio (Figs. 4.7A, D, C, E, F). Cortisol dose-dependently upregulated *PTX3*, *RGS2*, *VNN2*, and *HSD11B1* and decreased *HSD11B2* without affecting *NR3C1* expression (Figs. 4.8A-F).

5.3.3. Cortisol and progestogen interaction in bovine luteinized granulosa cells

In the absence of endogenous P4, NA significantly increased *STAR* expression and tended to increase *CYP11A1* but decreased cortisol-induced *HSD3B1* expression (Figs. 4.9A-C). NA increased *PTX3*, *HSD11B2*, and *NR3C1* and tended to increase *RGS2*, whereas it decreased the expression of *VNN2* and HSD11B1 in the presence and absence of cortisol (Fig. 4.10).

5.4. Discussion

The ovary is a glucocorticoid target organ, and glucocorticoids have modulated its functions in various species [135]. In this study, the effects of cortisol on the expression of luteinization markers in bovine LGCs have been demonstrated.

Glucocorticoids act through the receptor NR3C1 to influence steroidogenesis in the ovary [135], and studies have shown that they regulate P4 production by modulating the expression of classical luteinizing markers: *STAR*, *CYP11A1*, and *HSD3B1* [128,130,131,136,137]. In the present study, bovine LGCs expressed considerable levels of *NR3C1*, which indicated the possible action of glucocorticoids. Cortisol had a differential influence on the gene expression of steroidogenic proteins as it downregulated *STAR* and increased *HSD3B1* expression in Day 6 and Day 12 LGCs. On the other hand, cortisol effects on *CYP11A1* expression seemed to be status-dependent, as it upregulated the expression in proliferating but not non-proliferating LGCs. Nevertheless, P4 production was not altered by cortisol in the present study. This may be due to the opposite effect of cortisol on *STAR* and *HSD3B1* expression. However, because STAR regulates a rate-limiting step in steroidogenesis [4,138,139], the results suggest that extended exposure to cortisol may negatively affect P4 production by luteinized GCs. Similarly, dexamethasone, a potent synthetic glucocorticoid, suppressed LH-induced *STAR* expression and P4 synthesis in GCs from rats [136]. On the contrary, glucocorticoids increased basal and FSH-induced P4 production in GCs from various species, including bovine [128–133,140,141]. These differences appear to be related to the status of the GCs, as glucocorticoids seem to upregulate P4 synthesis by non-luteinized GCs but downregulate that of luteinized GCs.

In this study, cortisol dose-dependently upregulated the expression of *PTX3*, *RGS2*, and *VNN2* in bovine LGCs *in vitro*. These results suggest that cortisol may play a role in the luteinization process of GCs, as these genes have previously been referred to as luteinizing markers in GCs. Because these genes were upregulated while the production of P4 remained unchanged, the results suggest that these genes may not be associated with P4 production. However, these genes may play a role in protecting the GCs during the inflammatory process of ovulation, as is the case in extraovarian cells and tissues [36–40,60–72]. Considering the anti-inflammatory actions of cortisol [142], which have also been suggested during the ovulatory process, the results also suggest that cortisol may protect LGCs and possibly CL from the adverse effects of inflammation by regulating *PTX3*,

RGS2, and *VNN2* expression. Similarly, studies in extraovarian cells have shown that glucocorticoids upregulate *PTX3* and *RGS2* and play anti-inflammatory roles [143–145]. Put together, these results suggest that cortisol also regulates the expression of *PTX3*, *RGS2*, and *VNN2* in the bovine LGCs.

In both Day 6 and Day 12 LGCs, cortisol dose-dependently upregulated HSD11B1 but downregulated *HSD11B2* expression. Because HSD11B1 mainly mediates the conversion of cortisone, the inert glucocorticoid, to the active cortisol, while HSD11B2 does the opposite, the results imply that cortisol may influence its production in bovine LGCs. Likewise, glucocorticoids have been shown to regulate the expression of *HSD11B1* and *HSD11B2* in cultured hippocampal cells [146] and the placenta [147,148]. Together with the *NR3C1* expression observed in the LGCs, these results imply that bovine LGCs can respond to cortisol and may regulate the production of P4 and cortisol.

In this study, treatment with cortisol showed clear effects on gene expression in both Day 6 and Day 12 LGCs. However, these effects were only observed under experimental conditions where locally produced P4 was either absent or gradually accumulating in the media over the 24 h treatment. The gradual accumulation of P4 levels allowed cortisol to predominantly influence gene expression in LGCs. However, when cortisol was concurrently treated with NA in the presence of trilostane, NA's effects clearly overrode those of cortisol. This therefore suggests that the cortisol effects observed in the present study may not be the case i*n vivo,* where LGCs produce large amounts of P4.

In summary, this study demonstrates that cortisol: 1) differentially regulates the gene expression of luteinizing markers: *STAR*, *CYP11A1*, *HSD3B1*, *PTX3*, *RGS2*, and *VNN2*; and 2) regulates *HSD11B1* and *HSD11B2* expression, thus may regulate its production in bovine LGCs.
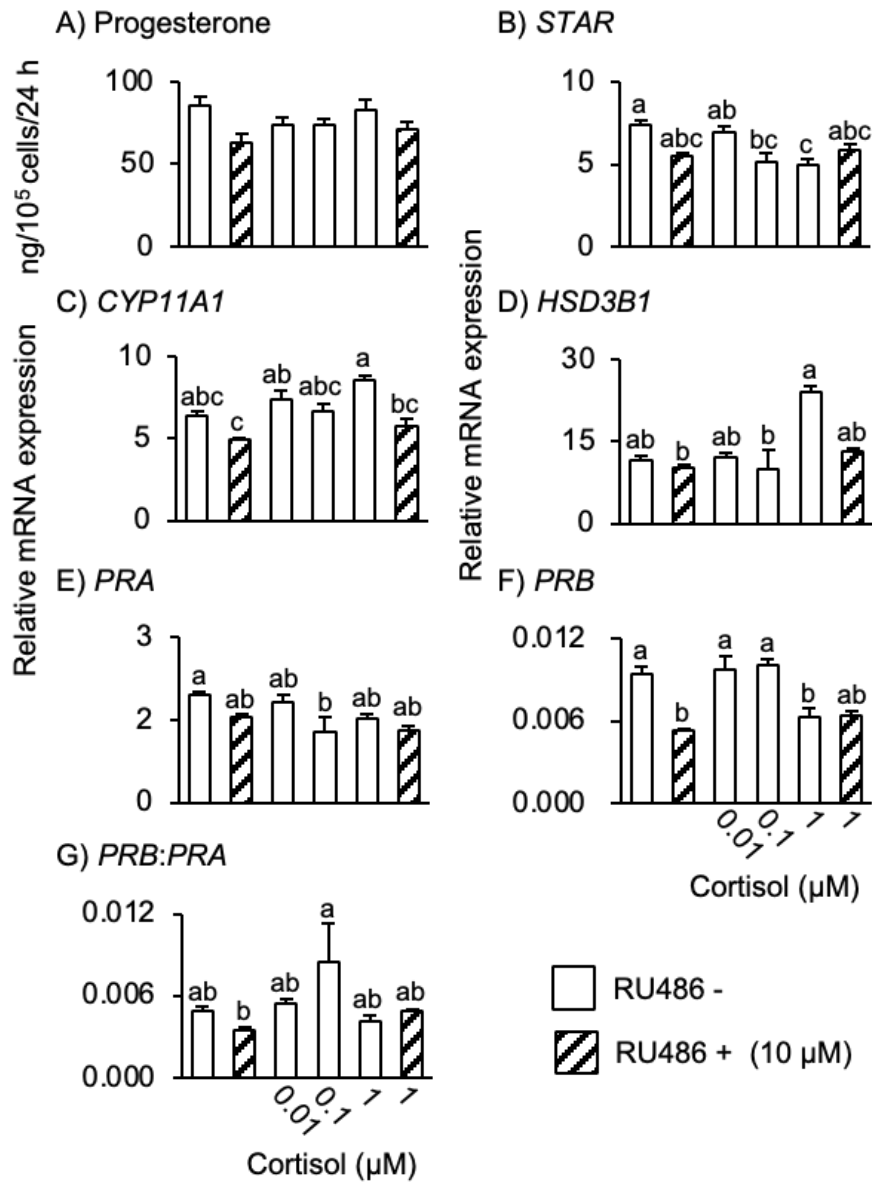
Figure 5.1. Effects of cortisol on progesterone production (A) and expression of *STAR* (B), *CYP11A1* (C), *HSD3B1*(D), *PRA* (E), *PRB* (F), and the ratio of *PRB* to *PRA* (G) in bovine luteinized granulosa cells (LGCs). Day 6 bovine LGCs were treated with/without cortisol (0, 0.01, 0.1 and 1 μM) and with/without 10 μM RU486 for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
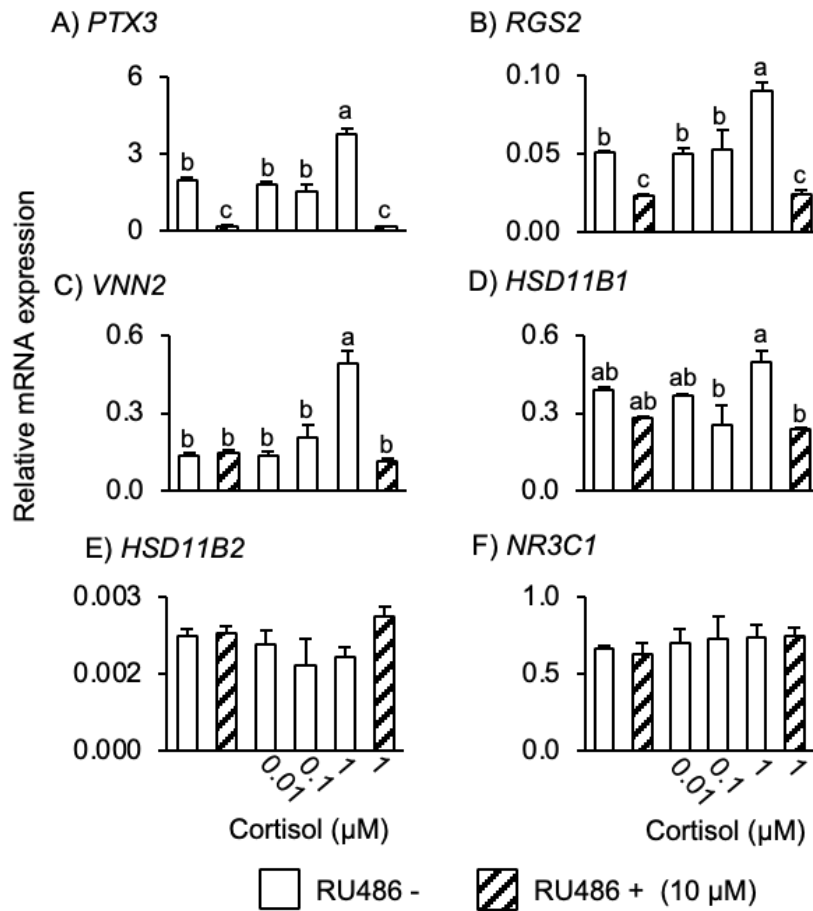
Figure 5.2. Effects of cortisol on *PTX3* (A), *RGS2* (B), *VNN2* (C), *HSD11B1* (D), *HSD11B2* (E), *NR3C1* (F) in bovine luteinized granulosa cells (LGCs). Day 6 bovine LGCs were treated with/without cortisol (0, 0.01, 0.1 and 1 μM) and with/without 10 μM RU486 for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
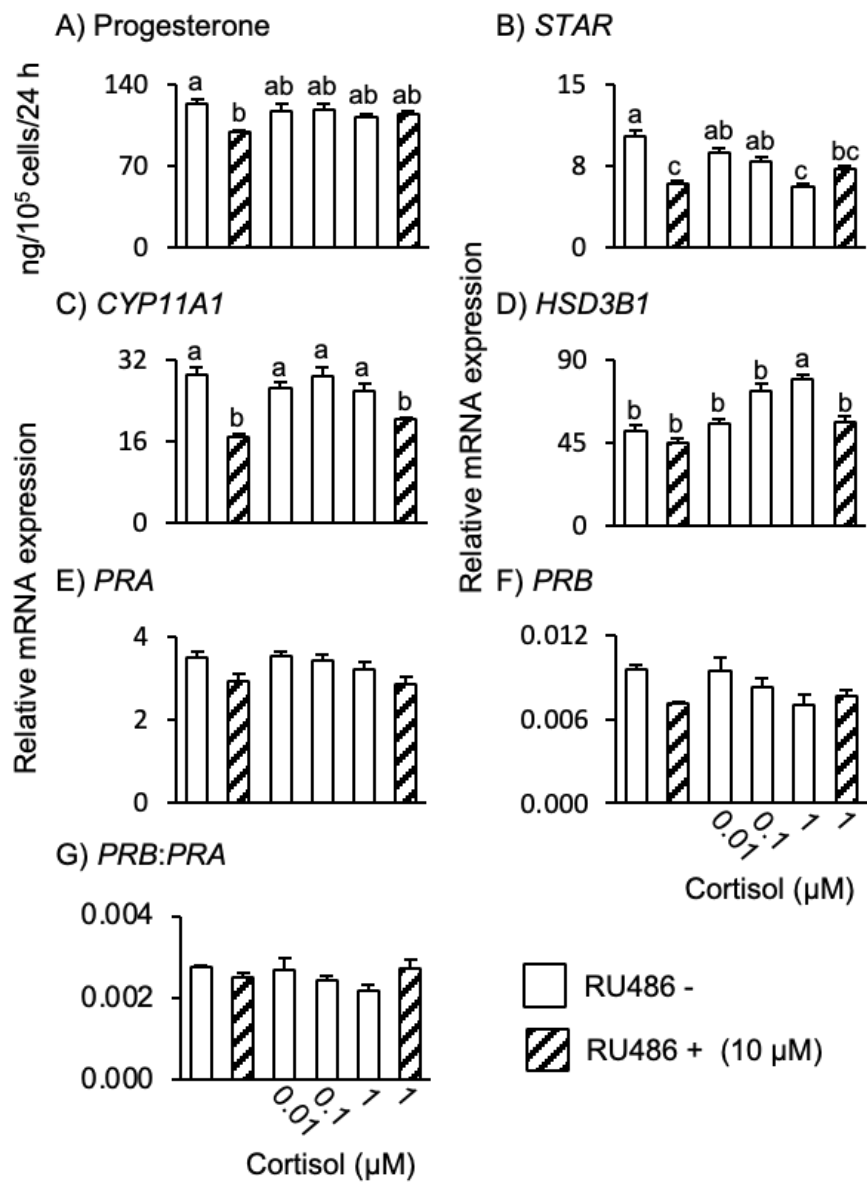
Figure 5.3. Effects of cortisol on progesterone production (A) and expression of *STAR* (B), *CYP11A1* (C), *HSD3B1*(D), *PRA* (E), *PRB* (F), and the ratio of *PRB* to *PRA* (G) in bovine luteinized granulosa cells (LGCs). Day 12 bovine LGCs were treated with/without cortisol (0, 0.01, 0.1 and 1 μM) and with/without 10 μM RU486 for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
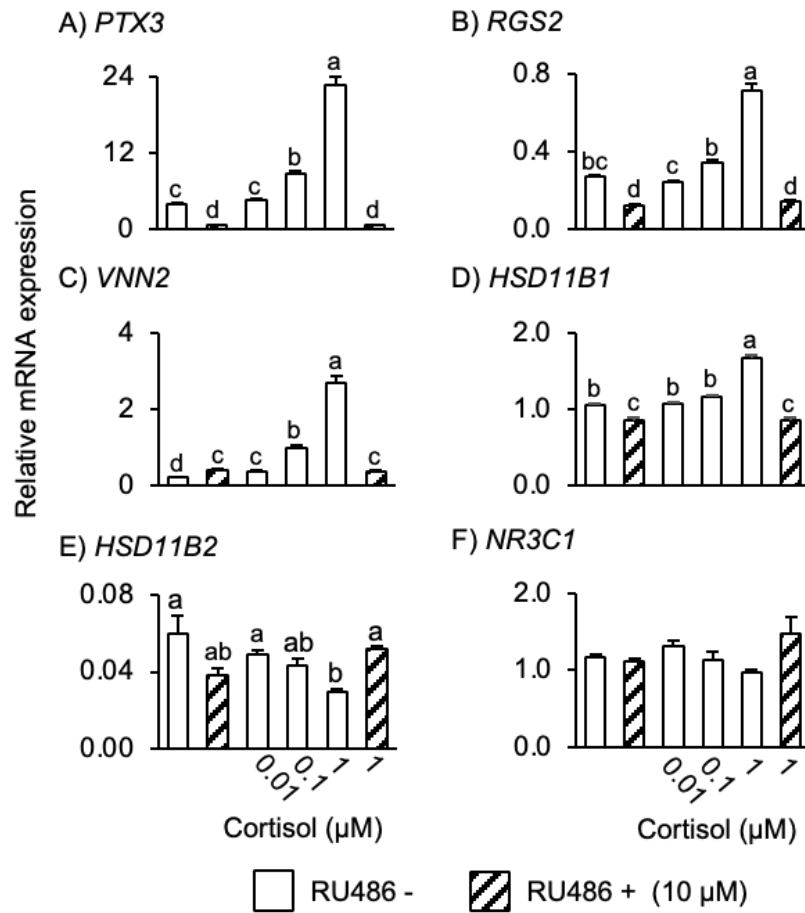
Figure 5.4. Effects of cortisol on *PTX3* (A), *RGS2* (B), *VNN2* (C), *HSD11B1* (D), *HSD11B2* (E), *NR3C1* (F) in bovine luteinized granulosa cells (LGCs). Day 12 bovine LGCs were treated with/without cortisol (0, 0.01, 0.1 and 1 μM) and with/without 10 μM RU486 for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).

Figure 5.5. Effects of cortisol on the expression of *STAR* (A), *CYP11A1* (B), *HSD3B1* (C), *PRA* (D), *PRB* (E),

and the ratio of *PRB to PRA* (F) in cultured bovine luteinized granulosa cells (LGCs) in the absence of

endogenous progesterone. Bovine LGCs were treated with/without trilostane and with/without cortisol (0,

0.01, 0.1 and 1 μM) for 24 h on Day 6 of culture. Data are expressed as mean ± SEM (n=4). Different

superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).

Figure 5.6. Effects of cortisol on the expression of *PTX3* (A), *RGS2* (B), *VNN2* (C), *HSD11B1* (D), *HSD11B2* (E), and *NR3C1* (F) in cultured bovine luteinized granulosa cells (LGCs) in the absence of endogenous progesterone. Bovine LGCs were treated with/without trilostane and with/without cortisol (0, 0.01, 0.1 and 1 μM) for 24 h on Day 6 of culture. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
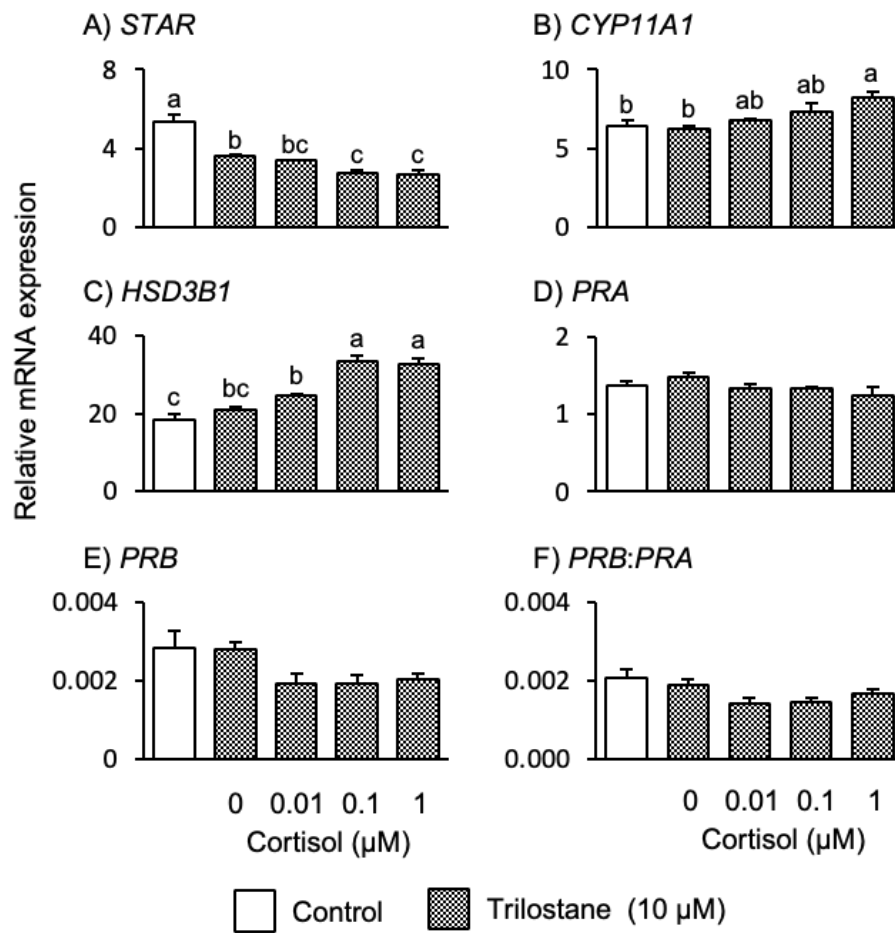
Figure 5.7. Effects of cortisol on the expression of *STAR* (A), *CYP11A1* (B), *HSD3B1* (C), *PRA* (D), *PRB* (E), and the ratio of *PRB to PRA* (F) in cultured bovine luteinized granulosa cells (LGCs) in the absence of endogenous progesterone. Bovine LGCs were treated with/without trilostane and with/without cortisol (0, 0.01, 0.1 and 1 μM) for 24 h on Day 12 of culture. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
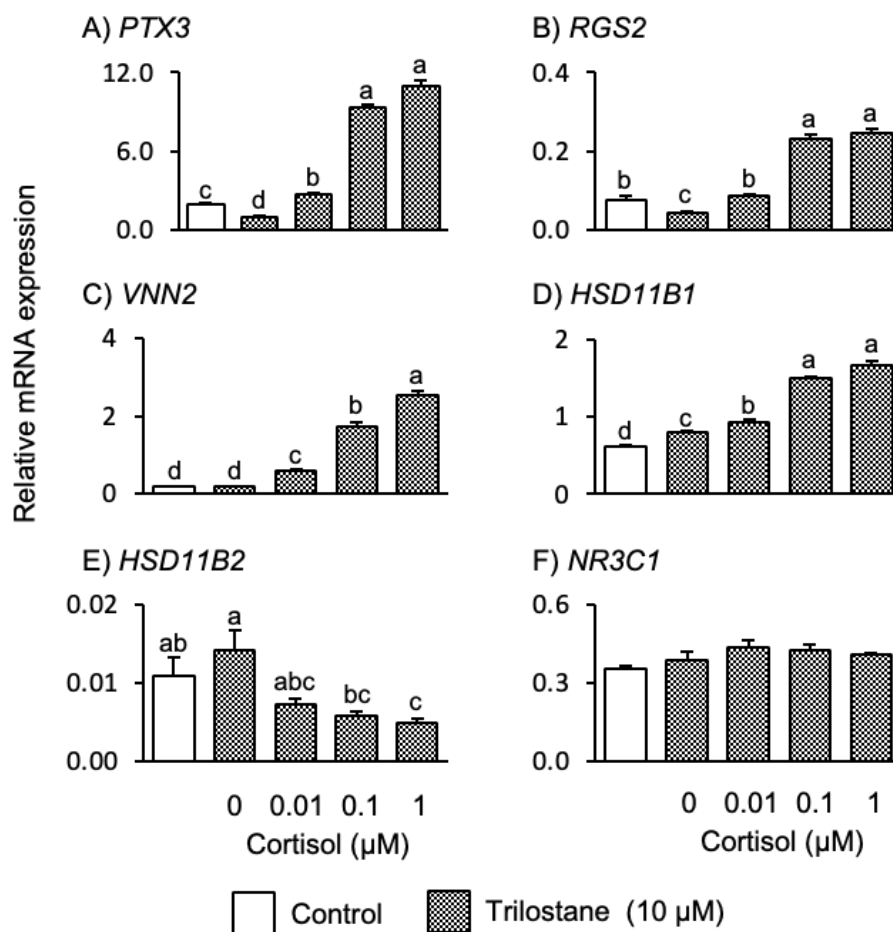
Figure 5.8. Effects of cortisol on the expression of *PTX3* (A), *RGS2* (B), *VNN2* (C), *HSD11B1* (D), *HSD11B2* (E), and *NR3C1* (F) in cultured bovine luteinized granulosa cells (LGCs) in the absence of endogenous progesterone. Bovine LGCs were treated with/without trilostane and with/without cortisol (0, 0.01, 0.1 and 1 μM) for 24 h on Day 12 of culture. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences ($P < 0.05$, Tukey's multiple comparison test).
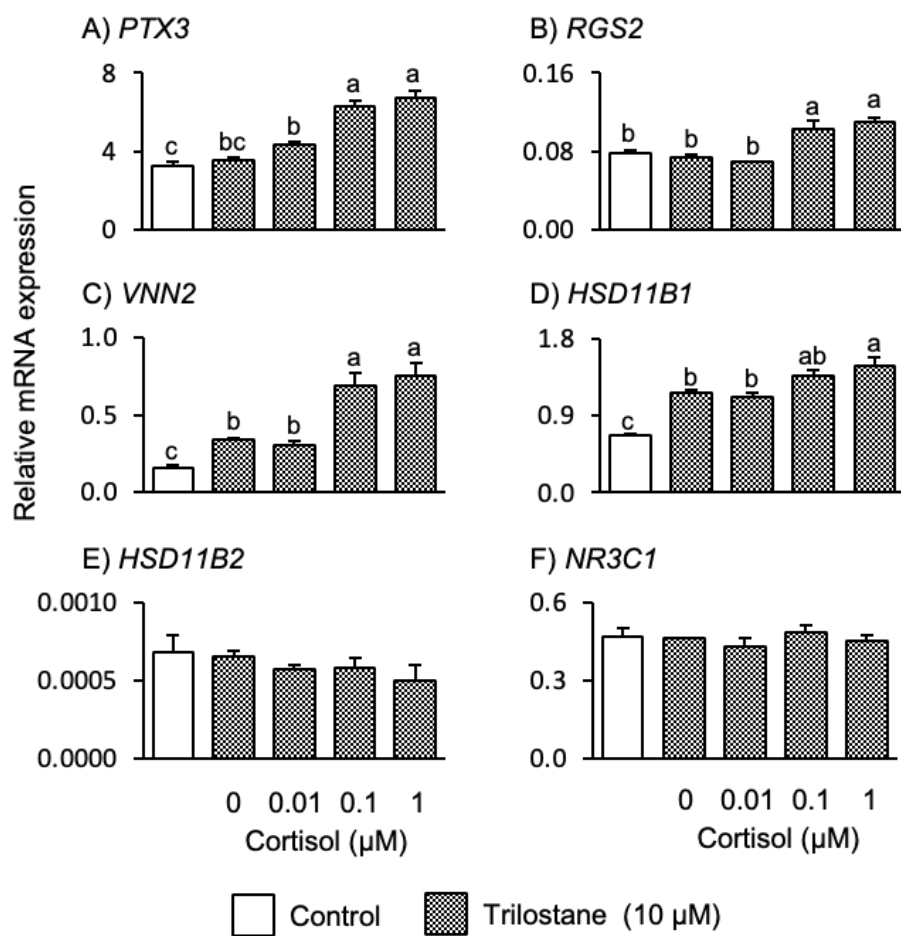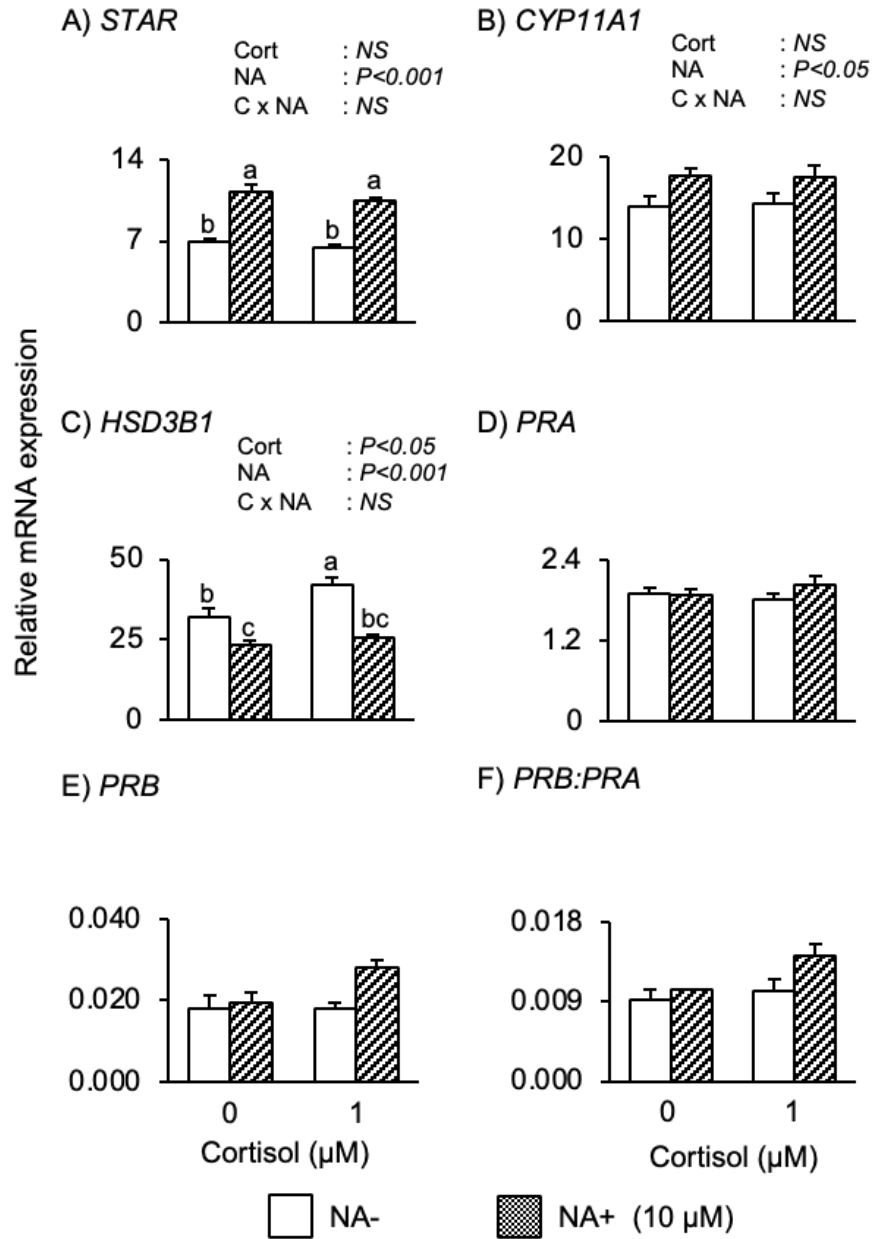
Figure 5.9. Effects of cortisol and nomegestrol acetate (NA) on the expression of *STAR* (A), *CYP11A1* (B), *HSD3B1* (C), *PRA* (D), *PRB* (E) and *PRB* to *PRA* ratio (F) in cultured bovine luteinized granulosa cells (LGCs) in the absence of endogenous progesterone. Day-6 bovine LGCs were treated with trilostane and with/without cortisol (Cort: 1 µM) and nomegestrol acetate (10 µM) for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).

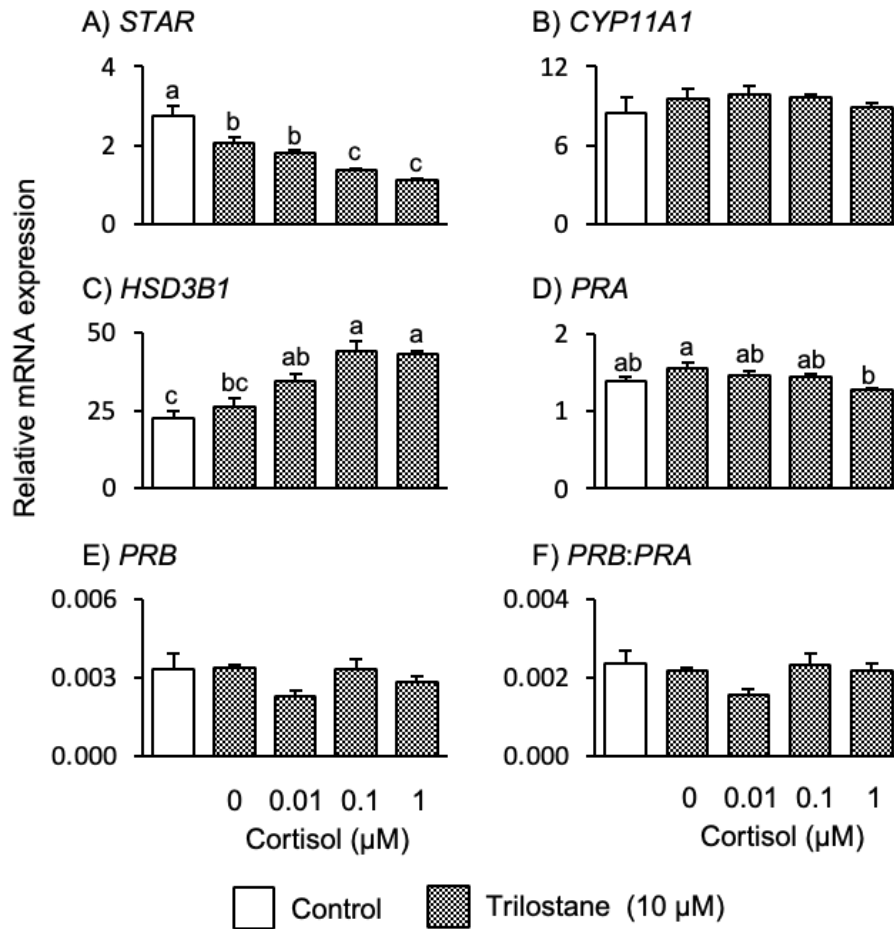**Figure 5.10.** Effects of cortisol and nomegestrol acetate (NA) on the expression of *PTX3* (A), *RGS2* (B), *VNN2* (C), *HSD11B1* (D), *HB11B2* (E) and *NR3C1* (F) in cultured bovine luteinized granulosa cells (LGCs) in the absence of endogenous progesterone. Day-6 bovine LGCs were treated with trilostane and with/without cortisol (Cort: 1 μM) and nomegestrol acetate (10 μM) for 24 h. Data are expressed as mean ± SEM (n=4). Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison test).
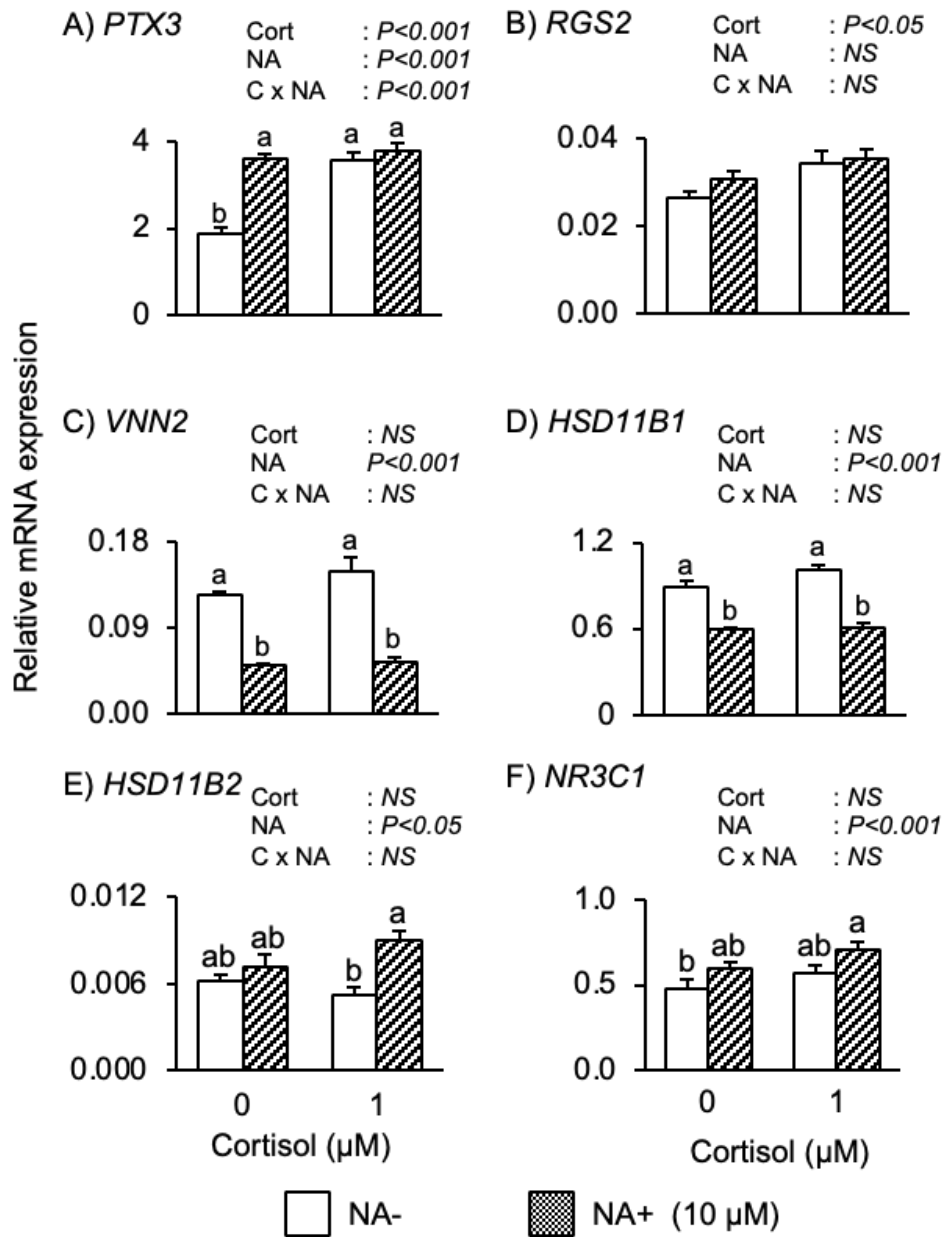
CHAPTER 6. General Discussion

During the peri-ovulatory period, P4 production and expression of luteinizing markers, *PTX3*, *RGS2*, and *VNN2*, by GCs undergoing luteinization increase. At the same time, there is an increase in HSD11B1-mediated cortisol production from cortisone [73–76] and possibly CYP21A2 and CYP11B1-mediated cortisol production from P4 [81–84,86]. However, the interaction of these C_{21} steroids and their effects on the above luteinizing markers have not been established. This study investigated the mutual effects of P4 and cortisol using cultured bovine LGCs.

6.1. Cortisol production in bovine luteinized granulosa cells

In any given cell, the expression of CYP21A2 and CYP11B1 enzymes is a prerequisite for the de novo production of cortisol [77,78]. In the present study, the bovine LGCs barely expressed and did not express CYP21A2 or CYP11B1, respectively. Although the LGCs produced quite an amount of P4, a precursor for cortisol, they were not able to produce cortisol via the de novo pathway as cortisol was not detected in the culture media. Moreover, even when the LGCs were challenged with ACTH and Ang II, de novo production of cortisol was still not detected. Nevertheless, the bovine LGCs were capable of HSD11B1-mediated cortisol production, just as is reported elsewhere in GCs, luteal cells, and CL.

Over the years, research has garnered evidence for extra-adrenal production of corticosteroids in various organs, including the ovary [77,78,81,82,84,86,149]. The ovary produces a large amount of P4, a precursor for de novo cortisol production, and the expression of CYP21A2 and CYP11B1 in the ovary has been demonstrated. Therefore, it is possible that P4 may be metabolized to cortisol in the ovary. However, extra-adrenal production of corticosteroids is a major debate among researchers. Similarly, there is a huge debate on their production in the ovary, as there seem to be differences in findings both within and among species. On the other hand, just like in many other organs, the interconversion of glucocorticoids via the HSD11B1/HSD11B2 pathways in the ovary is well accepted among researchers [75,90,92,106,126,150,151]. In this study, the results clearly indicated that bovine LGCs are capable of producing cortisol via the HSD11B1 pathway and not via the CYP21A2/CYP11B1 pathway.

6.2. Effects of progestogens on cortisol production and gene expression in bovine luteinized granulosa cells

Studies have shown the regulation of HSD11B1-mediated cortisol production in GCs and LGCs by factors such as gonadotropins and cytokines [106,107]. However, its regulation by P4, in LGCs, is not established. This study found that P4 and the synthetic progestogen, nomegestrol acetate, downregulate the conversion of cortisone to cortisol in bovine LGCs by regulating *HSD11B1* and probably, *HSD11B2* expression. This was clearly observed when local P4 production was suppressed using trilostane, HSD11B1 expression and the conversion of cortisone to cortisol were upregulated, whereas *HSD11B2* expression was downregulated. These effects were reversed when progestogens were concomitantly added to the culture medium.

It is clear that the ovary is a target organ for glucocorticoids, as various ovarian cells, including LCGs, express the glucocorticoid receptor NR3C1, and their direct effects on these cells have been reported [90,92,127,150]. Therefore, the ovary is constantly stimulated by circulating glucocorticoids, whose levels vary in response to diurnal rhythm, stress, and blood glucose levels. Excessive glucocorticoid secretion from the adrenal glands is linked to ovarian dysfunction in humans, and this appears to be the result of both direct glucocorticoid action on the ovary and indirect action through the adrenal-hypothalamo-pituitary axis [152]. Given that the ovary may not necessarily have the same physiological needs for glucocorticoids as other parts of the body, the ovarian expression of HSD11B1 and HSD11B2 is a mechanism by which glucocorticoid levels are modified to satisfy the changing ovarian physiological needs throughout the ovarian cycle. The regulation of *HSD11B1* and *HSD11B2* expression and cortisol production from cortisone by progestogens that was observed in the present study, therefore, suggests the presence of yet another regulatory mechanism or a switch that may function to maintain homeostatic glucocorticoid levels in LGCs and possibly in the CL.

In this study, progestogens also differentially regulated the luteinizing markers. In the presence of locally produced cortisol, progestogen upregulated *STAR* and *CYP11A1* but downregulated *HSD3B1*, *PTX3*, *RGS2* and *VNN2* expression. The downregulation of *PTX3*, *RGS2* and *VNN2* may be linked to the downregulation of

cortisol production, as indicated by their correlations. Altogether, these results suggest that P4 acts in autocrine manner to regulate HSD11B1-mediated cortisol production and the expression of luteinizing markers.

6.3. Cortisol actions in luteinized granulosa cells

Although the elevation of cortisol concentration in preovulatory follicles has been reported [73–76], its function during this period is not well known. Nonetheless, anti-inflammatory roles for cortisol during this period have been suggested [76,90,92,126]. The influence of glucocorticoids on P4 production has been demonstrated [127–134] in GCs, but it is not well known if this is the case in LGCs. In this study, cortisol appeared to differentially regulate the expression of steroidogenic genes but had no influence on P4 production in bovine LGCs. Cortisol upregulated *CYP11A1*, *HSD3B1*, and *HSD11B1*, but downregulated *STAR* and *HSD11B2* expression. Cortisol's effects on *HSD11B*s suggested that it may promote its own production in bovine LGCs. However, due to the simplicity of the experimental designs used in this study, this could not be elucidated. Therefore, further studies are necessary to verify this hypothesis. On the other hand, cortisol's influence on luteinization was inconclusive, as it had differential effects on the luteinization markers and did not influence P4 production. Cortisol also upregulated *PTX3*, *RGS2*, and *VNN2*. Because P4 production did not change with the upregulation of *PTX3*, *RGS2*, and *VNN2*, the results suggest that the function of the genes in bovine LGCs may not be linked to P4 production. This, however, requires further studies to verify this hypothesis.

6.4. Interaction of cortisol with progesterone in bovine luteinized granulosa cells

Interestingly, cortisol and progestogens exerted opposite effects on the expression of *STAR*, *HSD3B1*, *HSD11B1*, *HSD11B2*, *RGS2,* and *VNN2* (Fig. 6.1). Both steroids exerted clear effects under the experimental conditions where one steroid was administered in the absence or presence of the endogenous other, which gradually accumulated in the culture medium during the experiments. However, when concurrently administered, the effects of progestogens predominated those of cortisol. Therefore, it suffices to hypothesize that the effects of cortisol may not be evident in vivo, where P4 production far exceeds cortisol production, which is fairly limited by the availability of endocrine-derived cortisone. Moreover, cortisol levels in the follicular fluid and plasma are

generally between 0.01 to 0.1 μM, and are unlikely to reach 1 μM [74,85]. Put together, this suggests that P4 predominantly regulates gene expression and cortisol production in bovine LGCs.

6.4. Summary

This study shows that LGCs can produce cortisol and that its production is downregulated by high levels of P4 and possibly upregulated by cortisol if the P4 level is low enough to allow the selective action of cortisol. Additionally, the two C_{21} steroids differentially regulate the expression of *STAR*, *CYP11A1*, *HSD3B1*, *HSD11B*s, *PTX3*, *RGS2*, and *VNN2* in bovine LGCs. Further studies are necessary to determine if this is the case *in vivo* and to understand the physiological significance of this phenomenon during luteinization.
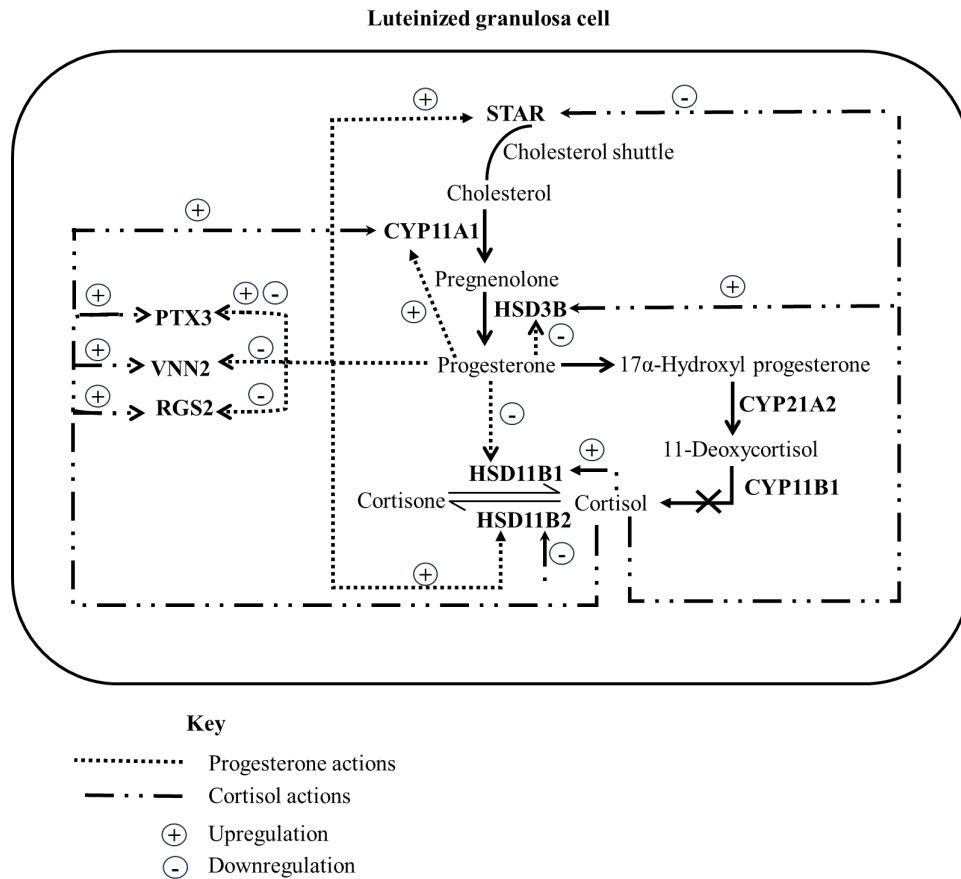
Figure 6.1. Summary of the effects of progesterone and cortisol on gene expression and cortisol production in luteinized granulosa cells.

References

[1]   **Sunderland SJ, Crowe MA, Boland MP, Roche JF, Ireland JJ**. Selection, dominance and atresia of follicles during the oestrous cycle of heifers. Reproduction 1994;101:547–55.

[2]   **Smith MF, McIntush EW, Smith GW.** Mechanisms associated with corpus luteum development. J Anim Sci 1994;72:1857–72.

[3]   **Sanderson JT.** The Steroid Hormone Biosynthesis Pathway as a Target for Endocrine-Disrupting Chemicals. Toxicological Sciences 2006;94:3–21.

[4]   **Niswender GD.** Molecular control of luteal secretion of progesterone. Reproduction 2002;123:333–9.

[5]   **Der Schoot P Van, Bakker GH, Klijn JGM**. Effects of the Progesterone Antagonist RU486 on Ovarian Activity in the Rat. Endocrinology 1987;121:1375–82.

[6]   **Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS**. Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. Proc Natl Acad Sci U S A 2000;97:4689–94.

[7]   **Robinson NA, Leslie KE, Walton' JS**. Effect of Treatment with Progesterone on Pregnancy Rate and Plasma Concentrations of Progesterone in Holstein Cows. J Dairy Sci 1989;72:202–7.

[8]   **Demayo FJ, Lyson JP, Demayo FJ, Lydon JP**. 90 YEARS OF PROGESTERONE: New insights into progesterone receptor signaling in the endometrium required for embryo implantation. J Mol Endocrinol 2020;65:T1–14.

[9]   **Peluso JJ, Pappalardo A**. Progesterone Mediates its Anti-Mitogenic and Anti-Apoptotic Actions in Rat Granulosa Cells Through a Progesterone-Binding Protein With Gamma Aminobutyric Acid A Receptor-Like Features. Biol Reprod 1998;58:1131–7.

[10]   **Luciano AM, Peluso JJ**. Effect of in Vivo Gonadotropin Treatment on the Ability of Progesterone, Estrogen, and Cyclic Adenosine 5′-Monophosphate to Inhibit Insulin-Dependent Granulosa Cell Mitosis in Vitro. Biol Reprod 1995;53:664–9.

[11]   **Chaffkin LM, Luciano AA, Peluso JJ**. The role of progesterone in regulating human granulosa cell proliferation and differentiation in vitro. J Clin Endocrinol Metab 1993;76:696–700.

[12]   **Rothchild I.** The Regulation of the Mammalian Corpus Luteum. Recent Prog Horm Res 1981;37:183–298. https://doi.org/10.1016/B978-0-12-571137-1.50009-8.

[13]   **Peluso JJ, Pappalardo A, Losel R, Wehling M**. Expression and Function of PAIRBP1 Within Gonadotropin-Primed Immature Rat Ovaries: PAIRBP1 Regulation of Granulosa and Luteal Cell Viability. Biol Reprod 2005;73:261–70.

[14]   **Telleria CM, Stocco CO, Stati AO, Deis RP**. Progesterone receptor is not required for progesterone action in the rat corpus luteum of pregnancy☆. Steroids 1999;64:760–6.

[15]   **Goyeneche AA, Deis RP, Gibori G, Telleria CM**. Progesterone Promotes Survival of the Rat Corpus Luteum in the Absence of Cognate Receptors. Biol Reprod 2003;68:151–8.

[16]   **Rekawiecki R, Nowik M, Kotwica J**. Stimulatory effect of LH, PGE2 and progesterone on StAR protein, cytochrome P450 cholesterol side chain cleavage and 3β hydroxysteroid dehydrogenase gene expression in bovine luteal cells. Prostaglandins Other Lipid Mediat 2005;78:169–84.

[17]   **Schreiber JR, Nakamura K, Erickson GF**. Progestins inhibit FSH-stimulated steroidogenesis in cultured rat granulosa cells. Mol Cell Endocrinol 1980;19:165–73.

[18]   **Makrigiannakis A, Coukos G, Christofidou-Solomidou M, Montas S, Coutifaris C**. Progesterone Is an Autocrine/Paracrine Regulator of Human Granulosa Cell Survival in Vitro. Ann N Y Acad Sci 2000;900:16–25.

[19]   **Svensson EC, Markstrom E, Andersson M, Billig H**. Progesterone Receptor-Mediated Inhibition of Apoptosis in Granulosa Cells Isolated from Rats Treated with Human Chorionic Gonadotropin. Biol Reprod 2000;63:1457–64.

[20] **Shao R, Markström E, Friberg PA, Johansson M, Billig H**. Expression of Progesterone Receptor (PR) A and B Isoforms in Mouse Granulosa Cells: Stage-Dependent PR-Mediated Regulation of Apoptosis and Cell Proliferation. Biol Reprod 2003;68:914–21.

[21] **O'Malley BW**. A Life-Long Search for the Molecular Pathways of Steroid Hormone Action. Molecular Endocrinology 2005;19:1402–11.

[22] **Richards JAS, Russell DL, Robker RL, Dajee M, Alliston TN**. Molecular mechanisms of ovulation and luteinization. Mol Cell Endocrinol 1998;145:47–54.

[23] **Russell DL, Robker RL**. Molecular mechanisms of ovulation: co-ordination through the cumulus complex. Hum Reprod Update 2007;13:289–312.

[24] **Honda T, Fujiwara H, Yamada S, Fujita K, Nakamura K, Nakayama T, Higuchi T, Ueda M, Maeda M, Mori T**. Integrin alpha5 is expressed on human luteinizing granulosa cells during corpus luteum formation, and its expression is enhanced by human chorionic gonadotrophin in vitro. Mol Hum Reprod 1997;3:979–84.

[25] **Robker RL, Richards JS**. Hormone-induced proliferation and differentiation of granulosa cells: A coordinated balance of the cell cycle regulators cyclin D2 and p27(kip1). Molecular Endocrinology 1998;12:924–40.

[26] **Shimizu T, Sudo N, Yamashita H, Murayama C, Miyazaki H, Miyamoto A**. Histone H3 acetylation of StAR and decrease in DAX-1 is involved in the luteinization of bovine granulosa cells during in vitro culture. Mol Cell Biochem 2009;328:41–7.

[27] **Pescador N, Stocco DM, Murphy BD**. Growth Factor Modulation of Steroidogenic Acute Regulatory Protein and Luteinization in the Pig Ovary 1. Biol Reprod 1999;60:1453–61.

[28] **Bagnjuk K, Mayerhofer A**. Human Luteinized Granulosa Cells—A Cellular Model for the Human Corpus Luteum. Front Endocrinol (Lausanne) 2019;10:452.

[29] **Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW**. Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 2000;80:1–29.

[30]   **Bao B, Garverick HA**. Expression of Steroidogenic Enzyme and Gonadotropin Receptor Genes in Bovine Follicles during Ovarian Follicular Waves: A Review. J Anim Sci 1998;76:1903–21.

[31]   **Shirasuna K, Watanabe S, Yamamoto D, Hayashi M, Nagai K, Miyamoto A**. Bovine endothelial cells interact with fully-luteinized, but not luteinizing, granulosa cells in the mRNA expression of endothelin-1 system in response to prostaglandin F2α. Reproduction in Domestic Animals 2007;42:637–42.

[32]   **Baufeld A, Vanselow J**. Increasing cell plating density mimics an early post-LH stage in cultured bovine granulosa cells. Cell Tissue Res 2013;354:869–80.

[33]   **Baufeld A, Vanselow J**. Lactate promotes specific differentiation in bovine granulosa cells depending on lactate uptake thus mimicking an early post-LH stage. Reproductive Biology and Endocrinology 2018;16.

[34]   **Baufeld A, Koczan D, Vanselow J**. Induction of altered gene expression profiles in cultured bovine granulosa cells at high cell density. Reproductive Biology and Endocrinology 2017;15.

[35]   **Christenson LK, Gunewardena S, Hong X, Spitschak M, Baufeld A, Vanselow J**. Research Resource: Preovulatory LH Surge Effects on Follicular Theca and Granulosa Transcriptomes. Molecular Endocrinology 2013;27:1153–71.

[36]   **Bottazzi B, Garlanda C, Salvatori G, Jeannin P, Manfredi A, Mantovani A**. Pentraxins as a key component of innate immunity. Curr Opin Immunol 2006;18:10–5.

[37]   **Bottazzi B, Bastone A, Doni A, Garlanda C, Valentino S, Deban L, Maina V, Cotena A, Moalli F, Vago L, Salustri A, Romani L, Mantovani A**. The long pentraxin PTX3 as a link among innate immunity, inflammation, and female fertility. J Leukoc Biol 2006;79:909–12.

[38]   **Garlanda C, Hirsch E, Bozza S, Salustri A, De Acetis M, Nota R, Maccagno A, Riva F, Bottazzi B, Peri G, Doni A, Vago L, Botto M, De Santis R, Carminati P, Siracusa G, Altruda F, Vecchi A**, et al. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. Nature 2002;420:182–6.

[39]   **Ravizza T, Moneta D, Bottazzi B, Peri G, Garlanda C, Hirsch E, Richards GJ, Mantovani A, Vezzani A**. Dynamic induction of the long pentraxin PTX3 in the CNS after limbic seizures: evidence for a protective role in seizure-induced neurodegeneration. Neuroscience 2001;105:43–53.

[40]   **Mantovani A, Garlanda C, Bottazzi B**. Pentraxin 3, a non-redundant soluble pattern recognition receptor involved in innate immunity. Vaccine 2003;21:S43–7.

[41]   **Garlanda C, Maina V, Martinez de la Torre Y, Nebuloni M, Locati M**. Inflammatory Reaction and Implantation: the New Entries PTX3 and D6. Placenta 2008;29:129–34.

[42]   **Romero JJ, Antoniazzi AQ, Smirnova NP, Webb BT, Yu F, Davis JS, Hansen TR**. Pregnancy-associated genes contribute to antiluteolytic mechanisms in ovine corpus luteum. Physiol Genomics 2013;45:1095–108.

[43]   **Garlanda C, Bottazzi B, Bastone A, Mantovani A**. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Annu Rev Immunol 2005;23:337–66.

[44]   **Pepperl DJ, Shah-Basu S, VanLeeuwen D, Granneman JG, MacKenzie RG**. Regulation of RGS mRNAs by cAMP in PC12 Cells. Biochem Biophys Res Commun 1998;243:52–5.

[45]   **Riddle EL, Schwartzman RA, Bond M, Insel PA**. Multi-tasking RGS proteins in the heart: The next therapeutic target? Circ Res 2005;96:401–11.

[46]   **Miles RR, Sluka JP, Santerre RF, Hale L V., Bloem L, Boguslawski G, Thirunavukkarasu K, Hock JM, Onyia JE**. Dynamic Regulation of RGS2 in Bone: Potential New Insights into Parathyroid Hormone Signaling Mechanisms. Endocrinology 2000;141:28–36.

[47]   **Heximer SP, Cristillo AD, Forsdyke DR**. Comparison of mRNA Expression of Two Regulators of G-Protein Signaling, RGS1/BL34/1R20 and RGS2/G0S8, in Cultured Human Blood Mononuclear Cells.

[48]   **Kach J, Sethakorn N, Dulin NO**. A finer tuning of G-protein signaling through regulated control of RGS proteins. American Journal of Physiology-Heart and Circulatory Physiology 2012;303:19–35.

[49]   **Zhang P, Mende U**. Functional role, mechanisms of regulation, and therapeutic potential of regulator of G protein signaling 2 in the heart. Trends Cardiovasc Med 2014;24:85–93.

[50]   **Granjeaud S, Naquet P, Galland F**. An ESTs description of the new Vanin gene family conserved from fly to human. Immunogenetics 1999;49:964–72.

[51]   **Galland F, Malergue F, Bazin H, Mattei MG, Aurrand-Lions M, Theillet C, Naquet P**. Two Human Genes Related to Murine Vanin-1 Are Located on the Long Arm of Human Chromosome 6. Genomics 1998;53:203–13.

[52]   **Wojtczak L, Slyshenkov VS**. Protection by pantothenic acid against apoptosis and cell damage by oxygen free radicals - The role of glutathione. BioFactors 2003;17:61–73.

[53]   **Wilson MJ, Jeyasuria P, Parker KL, Koopman P**. The Transcription Factors Steroidogenic Factor-1 and SOX9 Regulate Expression of Vanin-1 during Mouse Testis Development. Journal of Biological Chemistry 2005;280:5917–23.

[54]   **Bailey CDC, Johnson GVW**. The protective effects of cystamine in the R6/2 Huntington's disease mouse involve mechanisms other than the inhibition of tissue transglutaminase. Neurobiol Aging 2006;27:871–9.

[55]   **Roisin-Bouffay C, Castellano R, Valéro R, Chasson L, Galland F, Naquet P**. Mouse vanin-1 is cytoprotective for islet beta cells and regulates the development of type 1 diabetes. Diabetologia 2008;51:1192–201.

[56]   **Varani S, Elvin JA, Yan C, DeMayo J, DeMayo FJ, Horton HF, Byrne MC, Matzuk MM**. Knockout of Pentraxin 3, a Downstream Target of Growth Differentiation Factor-9, Causes Female Subfertility. Molecular Endocrinology 2002;16:1154–67.

[57]   **Tranguch S, Chakrabarty A, Guo Y, Wang H, Dey SK**. Maternal Pentraxin 3 Deficiency Compromises Implantation in Mice. Biol Reprod 2007;77:425–32.

[58]   **Zhu Y, Jiang YH, He YP, Zhang X, Sun ZG, Jiang MX, Wang J.** Knockdown of regulator of G-protein signalling 2 (Rgs2) leads to abnormal early mouse embryo development in vitro. Reprod Fertil Dev 2015;27:557–66.

[59]   **Girard A, Dufort I, Sirard MA**. The effect of energy balance on the transcriptome of bovine granulosa cells at 60 Days postpartum. Theriogenology 2015;84:1350-1361.e6.

[60]   **Basavaraja R, Madusanka ST, Shrestha K, Przygrodzka E, Kaczmarek MM, Meidan R**. Pentraxin-3 mediates prosurvival actions of interferon tau in bovine luteinized granulosa cells. Reproduction 2020;160:603–12.

[61]   **Norata GD, Garlanda C, Catapano AL**. The long pentraxin PTX3: A modulator of the immunoinflammatory response in atherosclerosis and cardiovascular diseases. Trends Cardiovasc Med 2010;20:35–40.

[62]   **Mantovani A, Garlanda C, Doni A, Bottazzi B**. Pentraxins in innate immunity: From C-reactive protein to the long pentraxin PTX3. J Clin Immunol 2008;28:1–13.

[63]   **Camaioni A, Klinger FG, Campagnolo L, Salustri A**. The Influence of Pentraxin 3 on the Ovarian Function and Its Impact on Fertility. Front Immunol 2018;9.

[64]   **Kim Y, Ghil S**. Regulators of G-protein signaling, RGS2 and RGS4, inhibit protease-activated receptor 4-mediated signaling by forming a complex with the receptor and Gα in live cells. Cell Communication and Signaling 2020;18:1–13.

[65]   **Oliveira-dos-Santos AJ, Matsumoto G, Snow BE, Bai D, Houston FP, Whishaw IQ, Mariathasan S, Sasaki T, Wakeham A, Ohashi PS, Roder JC, Barnes CA, Siderovski DP, Penninger JM**. Regulation of T cell activation, anxiety, and male aggression by RGS2. Proc Natl Acad Sci U S A 2000;97:12272–7.

[66]   **Jang HS, Kim JI, Noh M, Rhee MH, Park KM**. Regulator of G protein signaling 2 (RGS2) deficiency accelerates the progression of kidney fibrosis. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 2014;1842:1733–41.

[67]   **George T, Chakraborty M, Giembycz MA, Newton R**. A bronchoprotective role for Rgs2 in a murine model of lipopolysaccharide-induced airways inflammation. Allergy Asthma Clin Immunol 2018;14.

[68]   **George T, Bell M, Chakraborty M, Siderovski DP, Giembycz MA, Newton R**. Protective Roles for RGS2 in a Mouse Model of House Dust Mite-Induced Airway Inflammation. PLoS One 2017;12.

[69]   **Dong H, Zhang Y, Wang J, Kim DS, Wu H, Sjögren B, Gao W, Luttrell L, Wang H**. Regulator of G protein signaling 2 is a key regulator of pancreatic β-cell mass and function. Cell Death & Disease 2017 8:5 2017;8:e2821–e2821.

[70]   **Aurrand-Lions M, Galland F, Bazin H, Zakharyev VM, Imhof BA, Naquet P**. Vanin-1, a novel GPI-linked perivascular molecule involved in thymus homing. Immunity 1996;5:391–405.

[71]   **Watanabe T, Sendo F**. Physical association of β2 integrin with GPI-80, a novel glycosylphosphatidylinositol-anchored protein with potential for regulating adhesion and migration. Biochem Biophys Res Commun 2002;294:692–4.

[72]   **Sendo F, Suzuki K, Watanabe T, Takeda Y, Araki Y**. Modulation of leukocyte transendothelial migration by integrin-associated glycosyl phosphatidyl inositol (GPI)-anchored proteins. Inflammation Research 1998;47.

[73]   **Harlow CR, Jenkins JM, Winston RML**. Increased follicular fluid total and free cortisol levels during the luteinizing hormone surge. Fertil Steril 1997;68:48–53. https://doi.org/10.1016/S0015-0282(97)81474-4.

[74]   **Acosta TJ, Tetsuka M, Matsui M, Shimizu T, Berisha B, Schams D, Miyamoto A**. In vivo evidence that local cortisol production increases in the preovulatory follicle of the cow. Journal of Reproduction and Development 2005;51:483–9.

[75]   **Tetsuka M, Milne M, Simpson GE, Hillier SG**. Expression of 11β-hydroxysteroid dehydrogenase, glucocorticoid receptor, and mineralocorticoid receptor genes in rat ovary. Biol Reprod 1999;60:330–5.

[76]   **Yong PYK, Thong KJ, Andrew R, Walker BR, Hillier SG**. Development-related increase in cortisol biosynthesis by human granulosa cells. J Clin Endocrinol Metab 2000;85:4728–33.

[77]   **Davies E, MacKenzie SM**. Extra-adrenal production of corticosteroids. Clin Exp Pharmacol Physiol 2003;30:437–45.

[78]   **Taves MD, Gomez-Sanchez CE, Soma KK**. Extra-adrenal glucocorticoids and mineralocorticoids: Evidence for local synthesis, regulation, and function. Am J Physiol Endocrinol Metab 2011;301.

[79]   **Raff H, Carroll T**. Cushing's syndrome: from physiological principles to diagnosis and clinical care. J Physiol 2015;593:493.

[80]   **Dickmeis T**. Glucocorticoids and the circadian clock. Journal of Endocrinology 2009;200:3–22.

[81]   **Fru KN, Vandevoort CA, Chaffin CL**. Mineralocorticoid synthesis during the periovulatory interval in macaques. Biol Reprod 2006;75:568–74..

[82]   **Amin M, Simerman A, Cho M, Singh P, Briton-Jones C, Hill D, Grogan T, Elashoff D, Clarke NJ, Chazenbalk GD, Dumesic DA**. 21-Hydroxylase-derived steroids in follicles of nonobese women undergoing ovarian stimulation for in vitro fertilization (IVF) positively correlate with lipid content of luteinized granulosa cells (LGCs) as a source of cholesterol for steroid synthesis. J Clin Endocrinol Metab 2014;99:1299–306.

[83]   **Robic A, Feve K, Louveau I, Riquet J, Prunier A**. Exploration of steroidogenesis-related genes in testes, ovaries, adrenals, liver and adipose tissue in pigs. Animal Science Journal 2016;87:1041–7.

[84]   **Yazawa T, Uesaka M, Inaoka Y, Mizutani T, Sekiguchi T, Kajitani T, Kitano T, Umezawa A, Miyamoto K**. Cyp11b1 is induced in the murine gonad by luteinizing hormone/human chorionic gonadotropin and involved in the production of 11-ketotestosterone, a major fish androgen: Conservation and evolution of the androgen metabolic pathway. Endocrinology 2008;149:1786–92.

[85]   **Mukangwa M, Takizawa K, Aoki Y, Hamano S, Tetsuka M**. Expression of genes encoding mineralocorticoid biosynthetic enzymes and the mineralocorticoid receptor, and levels of mineralocorticoids in the bovine follicle and corpus luteum. J Reprod Dev 2020;66:75–81.

[86]   **Amweg AN, Rodríguez FM, Huber E, Marelli BE, Gareis NC, Belotti EM, Rey F, Salvetti NR, Ortega HH**. Detection and activity of 11 beta hydroxylase (CYP11B1) in the bovine ovary. Reproduction 2017;153:433–41.

[87]   **Whorwood CB, Franklyn JA, Sheppard MC, Stewart PM**. Tissue localization of 11β-hydroxysteroid dehydrogenase and its relationship to the glucocorticoid receptor. J Steroid Biochem Mol Biol 1992;41:21–8.

[88]   **Okuda K, Korzekwa A, Shibaya M, Murakami S, Nishimura R, Tsubouchi M, Woclawek-Potocka I, Skarzynski DJ**. Progesterone is a suppressor of apoptosis in bovine luteal cells. Biol Reprod 2004;71:2065–71.

[89] **Komiyama J, Nishimura R, Lee HY, Sakumoto R, Tetsuka M, Acosta TJ, Skarzynski DJ, Okuda K**. Cortisol is a suppressor of apoptosis in bovine corpus luteum. Biol Reprod 2008;78:888–95.

[90] **Whirledge S, Cidlowski JA**. Glucocorticoids, stress, and fertility. Minerva Endocrinol 2010;35:109–25.

[91] **Duffy DM, Ko C, Jo M, Brannstrom M, Curry TE**. Ovulation: Parallels with inflammatory processes. Endocr Rev 2019;40:369–416.

[92] **Hillier SG, Tetsuka M**. An anti-inflammatory role for glucocorticoids in the ovaries? J Reprod Immunol 1998;39:21–7.

[93] **Sasson R, Amsterdam A**. Pleiotropic anti-apoptotic activity of glucocorticoids in ovarian follicular cells. Biochem Pharmacol 2003;66:1393–401.

[94] **Sasson R, Winder N, Kees S, Amsterdam A**. Induction of apoptosis in granulosa cells by TNFα and its attenuation by glucocorticoids involve modulation of Bcl-2. Biochem Biophys Res Commun 2002;294:51–9.

[95] **Sasson R, Tajima K, Amsterdam A**. Glucocorticoids protect against apoptosis induced by serum deprivation, cyclic adenosine 3′,5′-monophosphate and p53 activation in immortalized human granulosa cells: Involvement of Bcl-2. Endocrinology 2001;142:802–11.

[96] **Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL**. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 2012;13:134.

[97] **Rekawiecki R, Kowalik MK, Kotwica J**. Cloning and expression of progesterone receptor isoforms A and B in bovine corpus luteum. Reprod Fertil Dev 2015;27:1029–37.

[98] **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F**. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3.

[99] **Murphy BD. Luteinization**. The Ovary: Second Edition 2004:185–99.

[100]   Rábano M, Peña A, Brizuela L, Macarulla JM, Gómez-Muñoz A, Trueba M. Angiotensin II-stimulated cortisol secretion is mediated by phospholipase D. Mol Cell Endocrinol 2004;222:9–20.

[101]   **Espey LL, Richards JAS**. Temporal and Spatial Patterns of Ovarian Gene Transcription Following an Ovulatory Dose of Gonadotropin in the Rat. Biol Reprod 2002;67:1662–70.

[102]   **Sayasith K, Sirois J, Lussier JG**. Expression, regulation, and promoter activation of vanin-2 (VNN2) in bovine follicles prior to ovulation. Biol Reprod 2013;89.

[103]   **Sayasith K, Sirois J, Lussier JG**. Expression and regulation of regulator of G-protein signaling protein-2 (RGS2) in equine and bovine follicles prior to ovulation: Molecular characterization of RGS2 transactivation in bovine granulosa cells. Biol Reprod 2014;91.

[104]   **Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazi B, Doni A, Bastone A, Mantovani G, Peccoz PB, Salvatori G, Mahoney DJ, Day AJ, Siracusa G, Romani L, Mantovani A**. PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. Development 2004;131:1577–86.

[105]   **Lussier JG, Diouf MN, Lévesque V, Sirois J, Ndiaye K**. Gene expression profiling of upregulated mRNAs in granulosa cells of bovine ovulatory follicles following stimulation with hCG. Reproductive Biology and Endocrinology 2017;15.

[106]   **Tetsuka M, Haines LC, Milne M, Simpson GE, Hillier SG**. Regulation of 11β-hydroxysteroid dehydrogenase type 1 gene expression by LH and interleukin-1β in cultured rat granulosa cells. Journal of Endocrinology 1999;163:417–23.

[107]   **Evagelatou M, Peterson SL, Cooke BA**. Leukocytes modulate 11β-hydroxysteroid dehydrogenase (11β-HSD) activity in human granulosa-lutein cell cultures. Mol Cell Endocrinol 1997;133:81–8.

[108]   **Rueda BR, Hendry IR, Hendry WJ, Stormshak F, Slayden OD, Davis JS**. Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. Biol Reprod 2000;62:269–76.

[109] **Svensson EC, Markstrom E, Andersson M, Billig H**. Progesterone receptor-mediated inhibition of apoptosis in granullosa cells isolated from rats treated with human chorionic gonadotropin. Biol Reprod 2000;63:1457–64.

[110] **Friberg PA, Larsson DGJ, Billig H**. Dominant Role of Nuclear Progesterone Receptor in the Control of Rat Periovulatory Granulosa Cell Apoptosis. Biol Reprod 2009;80:1160–7.

[111] **Pate JL**. Intercellular communication in the bovine corpus luteum. Theriogenology 1996;45:1381–97.

[112] **Rekawiecki R, Kowalik MK, Slonina D, Kotwica J**. Regulation of progesterone synthesis and action in bovine corpus luteum. Journal of Physiology and Pharmacology, vol. 59, 2008, p. 75–89.

[113] **Vandevoort CA, Overstreet JW, Lasley BL, Stewart DR**. Effects of Progesterone Receptor Blockers on Human Granulosa-Luteal Cell Culture Secretion of Progesterone, Estradiol, and Relaxin. Biol Reprod 2000;62:200–5.

[114] **Ruiz de Galarreta CM, Fanjul LF, Hsueh AJW**. Progestin regulation of progesterone biosynthetic enzymes in cultured rat granulosa cells. Steroids 1985;46:987–1002.

[115] **Caffrey JL, Nett TM, Abel JH, Niswender GD**. Activity of 3β-Hydroxy-Δ5-Steroid Dehydrogenase/Δ 5-Δ4-Isomerase in the Ovine Corpus Luteum. Biol Reprod 1979;20:279–87.

[116] **Anbo N, Suzuki A, Mukangwa M, Takahashi R, Muranishi Y, Tetsuka M**. Progesterone stimulates cortisol production in the maturing bovine cumulus-oocyte complex. Theriogenology 2022;189:183–91.

[117] **Ujioka T, Russell DL, Okamura H, Richards JS, Espey LL**. Expression of regulator of G-protein signaling protein-2 gene in the rat ovary at the time of ovulation. Biol Reprod 2000;63:1513–7.

[118] **Wu YL, Chuang HH, Kou YR, Lee TS, Lu SH, Huang YC, Nishi Y, Yanase T**. Regulation of LH receptor and PGF2α receptor signaling by the regulator of G protein signaling 2 (RGS2) in human and mouse granulosa cells. Chinese Journal of Physiology 2008;51:282–91.

[119] **Nagyova E, Kalous J, Nemcova L**. Increased expression of pentraxin 3 after in vivo and in vitro stimulation with gonadotropins in porcine oocyte-cumulus complexes and granulosa cells. Domest Anim Endocrinol 2016;56:29–35.

[120] **Ranta T, Knecht M, Darbon JM, Baukal AJ, Catt KJ**. Induction of granulosa cell differentiation by forskolin: Stimulation of adenosine 3',5'-monophosphate production, progesterone synthesis, and luteinizing hormone receptor expression. Endocrinology 1984;114:845–50.

[121] **Mamluk R, Wolfenson D, Meidan R**. LH receptor mRNA and cytochrome P450 side-chain cleavage expression in bovine theca and granulosa cells luteinized by LH or forskolin. Domest Anim Endocrinol 1998;15:103–14.

[122] **Adashi EY, Resnick CE**. Forskolin-induced differentiation of cultured rat granulosa cells: New evidence for an intermediary role of adenosine 3',5'-monophosphate in the mechanism of action of follicle-stimulating hormone. Endocrinology 1984;115:183–90.

[123] **Tetsuka M, Yamamoto S, Hayashida N, Hayashi KG, Hayashi M, Acosta TJ, Miyamoto A**. Expression of 11beta-hydroxysteroid dehydrogenases in bovine follicle and corpus luteum. J Endocrinol 2003;177:445–52.

[124] **Clemens JW, Robker RL, Kraust WL, Katzenellenbogen BS, Richards JS**. Hormone induction of progesterone receptor (PR) messenger ribonucleic acid and activation of PR promoter regions in ovarian granulosa cells: Evidence for a role of cyclic adenosine 3',5'-monophosphate but not estradiol. Molecular Endocrinology 1998;12:1201–14.

[125] **Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ**. Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Mol Cell Endocrinol 2001;179:97–103.

[126] **Rae MT, Niven D, Critchley HOD, Harlow CR, Hillier SG**. Antiinflammatory steroid action in human ovarian surface epithelial cells. Journal of Clinical Endocrinology and Metabolism 2004;89:4538–44.

[127] **Hsueh AJW, Erickson GF**. Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. Steroids 1978;32:639–48.

[128] **Adashi EY, Jones PBC, Hsueh AJW**. Synergistic Effect of Glucocorticoids on the Stimulation of Progesterone Production by Follicle-Stimulating Hormone in Cultured Rat Granulosa Cells. Endocrinology 1981;109:1888–94.

[129] **Schoonmaker JN, Erickson GF**. Glucocorticoid Modulation of Follicle-Stimulating Hormone-Mediated Granulosa Cell Differentiation. Endocrinology 1983;113:1356–63.

[130] **Yuan XH, Yang BQ, Hu Y, Fan YY, Zhang LX, Zhou JC, Wang YQ, Lu CL, Ma X**. Dexamethasone altered steroidogenesis and changed redox status of granulosa cells. Endocrine 2014;47:639–47.

[131] **Kashino C, Hasegawa T, Nakano Y, Iwata N, Yamamoto K, Kamada Y, Masuyama H, Otsuka F**. Involvement of BMP-15 in glucocorticoid actions on ovarian steroidogenesis by rat granulosa cells. Biochem Biophys Res Commun 2021;559:56–61.

[132] **Viveiros MM, Liptrap RM**. Glucocorticoid influence on porcine granulosa cell IGF-I and steroid hormone production in vitro. Theriogenology 1999;51:1027–43.

[133] **Kawate N, Inaba T, Mori J**. Effects of cortisol on the amounts of estradiol-17β and progesterone secreted and the number of luteinizing hormone receptors in cultured bovine granulosa cells. Anim Reprod Sci 1993;32:15–25.

[134] **Ben-Rafael Z, Benadiva CA, Garcia CJ, Flickinger GL**. Cortisol stimulation of estradiol and progesterone secretion by human granulosa cells is independent of follicle-stimulating hormone effects. Fertil Steril 1988;49:813–6.

[135] **Tetsuka M**. Actions of glucocorticoid and their regulatory mechanisms in the ovary: Review article. Animal Science Journal 2007;78:112–20.

[136] **Huang TJ, Li PS**. Dexamethasone inhibits luteinizing hormone-induced synthesis of steroidogenic acute regulatory protein in cultured rat preovulatory follicles. Biol Reprod 2001;64:163–70.

[137] **Michael TE, Pester LA, Curtis P, Shaw RW, Edwards CRW, Cooke BA**. Direct inhibition of ovarian steroidogenesis by cortisol and the modulatory role of 11 beta-hydroxysteroid dehydrogenase. Clin Endocrinol (Oxf) 1993;38:641–4.

[138] **Sugawara T, Kiriakidou M, McAllister JM, Holt JA, Arakane F, Strauss JF**. Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: A central role for steroidogenic factor 1. Steroids, vol. 62, Elsevier Inc.; 1997, p. 5–9.

[139] **Castillo J, Castellana B, Acerete L, Planas J V., Goetz FW, Mackenzie S, Tort L**. Stress-induced regulation of steroidogenic acute regulatory protein expression in head kidney of Gilthead seabream (Sparus aurata). Journal of Endocrinology 2008;196:313–22. https://doi.org/10.1677/JOE-07-0440.

[140] **Hsueh AJW, Erickson GF**. Glucocorticoid inhibition of fsh-induced estrogen production in cultured rat granulosa cells. Steroids 1978;32:639–48.

[141] **Ben-Rafael Z, Benadiva CA, Garcia CJ, Flickinger GL**. Cortisol stimulation of estradiol and progesterone secretion by human granulosa cells is independent of follicle-stimulating hormone effects. Fertil Steril 1988;49:813–6.

[142] **Cain DW, Cidlowski JA**. Immune regulation by glucocorticoids. Nat Rev Immunol 2017;17:233.

[143] **Zhang J, Koussih L, Shan L, Halayko AJ, Tliba O, Gounni AS**. Glucocorticoids regulate pentraxin-3 expression in human airway smooth muscle cells. PLoS One 2019;14.

[144] **Holden NS, Bell MJ, Rider CF, King EM, Gaunt DD, Leigh R, Johnson M, Siderovski DP, Heximer SP, Giembycz MA, Newton R**. β 2-Adrenoceptor agonist-induced RGS2 expression is a genomic mechanism of bronchoprotection that is enhanced by glucocorticoids. Proc Natl Acad Sci USA 2011;108:19713–8.

[145] **Holden NS, George T, Rider CF, Chandrasekhar A, Shah S, Kaur M, Johnson M, Siderovski DP, Leigh R, Giembycz MA, Newton R**. Induction of Regulator of G-Protein Signaling 2 Expression by Long-Acting β2-

Adrenoceptor Agonists and Glucocorticoids in Human Airway Epithelial Cells. Journal of Pharmacology and Experimental Therapeutics 2014;348:12–24.

[146]   **Rajan V, Edwards CRW, Seckl JR**. 11 beta-Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. J Neurosci 1996;16:65–70.

[147]   **Sun K, He P, Yang K**. Intracrine induction of 11β-hydroxysteroid dehydrogenase type 1 expression by glucocorticoid potentiates prostaglandin production in the human chorionic trophoblast. Biol Reprod 2002;67:1450–5.

[148]   **Clarke KA, Ward JW, Forhead AJ, Giussani DA, Fowden AL**. Regulation of 11 beta-hydroxysteroid dehydrogenase type 2 activity in ovine placenta by fetal cortisol. J Endocrinol 2002;172:527–34.

[149]   **MacKenzie SM, Fraser R, McConnell J, Davies E**. Local renin-angiotensin systems and their interactions with extra-adrenal corticosteroid production. JRAAS - Journal of the Renin-Angiotensin-Aldosterone System 2002;3:214–21.

[150]   **Whirledge S, Cidlowski JA**. Glucocorticoids and Reproduction: Traffic Control on the Road to Reproduction. Trends in Endocrinology & Metabolism 2017;28:399–415.

[151]   **Thomas FJ, Thomas MJ, Tetsuka M, Mason JI, Hillier SG**. Corticosteroid metabolism in human granulosa-lutein cells. Clin Endocrinol (Oxf) 1998;48:509–13.

[152]   **Michael AE, Cooke BA**. A working hypothesis for the regulation of steroidogenesis and germ cell development in the gonads by glucocorticoids and 11 beta-hydroxysteroid dehydrogenase (11 beta HSD). Mol Cell Endocrinol 1994;100:55–63.

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