Expression of genes involved in prostaglandin E₂ and progesterone production in bovine cumulus–oocyte complexes during in vitro maturation and fertilization

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Abstract

Prostaglandin E₂ (PGE₂) and progesterone appear to be critical mediators of cumulus expansion and the resumption of oocyte meiosis. The aim of this study was to identify the types of prostaglandin E synthase (PTGES) expressed in the bovine cumulus–oocyte complex (COC), to characterize their temporal expression during the periconceptional interval using an in vitro model of maturation (IVM) and fertilization (IVF), and to compare their expression with the level of steroidogenic gene expression. Real-time RT-PCR analysis revealed that enzymes related to the PGE₂ biosynthesis pathway were mainly expressed during IVM. Transcripts encoding PTGES1–3 were detected in bovine COCs. Only the expression of PTGES1 significantly increased during IVM whereas that of PTGES2 and PTGES3 remained unchanged. The induction of PTGES1 expression paralleled the induction of prostaglandin G/H synthase-2 (PTGS2) expression and the amounts of PGE₂ secreted by maturing COCs. Concomitantly, cholesterol side chain cleavage cytochrome P450 expression was significantly upregulated in maturing COCs and the high level of expression persisted in fertilized COCs. The expression of the StAR protein remained constant during IVM and then decreased significantly during IVF. Expression of the progesterone catabolic-related enzyme, 20α-hydroxysteroid dehydrogenase significantly decreased throughout the periconceptional interval. This was associated with a rising level of progesterone released by COCs in the culture media. In conclusion, our results suggest that the periconceptional differentiation of the bovine COC includes the transient induction of PGE₂ biosynthetic activity via the PTGS2/PTGES1 pathway during the maturation period and the increasing ability to produce progesterone from the immature to the fertilized stages.


Introduction

In the preovulatory follicle, terminal differentiation of the cumulus oophorus plays a crucial role in the ability of oocyte to resume meiosis and subsequently develop after fertilization (Hashimoto et al. 1998, Tanghe et al. 2002, Luciano et al. 2005, Gutnisky et al. 2007). The cumulus oophorus is a group of follicular somatic cells closely associated with the oocyte to form the cumulus–oocyte complex (COC; Makabe et al. 1992, Motta et al. 1995). Before ovulation, and at the same time as the progression of oocyte meiosis to metaphase II, the cumulus cell (CC) mass undergoes a process called ‘mucification’ or ‘cumulus expansion’. The CCs synthesize and assemble a highly viscoelastic extracellular matrix (ECM) in which hyaluronan is the principal component (Salustri et al. 1989). At ovulation, a metaphase II oocyte surrounded by an expanded cumulus is released from the ruptured follicle into the oviduct. In the majority of species, cumulus cells are still associated with the egg at the time of fertilization. The number of CCs in the female genital tract gradually declines to end in complete denudation of the zona pellucida covering the early embryo (humans: Motta et al. 1995, cattle: Familiari et al. 1998, and mice: Fowler et al. 1986). In cattle, the CC mass is rapidly reduced after ovulation. Nevertheless, a scattered population of CC clusters remains attached to the zona pellucida until the first stages of embryo development (Hyttel et al. 1988, Familiari et al. 1998).

A complex crosstalk mediated by gap junctions and paracrine signaling occurs between the oocyte and surrounding cumulus cells (Canipari 2000). Bidirectional communications between both compartments of the COC enable each of them to coordinate their differentiation (Epig 2001, Matzuk et al. 2002). Oocyte factors regulate the development and function of its somatic...
microenvironment, and appropriately developed surrounding somatic cells in turn promote the development of a competent oocyte (Su et al. 2004). Such concerted development is required to produce a fertilizable oocyte fully competent to undergo subsequent embryo development.

Prostaglandins (PGs) appear to be involved in cumulus-oocyte coupling. During the preovulatory period, prostaglandin G/H synthase-2 (PTGS2)-derived PGs produced by the follicular somatic compartment appear to constitute critical mediators of the cumulus expansion and oocyte maturation processes (Eppig 1981, Hizaki et al. 1999). The genetic or chemical alteration of PTGS2 activity leads to defects in cumulus expansion and oocyte maturation completion as well as severe fertilization failure (Murdoch et al. 1993, Lim et al. 1997). The production of PG starts by the conversion of arachidonic acid into prostaglandin H₂ (PGH₂) by PTGS, also known as cyclooxygenases (Murakami & Kudo 2004). The unstable intermediate, PGH₂, is then converted into various PGs by specific terminal PG synthases. Two PTGS isoforms, PTGS1 and PTGS2, are found in mammals. Unlike the constitutively expressed isoenzyme PTGS1, the PTGS2 isoform is considered to be inducible. The follicular expression of PTGS2 is upregulated by the preovulatory surge of gonadotropins. Some hours prior to ovulation, PTGS2 expression increases dramatically in the cumulus and mural granulosa cells of different mammalian species (Sirois 1994, Liu et al. 2002, McKenzie 2004). This increase in PTGS2 expression coincides with rise in the intrafollicular levels of PGs. PGE₂ is the main PG secreted following the induction of PTGS2 by maturing COCs (Takahashi et al. 2006). To date, three forms of PGE synthase (PTGES) are known: PTGES1 (or microsomal PGES-1), PTGES2 (or microsomal PGES-2), and PTGES3 (or cytosolic PGES; Kudo & Murakami 2005). The inducible membrane-bound enzyme PTGES1 is colocalized with PTGS2 in the perinuclear membrane. PGE₂ generation by PTGES1 occurs predominantly via the PTGS2-dependant pathway. Another membrane-associated PTGES2 may be coupled with both PTGS1 and PTGS2. The third form, PTGES3, is not associated with intracellular membranes. This cytosolic enzyme is constitutively expressed and functionally linked to PTGS1 with respect to the promotion of PGE₂ production. Very little is known about the expression of PTGES in ovarian follicles. In primates, mural granulosa and theca cells of periovulatory follicles express the three forms of PTGES (Duffy et al. 2005), of which only PTGS1 exhibits a gonadotropin-stimulated expression. The gonadotropin-dependent induction of PTGS1 has also been observed in bovine and mouse mural granulosa cells (Filion et al. 2001, Sun et al. 2006). However, PTGES have never been studied in the COC at any stage of differentiation, despite the proven importance of PGE₂ to the developmental competence of oocytes in mammals.

Studies in mice have suggested that prostaglandins interact with the steroid biosynthetic pathway during preovulatory differentiation of the COC (Élvin et al. 2000, Shimada et al. 2006). The PTGS2/PGE₂ biosynthesis pathway may at least partly regulate the induction of genes involved in progesterone production. Both progesterone and prostaglandins seem to be involved in the terminal differentiation of the COC. In vitro CC expansion and the resumption of oocyte meiosis are blocked by adding progesterone synthesis inhibitors or receptor antagonists to the maturation medium (pig: Shimada & Terada 2002, Shimada et al. 2004 and cattle: Wang et al. 2006). Like mural granulosa cells, CCs exhibit dramatic changes in steroidogenic gene expression and steroidogenesis during the preovulatory period in mice (Su et al. 2006). The expression of CYP11A1 and STAR (encoding the cholesterol side chain cleavage cytochrome P450 and the StAR protein respectively) is induced by the gonadotropin surge whereas the expression of CYP19A1 encoding aromatase is inhibited. These changes to the CC expression of steroidogenic enzymes are accompanied by an increase in progesterone production. Moreover, in vivo studies in the rat have suggested that the strong steroidogenic activity of the CC mass may be maintained in the postovulatory COC (Schuetz & Dubin 1981, Goldschmit et al. 1989). The ability of maturing bovine COCs to secrete progesterone in culture systems has been reported (Armstrong et al. 1996, Mingoti et al. 2002, Schoenfelder et al. 2003). However, neither the pattern of steroidogenic gene expression nor its relationship with PGE biosynthetic activity within the periconceptional COC has been described in cattle.

The aim of this study was thus to identify the form(s) of PTGES present in bovine COCs, to characterize the temporal expression patterns of enzymes involved in the PGE biosynthesis pathway during the periconceptional period, and to compare these with steroidogenic gene expression. In addition, we examined the expression of 20α-hydroxysteroid dehydrogenase (20α-HSD) because the progesterone secretion may also be regulated at the level of its catabolism (Moon et al. 1985). The 20α-HSD enzyme catalyzes the conversion of progesterone into its inactive form, 20α-hydroxyprogesterone. We used a well-established model for in vitro maturation (IVM) inducing differential levels of oocyte maturation, cumulus expansion, and PTGS2 expression (Nuttinck et al. 2002, Vigneron et al. 2003). Differential expression of the studied enzymes was analyzed in relation to the rate of in vitro fertilization (IVF) and subsequent embryo development.

Results

Enzymes of the PTGS2/PGE₂ biosynthesis pathway and PGE₂ production

Immature, matured, and fertilized COCs were harvested in order to identify the forms of PTGES expressed in the COC, and to investigate the stage-dependent changes to


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the expression of PTGS2/PGE₂ biosynthesis pathway during the periconceptional interval. Analysis using real-time RT-PCR revealed that the enzymes involved in PGE₂ synthesis were mainly expressed during IVM. The transcripts coding for the three forms of PTGES were detected in COCs. As shown in Fig. 1, the expression of PTGS2 and PTGES1 significantly increased during IVM (8- and 3.4-fold the level at 0 h respectively; \( P < 0.05 \)) whereas the level of PTGES2 and PTGES3 expression remained unaffected by the culture period. At the end of the IVF period, fertilized COCs exhibited a minimum level of PTGES expression and PTGES mRNA became undetectable. The level of \( \beta \)-actin mRNA expression did not significantly change from the immature to the fertilized stages (data not shown). We had previously shown that the addition of EGF to the maturation medium significantly increased the level of PTGS2 expression (Nuttinck et al. 2002). In the present work, we aimed to determine whether modification to maturation conditions resulted in parallel alterations of PTGES1 and PTGS2 expression at both the mRNA and protein levels. The presence of 10 ng/ml EGF in the maturation medium gave rise to a significant increase in the expression of PTGES1 mRNA and protein (2.9- and 5.8-fold the level in TCM199 alone respectively; \( P < 0.05 \)), concomitant with the increase in PTGS2 expression (Fig. 2). To determine whether the increase in PTGS2/PTGES1 expression was associated with an increase in PGE₂ secretion by maturing COCs, the concentrations of PGE₂ in maturation media supplemented or not with EGF were measured after 24 h of culture. PGE₂ production by maturing COCs was significantly higher when EGF was added to the culture medium (2.03 ± 0.35 pg versus 1.15 ± 0.17 pg of PGE₂/COC, \( P < 0.05 \)).

**Steroidogenic enzymes and progesterone production**

Immature, matured, and fertilized COCs were harvested in order to investigate the expression of steroidogenic enzymes in the COC during the periconceptional interval. The transcripts coding for STAR and CYP11A1 were detected in COCs as early as the immature stage (Fig. 3). The level of the transcript encoding STAR remained unchanged during the IVM period and then significantly decreased (\( P < 0.05 \)) to become weakly expressed after the 24 h IVF period. The level of CYP11A1 expression significantly increased during IVM (1.7-fold higher than the level at 0 h, \( P < 0.05 \)) and this rise was sustained in fertilized COCs. To investigate the ability of COCs to produce progesterone during the periconceptional interval, the amounts of progesterone accumulating in culture media during IVM and IVF were determined. The level of progesterone production exhibited by COCs during IVF was 2.2-fold higher than the level observed during IVM (313.3 ± 17.5 pg versus 144.1 ± 14.7 pg progesterone/COC, \( P < 0.01 \)). To compare the level of expression of steroidogenesis-related enzymes with the level of induction of the PTGS2/PGE₂ biosynthesis pathway, differential IVM conditions were employed. In the absence of EGF, COCs exhibited a significant reduction in STAR expression (13.6-fold lower than the level in the presence of EGF, \( P < 0.05 \)). Concomitantly, the rise in CYP11A1 expression did not occur during the 24 h IVF period (Fig. 4). In addition, the level of progesterone secreted into the maturation medium was significantly lower by about 30% in the absence of EGF (107.5 ± 3.9 pg versus 144.1 ± 14.7 pg progesterone/COC, \( P < 0.05 \)).

**Expression of 20α-HSD (AKR1B5)**

In order to investigate progesterone catabolism in the periconceptional COC, the level of 20α-HSD expressed by immature, matured, and fertilized COCs was quantified. The transcript encoding 20α-HSD was strongly expressed in immature COCs (Fig. 3). The high level of 20α-HSD expression declined significantly throughout IVM and IVF (2.3- and 9.2-fold lower than the level at 0 h respectively, \( P < 0.05 \)). To determine whether maturation conditions influenced the level of 20α-HSD expression, differential IVM conditions were applied. In the absence of EGF, the expression of 20α-HSD remained high at the end of the culture period. By contrast, the addition of EGF to the maturation medium led to a significant reduction in the expression of 20α-HSD mRNA and protein (3.7- and 2.1-fold lower than the level in TCM199 alone respectively; \( P < 0.05 \); Fig. 5).

**Developmental competence**

To evaluate the effect of COC differentiation during IVM on developmental competence, the COCs were fertilized and the embryos produced in vitro were cultured for 7 days. The presence of EGF in the maturation medium significantly increased (\( P < 0.05 \)) the proportion of fertilized oocytes (88.2% vs 75.5%), cleaved eggs (77.1% vs 66.8%), and blastocysts (31.2% vs 21.1%) compared with IVM in TCM199 alone. The number of COCs examined after IVM with or without EGF was 144 and 208 respectively.

**Discussion**

The present study constitutes the first report on the expression of the three PTGES in the bovine COC and their modulation during the periconceptional interval. During the maturation process, PGE₂ is essential for the successful resumption of oocyte meiosis and cumulus expansion (Eppig 1981, Hizaki et al. 1999, Takahashi et al. 2006). PGE₂ produced in the prevulatory follicle is synthesized from arachidonic acid by the sequential actions of PTGS2 and specific PTGES. We had previously reported the upregulation of PTGS2
expression in cumulus cells related to the de novo synthesis of PGE2 during maturation in cattle, but PTGES expression was not studied at that time (Nuttinck et al. 2002). The present study reports the pattern of PTGES1, PTGES2, and PTGES3 expression during terminal differentiation of the COCs from the immature to the fertilized stages. Strikingly, the expression of enzymes related to the PGE2 biosynthesis pathway occurred during the maturation process. Of the three forms of PTGES expressed in bovine COCs, only PTGES1 exhibited a marked upregulation of its expression. Our results provide evidence that in vitro maturing COCs exhibit similar patterns of PTGES expression to those previously described in mural granulosa cells during the preovulatory period (Duffy et al. 2005). During experiments performed under differential IVM conditions, we demonstrated that the induction of the mRNA and protein expression of PTGES1 directly paralleled the induction of PTGS2 expression and the amounts of PGE2 released by maturing COCs in the culture media. This result suggests that the membrane-associated PTGES, PTGES1, is functionally coupled with PTGS2 to promote PGE2 production in bovine COCs during the maturation process. The coordinated induction of both PTGS2 and PTGES1 gene expression was recently described in endometrial and myometrial tissues (Parent & Fortier 2005, Astle et al. 2007). Nevertheless, the molecular mechanisms involved in the control of these two genes are still to be established. PTGES3 is thought to be functionally linked to the PTGS1 isoform of cyclooxygenase to produce PGE2 (Kudo & Murakami 2005). In the present study, we detected a transcript encoding PTGES3 despite the absence of PTGS1 expression in the COCs (Nuttinck et al. 2002). Further studies will be needed to clarify the possible contribution of PTGES2 and PTGES3 to the PGE2 production that occurs during maturation.

Like PGE2, progesterone is thought to be required for successful terminal differentiation of the COC (Shimada & Terada 2002, Shimada et al. 2004, Wang et al. 2006). The present study reports the expression patterns of steroidogenesis-related enzymes in periconceptional COCs from the immature to the fertilized stages. We showed that transcripts encoding STAR and CYP11A1 are expressed in bovine COCs as early as the immature stage. CYP11A1 and STAR control the rate-limiting step in steroid biosynthesis. STAR transports cholesterol to the inner mitochondrial membrane where CYP11A1 converts cholesterol into pregnenolone. The expression of CYP11A1 mRNA was markedly upregulated during the IVM period, and a high level of CYP11A1 expression was sustained during the IVF period. Interestingly, the amount of progesterone secreted by COCs (already substantial during IVM) continued to increase during IVF whereas the level of CYP11A1 expression remained constant throughout this period. Progesterone secretion may be regulated not only at level of its biosynthesis but also at level of its catabolism. Progesterone catabolism is

Figure 1 Stage-dependent changes to (A) PTGS2, (B) PTGES3, (C) PTGES1 and (D) PTGES2 mRNA expression in bovine COCs during the periconceptional interval. Isolated COCs were subjected to in vitro maturation in the presence of EGF (10 ng/ml) and to in vitro fertilization. The expression levels of target genes were measured in immature, matured, and fertilized COCs by real-time quantitative RT-PCR analysis. Each transcript level was normalized to β-actin expression. Data are presented as means ± S.E.M. of four replicates. Within each panel, groups with different superscripts are different (P<0.05).
known to occur predominantly via the activity of 20α-HSD and 5α-reductase enzymes (Moon et al. 1985). Here, we demonstrate that the level of 20α-HSD mRNA expression, which was high in immature COCs, dramatically decreased throughout the IVM and IVF periods to reach its lowest levels in fertilized COCs. A previous in vitro study of porcine COCs had reported that 20α-HSD activity was closely related to oocyte nuclear status (Takano & Niimura 2002). The 20α-HSD activity present in immature COCs decreased during IVM to disappear at the metaphase II stage. Thus, we hypothesize that the increasing level of progesterone production observed in periconceptional COCs results from an enhancement of progesterone biosynthesis as well as a reduction in its catabolic activity. Further studies using specific microassays of steroids in culture media will be needed to determine the respective levels of progesterone metabolites released by COCs during the periconceptional interval. Another outcome of this study was that the level of STAR mRNA expression was maintained at a constant level during IVM and then decreased during the IVF period. An in vivo study performed by Su et al. (2006) demonstrated the simultaneous and dramatic upregulation of STAR and CYP11A1 expression in mice COCs after the endogenous surge of luteinizing hormone (LH). During our in vitro experiments, only CYP11A1 expression was upregulated during the maturation process. This could have been due to insufficient triggering stimuli linked to an IVM model in which COCs were deprived of their follicular environment.

In a previous study, we reported that the addition of EGF to the maturation medium led to a higher level of PTGS2 expression in cumulus cells and this was associated with higher proportion of both expanded cumulus cells and oocytes at metaphase II stage after 24 h of culture (Nuttinck et al. 2002). The present study completes our previous results. The IVM of COCs in the presence of EGF resulted in COCs being more capable of producing PGE2 and progesterone during the periconceptional interval, this was related to oocytes being more able to be fertilized and to reach the blastocyst stage in vitro. Exogenous EGF could mimic some effects of the EGF-like peptides expressed by follicular somatic cells following induction of the preovulatory surge of LH. Indeed, amphiregulin, epiregulin, and β-cellulin (all members of the EGF family) have been shown to mediate, at least partially, the LH stimulation of oocyte maturation and cumulus expansion (Park et al. 2004, Tsafriri et al. 2005, Downs & Chen 2008). A recent study performed in mice by Shimada et al. (2006) suggested that amphiregulin-dependent pathways regulated genes not only involved in the induction of cumulus expansion such as PTGS2 but also involved in steroidogenesis. The LH-induced expression of amphiregulin and the subsequent synthesis of de novo PGE2 could share the regulation of expression of steroidogenesis-related genes.

**Figure 2** Effect of in vitro maturation conditions on PTGS2 and PTGES1 expression in bovine COCs. The levels of transcript encoding (A) PTGS2 and (B) PTGES1 were measured at 0 and 24 h of culture in the absence or presence of EGF (10 ng/ml) by real-time quantitative RT-PCR analysis. Each transcript level was normalized to β-actin expression. Data are presented as means±S.E.M. of four replicates. (C) The PTGES1 protein was immunodetected in COCs at 0 and 24 h of culture in the absence or presence of EGF (10 ng/ml) by Western blotting. A representative blot of one experiment is presented. Lanes 1–2: 10 and 5 μg solubilized liver extracts, lanes 3–5: solubilized extracts corresponding to ten COCs. The intensity of the bands was quantified by densitometric analysis. Data are presented as means±S.E.M. of three replicates. Within each panel, groups with different superscripts are different (P<0.05).
enzymes. Steroid hormones, and particularly progesterone, have been described as transcriptional factors (Ballare et al. 2006, Boonyaratanakornkit et al. 2007). The rising level of progesterone secreted in the oocyte microenvironment during the periconceptional interval could be involved in the complex regulation of gene expression, which occurs in the egg during its early development. Recently, progesterone has been shown to participate in inducing target genes by modifying the chromatin and preparing it for transcription (Vicent et al. 2006). For the egg, epigenetic modifications represent an important way of regulating developmental-specific gene expression (Young & Beaujean 2004). More studies will be needed to explore the effects exerted by several lipid mediator pathways (e.g. prostanoids and steroid hormones) during the periconceptional interval on the epigenetic regulation of embryo gene expression.

In conclusion, we have shown that bovine COCs express the three PTGES and we have described the pattern of their expression during the periconceptional interval. The synthesis of PGE2 induced in maturing COCs mainly occurred via the PTGS2/PTGES1 pathway. The transitional induction of PGE2 biosynthetic activity during the maturation process was concomitant with a rising level of progesterone production throughout the periconceptional interval. The increasing level of progesterone production in the COCs seemed to result from a coordinated regulation of progesterone biosynthesis and catabolic activities. Changes to PGE2 and progesterone metabolism represent important features of terminal COC differentiation and could impact the developmental competence of the oocyte. Greater knowledge of cumulus physiology may prove useful to understanding the molecular mechanisms involved in interactions between

**Figure 3** Stage-dependent changes to (A) STAR, (B) CYP11A1 and (C) 20α-HSD mRNA expression in bovine COCs during the periconceptional interval. Isolated COCs were subjected to in vitro maturation in the presence of EGF (10 ng/ml) and to in vitro fertilization. The expression levels of target genes were measured in immature, matured, and fertilized COCs by real-time quantitative RT-PCR analysis. Each transcript level was normalized to β-actin expression. Data are presented as means ± S.E.M. of four replicates. Within each panel, groups with different superscripts are different (P<0.05).

**Figure 4** Effect of in vitro maturation conditions on STAR and CYP11A1 expression in bovine COCs. The levels of transcript encoding (A) STAR and (B) CYP11A1 were measured at 0 and 24 h of culture in the absence or presence of EGF (10 ng/ml) by real-time quantitative RT-PCR analysis. Each transcript level was normalized to β-actin expression. Data are presented as means ± S.E.M. of four replicates. Within each panel, groups with different superscripts are different (P<0.05).
the oocyte and its surrounding somatic cells during the periconceptional period and how these interactions affect subsequent embryo development.

Materials and Methods

COC collection and culture conditions

Bovine ovaries were collected at the slaughterhouse and COCs were aspirated from 3 to 6 mm antral follicles. Only oocytes surrounded by more than three compact layers of cumulus cells were selected. COCs differentially matured in vitro were obtained as described previously (Nuttinck et al. 2002). Briefly, COCs were cultured in a defined maturation medium consisting of TCM199 (Sigma) alone or supplemented with 10 ng/ml epidermal growth factor (mouse EGF; Sigma) for 24 h at 39 °C in a water-saturated atmosphere under 5% carbon dioxide. The addition of EGF to the maturation media constituted the standard condition. Immature and in vitro matured COCs were harvested at 0 and 24 h of culture respectively. Samples were stored in groups of ten COCs at −80 °C until RT-PCR or Western blot analysis. Maturation media incubated with or without COCs were collected for immunoassays of PGE2 and progesterone. After IVM under differential conditions, COCs underwent IVF. They were transferred in groups of 25 COCs to 500 μl fertilization medium containing 5 × 105 motile spermatozoa (Parrish et al. 1986). Twenty-four hours later, some of the COCs fertilized in vitro after maturation in the presence of EGF were stored at −80 °C in groups of ten, called fertilized COCs, until RT-PCR or Western blot analysis. Fertilization media incubated with or without COCs were centrifuged and the supernatants were collected for immunoassays of PGE2 and progesterone. Resting presumptive zygotes were denuded to assess the fertilization rate. In order to evaluate their developmental competence, fertilized oocytes obtained after differential maturation were transferred into 25 μl droplets (under paraffin oil) of modified synthetic oviduct fluid (Holm et al. 1999) supplemented with 16 mg/ml BSA. Embryos were cultured for 7 days at 39 °C in a water-saturated atmosphere under 5% CO2/5% O2/90% N2. Cleaved oocytes and blastocysts were recovered on days 1 and 7 of embryo culture respectively.

Production of cDNA for standard curves in real-time PCR

Total RNA (1 μg) from bovine endometrium, corpus luteum, and liver was reverse transcribed in a 20 μl mixture using oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen, Life Technologies). cDNAs for all studied enzymes as well as for β-actin were generated by conventional PCR on a thermal block cycler (Biometra, Göttingen, Germany). PCRs were performed by adding 1 μl RT product to the PCR mix, which for all the factors examined contained 0.5 unit of TAQ polymerase (Qbiogen, Illkirch, France), 2.5 μl 10× buffer with MgCl2 (Qbiogen), 0.5 μM specific downstream and upstream primers, and 0.1 mM of each dNTPs (total volume 25 μl). The primer sequences were either designed using the GCG Wisconsin software package (Genetics Computer Group, Madison, WI, USA) or obtained from the literature (Table 1). The PCR protocol involved a single denaturation step of 94 °C (1 min), followed by 40 amplification cycles of 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. PCR was completed by a 10-min extension time at 72 °C. All PCR products were cloned into a plasmid vector (pCR4-TOPO; Invitrogen, Life Technologies) using a standard procedure (TOPO TA Cloning; Invitrogen, Life Technologies). All fragments were sequenced to confirm their identity. Appropriate cDNAs to be used as standards in real-time PCR quantification were generated by PCR under the same conditions as those...
described above and 1 ng plasmid was added as the DNA matrix. All cDNAs were stored at −20°C after purification through a column (GeneClean turbo kit, Bio 101; Qbiogene, Carlsbad, CA, USA) until required for use in real-time PCR.

Quantification of transcripts in COCs using real-time PCR

Levels of mRNA for all studied enzymes and β-actin were analyzed using real-time RT-PCR with an ABI Prism 7000HT (Applied Biosystems, Les Ulis, France). For each treatment, four pools of ten COCs were analyzed. Total RNA was extracted using RNA Plus (Qbiogen), in accordance with the manufacturer’s protocol. First-strand cDNA synthesis was carried out from total RNA using an oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen, Life technologies). mRNA amplifications were performed using the SYBR Green Master Mix (Applied Biosystems). All reactions used cDNA amount equivalent to 0.25 COC, 0.3 μM of each primer, and an annealing temperature of 60°C. The primers designed were based on ovine or bovine cDNA sequences (Table 2) using Design software (Primer Express, Applied Biosystems). For all factors, five log dilutions of the appropriate purified cDNA (produced as described above) were included in each assay and used to generate a standard curve. Following PCR, a melting curve was performed on the amplified products to ensure that only specific PCR amplicons were obtained and quantified. The expression of all target mRNAs was determined in each sample during independent assays. PCRs were set up in duplicate. The median value of PCR duplicates was considered and then expressed as a ratio to β-actin mRNA for each sample. Data are presented as means ± S.E.M. of four replicates.

Table 1 Prime pairs used for conventional RT-PCRs.

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<td>Burns et al. (1997)</td>
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<td>484</td>
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Table 2 Prime pairs used for real-time RT-PCRs.

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<tr>
<td>PTGES3</td>
<td>Orisaka et al. (2006)</td>
<td>Downstream</td>
<td>101</td>
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<td>CYP11A1</td>
<td>Orisaka et al. (2006)</td>
<td>Upstream</td>
<td>136</td>
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<td>STAR</td>
<td>(2001)</td>
<td>Downstream</td>
<td>103</td>
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<tr>
<td>20α-HSD (AKR1B5)</td>
<td>(2003)</td>
<td>Upstream</td>
<td>207</td>
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in 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham) for 2 h at 4 °C. The membranes were saturated overnight at 4 °C in 50 mM Tris–HCl (pH 7.5), 250 mM NaCl, and 0.1% Tween 20 (TBS-T buffer) containing 5% fat-free dry milk. They were then incubated for 1 h at room temperature in the same buffer with either a 1:500 dilution of anti-human mPGES-1 (Cayman Chemical, Spi-Bio, Massy, France) or a 1:2000 dilution of anti-bovine 20α-HSD (AKR1B5; gift from Prof. Michel Fortier, Laval, Québec; Madore et al. 2003) rabbit polyclonal antibodies. After extensive washes in TBS-T buffer, blots were further incubated for 1 h at room temperature with a dilution at 1:100 000 of donkey anti-rabbit IgG conjugated with horseradish peroxidase (Jackson products, Interchim, Montluçon, France) in TBS-T buffer. Any excess of the secondary antibody was eliminated by extensive washes in TBS-T buffer (five consecutive 10-min washes). In order to normalize the amounts of mPGES-1 and 20α-HSD (AKR1B5), 5 and 10 μg total protein extract from liver were used as a reference and run in parallel with the samples. Molecular weights were determined using prestained protein standards (ProSieve, BMA product, Tebu-bio, Le Perray en Yvelines, France). The chemiluminescent substrate was used according to the manufacturer’s instructions (ECL + Kit, Amersham) and the fluorescence from immunoreactive proteins was captured with an LAS-1000 plus camera (Fujifilm). Relative quantification of the signals was performed by densitometric analysis using the Advanced Image Data Analyzer software (Fujifilm). Three replicates of ten COCs per treatment were included in each assay.

**RIA of PGE₂**

The amounts of PGE₂ produced by COCs during the 24-h IVM or IVF period were measured in the culture media. After removing the COCs, pools of 2 ml culture medium corresponding to four dishes were assayed. The same volume of maturation or fertilization medium, incubated concurrently without COC, was used as a negative control. For each treatment, four replicates were included in the assay. All samples were stored at −80 °C until RIA analysis. Prostaglandins were extracted and quantified according to the procedure described previously (Charpigny et al. 1997). All samples were analyzed during the same assay and the intra-assay coefficient of variation was 8%. Results were expressed in pg of PGE₂ produced by COC during the 24 h period of IVM or IVF (means ± S.E.M).

**Progesterone assay**

Progesterone that accumulated in maturation or fertilization culture media during the 24-h culture period was determined by ELISA using the Biotechnov Ovuchek plasma ELISA kit (Biotechnov, St-Hyacinthe, QC, Canada), in accordance with the manufacturer’s protocol. The range of the standard curve was 0.5–10 ng/ml and the sensitivity of the procedure was 0.5 ng/ml. Intra- and inter-assay coefficients of variation were 10 and 15% respectively. The samples of culture media were harvested and pooled as described previously. Results were expressed in pg of progesterone produced by COC during the 24 h IVM or IVF period (means ± S.E.M.).

**Statistical analysis**

Differences in mean mRNA and protein expression of the factors examined were analyzed for the effects of IVF conditions (in the absence or presence of EGF) or the differentiation stage (immature, matured, and fertilized COCs) using one-way ANOVA followed by Tukey’s post hoc tests. Concentrations of PGE₂ and progesterone in the culture media were also analyzed by ANOVA. Data are presented as means ± S.E.M. and significant differences reported at the P<0.05 level. Rates of fertilization, cleavage, and development to the blastocyst stage were compared by χ² analysis. Data from three or four replicates were pooled to calculate percentages based on the total number of oocytes subjected to IVM.

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**References**


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