Gene expression profiling of bovine preovulatory follicles: gonadotropin surge and prostanoid-dependent up-regulation of genes potentially linked to the ovulatory process

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Abstract

The molecular mechanisms of ovulation and luteinization have not been well established, partially due to lack of a comprehensive understanding of functionally significant genes up-regulated in response to an ovulatory stimulus and the signaling pathways involved. In the present study, transcripts increased in bovine preovulatory follicles following a GnRH-induced LH surge were identified using microarray technology. Increased expression of 368 and 878 genes was detected at 12 (368 genes) and 20 h (878 genes) following GnRH injection. The temporal, cell specific and prostanoid-dependent regulation of selected genes (ADAM10, DBI, CD36, MTSS1, TFG, and RABGAP1) identified from microarray studies and related genes (ADAM17 and AREG) of potential significance were also investigated.

Expression of mRNA for DBI and CD36 was simultaneously up-regulated in theca and granulosa cells (GC) following the LH surge, whereas temporal regulation of ADAM10, MTSS1, TFG, and RABGAP1 was distinct in the two cell compartments and increased granulosa TFG and RABGAP1 mRNA were prostanoid dependent. AREG mRNA was increased in theca and GCs at 12 and 24 h following GnRH injection. ADAM17 mRNA was increased in theca, but reduced in GCs 24 h following GnRH injection. The increased ADAM17 and AREG mRNA were prostanoid dependent. ADAM10 and ADAM17 protein were increased specifically in the apex but not the base of preovulatory follicles and the increase in ADAM17 was prostanoid dependent. Results reveal novel information on the regulation of preovulatory gene expression and suggest a potential functional role for ADAM10 and ADAM17 proteins in the region of follicle rupture.

Introduction

As a prerequisite for fertilization and embryonic development, ovulation has been a topic of continuous scientific interest over centuries. Ovulation is a very complex process and absolutely dependent upon appropriate timing of preovulatory follicular development and initiation of luteinization, cumulus expansion, and follicle rupture in response to the LH surge (Tsafiri & Reich 1999, Ny et al. 2002, Richards et al. 2002, Curry & Osteen 2003, Richards 2005, 2007). The preovulatory follicle has been proposed to be a ‘gatekeeper’ of successful reproduction (Richards et al. 2002), with numerous LH surge-induced local factors and signaling pathways involved in the processes of ovulation and luteinization. However, our understanding of the intrafollicular mediators of ovulation and luteinization triggered by the preovulatory LH surge is incomplete.

Accumulating evidence in multiple species including mouse (Lim et al. 1997, Davis et al. 1999), rat (Mikuni et al. 1998, Gaytan et al. 2002), sheep (Murdoch et al. 1986), pig (Downey & Ainsworth 1980), monkey (Duffy & Stouffer 2002), and cattle (Peters et al. 2004) indicates that intrafollicular synthesis of prostanoids in response to the LH surge is critical for the ovulatory process. The cyclooxygenase (COX)-2 enzyme, which catalyzes the rate limiting step in intrafollicular prostanoid synthesis, is induced within the granulosa layer of rat (Sirois et al. 1992), equine (Sirois & Dore 1997), and bovine preovulatory follicles (Sirois 1994, Tsai et al. 1996) in response to the LH surge. COX inhibitors such as indomethacin inhibit ovulation in cattle (De Silva & Reeves 1985, Peters et al. 2004). Moreover, mice with targeted disruption of the COX-2 gene are infertile and unable to ovulate potentially due to the defects in cumulus expansion and follicle rupture (Dinchuk et al. 1995, Davis et al. 1999).

We have shown that intrafollicular administration of the prostanoid synthesis inhibitor indomethacin blocks follicular rupture and LH surge-induced regulation of key mediators of extracellular matrix remodeling linked to the ovulatory process in cattle (Li et al. 2006).
However, knowledge of the intrafollicular mediators of prostanoid action critical for normal progression of ovulatory follicles through the preovulatory period is incomplete. The objective of the present study was to identify genes up-regulated in bovine preovulatory follicles in response to an ovulatory stimulus (GnRH injection) using microarray technology and to investigate the temporal, cell specific and prostanoid-dependent regulation of the expression of select genes identified from microarray analysis and related genes of potential significance to the ovulatory process. In this study, six candidate genes (ADAM10, DBI, CD36, MTSS1, TFG, and RABGAP1) from microarray gene lists and three related genes (ADAM17, AREG, and BTC) were selected for further study because: 1) the regulation of expression of such genes during the bovine preovulatory process has not been investigated; 2) selected genes have known functions that are potentially important for follicular development and/or the ovulatory process; and (or) 3) selected genes are members of known gene families potentially implicated in preovulatory follicle development and/or ovulation in other species.

Results

Gonadotropin surge induced up-regulation of gene expression in bovine preovulatory follicles

Microarray analysis was performed to identify genes with up-regulated mRNA expression in bovine follicles following the LH surge. Our results revealed changes in bovine preovulatory follicular gene expression in response to an ovulatory stimulus. Increased mRNA abundance for 368 genes was detected at 12 h post-GnRH injection (Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/) and for 878 genes at 20 h post-GnRH injection (Supplementary Table 2, which can be viewed online at www.reproduction-online.org/supplemental/) relative to the 0 h time point (false discovery rate (FDR) = 10%). Six candidate genes (ADAM10, DBI, CD36, MTSS1, TFG, and RABGAP1) were selected for further analysis from the above gene lists based on results of annotation and gene ontology analysis (data not shown). Properties of genes selected for further analysis (from microarray gene lists) potentially relevant to preovulatory follicular development and/or follicle rupture are summarized below. ADAM10 belongs to the ADAM family of transmembrane proteinases linked to processing of EGF family ligands (Blobel 2005) that mediate LH action (cumulus expansion, oocyte maturation, ovulation) during the periovulatory period in mice (Park et al. 2004, Conti et al. 2006). DBI is present in the rat ovary (Bovolin et al. 1990, Toranzo et al. 1994) and is a known stimulator of steroidogenesis/pregnenolone biosynthesis (Besman et al. 1989, Papadopoulos et al. 1991). CD36 is a multifunctional scavenger receptor that binds known autocrine growth factors that can regulate cell growth, adhesion, and angiogenesis (Febrario et al. 2001). MTSS1 is a sonic hedgehog responsive actin binding protein (Callahan et al. 2004) and a functional hedgehog signaling system in murine follicles has been reported (Wijgerde et al. 2005, Russell et al. 2007). TFG is involved in promoting NF-kappaB activity (Miranda et al. 2006), which is potentially related to cumulus expansion in mice (Takahashi et al. 2006). RABGAP1 is a GTPase that may have novel functions in the ovary.

The LH surge-induced up-regulation of mRNA expression for the six candidate genes identified by microarray analysis using Animal model 1 (increased expression in whole follicles at 12 or 20 h post-GnRH injection versus 0 h) was subsequently confirmed by real-time PCR analysis using the same samples. Increased mRNA expression for ADAM10 (> twofold increase), DBI (> fivefold increase), CD36 (~ threefold increase), and TFG (~ 2.5 fold increase) at 12 h following GnRH injection and for MTSS1 (> twofold increase), and RABGAP1 (~ twofold increase) at 20 h post-GnRH injection was observed (Fig. 1).

Figure 1 Quantitative real-time PCR analysis of changes in mRNA abundance for selected genes identified by microarray analysis in bovine preovulatory follicles following the gonadotropin surge (Animal model 1). Abundance of (A) ADAM10, (B) DBI, (C) CD36, (D) TFG, (E) MTSS1, and (F) RABGAP1 mRNAs was normalized relative to that of RPL19 mRNA. Relative mRNA abundance at 0 and 12 or 20 h (n=4 per time point) was calculated using ∆∆Ct method. Data are shown as LS mean±S.E.M. Time points without a common superscript are different at P<0.05.
Temporal and cell specific regulation of preovulatory follicular ADAM10, DBI, CD36, TFG, MTSS1, and RABGAP1 mRNA in response to the preovulatory gonadotropin surge

Because the intrafollicular cell types responsible for the increases in mRNA for above genes in bovine preovulatory follicles are unknown, we examined the effect of the preovulatory LH surge on mRNA abundance for these genes in granulosa cells (GC) and thecal tissue (TC) collected 0, 12, and 24 h post-GnRH injection (using Animal model 2). Messenger RNAs for ADAM10, DBI, CD36, TFG, MTSS1, and RABGAP1 were detected in both GC and TC. In the GC compartment, CD36 and DBI mRNAs were increased at 12 h post-GnRH injection and remained elevated at 24 h (P<0.05; Fig. 2A and B). By contrast, transcript abundance for ADAM10 and MTSS1 in GC was transiently elevated at 12 h and returned to pre LH surge levels by 24 h after GnRH injection (P<0.05; Fig. 2C and D) and abundance of GC TFG and RABGAP1 mRNAs was increased only at 24 h post-GnRH injection (P<0.05; Fig. 2E and F). In the TC compartment, CD36, DBI, ADAM10, and TFG mRNAs were elevated at both time points examined (P<0.05; Fig. 2A–C and E). Increased expression of MTSS1 and RABGAP1 mRNAs was specific to GC as no change in TC mRNA for either gene was observed following GnRH injection (P>0.05; Fig. 2D and F). We acknowledge there were subtle differences in mRNA expression results obtained using samples from Animal model 1 versus Animal model 2. Such differences are likely attributed to variation in time of sample collection (20 vs 24 h) post-GnRH injection, nature of the sample (whole follicles versus isolated TC and GC) and (or) differences in molecular techniques utilized. Given differences in the time points for collection and nature of the samples, subsequent biological conclusions about temporal regulation of mRNA expression were drawn from the latter studies using isolated theca and GCs which allows more specific and functional hypotheses for future studies to be formulated.

Effect of intrafollicular inhibition of prostanoid synthesis (indomethacin injection) on ADAM10, DBI, CD36, TFG, MTSS1, and RABGAP1 mRNA abundance in GC and TC

Given the established role of intrafollicular prostanoids in promoting follicle rupture, intrafollicular administration of indomethacin following GnRH injection to induce the LH surge was used to examine whether up-regulation of ADAM10, DBI, CD36, MTSS1, TFG, and RABGAP1 was prostanoid dependent (Animal model 2). We have previously shown that intrafollicular injection of indomethacin blocks the LH surge-induced increase in follicular fluid (FF) PGE2 characteristic of the ovulatory process (Li et al. 2006). Results of the present study indicate that the LH surge-induced up-regulation of TFG and RABGAP1 transcripts was reduced in GC of indomethacin-treated follicles versus control follicles collected 24 h post-GnRH injection (P<0.05; Fig. 3D and F), whereas mRNA abundance for ADAM10, DBI, CD36, and MTSS1 was not altered (P>0.05; Fig. 3A–C and E).

Regulation of bovine preovulatory follicular ADAM17 and AREG mRNAs following the LH surge

Extensive evidence in rodents supports a key role of EGF-like growth factors in mediating LH action in preovulatory follicles (Ben-Ami et al. 2006, Conti et al. 2006, Hsieh et al. 2007, Panigone et al. 2008). Moreover, ADAMs, such as ADAM10 function as sheddases and play critical roles in releasing such EGF receptor ligands (Blobel 2005). Hence, the preovulatory expression and prostanoid-dependent regulation of another prominent ADAM family member with sheddase activity (ADAM17), and of betacellulin (BTC) and amphiregulin (AREG), members of the EGF-like growth factor family which are critical mediators of LH action (Park et al. 2004), were also investigated (Animal model 2).
ADAM17 mRNA was increased at 24 h post-GnRH injection in the TC but not the GC (P<0.05; Fig. 4A). Up-regulation of AREG mRNA following the LH surge was detected in the GC and TC at both 12 and 24 h (P<0.05; Fig. 4B), but AREG mRNA was reduced in the GC at 24 h relative to the 12 h time point. We acknowledge that changes in transcript levels for ADAM17 and AREG were not detected using microarray screening. However, AREG is not represented on the array utilized and ADAM17 mRNA may not be changed in whole ovarian tissues collected at 20 h time point (used for microarray studies), since it was down-regulated in GC but up-regulated in TC at 24 h.

Results also indicate that the LH surge-induced up-regulation of ADAM17 and AREG mRNA is prostanoid dependent. Intrafollicular indomethacin administration resulted in significantly reduced mRNA abundance for ADAM17 in GC (P<0.05; Fig. 4C), and AREG in both GC and TC (P<0.05; Fig. 4D) 24 h post-GnRH injection. Messenger RNA for BTC was undetectable in both GC and TC compartments (data not shown).

**Temporal, spatial, and hormonal regulation of preovulatory follicular expression of ADAM10 and ADAM17 proteins**

Western blot analysis was performed (using samples collected from Animal model 2) to determine whether changes in abundance of ADAM10 (post-GnRH injection) and ADAM17 (post-GnRH injection and INDO administration) proteins mirror changes in mRNA abundance and if the regulation is prostanoid dependent and specific to the follicular apex, the region of active proteolysis prior to follicle rupture (Murdoch & McCormick 1992). For ADAM10, a prominent immunoreactive band of ≈ 80 000 Mr was detected in homogenates of both the apex and base of bovine preovulatory follicle (Fig. 5A and B). Abundance of ADAM10 protein was increased in homogenates of the preovulatory follicle apex at both 12 and 24 h following GnRH injection (relative to 0 h time point), but was decreased in extracts of the preovulatory follicle base collected at 24 h. For ADAM17, a prominent form of 80 000 Mr, corresponding to the active form (Moss et al. 1997) was detected (Fig. 5C and D) and a second immunoreactive band of 120 000 Mr was also detected, but not significantly regulated (Fig. 5C and D). Furthermore, ADAM17 protein (80 000 Mr) was also increased specifically in the apex of preovulatory follicles at 24 h post-GnRH injection (Fig. 5C), and such regulation was prostanoid dependent (Fig. 5D).
Temporal and prostanoid-dependent regulation of ADAM17 protein were not observed in the base of preovulatory follicles (data not shown).

Discussion

Key events/features accompanying the ovulatory process include vascular changes (Tsafriri & Reich 1999), proteolytic degradation of the follicle wall (Tsafriri 1995, Tsafriri & Reich 1999, Ny et al. 2002, Curry & Osteen 2003), oocyte nuclear maturation and acquisition of developmental competence, expansion of the cumulus cells (Park et al. 2004), and luteinization of the steroidogenic cell compartments (Stocco et al. 2007). The preovulatory LH surge is the endocrine signal initiating such changes, and a role for intrafollicular prostanoids in mediating the ovulatory process has long been established (Downey & Ainsworth 1980, Murdoch et al. 1986, Davis et al. 1999, Duffy & Stouffer 2002, Gaytan et al. 2002, Peters et al. 2004). However, the LH surge-induced changes in follicular global gene expression, and the role of intrafollicular prostanoids in mediating such changes, have not been fully characterized, especially in monotocous species. In the present study, microarrays were used as a screening tool to identify LH surge-induced changes in gene expression in bovine preovulatory follicles. A significant number of genes were up-regulated at 12 and 20 h post-GnRH injection representing a multitude of functional categories (e.g. cell growth and maintenance, metabolism, signal transduction, etc.). Six candidate genes from microarray data were subjected to further analysis along with three related genes of potential functional significance. Our results provide novel information on temporal and cell specific regulation of ADAM10, DBI, CD36, MTTS1, TFG, RABGAP1, ADAM17, and AREG in bovine preovulatory follicles in response to the preovulatory LH surge and the potential involvement of prostanoids in such regulation, providing novel insight into preovulatory regulation of genes potentially important for the ovulatory process.

Ovulation is accompanied by broad-spectrum proteolysis mediated by a vast number of proteinase families, such as the matrix metalloproteinases, plasminogen activators /plasmin, and members of the disintegrin and metalloproteinase domain with thrombospondin (TSP) motif (ADAMTS) gene family (Ny et al. 2002, Curry & Osteen 2003, Ohnishi et al. 2005, Ben-Ami et al. 2006). Experimental evidence supports a potential involvement of ADAMTS family members in follicular growth and the ovulatory process (Espey et al. 2000, Richards et al. 2002, Boerboom et al. 2003, Madan et al. 2003, Richards 2005, Brown et al. 2006). Preovulatory regulation of ADAMTS family members in mice has also been reported (Richards 2005). Furthermore, ADAMTS1 deficiency in mice results in subfertility with defects in follicular development and ovulation (Shindo et al. 2000, Mittaz et al. 2004, Brown et al. 2006). While preovulatory regulation and/or function of ADAMTS family members, particularly ADAMTS1, has been investigated in multiple species, less is known about regulation and the potential functional roles of ADAM family members during the preovulatory period. ADAMs are type I transmembrane proteins with both metalloproteinase and disintegrin domains, but lack TSP motifs characteristic of ADAMTS molecules (Tang 2001). ADAMs are implicated in cell–cell and cell–matrix interactions and shedding of membrane-bound precursors such as EGF family ligands (Blobel 2005). Results of the present studies demonstrated pronounced temporal and spatial regulation of ADAM10 and ADAM17 mRNA and protein in bovine preovulatory follicles in response to an ovulatory stimulus. Increased expression of EGF family ligands accompanies the cascade of events resembling an inflammatory and/or tissue remodeling process during the preovulatory period, and shedding and action of

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such paracrine signals is critical for LH actions including cumulus expansion, oocyte maturation, and ovulation (Park et al. 2004, Conti et al. 2006).

The precise functional role of ADAM10 in bovine preovulatory follicles during the window from the LH surge to ovulation is not known. Despite the temporal and spatial regulation of ADAM10 transcripts and protein, effects of INDO injection on ADAM10 mRNA abundance were not observed, suggesting that preovulatory regulation of ADAM10 expression is likely prostanoid independent. ADAM10 is the main sheddase involved in activation and release of BTC extracellularly from the cell membrane (Sahin et al. 2004). A role for ADAM10 in BTC activation in bovine follicles is unlikely since follicular BTC mRNA was undetectable in the present study. However, a role for ADAM10 in regulation of endothelial cell permeability has been reported (Schulz et al. 2008). Given the fact that ADAM10 protein abundance was increased specifically in the apex of preovulatory follicles following GnRH injection, a potential role for ADAM10 in promoting increased vasodilation characteristic of the ovulatory process (Tsafiri & Reich 1999) seems plausible. Characterization of the molecular mechanisms underlying the LH surge-induced up-regulation of ADAM10 protein specifically at the site of ovulation is of further interest.

Abundance of mRNA and protein for ADAM17 in bovine preovulatory follicles was also increased following GnRH injection, but a functional role for ADAM17 has not been directly established. However, evidence in other systems indicates that ADAM17 is a major convertase/sheddase for AREG (Sahin et al. 2004). Thus, the observed temporal and cell specific regulation of ADAM17 and AREG transcript levels in the present studies suggests that ADAM17 may help promote shedding/release of AREG in the theca layer of bovine preovulatory follicles. Increased AREG mRNA was observed in the theca layer at 12 and 24 h post-GnRH injection and accompanied by an increase in ADAM17 mRNA and protein at the 24 h time point and the increase in ADAM17 protein was specific to the follicle apex. Furthermore, increased expression of both AREG and ADAM17 in the theca layer was prostanoid dependent. It is unclear whether potential paracrine actions of AREG are mediated locally in the theca or also on neighboring GC, but interesting to note that the observed up-regulation of ADAM17 and AREG was specific to the theca layer, as GC ADAM17 and AREG expression was, in fact, decreased at the same 24 h time point. Although the functional significance of the putative ADAM17–AREG system in bovine preovulatory follicles remains to be established, a role for ADAM17–released AREG in the regulation of theca and or GC function is plausible.

Temporal, cell specific and prostanoid-dependent up-regulation of TFG and RABGAP1 mRNAs were noted in the present studies. TFG was originally identified as part of an oncogenic fusion gene (TRK–T3) containing 1412 nucleotides of NTRK1, which is preceded by TFG (598 nucleotides). Moreover, TFG is a fusion partner for anaplastic lymphoma kinase in anaplastic large cell lymphoma in humans (Hernandez et al. 1999). While the role of TFG in the regulation of normal cellular function was previously unknown, TFG has been shown to interact with SHP-1 phosphatase and regulate its activity (Roccati et al. 2005). Furthermore, TFG enhances the effect of TNF-α, TANK, TNF receptor-associated factor (TRAF) 2, and TRAF6 in inducing NF-kappaB activity (Miranda et al. 2006). Interestingly, PGE2-dependent stimulation of cumulus expansion in mice is NF-kappaB dependent (Takahashi et al. 2006), implying a potential functional link between NF-kappaB activity and key prostanoid-dependent events during the preovulatory period. By contrast, the Rab proteins function as regulators of vesicular transport (Ng & Tang 2008) and their functions are regulated at the level of protein expression, localization, membrane association, and activation (Novick & Brenwald 1993, Pfeffer 1994, Stein et al. 2003). The potential functional role of RABGAP1 at the level of transcription in bovine preovulatory follicles is not known.

While observed prostanoid-dependent regulation of a subset of genes examined (ADAM17, TFG, RABGAP1, and AREG) is of potential significance to the ovulatory process, proovulatory regulation of several genes was observed to be prostanoid independent (DBI, CD36, MTSS1, and ADAM10) and may be functionally associated with other key events during the preovulatory period. For example, observed up-regulation of DBI expression in bovine preovulatory follicles may have functional significance to the changes in steroidogenic capacity characteristic of the preovulatory period. As the endogenous ligand for benzodiazepine (BZD) receptors (BZR), DBI can displace BZD from BZR. DBI can stimulate in vivo steroidogenesis via binding to peripheral BZR resulting in increased pregnenolone synthesis (Besman et al. 1989, Papadopoulos et al. 1991). DBI expression in the rat ovary has been reported previously (Bovolin et al. 1990, Toranzo et al. 1994). In the current study, DBI mRNA was increased in both the GC and TC compartments of bovine preovulatory follicles in response to an ovulatory stimulus. However, to our knowledge BZR expression in the bovine ovary has not been reported. It will be interesting to determine whether the increase in DBI makes a functional contribution to the increase in progesterone production accompanying the luteinization processes in bovine preovulatory follicles (Li et al. 2007).

Increased expression of CD36 in GC and TC at 12 and 24 h post-GnRH injection was also noted in the current studies. As a transmembrane glycoprotein, CD36 is a multifunctional scavenger receptor associated with cellular adhesion, antigen presentation,
clearance of apoptotic cells, transportation of fatty acids, platelet aggregation, and inhibition of angiogenesis (Febbraio et al. 2001). Ligands for CD36 include oxidized low density lipoprotein (LDL) > (Endemann et al. 1993), fatty acids (Febbraio et al. 2001), anionic phospholipids (Febbraio et al. 2001), collagen (Tandon et al. 1989, Febbraio et al. 2001), and TSP (Greenwald et al. 1992, Febbraio et al. 2001). Among these ligands, TSP-1 and -2 act as autocrine growth factors and are implicated in diverse physiological events including cell growth, adhesion and migration, platelet aggregation, and regulation of angiogenesis (Lahav 1993). In the rat ovary, both TSP-1 and CD36 are localized primarily to the GC layer of antral follicles, while TSP-2 is expressed later during the ovulatory/early luteal phase (Petrik et al. 2002). In the bovine, TSPs are co-expressed with CD36 on GC of follicles (Greenaway et al. 2005). The specific functional role of CD36 in bovine preovulatory follicles remains to be elucidated.

Preovulatory follicular expression of MTSS1 was investigated in the present studies. MTSS1, whose expression is down-regulated or absent in several metastatic cell lines (Nixdorf et al. 2004, Loberg et al. 2005), is a sonic hedgehog responsive actin binding protein involved in the regulation of actin filament assembly and is a potentiator of Gli-dependent transcription (Callahan et al. 2004). Interestingly, MTSS1 was transiently up-regulated in the granulosa layer of bovine follicles at 12 h post-GnRH injection. While a functional hedgehog signaling pathway in mouse follicles has been identified (Wijgerde et al. 2005, Russell et al. 2007) and effects on GC proliferation established (Russell et al. 2007), the expression of hedgehog signaling components and their targets are down-regulated in mouse preovulatory follicles following hCG injection (Wijgerde et al. 2005). Hence, the significance of preovulatory regulation of MTSS1 expression in bovine follicles remains unclear.

In summary, results of the present studies demonstrate temporal, cell specific and/or prostanoid-dependent regulation of ADAM10, DBI, CD36, MTSS1, TFG, RABGAP1, ADAM17, and AREG mRNA expression in bovine preovulatory follicles in response to the LH surge. Results also demonstrate an increase in ADAM10 and ADAM17 proteins specifically in the apex of preovulatory follicles prior to follicle rupture, suggestive of a potential local role in the region of active proteolysis characteristic of the ovulatory process (Murdoch & McCormick 1992) and cooperation of prostanoid-dependent and independent processes culminating in ovulation. A direct link between the local actions of the above molecules and the complex ovulatory events needs to be established. Such knowledge will further enhance understanding of the functional significance of prostanoid-dependent and -independent gene expression induced during the preovulatory period.

Materials and Methods

Animal models and sample collection

All animal procedures were approved by the All University Committee on Animal Use and Care at Michigan State University.

Animal model 1

For microarray experiments, animal treatments, and sample collections were described elsewhere (Li et al. 2004). Briefly, cross-bred beef heifers (n=12) received two injections of prostaglandin F2α (PGF2α) to synchronize estrus. On day 6 post estrus, animals received two injections of PGF2α to regress corpora lutea (CL), and the animals were then injected with 100 µg GnRH to induce a gonadotropin surge 30 h after the first PGF2α injection. Ovaries containing the preovulatory follicles were collected by colpotomy (Drost et al. 1992) at 0, 12, and 20 h after GnRH injection and processed for RNA isolation (n=4 per time point).

Animal model 2

For studies of temporal, cell specific and prostanoid-dependent regulation of genes of interest in bovine follicles, animal treatments, and sample collections were described elsewhere (Li et al. 2006). Briefly, Holstein cows were synchronized using the Ovsynch (GnRH-7d-PGF2α-36h-GnRH) procedure with a slight modification (Pursley et al. 1995, 1997). GnRH was injected to start a new wave of follicular growth and thus a new dominant follicle. Then, PGF2α was given to regress the CL 6.5 days later. A second GnRH injection was given 36 h later to induce a gonadotropin surge resulting in ovulation of the new dominant follicle. Synchronized ovulation of the dominant follicle occurs an average of 29 h after the second GnRH injection (Pursley et al. 1995).

To determine prostanoid-dependent regulation of preovulatory gene expression, indomethacin, in a volume of 100 µl (INDO; 200 µg/ml final intrafollicular concentration) or the same volume of diluent (PBS; CON) were injected into preovulatory follicles immediately after the second GnRH injection using ultrasound-mediated intrafollicular injection procedures (Peters et al. 2004). Dose of INDO selected was determined previously based on results of preliminary dose response experiments (Li et al. 2006). Daily ultrasound analyses were performed after the first GnRH injection until the time of follicle collection.

Ovaries containing preovulatory follicles were collected by colpotomy (Drost et al. 1992) at 0 (n=5), 12 (CON; n=3), and 24 h (INDO and CON; n=5 each) after the second GnRH injection. Because the induction of the COX-2 enzyme (Sirois et al. 1994) and accompanying increase in FF PGE2 (Peters et al. 2004) occur 18 h post-gonadotropin injection, INDO-treated follicles were not collected at 12 h post-GnRH injection. Follicles were dissected as described elsewhere (Bakke et al. 2002, Li et al. 2004) and then sagittally cut into several portions for GC and TC isolation and protein extraction. Cumulus oocyte complexes were not retrieved prior to processing of follicles and sample preparation. GCs were scraped and collected by centrifugation, while TC was
isolated by dissection from a portion of the follicle. One milliliter TRizol (Life Technologies Inc.) was added to the GC immediately after collection. Both GC and TC were snap frozen in liquid nitrogen and stored at −80 °C for total RNA isolation and real-time PCR assay. Follicle apex and base were separated from another portion of the same follicles and snap frozen and kept at −80 °C until protein isolation and Western blot analysis.

RNA isolation

Total RNA was extracted from either whole follicles or GC and TC using TRizol reagent (Life Technologies Inc.) according to the manufacturer’s instruction. Quantification and quality analysis of total RNA isolated from the above samples were determined by using Bio-analyzer 2100 RNA 6000 nanochip (Agilent Technologies, Walbronn, Germany).

cDNA labeling and hybridization

Microarray experiments were performed in the Center for Animal Functional Genomics at Michigan State University using a loop design. For each time point, 15 µg total RNA was reverse transcribed with superscript III reverse transcriptase (Invitrogen) in the presence of exogenous polyadenylated RNA for the λ Q-gene using Oligo-dT primers. The cDNA was coupled with either Cy3 or Cy5 dyes (Amersham). Labeled cDNA was purified to remove unincorporated dyes, combined and concentrated to ~ 10 µl using Microcon columns (Millipore, Billerica, MA, USA). Hybridization buffer (Slidehybe #3; Ambion, Austin, TX, USA) was added to the probe to achieve a final volume of 120 µl and denatured for 5 min at 70 °C. Then, the mixture was applied to microarray slides for hybridization. A previously described bovine cDNA probe to achieve a final volume of 120 µl and denatured for 5 min at 70 °C. Then, the mixture was applied to microarray slides for hybridization. A previously described bovine cDNA array containing ESTs representing ~15 200 unique genes (Suchyta et al. 2003) was utilized. Slides were hybridized for 18 h using step down temperatures from 65 to 42 °C in a sealed GeneTAC HybStation hybridization chamber (Genomic Solutions Inc., Ann Arbor, MI, USA). The slides were serially washed with medium stringency buffer, high stringency buffer and post wash buffer in the hybstation. Then, slides were immediately rinsed in 2× SSC and deionized water, dried by brief centrifugation and further cleaned with compressed air and scanned using GeneTAC LSIV microarray scanner and GeneTAC LS software (Genomic solutions). GeneTAC Integrator 4.0 software was used to process array images, align spots, integrate robot-spotting files with the microarray image, and to export spot intensity data in a Microsoft Excel format as described previously (Suchyta et al. 2003, Yao et al. 2004).

Microarray data analysis

The fluorescence intensities of Cy3 and Cy5 for each gene spots were transformed using the LOESS procedure (Yang et al. 2002) and the median intensity of the background was subtracted. To identify genes with increased mRNA abundance at the 12 or 20 h time point post-GnRH injection (relative to the 0 h control), data were analyzed using the significance analysis of microarrays program (version 1.21; Tusher et al. 2001). The cut-off for significance was determined by a tuning parameter, δ, which is chosen based on a desired FDR of <10%. Initial annotation and gene symbols for features on the array were obtained using the Gene Links toolkit (http://cafg.msu.edu). Ontological classification and further annotation of genes encoding for transcripts showing increased abundance following GnRH injection were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; Dennis et al. 2003).

Quantitative real-time PCR

One microgram total RNA from each sample was incubated for 15 min at 25 °C with 1 U DNase I (Invitrogen) before RT (Li et al. 2004) to eliminate possible genomic DNA contamination. The concentration of the resulting cDNA in each sample was determined by spectrophotometry. Each sample was diluted to 100 and 10 ng/µl in nuclease free water (Ambion). Real-time PCR primers (Table 1) were designed using Primer Express software (Primer Express, Applied Biosystems, Foster City, CA, USA). The optimal primer ratio and amount of cDNA used were determined in a preliminary experiment. Amplifications were performed in 96-well plates (Applied Biosystems) in 25 µl reaction volumes containing 12.5 µl of SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA, appropriate amount of primers for each gene, and nuclease free water. Non-template controls (RNase-free water) for each primer set were included. The real-time PCR was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the thermal cycler program.

<table>
<thead>
<tr>
<th>Table 1 Real-time PCR primers.</th>
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<tr>
<td>Clone number</td>
</tr>
<tr>
<td>BE485931</td>
</tr>
<tr>
<td>AW658375</td>
</tr>
<tr>
<td>BE231421</td>
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<tr>
<td>AW632222</td>
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consisting of 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantification of RNA transcript abundance was performed using the comparative threshold cycle (C_t) method established by Livak & Schmittgen (2001). The abundance of transcripts for each gene was normalized to the endogenous reference (RPL19 mRNA). The mean C_t of each gene including the endogenous control was calculated for each sample from duplicate wells. ΔC_t was produced by subtracting the mean C_t of RPL19 from the C_t of each target gene. Fold changes in mRNA expression of target genes in 12 and 24 h samples relative to 0 h were determined by using the formula 2^−ΔΔC_t, where ΔΔC_t=ΔC_t 12 or 24 h sample−ΔC_t 0 h sample. The mean ΔC_t of 0 h samples was used as the reference sample.

Protein extraction and Western blot analysis

Homogenates of follicle apex and base were prepared as described previously (Bakke et al. 2002). The supernatants were collected and protein concentration determined using Lowry method (Lowry et al. 1951). Aliquots of proteins were frozen at 20 °C until analyzed. Western blot analysis was conducted for quantification of ADAM10 and ADAM17 proteins using the protocol described previously (Bakke et al. 2004, Li et al. 2004). Membranes were stripped and reprobed with the actin antibody, and relative density was calculated against actin density units for further quantification (Bakke et al. 2004). Rabbit anti-ADAM10 (Kuzbanian, MADM) polyclonal antibody (1:1000), rabbit anti-human actin MAB (1:10 000) and rabbit anti-ACTA1 polyclonal antibody (1:10 000), were obtained from Amersham Biosciences and GenoTech (St Louis, MO, USA). Differences in mRNA abundance and protein amounts among different time points/groups were determined by one-way ANOVA using the General Linear Model procedure of the Statistical Analysis System (Version 8, SAS Institute, Cary, NC, USA). Differences between individual groups were further analyzed with Tukey’s test. When heterogeneity of variance was observed, data were log transformed before analysis. Data are presented as least square (LS) mean ± S.E.M., and probability values of less than 0.05 were considered to be statistically significant.

Statistical analysis

Differences in mRNA abundance and protein amounts among different time points/groups were determined by one-way ANOVA using the General Linear Model procedure of the Statistical Analysis System (Version 8, SAS Institute, Cary, NC, USA). Differences between individual groups were further analyzed with Tukey’s test. When heterogeneity of variance was observed, data were log transformed before analysis. Data are presented as least square (LS) mean ± S.E.M., and probability values of less than 0.05 were considered to be statistically significant.

Declaration of interest

The authors declare there is no conflict of interest.

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