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Andressa Minussi Pereira Dau

SISTEMA RENINA-ANGIOTENSINA NAS CÉLULAS DA TECA E GRANULOSA DURANTE A OVULAÇÃO E LUTEINIZAÇÃO EM BOVINOS

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de Doutor em Medicina Veterinária

Orientador: Prof. Paulo Bayard Dias Gonçalves

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Aprovado em 10 de março de 2017:

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Paulo Bayard Dias Gonçalves, PhD (UFSM)
(Presidente/orientador)

___

Vilceu Bordignon, PhD (McGill)

___

Fernando Silveira Mesquita, PhD (UNIPAMPA)

___

Marcos Henrique Barreta, Dr (UFSC)

___

Rogério Ferreira, Dr (UDESC)

Santa Maria, RS
2017

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RESUMO

SISTEMA RENINA-ANGIOTENSINA NAS CÉLULAS DA TECA E GRANULOSA DURANTE A OVULAÇÃO E LUTEINIZAÇÃO EM BOVINOS

AUTOR: Andressa Minussi Pereira Dau
ORIENTADOR: Paulo Bayard Dias Gonçalves

O objetivo do presente trabalho foi investigar a função do receptor de (pro)renina [(P)RR] nas células da teca e da granulosa durante o período pré-ovulatório e luteinização em bovinos. No início do período pré-ovulatório, pró-renina reiniciou a meiose oocitária bloqueada tanto pelas metades foliculares, quanto por forscolina. Nas células da granulosa, pró-renina não aumentou a expressão de RNAm para epirregulina (EREG) que foi induzido por LH após 6 horas de cultivo. Pró-renina mais LH aumentaram a expressão de RNAm para anfirregulina (AREG) e prostaglandina endoperoxidase sintetase-2 (PTGS2). Contudo, a ausência do efeito de pró-renina para estimular o RNAm para EREG, AREG e PTGS2 nas células da granulosa foi evidenciada utilizando as diferentes combinações de tratamento com pró-renina e/ou alisquireno [inibidor do (P)RR] e/ou LH. O tratamento das células da granulosa com LH e antagonista de EGFR (AG1478) não regularam o RNAm para pró-renina e (P)RR após 6 horas de cultivo. Esse resultado foi confirmado *in vivo*, utilizando um modelo de tratamento intrafolicular com AG1478 e GnRH intramuscular em vacas. Por fim, (P)RR e o RNAm para pró-renina e genes prófibróticos aumentaram nas células da granulosa a partir das 12 horas após tratamento de vacas com GnRH. Nas células da teca, a expressão de (P)RR aumentaram 6 horas após tratamento de vacas com GnRH. O estimulo de LH sobre o transcrito de (P)RR foi confirmado *in vitro*. O tratamento intrafolicular com alisquireno não reduziu a taxa de ovulação. No nosso cultivo de células da teca, a expressão de RNAm para AREG e EREG não foi significativa e ADAM17 não foi estimulado por pró-renina. Injeção intrafolicular com AG1478 não regulou (P)RR estimulado por LH, mas aumentou a proteína para CYP17A1. Pró-renina não induziu a síntese de androstenediona e testosterona no nosso sistema de cultivo. No corpo lúteo, RNAm para pró-renina e (P)RR foi aumentado no dia 10 do ciclo estral comparado ao dia 5 e não foram regulados por prostaglandina *in vivo*, como observado para os genes pró-fibróticos. O tratamento intrafolicular com alisquireno diminuiu os níveis de progesterona plasmática em vacas que ovularam. O papel de pró-renina na síntese de progesterona através de (P)RR também foi evidenciado *in vitro.* Ainda, pró-renina induziu a phosphorilação de ERK1/2 nas células luteais, embora o bloqueio de ERK1/2 (PD0325901) não inibiu completamente a síntese de progesterona induzida por pró-renina, como evidenciado pelo uso de AG1478. Em resumo, esses resultados demonstram que pró-renina e (P)RR são estimulados por LH no final do período pré-ovulatório e, portanto, não estão relacionados com os genes regulados por LH no início do processo ovulatório nas células da granulosa; (P)RR é estimulado por LH nas células da teca de forma independente de EGFR; e a pró-renina estimula a síntese de progesterona via (P)RR envolvendo a participação de ERK1/2 e EGFR neste processo. Em conclusão, (P)RR é regulado positivamente nas células da granulosa e da teca após o pico de LH e a pró-renina/(P)RR possui um importante papel no reinicio da meiose oocitária e na síntese de progesterona pelo corpo lúteo em bovinos.

Palavras chave: ATP6AP2. Pró-renina. RAS. Progesterona. Corpo lúteo. Ovulação.

ABSTRACT

RENIN-ANGIOTENSIN SYSTEM IN THE GRANULOSA AND TECA CELLS DURING OVULATION AND LUTEINIZATION IN BOVINES

AUTHOR: Andressa Minussi Pereira Dau
ADVISOR: Paulo Bayard Dias Gonçalves

The objective of present study was to investigate (Pro)renin receptor function in the theca and granulosa cells during the preovulatory period and luteinization in cattle. During the initial preovulatory period, prorenin induced the resumption of oocyte meiosis even in the presence of follicular hemisections or forskolin. In granulosa cells, pró-renina did not increase LH-induced epiregulin (EREG) mRNA after 6 h of culture. Treatment with prorenin plus LH increased amphiregulin (AREG) and prostaglandin synthase 2 (PTGS2) mRNA in granulosa cells. The absence of prorenin effect to stimulate EREG, AREG, and PTGS2 in granulosa cells was established using different combinations of treatments with prorenin and/or aliskiren ([P]RR inhibitor) and/or LH. Treatment of granulosa cells with LH plus EGFR antagonist (AG1478) did not regulate prorenin and (P)RR after 6 h of culture. This result was confirmed *in vivo* using a model of intrafollicular treatment with AG1478 and intramuscular treatment with GnRH. Finally, (P)RR protein and transcripts for prorenin and pro-fibrotic genes increased in the granulosa cells from 12 h post-GnRH. In the theca cells, (P)RR mRNA and protein increased 6 h after treatment of cows with GnRH. The LH effect to stimulate (P)RR transcript was confirmed *in vitro.* Intrafollicular treatment with aliskiren did not reduce the ovulation rate. In cultured theca cells, AREG and EREG mRNA were not significantly expressed and ADAM17 was not stimulated by prorenin. Intrafollicular injection of AG1478 did not regulate LH-induced (P)RR, although increased CYP17A1 protein. Prorenin did not induce androstenedione and testosterone synthesis in cultured theca cells. In the corpus luteum, prorenin and (P)RR mRNA were increased at day 10 of estrous cycle compared to day 5, but were not regulated by prostaglandin *in vivo,* as observed for profibrotic genes. Intrafollicular treatment with aliskiren reduces serum progesterone levels in cows that ovulated. Prorenin role in progesterone synthesis through (P)RR was also evidenced *in vitro.* Moreover, prorenin induced ERK1/2 phosphorylation in luteal cells, although ERK1/2 inhibition (PD0325901) did not completely inhibit prorenin-induced progesterone synthesis, as evidenced using AG1478. In summary, these results demonstrate that prorenin and (P)RR are stimulated by LH at the end of the preovulatory period and, therefore, they are not related to genes regulated by LH at the initial ovulatory process in granulosa cells; (P)RR is stimulated by LH in the theca cells independently of EGFR; and prorenin stimulate progesterone synthesis through (P)RR, which involves ERK1/2 and EGFR participation. In conclusion, (P)RR is upregulated in granulosa and theca cells after gonadotropins peak and prorenin/(P)RR play an important role in the resumption of oocyte meiosis and on progesterone synthesis in the corpus luteum in cattle.

Keywords: ATP6AP2. Prorenin. RAS. Progesterone. Corpus Luteum. Ovulation.

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1. INTRODUÇÃO

O pico de gonadotrofinas (LH e FSH) desencadeia uma série de mecanismos intracelulares e extracelulares que culminam na ovulação de um oócito apto para ser fecundado (ESPEY, 1980; RUSSELL e ROBKER, 2007). As células foliculares, por sua vez, sofrerão modificações estruturais e moleculares, após a ovulação, que compreendem o processo de luteinização, a formação do corpo lúteo, o qual possui a principal função de secretar progesterona (P4) e manter a gestação (ROBINSON *et al.*, 2008). Dessa forma, a elucidação sobre os mecanismos celulares envolvidos na ovulação e luteinização tem um importante papel para preencher lacunas ainda existentes na fisiologia básica envolvida nestes processos e, assim, servir como base para a resolução de problemas reprodutivos e aplicação de biotecnologias reprodutivas.

O sistema renina angiotensina (RAS) possui uma participação efetiva nos eventos reprodutivos nas diferentes espécies (GONCALVES *et al.*, 2012). A angiotensina II (Ang II), principal efetor do RAS, no folículo pré-ovulatório de bovinos possui papel essencial para a ovulação e tem sua ação mediada pelo receptor de Ang II tipo 2 (AGTR2) (FERREIRA *et al.*, 2007). Ainda no período pré-ovulatório de bovinos, a Ang II participa do reinicio da meiose oocitária (GIOMETTI *et al.*, 2005; BARRETA *et al.*, 2008; SIQUEIRA, L. C. *et al.*, 2012). Nas células da granulosa, Ang II atua como co-fator de LH para induzir a expressão de RNAm para genes importantes para o processo ovulatório, como metaloproteinase de membrana ADAM17, anfirregulina (AREG), epirregulina (EREG) e prostaglandina endoperoxidase sintetase-2 (PTGS2) (PORTELA *et al.*, 2011). Ang II também induz a síntese de P4 no corpo lúteo inicial de bovinos (KOBAYASHI *et al.*, 2001). Esse conjunto de dados evidencia a importância de Ang II durante o período peri-ovulatório em bovinos.

A pró-renina também é um componente do RAS e foi considerado por muitos anos apenas como precursor da renina, a qual cliva angiotensinogênio (AOG) para formar a angiotensina I (Ang I), que por sua vez, é clivada para formar a Ang II. Em 2002, foi identificado um receptor de (pro)renina [(P)RR], também conhecido por ATP6AP2, o qual possui a capacidade de mediar as ações de renina e de pró-renina formando uma via de sinalização intracelular independente da Ang II (NGUYEN *et al.*, 2002; URAOKA *et al.*, 2009). A ligação de pró-renina ou renina ao seu receptor também participa da formação da Ang I e, consequentemente, a pró-renina atua na via dependente da Ang II (NGUYEN *et al.*,

2002; URAOKA *et al.*, 2009; FERRI *et al.*, 2011). Além de a pró-renina possuir uma maior afinidade ao (P)RR comparado a renina, as concentrações de pró-renina, e não de renina, aumentam consideravelmente no fluido folicular em resposta ao LH (HAGEMANN *et al.*, 1994; NABI *et al.*, 2009). Dessa forma, o papel de pró-renina, independente da renina e da Ang II, em eventos reprodutivos do período peri-ovulatório foi postulado desde a década de 80, mas apenas ganhou força com a identificação do (P)RR em células mesangiais em 2002.

Uma função para o (P)RR foi evidenciado na placenta de mulheres no início da gestação (PRINGLE *et al.*, 2011). Em bovinos, nosso grupo de pesquisa evidenciou uma possível participação de (P)RR durante a divergência folicular (FERREIRA, GASPERIN, SANTOS, *et al.*, 2011). Recentemente, demonstramos um papel de pró-renina no reinicio da meiose via (P)RR, independente da Ang II, e identificamos a presença de (P)RR nas células da teca, da granulosa e do corpo lúteo de bovinos (DAU *et al.*, 2016). A ligação de pró-renina ao seu receptor aumenta a quantidade de Ang II, induz a fosforilação de receptor de fator de crescimento epidermal (EGFR), estimula a expressão de genes pró-fibroticos, como fator de crescimento e transformação – beta 1 (TGF-β1), inibidor do fator ativador de plasminogênio tipo I (PAI-1), colágeno tipo I (COL-1) e fibronectina (FN-1) via fosforilação de quinases reguladas por sinal extracelular 1/2 (ERK1/2) em células não reprodutivas (NGUYEN *et al.*, 2002; URAOKA *et al.*, 2009; FERRI *et al.*, 2011; LIU *et al.*, 2011; SHIBAYAMA *et al.*, 2013). Esses mesmos fatores estimulados pela pró-renina/(P)RR, em células não reprodutivas, são descritos para ovulação e luteinização em bovinos (SPICER e STEWART, 1996; KOBAYASHI *et al.*, 2001; CASEY *et al.*, 2005; TAJIMA *et al.*, 2005; FERREIRA *et al.*, 2007; HOU *et al.*, 2008; LI, Q. *et al.*, 2009; PORTELA *et al.*, 2011; MARONI e DAVIS, 2012). Entretanto, a função de (P)RR nas células foliculares de bovinos durante o período peri-ovulatório ainda precisa ser elucidado.

Os objetivos do presente trabalho foram investigar o papel de (P)RR na ovulação de bovinos; avaliar se LH estimula (P)RR nas células foliculares e determinar o envolvimento de (P)RR com genes regulados pelo LH nas células da teca e da granulosa. Além disso, investigamos o papel de pró-renina via (P)RR no reinicio da meiose oocitária e na síntese de P4 no corpo lúteo bovino.

2. REVISÃO BIBLIOGRÁFICA

2.1 OVULAÇÃO

O processo ovulatório é ativado por gonadotrofinas liberadas pela adeno-hipófise após estímulo de GnRH sintetizado no hipotálamo (ESPEY, 1980; RUSSELL e ROBKER, 2007). O LH é liberado de forma pulsátil, sendo que a frequência aumenta e a amplitude dos pulsos diminui de acordo com a redução dos níveis de P4 e aumento de estrógeno (E2) produzido pelo folículo dominante (BAIRD *et al.*, 1976; BAIRD *et al.*, 1981). Em bovinos, o pico de LH ocorre em torno de 2 horas após aplicação do análogo de GnRH (KOMAR *et al.*, 2001). Os folículos tornam-se capazes de ovular a partir do momento em que se tornam dominantes (≥10 mm de diâmetro); e sua responsividade ao pico de LH aumenta a partir de folículos maiores (≥12mm de diâmetro) em Bos taurus (SARTORI *et al.*, 2001). O receptor de LH (LH-R), membro da superfamilia de receptor ligado a proteína G, além de estar presente nas células da teca, possui seus níveis aumentados em folículos pré-ovulatórios nas células da granulosa de ratos (PENG *et al.*, 1991), murinos (EPPIG *et al.*, 1997) e bovinos (ROBERT *et al.*, 2003). Em todas as fases do desenvolvimento folicular há expressão de diferentes isoformas de transcrito para LH-R nas células da granulosa, entretanto apenas os transcritos de LH-R em folículos dominantes são capazes de formar uma proteína funcional (ROBERT *et al.*, 2003).

O pico de LH estimula fatores de crescimento semelhantes ao fator de crescimento epidermal (EGF), dentre os quais inclui-se a AREG, EREG e β-celulina (BTC) (PARK *et al.*, 2004). A transativação do EGFR ocorre nas células da granulosa pela enzima proteolítica ADAM17 (ou TACE, enzima conversora do TNF) que libera o domínio desses fatores (AREG e EREG) e regula fosforilação de proteína quinase ativada por mitógeno (MAPK) (YAMASHITA *et al.*, 2007; PANIGONE *et al.*, 2008; YAMASHITA *et al.*, 2009; YAMASHITA e SHIMADA, 2012). A fosforilação de ERK1/2 nas células da granulosa é essencial para que ocorra a ovulação, uma vez que em camundongo *Knockout* condicional para ERK1/2 nas células da granulosa foram observados oócitos inclusos e ausência de corpo lúteo após superovulação (FAN *et al.*, 2009). Contudo, a ablação do EGFR nas células da granulosa em camundongos não bloqueia completamente nem a atividade de MAPK e, consequentemente, nem a ovulação (PANIGONE *et al.*, 2008). Dessa forma, sugere-se a

existência de rotas alternativas envolvendo, por exemplo, os componentes do RAS, além dos fatores semelhantes ao EGF, para ativação de MAPK e sinalização para a regulação gênica dos eventos liderados pelo pico pré-ovulatório de LH.

A prostaglandina é originada do ácido araquidônico pela via PTGS2. Em resposta ao pico de LH, a isoforma PTGS2 é aumentada nas células da granulosa (LIU *et al.*, 1997; RICHARDS, 1997), e as concentrações das prostaglandinas E2 (PGE2) e F2 (PGF2) são elevadas no fluído folicular (SIROIS, 1994; LIU *et al.*, 1997). A PTGS2 está envolvida com a cascata de fatores semelhantes ao EGF induzida por LH, em que a ativação de EGFR induz um aumento no RNAm de PTGS2 em folículos pré-ovulatórios (SASAKI *et al.*, 1998; PARK *et al.*, 2004). Prostaglandina E2 (PGE2) possui efeitos similares ao LH, participando do processo de ovulação por induzir a síntese de fatores de crescimento semelhantes ao EGF via AMPc/PKA e MAPK (BEN-AMI *et al.*, 2006; SHIMADA *et al.*, 2006).

Após o pico de LH, a concentração de P4 torna-se maior em relação ao estradiol E2 no ovário, dando início ao processo de luteinização (KOMAR *et al.*, 2001). Os níveis de E2 no fluido folicular diminuem gradativamente a partir de 3 horas após GnRH (hora 0) em bovinos (TONELLOTTO DOS SANTOS *et al.*, 2012), reduzindo em torno de 70% na hora 12 (KOMAR *et al.*, 2001). A redução da concentração de E2 intrafolicular ocorre pela queda de enzimas esteroidogênicas como 17α-hidroxilase (17α-OH), o que reflete na reduzida produção de andrógeno e aromatase (CYP19A1), e consequentemente, na menor capacidade de conversão de andrógeno para E2 nas células da granulosa (KOMAR *et al.*, 2001). A síntese P4, por sua vez, é controlada pela 3β-hidroxiesteróide desidrogenase (HDS3B2), que quando inibida por trilostano intrafolicular em vacas induzidas com GnRH, apesar de resultar em menores concentrações de P4 no fluido folicular, não afeta a ovulação (Li *et al.*, 2007). Em camundongos *knockout* para receptor de P4, entretanto, a P4 foi essencial para ovulação (Lydon *et al.*, 1995). Em bovinos, a fosforilação de ERK1/2, estimulada por LH, nas células da teca promove a síntese de andrógenos e regula a síntese de P4 (TAJIMA *et al.*, 2005; FUKUDA *et al.*, 2009). O tratamento *in vitro* de células da teca com EGF, por sua vez, reduz a síntese de androstenediona induzida por LH (STEWART *et al.*, 1995). Juntos, esses resultados em bovinos sugerem que ERK1/2 pode estar mediando a esteroidogênese nas células da teca imediatamente após o pico de LH e EGFR no final do período pré-ovulatório bovino.

2.2 ESTEROIDOGÊNESE

A esteroidogênese consiste na produção de importantes esteroides, mediada por enzimas esteroidogênicas, nas células da teca e da granulosa de forma interativa. O colesterol é transportado pela proteína reguladora da esteroidogênese (*STAR*) para dentro da mitocôndria, onde é convertido para pregnenolona pela enzima P450scc (CYP11A1) (MILLER, 2007). A pregnenolona, por sua vez, pode ser convertida pela HDS3B2 para P4 ou, então, transformada em 17α-hidroxipregnenolona pela enzima 17α-hidroxilase (CYP17A1) nas células da teca. Contudo, nas células da granulosa a P4 sintetizada é secretada e não metabolizada em andrógeno (STOKLOSOWA, 1989). Isso é explicado pelas diferentes enzimas presentes em cada célula, uma vez que as células da teca possuem grande quantidade de CYP17A1, enquanto a mesma é ausente nas células da granulosa. Dessa forma, tanto as células da teca quanto as da granulosa são capazes de produzir pregnenolona e P4 a partir do colesterol, uma vez que ambas apresentam StAR, CYP11A1 e HSD3B2 (KING e LAVOIE, 2012). Nas células da teca, portanto, a partir da 17α-hidroxiprogesterona, o principal hormônio produzido é a androstenediona pelas atividades das enzimas CYP17A1 e HSD3B2. A androstenediona pode ser convertida em testosterona por isoenzimas da 17β-hidroxisteroide deidrogenase (HSD17B). Ademais, a androstenediona e testosterona são secretados pelas células da teca e, então, grande parte destes andrógenos são absorvidos pelas células da granulosa. As células da granulosa, ao contrário das células da teca, apresentam a enzima aromatase (CYP19A1), a qual converte androstenediona em estrona (transformada em estradiol pela enzima HSD17B) ou, alternativamente, testosterona em estradiol (TREMBLAY *et al.*, 1989; SIMPSON *et al.*, 1994). Logo, as células da teca expressam enzimas necessárias para converter colesterol para andrógeno, entretanto, a conversão de andrógenos para E2 deve ocorrer nas células da granulosa.

A capacidade esteroidogênica nas células pode ser mediada pela transcrição dos genes das enzimas esteroidogênicas, entre as quais, a STAR, que determina a habilidade que uma célula esteroidogênica tem para responder a um determinado estímulo (MILLER, 2007). A CYP11A1 é a enzima limitante para a produção de esteroides, determinando a capacidade quantitativa das células esteroidogênicas. A capacidade qualitativa, entretanto, é verificada pela enzima CYP17A1, cuja presença nas células da teca determina a conversão da P4 em androstenediona (BREMER e MILLER, 2008). Além disso, a ablação de CYP17A1 no ovário de ratos reduz a produção de 17α-hidroxiprogesterona, e, consequentemente, de testosterona e androstenediona (LI, Y. *et al.*, 2009).

O estímulo da síntese de andrógenos (BAIRD *et al.*, 1976; BAIRD *et al.*, 1981) e da expressão gênica de StAR e das enzimas esteroidogênicas CYP11A1, HSD3B2 e CYP17A1

nas células da teca de mamíferos ocorre, dentre outros mediadores, em resposta ao pico de LH (MAGOFFIN e WEITSMAN, 1993a; b; c; CAMPBELL *et al.*, 1998; RONEN-FUHRMANN *et al.*, 1998). Nas células da granulosa, após o pico gonadotrófico, enquanto ocorre uma redução gradativa de CYP19A1 (KOMAR *et al.*, 2001), há um aumento na expressão do RNAm de StAR, CYP11A1 e HSD3B2 (JONES *et al.*, 1983; RONEN-FUHRMANN *et al.*, 1998; LEE *et al.*, 2012). A insulina e/ou IGF-1 também induz um aumento na esteroidogênese das células foliculares: aumentando a expressão de RNAm de CYP19A1, CYP11A1, HSD3B2 e a secreção de E2 pelas células da granulosa bovina cultivadas *in vitro* por 48 horas acrescidas com FSH (SPICER *et al.*, 2002; SPICER e AAD, 2007; MANI *et al.*, 2010), bem como induzindo de forma dose-dependente aumento sobre a produção de esteroides e expressão de RNAm de HSD3B2, CYP11A1 e CYP17A1 estimulado pelo LH em células da teca de ratos (MAGOFFIN e WEITSMAN, 1993a; b; c), suínos (MORLEY *et al.*, 1989), ovinos (CAMPBELL *et al.*, 1998) e bovinos (STEWART *et al.*, 1995) submetidas a cultivos *in vitro*. Contudo, a regulação da esteroidogênese gonadal por mediadores das gonadotrofinas não está completamente elucidada e necessita ser melhor explorada.

2.3 LUTEINIZAÇÃO E LUTEÓLISE

A formação do corpo lúteo ocorre pela transição morfológica e molecular das células foliculares após a ovulação e é caracterizado pela produção de P4, necessária para mantença da gestação (ROBINSON *et al.*, 2008). A medida que a inadequada síntese de P4 pode ter consequências críticas sobre a fertilidade e o desenvolvimento embrionário inicial, a luteinização é considerada um importante processo no ciclo reprodutivo (MANN e LAMMING, 2001). Nas vacas, a fase luteal corresponde a 14 dias do ciclo estral bovino (período entre duas ovulações com duração média de 21 dias); e caracteriza-se pelo período de secreção de P4 pelo corpo lúteo (CL), o qual inicia com a transição de células foliculares para luteínicas após o pico de LH e, no caso da ausência de embrião, termina com o processo de regressão estrutural e funcional do CL denominado luteólise (REKAWIECKI *et al.*, 2008).

A transição folículo-luteal é resultado de uma ação coordenada de fatores autócrinos/parácrinos a partir da sinalização do LH. As células da teca e da granulosa cessam a produção de andrógenos e E2, respectivamente, e começam a sintetizar P4 (RICHARDS, 1980). Dessa forma, a expressão de enzimas necessárias para conversão de colesterol em P4 aumentam (CYP11A1 e HSD3B2) e enzimas que convertem P4 em E2 diminuem (CYP19A1 e HSD17B) (JONES *et al.*, 1983; TREMBLAY *et al.*, 1989; SIMPSON *et al.*, 1994; RONEN-

FUHRMANN *et al.*, 1998; LEE *et al.*, 2013). Além disso, as células da teca e da granulosa se reorganizam junto aos fibroblastos, às células endoteliais, da parede vascular (pericitos) e do sistema imune para a formação do corpo lúteo (JABLONKA-SHARIFF *et al.*, 1993; SMITH *et al.*, 1994; NISWENDER *et al.*, 2000; ROBINSON *et al.*, 2009). As células foliculares, por sua vez, sofrem alterações morfológicas, em que as células da granulosa transformam-se em células luteais grandes e as células da teca dão origem às células luteais pequenas (DONALDSON e HANSEL, 1965).

A proliferação das células, durante a luteinização, ocorre rapidamente a partir de sucessivas mitoses (JABLONKA-SHARIFF *et al.*, 1993) e é requisito para neovascularização que culmina na extensa rede capilar do CL (REYNOLDS *et al.*, 1992). A proliferação celular e a vascularização local influenciam na capacidade do CL de sintetizar P4, bem como substâncias vasoativas, fatores de crescimento e angiogênicos (ACOSTA e MIYAMOTO, 2004). Os fatores que regulam esses mecanismos locais tratam-se de uma complexa rede de fatores de crescimento angiogênicos e peptídeos vasoativos, muitos dos quais ainda necessitam ser explorados (ROBINSON *et al.*, 2009).

A luteólise deve ocorrer, na ausência de concepção, ao término da vida lútea, caracterizando o momento em que há alto nível de E2 e quedas na concentração de P4 momentos antes da ovulação. A luteólise ocorre em duas fases: funcional pela diminuição da secreção de P4 (MCGUIRE *et al.*, 1994) e estrutural pela perda de tecido luteal, incluindo a degeneração de seus capilares no ovário e, consequentemente, a diminuição do fluxo sanguíneo para o CL (PATE, 1994; NISWENDER *et al.*, 2000). Esse mecanismo é desencadeado pela PGF2α, que é liberada pelo endométrio em resposta à ligação entre a ocitocina luteal e seus receptores no endométrio. A sinalização de ocitocina para liberação da PGF2α ocorre pela ausência do interferon-tau liberado pelo embrião de ruminantes (SPENCER *et al.*, 2004). Portanto, na presença de um concepto, o CL deve permanecer ativo mantendo alto os níveis de P4 importantes para mantença da gestação.

A involução estrutural do CL envolve, dentre outros processos, principalmente a apoptose (DAVIS e RUEDA, 2002). A morte celular programada ocorre a partir da ativação de uma vasta rede de receptores de citocinas, segundos mensageiros e proteínas (ORRENIUS *et al.*, 1992; ANTONSSON, 2001; KORZEKWA *et al.*, 2008). O ligante Fas, pertencente à super-família TNF, quando ligado ao seu receptor (Fas) induz apoptose durante a luteólise (SAKAMAKI *et al.*, 1997; OKUDA e SAKUMOTO, 2003). Os membros da família de proteínas Bcl-2 regulam a via mitocondrial de morte celular, na qual a taxa de BAX (pró-apoptótico) em relação ao anti-apoptótico Bcl2 determina a indução da apoptose

(ANTONSSON, 2001). Ainda, proteínas da família Bcl-2 induzem a ativação das caspases e, subsequente, liberação mitocondrial de fatores apoptóticos (DAVIS e RUEDA, 2002). Dentre os membros da família de proteases de cisteína (caspases), a caspase-3 é considerada o principal efetor da apoptose durante a luteólise, uma vez que, camundongos com ablação da caspase-3 apresentam ausência de regressão luteal, bem como atraso da degradação do DNA (CARAMBULA *et al.*, 2002). Portanto, a PGF2α deve ativar citocinas pró-apoptóticas, que atuam na via de apoptose dependente de caspase-3, para induzir a luteólise (CARAMBULA *et al.*, 2003). Ademais, oxido nítrico (NO) também está envolvido na regressão do CL, mediando a ação da PGF2α tanto na fase funcional por inibir a secreção P4, quanto na fase estrutural da luteólise por aumentar, além da mobilização intracelular de cálcio, a expressão de Fas, Bax e caspase-3, bem como a fragmentação do DNA (KORZEKWA *et al.*, 2006; ACOSTA *et al.*, 2009).

2.4 RAS: PRÓ-RENINA E RECEPTOR DE (PRO)RENINA

O Sistema Renina-Angiotensina (RAS) é um dos principais mecanismos de ajuste e manutenção da pressão sanguínea. Nesse sistema, a renina, ativada após clivagem de um prosegmento de 43 aminoácidos (aa) da pró-renina, é sintetizada nos rins e liberada na circulação, na qual atua clivando angiotensinogênio (AOG) para formar Ang I (SEQUEIRA LOPEZ e GOMEZ, 2010). A glicoproteína denominada AOG é sintetizada primariamente no fígado e secretada na circulação em função da variação da pressão sanguínea e atua como precursor de Ang I (MENARD *et al.*, 1983; DESCHEPPER, 1994). A Ang I trata-se de um decapeptídeo que quando clivado pela enzima conversora da angiotensina (ECA) forma o octopeptídeo Ang II (SKEGGS *et al.*, 1956; WEI *et al.*, 1991). A Ang II é considerada o principal efetor do RAS e atua via seus receptores de angiotensina tipo 1 (AGTR1) e tipo 2 (AGTR2) (HALL, 2003; HUNYADY e CATT, 2006; PORRELLO *et al.*, 2009).

A pró-renina, membro da superfamília de aspartil proteases, deriva da pré-pró-renina e contém um prosegmento de 43 aa que está associado ao N-terminal lobular bi-homólogo da renina (340aa) (DO *et al.*, 1987; WU *et al.*, 2008). Inicialmente, a pré-pró-renina sofre uma clivagem de 23 aminoácidos para formação da pró-renina; e a mesma por sua vez, tem seu prosegmento clivado por calicreína, catepsina-B e convertases para formação da renina (DO *et al.*, 1987; PITARRESI *et al.*, 1992; WU *et al.*, 2008). Enquanto a síntese de pró-renina também ocorre em tecidos extra-renais, incluindo o ovário (DO *et al.*, 1987; ITSKOVITZ *et al.*, 1987; ITSKOVITZ *et al.*, 1992), a formação de renina se restringe às células

justaglomerulares nos rins, onde a mesma então permanece, estocada em grânulos (SEALEY *et al.*, 1977; PERSSON, 2003; KROP *et al.*, 2008). Apenas uma pequena parcela da pró-renina produzida nos rins é convertida para renina e, portanto, a maior parte de pró-renina é liberada continuamente na circulação (PRATT *et al.*, 1987). Por isso, em geral são encontradas maiores concentrações de pró-renina do que de renina no plasma.

A pró-renina, além de ser ativada pela clivagem e consequente formação da renina, também desempenha suas funções pela ligação ao (P)RR/ATPAP2 (NGUYEN *et al.*, 2002), pelo qual a pró-renina apresenta afinidade duas a três vezes maior que a renina (NABI *et al.*, 2009). Nesse caso, a ativação decorre da mudança conformacional do prosegmento da pró-renina, expondo o seu sítio ativo sem haver perda estrutural por clivagem (PITARRESI *et al.*, 1992; SUZUKI *et al.*, 2003). A ligação pró-renina-(P)RR estimula a clivagem de AGT para formação de Ang I, mas também ativa uma cascata de sinalização intracelular pela fosforilação de ERK1/2 independentemente da Ang II (URAOKA *et al.*, 2009). Além disso, a pró-renina quando ligada ao seu receptor induz fosforilação de EGFR (LIU *et al.*, 2011; SHIBAYAMA *et al.*, 2013) e estimula expressão de RNAm de moléculas pró-fibróticas como TGF-β1, PAI-1, COL-1 e FN-1 após ativação da ERK1/2 (NGUYEN *et al.*, 2002; HUANG *et al.*, 2006; FERRI *et al.*, 2011). Essa função está associada à natureza estrutural do (P)RR (350aa/ 35-37 KDa) que apresenta três domínios: extracelular (o sítio de ligação de pró-renina ou renina); transmembrana (que fixa o receptor à membrana celular); e cauda citoplasmática (responsável pela sinalização intracelular) (NGUYEN e CONTREPAS, 2008). Entretanto, (P)RR também pode ser encontrado como uma proteína de transmembrana integral ou no plasma na sua forma solúvel [(P)RRs; 28kDa]. O (P)RRs é liberado na circulação através de uma clivagem realizada por proteases, como furina (COUSIN *et al.*, 2009) e ADAM19 (desintegrina/metaloproteinase) (YOSHIKAWA *et al.*, 2011) . O (P)RRs apresenta domínio extracelular com capacidade de ligação à renina ou prorenina (COUSIN *et al.*, 2009) e tem sido relacionado com circulação materna e fetal (NARTITA *et al.*, 2016; TERADA *et al.*, 2017). Entretanto, mais estudos são necessários para esclarecer sobre a função do (P)RRs.

O bloqueio da interação entre pró-renina e (P)RR na sequência "*handle region*" pode ser realizado utilizando o peptídeo de 10 aa HRP (*handle region decoy peptide*) (SUZUKI *et al.*, 2003; ICHIHARA *et al.*, 2004; ICHIHARA *et al.*, 2006). No entanto, a eficiência desse bloqueio tem se mostrado questionável tanto *in vitro* como *in vivo* (BATENBURG *et al.*, 2007; FELDT, MASCHKE, *et al.*, 2008). Outra opção disponível é o alisquireno (inibidor ativo de renina), que já foi empregado com eficiência para reduzir expressivamente a ativação intracelular e extracelular estimulada pela ligação pró-renina-(P)RR (BISWAS *et al.*, 2010;

FERRI *et al.*, 2011; MA *et al.*, 2012), embora também tenha apresentado falhas em alguns experimentos (SARIS *et al.*, 2006; SAKODA *et al.*, 2010). Quanto à especificidade desses bloqueadores, deve-se destacar que o alisquireno inibe as atividades de renina livre e do (P)RR ligado à pró-renina ou à renina (BISWAS *et al.*, 2010), diferentemente de HRP, o qual atua apenas bloqueando a ligação pró-renina/(P)RR (SUZUKI *et al.*, 2003; ICHIHARA *et al.*, 2004; ICHIHARA *et al.*, 2006).

2.5 RECEPTOR DE (PRO)RENINA NO PERÍODO PERI-OVULATÓRIO

A ação local de RAS no ovário de mamíferos é proposto com base nos seguintes achados: o aumento das concentrações de Ang II e/ou pró-renina no fluido folicular em resposta ao pico de LH em ratos (HUSAIN *et al.*, 1987), coelhos (YOSHIMURA *et al.*, 1994), bovinos (HAGEMANN *et al.*, 1994; ACOSTA *et al.*, 2000) e humanos (GLORIOSO *et al.*, 1986; DO *et al.*, 1988); em ratas, mesmo após nefrectomia bilateral, as concentrações de Ang II permaneceram elevadas no fluido folicular (HUSAIN *et al.*, 1987); o pico de LH induziu o aumento dos níveis de AngII e de atividade de renina, e/ou, de enzimas com atividade semelhante à da renina no fluido folicular de ovários perfundidos de coelhas (YOSHIMURA *et al.*, 1994); na espécie bovina, demonstrou-se que o pico de LH induzido pelo análogo de GnRH, além de elevar a expressão de RNAm de AGTR2 e de ECA nas células da teca, aumenta a expressão do RNAm de AGT nas células da granulosa e a concentração de Ang II no fluído folicular (ACOSTA *et al.*, 2000; SHIMIZU *et al.*, 2007; SIQUEIRA *et al.*, 2013).

A Ang II, induzida por P4 e prostaglandina, atua sobre a retomada da meiose oocitária bovina (GIOMETTI *et al.*, 2005; BARRETA *et al.*, 2008; SIQUEIRA, L. C. *et al.*, 2012), o que também foi evidenciado em coelhas (YOSHIMURA *et al.*, 1996). Além disso, Ang II age como cofator de LH no estímulo de genes envolvidos nos processos de reinicio da meiose e/ou ovulação como: PTGS2, AREG, EREG e ADAM17 nas células da granulosa de folículos pré-ovulatórios bovinos (PORTELA *et al.*, 2011). Ademais, a indução da ovulação por hCG em coelhas (YOSHIMURA *et al.*, 1996) ou GnRH em vacas (FERREIRA *et al.*, 2007) é prejudicada por injeção intrafolicular de PD123,319 (antagonista específico de AGTR2) o que indica, fortemente, que a atuação de Ang II ocorre via AGTR2 nas células da teca e da granulosa durante o período pré-ovulatório.

A participação de pró-renina nos eventos fisiológicos ovarianos, independentemente de uma relação com os níveis sistêmicos de renina de origem renal, foi sugerida na década de

80. Nos referidos trabalhos, os autores relacionam a uma resposta ao pico de LH efeitos como: a elevação nos níveis de pró-renina (sem aumento de renina) na circulação (SEALEY *et al.*, 1985); e o aumento nas concentrações de pró-renina no fluido folicular em comparação proporcional à sua concentração plasmática (GLORIOSO *et al.*, 1986). Não obstante, cerca de 95% do total de renina detectado no fluido folicular corresponde a pró-renina sintetizada e liberada principalmente pelas células da teca do folículo ovariano (DO *et al.*, 1988; SCHULTZE *et al.*, 1989). Em função disso, a ativação de rotas fisiológicas, bem como a clivagem do AOG para produção de Ang I, via ligação de pró-renina/receptor, sem necessidade de sua conversão para renina, passou a ser considerada (ITSKOVITZ *et al.*, 1988).

A expressão do (P)RR foi evidenciada no coração, cérebro, placenta, pulmão, fígado, músculo esquelético e pâncreas humano (NGUYEN *et al.*, 2002). Nosso grupo de pesquisa ainda identificou a expressão de RNAm desse receptor nas células da teca e granulosa do ovário bovino (FERREIRA *et al.*, 2011). Recentemente, identificamos (P)RR por western blot no complexo cumulus-oócito, células da teca e da granulosa e corpo lúteo de bovinos (DAU *et al.*, 2016).

A pró-renina deve participar, via (P)RR, nos eventos peri-ovulatórios em mamíferos, tendo em vista que o pico de LH estimula a produção de pró-renina e a atividade de renina intrafolicular em coelhos (YOSHIMURA *et al.*, 1994), bovinos (HAGEMANN *et al.*, 1994) e humanos (GLORIOSO *et al.*, 1986). Recentemente, nosso grupo de pesquisa além de demonstrar a expressão de (P)RR nas células ovarianas de bovinos (FERREIRA, GASPERIN, SANTOS, *et al.*, 2011; DAU *et al.*, 2016), evidenciou sua função no reinicio da meiose oocitária (DAU *et al.*, 2016). A interação de pró-renina e (P)RR, além de estimular a produção de Ang II que promove a retomada da meiose oocitária e é essencial para a ovulação (GIOMETTI *et al.*, 2005; FERREIRA *et al.*, 2007), estimula a fosforilação de ERK1/2, independente de Ang II (NGUYEN *et al.*, 2002; URAOKA *et al.*, 2009). A ativação de ERK1/2 nas células da granulosa é essencial para a maturação do oócito e ovulação em camundongos (FAN *et al.*, 2009) e regula a esteroidogênese induzida por LH nas células da teca de bovinos (TAJIMA *et al.*, 2005; FUKUDA *et al.*, 2009). O (P)RR também tem sido relacionado ao EGFR (LIU *et al.*, 2011; SHIBAYAMA *et al.*, 2013), o qual está envolvido com os genes regulados por LH (STEWART *et al.*, 1995). Esse conjunto de evidências aponta para um papel determinante da pró-renina via (P)RR nas células foliculares durante o período pré-ovulatório. Entretanto, há diversos pontos a serem esclarecidos para a confirmação dos mecanismos envolvidos no postulado RAS local durante a ovulação de mamíferos.

O possível efeito da pró-renina/(P)RR na esteroidogênese e luteinização de mamíferos também deve ser considerado, tendo em vista que uma correlação temporal foi evidenciada em mulheres, indicando que a regulação de LH sobre E2 provavelmente estimule a síntese de pró-renina, aumentando, em sequência, a P4 plasmática (ITSKOVITZ *et al.*, 1987). Em vacas superovuladas, por sua vez, as concentrações de pró-renina e de renina no fluido folicular estão negativamente correlacionadas com a produção de E2 e positivamente correlacionadas com a concentração de P4 (HAGEMANN *et al.*, 1994). Isso sugere que a pró-renina presente no fluido folicular bovino deve atuar sobre o aumento de P4 em resposta ao pico de LH (KOMAR *et al.*, 2001; FORTUNE *et al.*, 2009).

Portanto, nossa hipótese é que assim como (P)RR participa no reinicio da meiose oocitária, possui efeito na ovulação e na luteinização nas células da teca e da granulosa de bovinos. Em função disso, nosso objetivo geral foi determinar a participação da pró-renina via (P)RR no período pré-ovulatório, na esteroidogenese e na luteinização em bovinos.

3. ARTIGO 1

TRABALHO ACEITO PARA PUBLICAÇÃO:

Bovine ovarian cells have (pro)renin receptors and prorenin induces resumption of meiosis in vitro

Andressa Minussi Pereira Dau, Eduardo Predebon da Silva, Paulo Roberto Antunes da Rosa, Felipe Tusi Bastiani, Karina Gutierrez, Gustavo Freitas Ilha, Fabio Vasconcellos Comim and Paulo Bayard Dias Gonçalves.

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Bovine ovarian cells have (pro)renin receptors and prorenin induces resumption of meiosis in vitro

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The work was carried out in the Laboratory of Biotechnology and Animal Reproduction, BioRep, Federal University of Santa Maria, Santa Maria, RS, Brazil.

Laboratory of Biotechnology and Animal Reproduction, BioRep, Federal University of Santa Maria, Roraima Avenue 1000, 97105-900 Veterinary Hospital, Santa Maria, RS, Brazil.

Corresponding author's details: PBD Gonçalves, Laboratório de Biotecnologia e Reprodução Animal, Hospital Veterinário, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil. Email address: bayard@ufsm.br

Abstract

The discovery of a receptor that binds prorenin and renin in human endothelial and mesangial cells highlights the possible effect of renin-independent prorenin in the resumption of meiosis in oocytes that was postulated in the 1980s.This study aimed to identify the (pro)renin receptor in the ovary and to assess the effect of prorenin on meiotic resumption. The (pro)renin receptor protein was detected in bovine cumulus-oocyte complexes, theca cells, granulosa cells, and in the corpus luteum. Abundant (pro)renin receptor messenger ribonucleic acid (mRNA) was detected in the oocytes and cumulus cells, while prorenin mRNA was identified in the cumulus cells only. Prorenin at concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M incubated with oocytes co-cultured with follicular hemisections for 15 h caused the resumption of oocyte meiosis. Aliskiren, which inhibits free renin and receptor-bound renin/prorenin, at concentrations of 10^{-7}, 10^{-5}, and 10^{-3} M blocked this effect (P<0.05). To determine the involvement of angiotensin II in prorenin-induced meiosis resumption, cumulus-oocyte complexes and follicular hemisections were treated with prorenin and with angiotensin II or saralasin (angiotensin II antagonist). Prorenin induced the resumption of meiosis independently of angiotensin II. Furthermore, cumulus-oocyte complexes cultured with forskolin (200 µM) and treated with prorenin and aliskiren did not exhibit a prorenin-induced resumption of meiosis (P<0.05). Only the oocytes' cyclic adenosine monophosphate levels seemed to be regulated by prorenin and/or forskolin treatment after incubation for 6 h. To the best of our knowledge, this is the first study to identify the (pro)renin receptor in ovarian cells and to demonstrate the independent role of prorenin in the resumption of oocyte meiosis in cattle.

Keywords: aliskiren, angiotensin II, saralasin, prorenin, oocyte, cumulus cell.

1. **Introduction**

Evidence is accumulating that supports possible roles for the (pro)renin receptor ([P]RR)-dependent system in the development of insulin resistance and hypertension. Interestingly, women with diabetes and hypertension often present with infertility problems [3, 28, 33]. Likewise, delays in oocyte maturation have been documented in animal models of diabetes [12, 14]. Findings from other studies carried out in the 1980s have suggested that prorenin has a role in the resumption of oocyte meiosis [29], but this remains unclear.

Oocytes are arrested during the first meiotic division (prophase I) by the follicular environment. The preovulatory luteinizing hormone (LH) surge induces the closure of gap junctions and a reduction in the inhibitory cyclic guanosine monophosphate (cGMP) signal from the cumulus cells to the oocyte in vivo. The low level of cGMP generated by the granulosa cells results in phosphodiesterase 3 activation, cyclic adenosine monophosphate (cAMP) hydrolysis, and the subsequent resumption of meiosis in the oocyte [15, 27, 38, 40, 45, 55]. The oocyte also resumes nuclear maturation when the cumulus-oocyte complex (COC) is removed from the follicular environment [42]. We have used forskolin (FSK) to maintain the high concentration of cAMP and to delay meiosis in an in vitro model [6, 13, 52]. Furthermore, follicular hemisections have been used to mimic the follicular environment and to delay the resumption of meiosis [4, 22, 43, 48, 51]. We have used these in vitro and in other in vivo models to study the role of angiotensin II (AngII) in the resumption of meiosis in cattle [1, 49, 4, 22].

The classic concept of the renin-angiotensin system (RAS) portrays prorenin as an enzymatically inactive precursor of renin, which is an aspartyl protease, and as being dependent on AngII to trigger the activation of intracellular signaling pathways.

In fact, plasma prorenin levels, but not plasma renin levels, increase after the LH surge [46]. Similarly, prorenin levels in the follicular fluid increase to about 12-times the concentrations detected in women's plasma after LH stimulation during in vitro fertilization procedures, and approximately 99% of the total renin identified in ovarian follicular fluid is prorenin [23].

The (P)RR was the first receptor identified that binds to an aspartyl protease [37]. The (P)RR acts within an extracellular-signal-regulated kinase (ERK1/2) pathway [54] that seems to be essential for the resumption of meiosis in mammals [16]. The (P)RR also stimulates the AngII pathway by binding to renin and prorenin, which promotes the cleavage of angiotensinogen to angiotensin I (AngI) [37, 54]. The presence of (P)RR messenger ribonucleic acid (mRNA) has been demonstrated in the bovine theca and granulosa cells during follicular dominance [18]. However, the presence of the (P)RR protein in ovarian follicular cells is yet to be confirmed, and the role of prorenin in the resumption of meiosis in oocytes is unknown. The aim of this study was to characterize the (P)RR in the cumulus-oocyte complex (COC), theca cells, granulosa cells, and corpus luteum (CL). Moreover, we evaluated the role of prorenin in the induction of oocyte meiotic resumption in cattle.

2. **Materials and methods**

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Care and Use Committee (no. 003/2012).

2.1. Chemicals

All of the chemicals used were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA) unless otherwise indicated.

2.2. Collection of the ovaries

Bovine ovaries at different stages of the estrous cycle were obtained from a local abattoir and transported to the laboratory in phosphate-buffered saline (PBS) at 4 ºC for mRNA and protein analyses [8, 9], or in a 0.9% NaCl solution containing penicillin (100 IU/ml) and streptomycin sulfate (50 μg/ml) at 30 ºC for the COC culture experiments [4].

2.3. Western blotting

Proteins from the COCs, theca cells, granulosa cells, and CL were extracted using radioimmunoprecipitation assay buffer. The proteins were boiled at 95 °C for 5 min, subjected to 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and the proteins were transferred onto nitrocellulose membranes. After blocking the membranes for 3 h using 5% skimmed milk in Tris-buffered saline (TBS) containing 0.1% Tween® 20 (TBS-T), the blots were incubated overnight with an antibody to the (P)RR (anti-ATP6IP2; diluted 1:1000; ab40790; Abcam plc, Cambridge, UK) at 4 °C while being agitated. Subsequently, the blots were washed three times for 5 min each time in TBS-T. The blots were then incubated with a goat anti-rabbit secondary antibody (diluted 1:2000; IgG-HRP; sc-2004; Santa Cruz Biotechnology, Inc., Dallas TX, USA) for 1 h while being agitated, which was followed by three washes for 5 min each in TBS-T. The immunoreactivity was detected using the Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions, and the images were visualized using the ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blots were incubated in a western blot stripping buffer, which comprised β-mercaptoethanol, 20% SDS, and 1 M Tris-HCl, at pH 6.8, for 1 h at 50 ºC. Then, the membranes were washed three times with TBS-T with each wash

lasting 20 min, and the membranes were re-blotted with an anti-beta actin antibody (diluted 1:5000; control; ab8227; Abcam plc, Cambridge, UK).

2.4. Ribonucleic acid extraction, reverse transcription, and the quantitative polymerase chain reaction

Total ribonucleic acid (RNA) was extracted using Trizol® (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. A NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to quantify the RNA and verify lack of contamination. Only RNAs with purity values of more than 1.8, based on the ratios of the absorbance at 260 and 280 nm, were used in the experiments. The integrity was verified in a 1.2% agarose gel that visualized the ribosomal RNA (rRNA). To generate the complimentary deoxyribonucleic acid (cDNA), the RNA (1 µg) was first treated with 0.2 U of deoxyribonuclease I (DNase I, Amplification Grade, Invitrogen Life Technologies, Waltham, MA, USA) and it was heated at 37 ºC for 5 min, then at 65 ºC for 10 min. Subsequently, the reverse transcription was performed using a QuantiTect Reverse Transcription Kit® (Qiagen, Venlo, Limburg, Netherlands) in accordance with the manufacturer's instructions.

The quantitative real-time polymerase chain reaction (RT-PCR) was conducted in a Step One Plus® instrument (Applied Biosystems, Foster, CA, USA) using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) and primers that were specific for bovine prorenin and the *(*P)RR [18]. After an initial denaturation step at 95 ºC for 3 min, 40 cycles at 95 ºC for 15 s were carried out, followed by 30 s at 60 ºC and 30 s at 72 ºC to amplify each transcript. The reaction was performed in duplicate, and the melting-curve was analyzed to determine the product's identity. The target mRNA concentration was normalized to the amplification of the constitutional gene GAPDH, which was the housekeeping gene [18]. The calculation

of the relative expression was performed as described by Pfaffl [41]. All of the primers were designed using Primer Express Software, version 3 (Life Technologies, Carlsbad, CA, USA), and the primers were synthesized by Invitrogen (Waltham, Massachusetts, USA). The primers used in the experiments were as follows: prorenin (F-GGGTGCCGTCCACCAA and R-TCCGTCCCATTCTCCACATAG), (P)RR (F-TGATGGTGAAAGGAGTGGACAA and R-TTTGCCACGCTGTCAAGACT) [18], and GAPDH (F-GATTGTCAGCAATGCCTCCT and R-GGTCATAAGTCCCTCCACGA) [18].

2.5. Preparation of the follicular hemisections

The follicular hemisections were obtained from transparent follicles that ranged in diameter from 2 to 5 mm. These follicles were isolated from the ovaries and halved as described by Richard & Sirard [43], washed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered tissue culture medium (TCM)-199 (Gibco Labs, Waltham, MA, USA), and incubated for 2 h before the COCs were added. Eight follicular hemisections were added to 200 µl of medium. This co-culture system was validated in our laboratory [4, 13, 22, 48, 51].

2.6. Oocyte recovery and in vitro cumulus-oocyte complex cultures

The COCs were aspirated from follicles that were 3–8 mm in diameter, and those that were categorized as grades 1 (>3 layers of compact cumulus cells and homogeneous ooplasm) or 2 (<3 layers of compact cumulus cells and ooplasm filling zona pellucida) were selected using a stereomicroscope in accordance with the method described by Leibfried & First [32]. The COCs (*n* = 20) were suspended in 200 µl of TCM-199 (Gibco Labs, Waltham, MA, USA) supplemented with 100 IU/ml of penicillin, 50 µg/ml of streptomycin sulfate, 0.2 mM of sodium pyruvate, 0.4% fatty acid-free bovine serum albumin, and 0.5 µg/ml of FSH (Folltropin®-V, Bioniche,

Ontario, CA, USA), as described previously [2, 4, 11, 13, 21, 48]. The COCs were cultured in the presence or absence of the follicular hemisections at 39 °C in an atmosphere of 5.0% CO_2 in air and under saturated humidity for 15 h [4, 13, 48]. At the end of the culture period, the oocytes were denuded by vortexing, fixed in 4.0% paraformaldehyde for 15 min, and the oocytes were transferred to 0.5% Triton-X-100. The evaluation of the resumption of meiosis was performed using 10 µg/ml of Hoechst 33342 (Life Technologies, Carlsbad, CA, USA) and fluorescence microscopy. The oocytes were classified according to the nuclear maturation stage as germinal vesicles, germinal vesicle breakdown (GVBD), and metaphase I (MI) after 15 h of culture.

2.7. Cyclic adenosine monophosphate and cyclic guanosine monophosphate measurements

cAMP and cGMP levels were measured in the oocytes and cumulus cells after incubation for 6 h. Pooled samples of 60 COCs were washed briefly in HEPES-buffered TCM-199 containing 1 mM 3-isobutyl-1-methylxanthine [44], and the COCs were denuded by vortexing for 5 min. The oocytes (n = 50) were extensively washed in PBS to obtain cumulus cell-free oocytes, and these were transferred to 100 µl of 0.1 M HCl. Those oocytes (n = 10) that did not denude were discarded. The HEPES-buffered TCM-199 containing the remaining cumulus cells was centrifuged at 12,000 *g* for 5 min and the pellet was resuspended in 100 µl of 0.1 M HCl. The cells were lysed for approximately 20 min on ice and stored at −80 ºC until the samples were assayed using a cAMP enzyme immunoassay (EIA) kit (No 581001; Cayman Chemical, Ann Arbor, MI, USA) or a cGMP EIA kit (Nº 581021; Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions. The experiment was performed in triplicate.

2.8. Experimental design

2.8.1. Characterization of the (pro)renin receptor protein in ovarian cells

The expression profile of the (P)RR protein was evaluated in COCs using follicles that were 3–8 mm in diameter (*n* = 200), theca cells and granulosa cells from follicles that were 4–5 mm in diameter (*n* = 6) and larger than 10 mm (*n* = 3) in diameter, and in the CL (*n* = 3). The COCs were aspirated and those categorized as grades 1 and 2 were selected using stereomicroscopy [32] and washed in PBS at 4 ºC. The follicles that were 4–5 mm in diameter were isolated from pairs of ovaries. The follicles that did not demonstrate obvious signs of atresia were dissected from the stromal tissue. The follicles were sectioned and washed extensively with PBS to obtain the theca cells only. The remaining PBS was filtered through a 40-µm nylon filter and it was centrifuged at 12,000 *g* for 2 min to obtain the granulosa cells from these follicles. Follicles that were larger than 10 mm and healthy, which was indicated by the presence of transparent follicular fluid and the absence of CL, were selected from the pairs of ovaries collected from a local abattoir and partially dissected to obtain the entire follicle with some stromal tissue, to avoid rupture of the follicle. The follicular fluid was removed and the granulosa cells were recovered by flushing with PBS. Then, the PBS containing the granulosa cells was filtered through a 40-µm nylon cell strainer and it was centrifuged at 12,000 *g* for 2 min. These follicles were hemisectioned and the theca cells were dissected from the stromal tissue, and scraping and washing with PBS at 4 ºC eliminated the granulosa cells. The absence of cross-contamination by theca and granulosa cells was confirmed using the RT-PCR to detect cytochrome P450 aromatase and 17a-hydroxylase (CYP17A1) mRNA, respectively [9, 10, 19]. The primers used were as follows: P450 (F-GTGTCCGAAGTTGTGCCTATT and R-GGAACCTGCAGTGGGAAATGA) [34],

and CYP17A1 (F-GAATGCCTTTGCCCTGTTCA and R-CGCGTTTGAACACAACCCTT) [10]. The bovine CLs that were macroscopically classified as mid-cycle, that is, at days 8–12 of the estrous cycle [36], were dissected from the ovaries and washed with PBS at 4 ºC. All of the samples were stored in PBS supplemented with 1.0% (v/v) protease and a phosphatase inhibitor at −80 ºC before undergoing western blot analysis of the (P)RR protein. The theca and granulosa cells were additionally stored in Trizol® (Invitrogen, Carlsbad, CA, USA) at −80 ºC before undergoing the RT-PCR for mRNA detection.

2.8.2. Characterization of prorenin and (pro)renin receptor messenger ribonucleic acid in the cumulus cells and oocytes

The COC were aspirated from follicles that were 1–3, 4–5, 6–8, and >8 mm in diameter and those categorized as grades 1 and 2 were selected [32]. The oocytes (n = 200) were denuded in HEPES-buffered TCM-199 medium by vortexing, washed in PBS, and stored in Trizol® (Invitrogen, Carlsbad, CA, USA) at −80 ºC. The TCM 199 medium containing the remaining cumulus cells was centrifuged and the pellet was stored in Trizol® (Invitrogen, Carlsbad, CA, USA) at −80 ºC. Aromatase mRNA was measured in the oocytes using RT-PCR to detect contamination by the cumulus cells. All samples found to be positive for P450 were discarded. Contamination of the cumulus cells with oocytes was ruled out by the presence of denuded oocytes with intact zonae pellucidae. The experiment was performed in quadruplicate.

2.8.3. Examination of the roles of prorenin and the (pro)renin receptor in the resumption of meiosis in oocytes

To determine the effect of prorenin on nuclear maturation, four control groups were prepared as follows: 1) COCs without follicular hemisections (positive control); 2) COCs with follicular hemisections (negative control); 3) COCs with follicular

hemisections treated with 10^{-11} M AngII (positive RAS control); and 4) COCs with follicular hemisections treated with 10^{-11} M AngII plus 10^{-5} M saralasin (Sar) (negative RAS control). To test the effect of prorenin on the nuclear maturation of oocytes, three doses of prorenin (10^{-10}, 10^{-9}, and 10^{-8} M) were added to the co-culture systems. The experiment was performed in triplicate using 20 COCs per group.

To analyze the functional requirements of the (P)RR in the resumption of meiosis, three control groups were prepared as follows: 1) COCs without follicular hemisections (positive control); 2) COCs with follicular hemisections (negative control); and 3) COCs with follicular hemisections and prorenin (10^{-10} M). The test groups comprised co-cultures of the COCs and follicular hemisections supplemented with 10^{-10} M of prorenin and three doses of aliskiren (10^{-7}, 10^{-5}, and 10^{-3} M), which is a direct renin inhibitor and potent inhibitor of the receptor-bound renin or prorenin [7]. The COCs were cultured without follicular hemisections and with two concentrations of aliskiren (10^{-5} and 10^{-7} M) to assess its cytotoxicity. Both experiments were performed in triplicate using 20 COCs per group.

To determine whether the effect of prorenin on the resumption of meiosis in oocytes is independent of AngII, the COC and follicular hemisection co-cultures were treated with AngII (10^{-11} M), AngII (10^{-11} M) with Sar (10^{-5} M), prorenin (10^{-10} M), prorenin (10^{-10} M) with aliskiren (10^{-7} M), AngII (10^{-11} M) with prorenin (10^{-10} M), and prorenin (10^{-10} M) with Sar (10^{-5} M). For the positive control group, the COCs were cultured without follicular hemisections. The experiment was performed in triplicate using 20 COCs per group.

2.8.4. Investigation of the induction of oocyte meiotic resumption by prorenin in the absence of follicular hemisections

The COCs were cultured in the absence (positive control) or presence (negative control) of 200 µM FSK and the cultures were supplemented with prorenin (10^{-10} M). Aliskiren (10^{-7} M) was added to the cultures that contained FSK and prorenin to determine whether prorenin affected the resumption of oocyte meiosis in the absence of follicular hemisections. The experiment was performed in triplicate using 20 COCs per group.

2.8.5. Examination of the induction of the resumption of meiosis by prorenin via the cyclic adenosine monophosphate and cyclic guanosine monophosphate pathways

In this experiment, the COCs were cultured in the presence or absence of FSK (200 µM) and prorenin (10^{-10} M) to verify the cAMP and cGMP levels in the oocytes and cumulus cells. We removed 180 COCs per group from culture after 6 h to measure the cAMP and cGMP levels in the oocytes (n = 150/group) and the cumulus cells (from 180 COCs/group). Other COCs (n = 60 COCs/group) were cultured for 15 h to assess nuclear maturation. This experiment was performed in triplicate.

2.9. Statistical analysis

The data obtained from the analysis of the mRNA expression levels and the evaluation of the cAMP and cGMP levels were tested for normality using the Shapiro-Wilk test, and normalized when necessary. The differences between the groups were analyzed using a multiple comparison least-squares means (LSMEANS) Student's *t*-test. The resumption of meiosis data were analyzed using the Categorical Data Analysis Procedure (PROC CATMOD analysis). The analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA), and the significance level adopted was 5%. The gene expression data are presented as the means ±

standard errors (SE) of the means, and the nuclear maturation data are presented as percentages.

3. **Results**

3.1. Characterization of the (pro)renin receptor protein in the bovine ovary

The presence of the (P)RR protein was examined in ovarian cells using western blotting. A band of approximately 42 kDa that corresponded to the (P)RR protein (predicted molecular weight: 39 kDa) was detected in the COCs, theca cells, and granulosa cells from both follicle size categories, namely, 4–5 mm and larger than 10 mm in diameter, and in the bovine CL (Fig. 1).

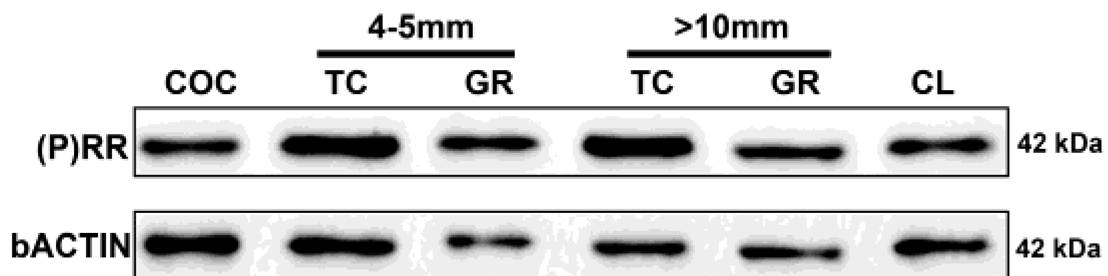


Figure 1 (Pro)renin receptor [(P)RR] protein expression in cumulus-oocyte complexes from follicles that were 3–8 mm in diameter, theca and granulosa cells from follicles that were 4–5 mm in diameter, theca and granulosa cells from follicles that were larger than 10 mm in diameter, and in the corpus luteum from bovine ovaries. Western blot images revealed a specific band at approximately 42 kDa for the (P)RR and at 42 kDa for β-actin. The Western blot was repeated at least three times.

3.2. Characterization of prorenin and (pro)renin receptor mRNA in the cumulus cells and oocytes

Quantitative RT-PCR characterized the abundance of prorenin and (P)RR transcripts in the cumulus cells and oocytes according to the development of the follicles. (P)RR mRNA was detected in the cumulus cells and oocytes from follicles that were 1–3, 4–5, 6–8, and > 8 mm in diameter. Prorenin mRNA was detected in the cumulus cells only. However, the prorenin (Fig. 2A) and (P)RR mRNA levels (Fig. 2B) in cumulus cells were not affected by follicular diameter ($P>0.05$). Similarly, the (P)RR transcript levels in the oocytes did not change significantly among the follicles of different sizes (Fig. 2C).

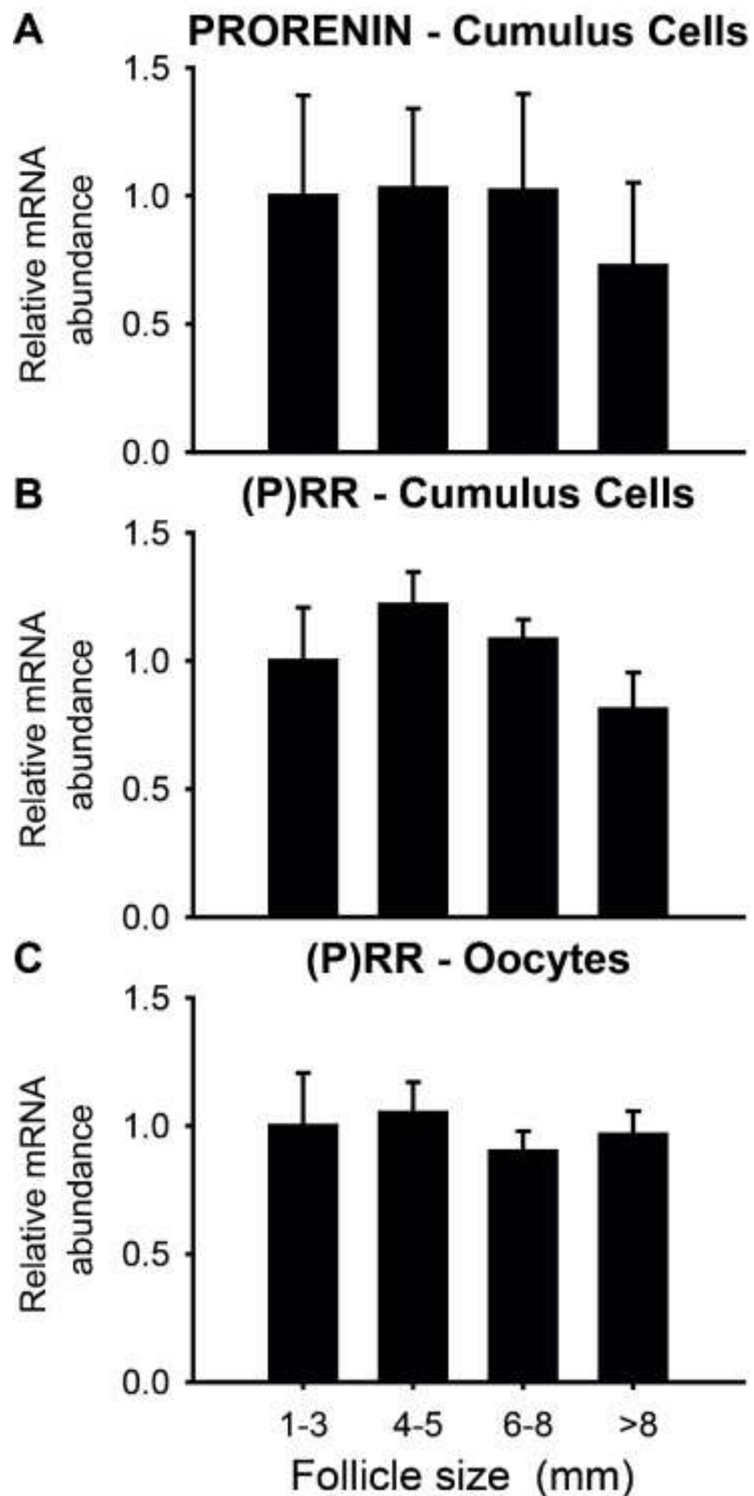


Figure 2 Characterization of the (pro)renin receptor (P)RR and prorenin messenger ribonucleic acid (mRNA) expression in cumulus cells and oocytes during follicle development. Prorenin mRNA expression in cumulus cells (A), (P)RR mRNA expression in cumulus cells (B), and oocytes (C). The experiment was performed in quadruplicate and 800 cumulus-oocyte complexes were examined in each group.

The statistical difference among the follicular sizes was evaluated at a level of significance of $P<0.05$.

3.3. Prorenin induces the resumption of oocyte meiosis

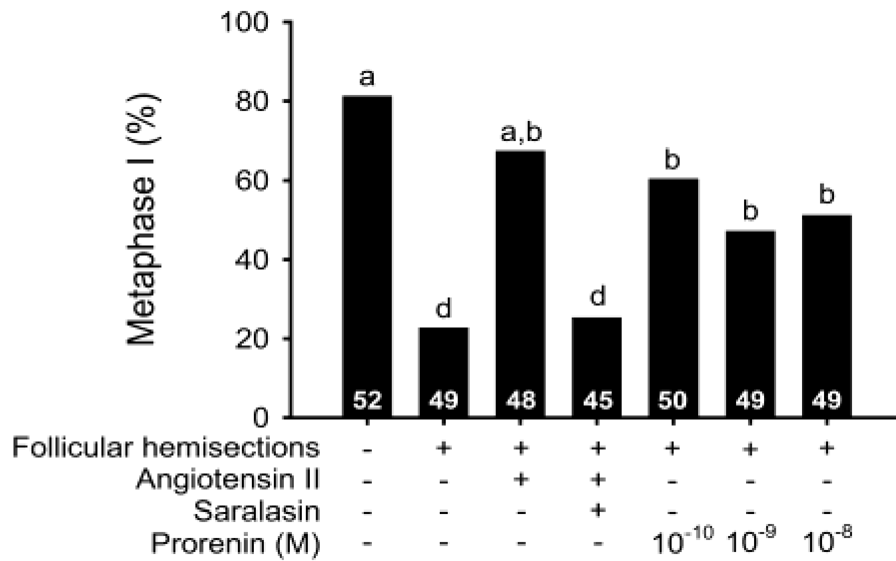
This experiment was designed to examine the effect of prorenin on the resumption of meiosis by examining the effects of AngII and Sar, which is a competitive AngII antagonist. AngII stimulated the resumption of meiosis, which was blocked by Sar. After a 15-h culture, prorenin at concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M significantly increased ($P<0.05$) the percentages of oocytes that reached the metaphase I (MI) stage to 60.0%, 46.8%, and 50.8%, respectively, compared with the negative control (22.3%) and the negative RAS control (24.9%; Fig. 3A). Prorenin treatment at concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M also induced the resumption of meiosis in oocytes that were co-cultured with follicular hemisections ($P>0.05$) as was observed in the positive RAS control group that involved AngII treatment and in which 67.0% of the oocytes reached MI (Fig. 3A).

The hypothesis that the (P)RR is required to induce the resumption of oocyte meiosis was tested using aliskiren, which is a direct renin inhibitor, at three concentrations (10^{-7}, 10^{-5}, and 10^{-3} M) and prorenin at 10^{-10} M. The proportion of oocytes that reached MI in the absence of the follicular hemisections (positive control group) was 74.0% and 78.6% of the oocytes reached MI in the presence of follicular hemisections and prorenin ($P>0.05$). Most of the oocytes did not acquire GVBD competence in the presence of follicular cells, without prorenin (negative control), in which 29.9% of the oocytes achieved MI ($P>0.05$), or in the presence of prorenin plus aliskiren at concentrations of 10^{-7}, 10^{-5}, and 10^{-3} M, where the percentages of oocytes that reached MI were 30.8%, 43.7%, and 40.3%, respectively (Fig. 3B).

A toxicity test was performed to investigate the harmful effects of aliskiren. COCs were incubated without follicular hemisections in the presence of 10^{-5}

M and 10^{-7} M aliskiren for 15 h. The percentages of oocytes treated with aliskiren at 10^{-5} M (*n* = 57 oocytes) and 10^{-7} M (*n* = 47 oocytes) that achieved MI were 69.9% and 69.2%, respectively, which reflected the percentage of oocytes that achieved MI (74.0%) in the control group (*n* = 57 oocytes) (data not shown; $P>0.05$). Therefore, there was no toxicity associated with aliskiren at the concentrations of 10^{-5} M and 10^{-7} M after the 15-h culture.

To evaluate whether prorenin induces the resumption of oocyte meiosis independently of the AngII pathway, the effects of prorenin and Sar were assessed on co-cultures of COCs with follicular cells. Prorenin at a concentration of 10^{-10} M induced 57.0% of the oocytes that had been cultured with follicular cells to reach MI, and this ability was retained after the oocytes had been treated with prorenin plus Sar at 10^{-5} M, with 48.7% of the oocytes reaching MI ($P>0.05$; Fig. 3C). To determine whether prorenin had an additive effect on the oocyte meiotic progression that was induced by AngII, prorenin plus AngII were added to the co-culture system. The percentage of the oocytes that reached MI after 15 h of incubation with prorenin and AngII (57.4%) did not differ significantly compared with those in the prorenin group (57.0%) and the AngII group (51.8%) (Fig. 3C).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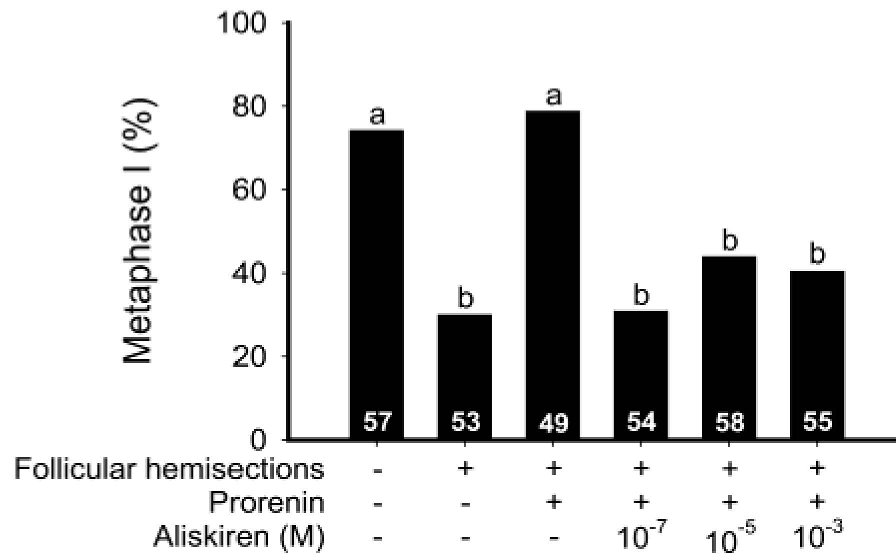
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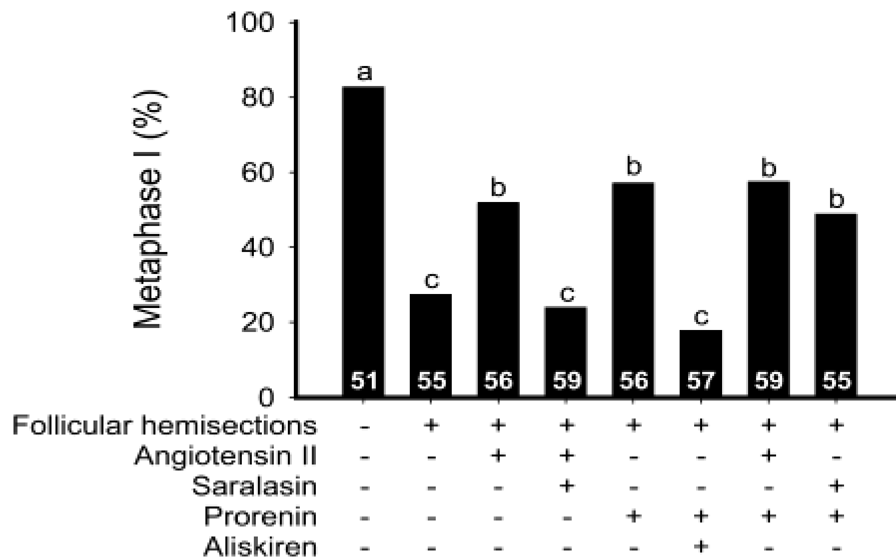
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Saralasin - - - + - - - +
Prorenin - - - - + + + +
Aliskiren - - - - - + - -

Figura 3 The dose-response effects of prorenin (A), aliskiren plus 10^{-10} M prorenin (B), and prorenin (10^{-10} M) plus angiotensin II (10^{-10} M) or prorenin (10^{-10} M) plus saralasin (10^{-10} M; C) on the resumption of meiosis after 15 h of bovine cumulus-oocyte complex and follicular hemisection co-culture. The experiment was performed in triplicate, and the number of oocytes examined for each treatment is indicated at the base of each bar. The different letters indicate the statistical differences between the groups ($P<0.05$).

3.4. Prorenin induces oocyte meiotic resumption without follicular hemisections

To determine whether prorenin is dependent on follicular cells for the induction of GVBD and oocyte progression to MI, FSK (200 μM) was used to block the resumption of meiosis instead of follicular hemisections. A higher percentage of oocytes incubated with prorenin reached MI (38.3%) than those incubated with FSK and without prorenin (18.9%) or with prorenin plus aliskiren (8.6%) for 15 h ($P<0.05$) (Fig. 4).

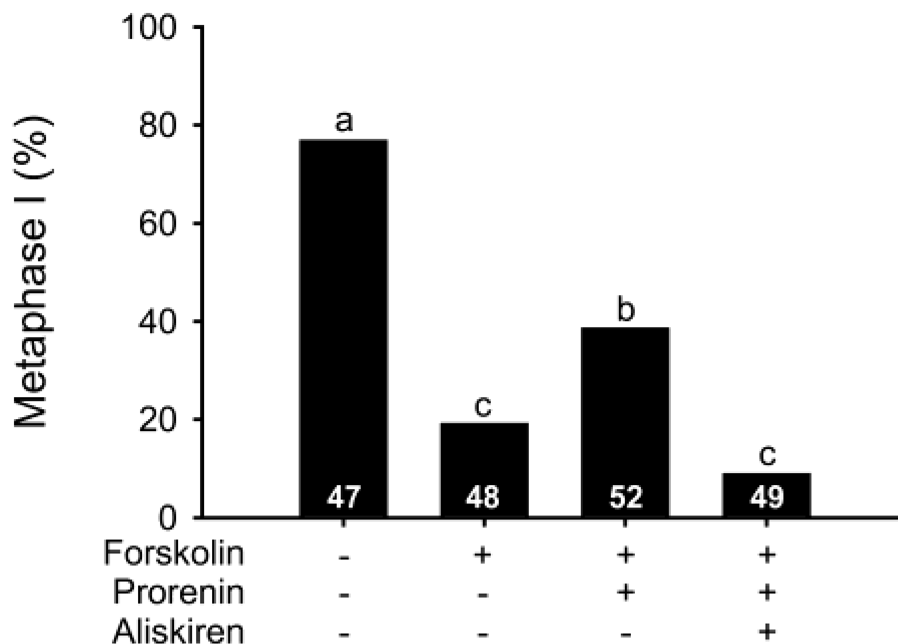

Figura 4 The effect of prorenin (10^{-10} M) on the resumption of forskolin (200 μM)-inhibited meiosis after 15 h of cumulus-oocyte complex culture. The experiment was performed in triplicate, and the number of oocytes examined for each treatment is indicated at

the base of each bar. The different letters indicate the statistical differences between the groups ($P<0.05$).

3.5. Prorenin seems to induce meiosis resumption through cyclic adenosine monophosphate pathways within the oocyte

This experiment was designed to assess the relationships between meiotic progression and the levels of cAMP and cGMP in the cumulus cells and in the oocytes after 6 h of incubation with and without FSK. A total of 60 COCs were cultured for 15 h to evaluate the nuclear maturation stages. The percentage of meiotic division was higher in the presence of prorenin (FSK plus prorenin) (49.9%) than in the absence of prorenin (FSK without prorenin) (25.5%) ($P<0.05$). The highest percentages of oocytes that reached MI were present in the control groups, specifically, oocytes incubated with prorenin and without FSK (78.3%) and oocytes incubated without FSK or prorenin (83.1%) (Fig. 5A). Oocytes treated with prorenin and FSK contained cAMP at levels that did not differ from the positive control or the prorenin groups ($P>0.05$). Compared with the control groups, the highest cAMP levels were observed in the oocytes cultured with FSK and without prorenin ($P<0.05$) (Fig. 5B). Cumulus cells incubated for 6 h with FSK, with FSK plus prorenin, without FSK or prorenin, or with prorenin alone had mean ± SE cAMP levels of 25.13 ± 11.32, 16.52 ± 6.14, 3.36 ± 2.38, and 8.42 ± 7.76 fmol/cumulus complex, respectively, which were not significantly different (data not shown). Cumulus cells incubated for 6 h with FSK, with FSK plus prorenin, without FSK or prorenin, or with prorenin alone had mean ± SE cGMP levels of 0.70 ± 0.32, 0.53 ± 0.12, 1.30 ± 0.67, and 6.02 ± 4.93 fmol/cumulus complex, respectively, which were not significantly different (data not shown). Oocytes incubated for 6 h with FSK, with FSK plus prorenin, without FSK or prorenin, or with prorenin alone had mean ± SE cGMP levels of 1.15 ± 0.18, 1.42 ± 0.19, 1.56 ± 0.24, and 1.21 ± 0.01 fmol/oocyte,

respectively, differences that were not significant (data not shown).

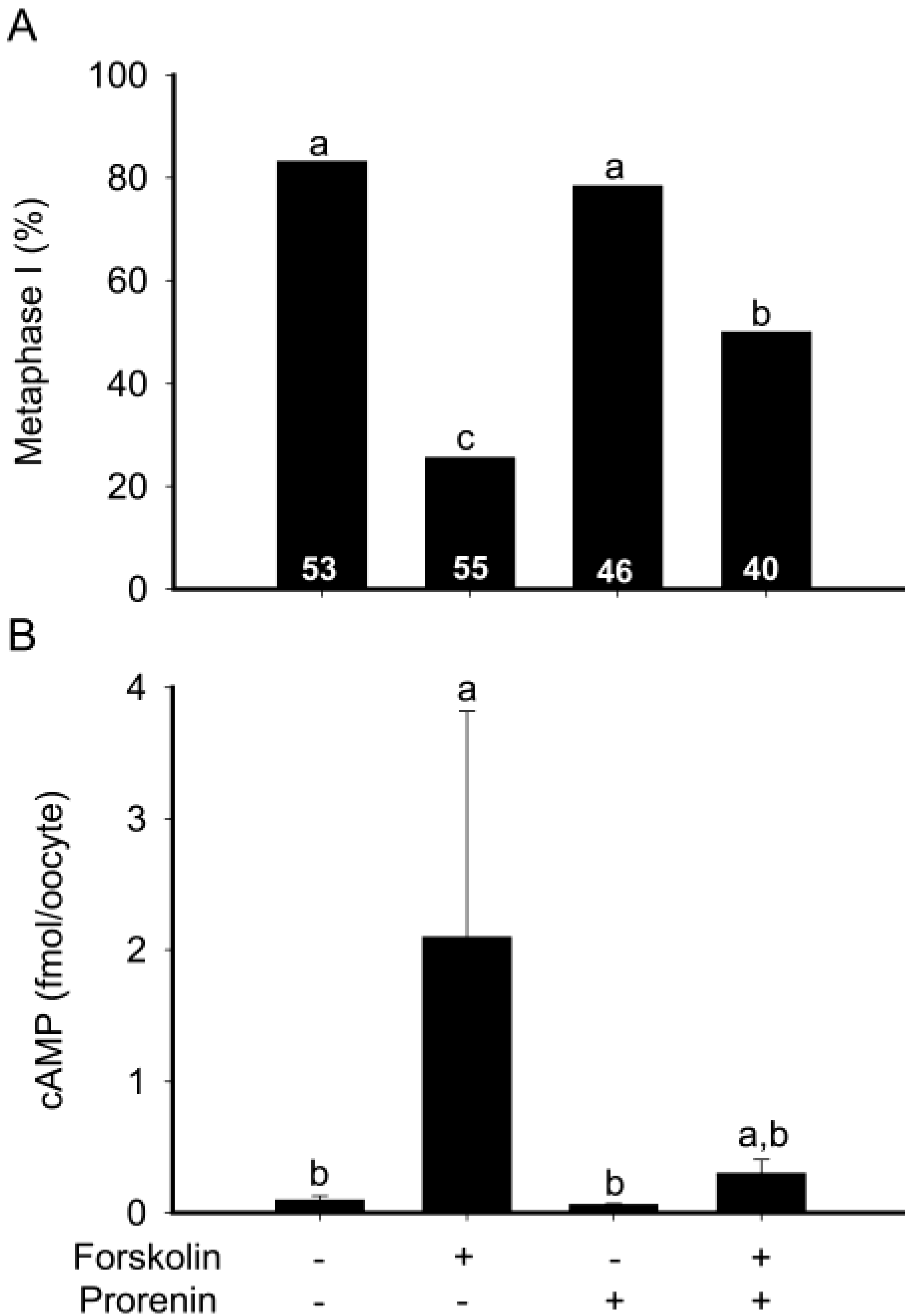

Figura 5 The effects of prorenin (10^{-10} M) on the resumption of meiosis after 15 h of culture (A) and the cyclic adenosine monophosphate levels within the oocytes after culturing the cumulus-oocyte complexes for 6 h (B). The different letters indicate the statistical differences between the groups ($P<0.05$). The experiment was performed in triplicate, and the number of oocytes examined in each treatment is indicated at the base of each bar.

4. **Discussion**

The most significant findings from this study are: (1) the (P)RR protein is present in all ovarian follicular cells; (2) (P)RR mRNA is transcribed in bovine oocytes and cumulus cells; (3) prorenin induces meiotic resumption in oocytes in a manner that is independent of the AngII pathway; and (4) prorenin induces the resumption of meiosis in oocytes that has been blocked by FSK.

The presence of the (P)RR protein in bovine CCOs, theca, granulosa, and luteal cells has been demonstrated by our group, which concurs with the previous identification of (P)RR mRNA in these follicular layers [18]. (P)RR mRNA was also observed in the COCs and prorenin mRNA in the bovine cumulus cells, but not in the oocytes. These transcripts were consistently maintained within follicles of different sizes, which supports the results obtained from superovulated heifers that showed that prorenin and active renin concentrations remain constant in the follicular fluid until the LH peak [24].

The prorenin concentrations used in the present study had been established by other researchers [54]. The concentrations tested were enough to increase the percentage of oocytes that reached MI compared with both the negative and negative RAS controls, but this effect was not dose-dependent. However, the ERK 1 and 2 were phosphorylated in a dose-dependent manner from 1 nM prorenin in endothelial cells cultured in vitro [54]. ERK 1/2 activation occurs from the first hours of maturation, and increases in accordance with the progression of meiosis in bovine oocytes [21]. Moreover, studies have evidenced that the activities of ERK 1/2 play pivotal roles in regulating the meiotic progression of oocytes [16, 17, 39]. Whether or

not prorenin induces the resumption of meiosis by activating the ERK 1/2 cascade remains to be determined.

Aliskiren inhibits not only the free and bound forms of renin, but also receptor-bound prorenin [7]. Thus, aliskiren blocks the intracellular and extracellular pathways activated by prorenin, impairing both ERK1/2 phosphorylation and AngI generated from angiotensinogen [20, 35]. In the present study, aliskiren at 10^{-7}, 10^{-5}, and 10^{-3} M inhibited prorenin's effect on the resumption of meiosis. These concentrations were used because 10^{-5} M aliskiren was sufficient to reduce prorenin-induced AngI production in human smooth muscle cells [20].

The binding of prorenin to the (P)RR leads to the conversion of angiotensinogen to AngI [20], and it activates intracellular ERK1/2 independently of AngII production [54]. However, prorenin did not act as a cofactor to AngII in the resumption of oocyte meiosis. In this study, Sar did not block the effect of prorenin on the resumption of oocyte meiosis that had been impaired by the follicular hemisections, which suggests that the role of prorenin in the resumption of bovine oocyte meiosis is independent of the AngII pathway. Another fact that must be mentioned with respect to oocyte meiotic resumption is the increase in intrafollicular prorenin levels that is induced by the preovulatory LH surge in vivo [23, 24]. This fact concurs with our results, which showed that prorenin induces the resumption of meiosis in bovine oocytes co-cultured with follicular hemisections in vitro.

The positive effect of AngII on oocyte meiotic resumption depends on the follicular cells [22]. To determine whether prorenin depends on the follicular cells to induce GVBD and progression to MI, FSK was used to block meiotic resumption instead of follicular hemisections. Prorenin induced the resumption of oocyte meiosis in a manner that was independent of the follicular hemisections. Interestingly, the

oocytes underwent GVBD and progressed to MI when they were cultured in the presence of prorenin and even in the presence of high levels of FSK, which increases intracellular cAMP levels. The findings from several studies have established that high levels of cAMP in oocytes are essential to maintain meiotic arrest in mammals [25, 26, 31, 45, 47]. Moreover, direct stimulation of adenylyl cyclase with 100 μM FSK delays the nuclear maturation of bovine oocytes [6]. In this study, prorenin induced the resumption of meiosis in the presence of 200 μM of FSK in the absence of follicular hemisections, cultured for 15 h.

Prorenin appeared to interact with the FSK-induced intra-oocyte cAMP, which was demonstrated by the following findings: (1) oocytes treated with prorenin and FSK resumed meiosis; (2) the cAMP levels in these oocytes tended to be lower than those in oocytes cultured with FSK alone; and (3) the cAMP levels did not differ from those in oocytes cultured without FSK. The levels of cAMP in the cumulus cells did not differ significantly in relation to their treatment with prorenin and/or FSK. Thus, the cellular mechanism that regulates the synthesis and degradation of cAMP seems to differ between oocytes and somatic cells. In addition, more cAMP accumulates in COC in response to FSK treatment compared with the amounts detected in zona-free bovine oocytes after 6 h of culture [6].

The concentrations of cGMP in the bovine COCs in the control group after 6 h of incubation were significantly lower compared with those observed after 3 h of incubation and immediately after collection [5]. In the same way, the cGMP levels decreased in rat oocytes during spontaneous nuclear maturation [53]. Thus, our hypothesis was that as well as inducing a resumption of meiosis, prorenin also lowers cGMP levels in bovine somatic cells and oocytes. However, prorenin did not have a significant effect on the cGMP levels in bovine cumulus cells and oocytes after 6 h of

incubation. Similarly, cGMP concentrations did not differ in response to the manipulation of the nitric oxide-cGMP pathway in bovine COCs after 6 h of incubation [5]. However, since the cGMP levels increased after 3 h of incubation with particular treatments [5], the effect of prorenin on the cGMP levels in bovine cumulus cells and oocytes cannot be ruled out.

The measurements of the cAMP and cGMP levels were performed after 6 h of incubation, because this is when GVBD is expected in vitro and in vivo, following the LH surge [30, 50]. The cAMP levels within oocytes have to be lower to resume meiosis [45]. Similarly, the percentages of oocytes that achieved MI in the prorenin and the positive control groups were 78.3% and 83.1%, respectively, after 15 h of incubation, and both groups maintained low levels of cAMP within the oocytes compared with the negative control group after 6 h of culture. The proportion of oocytes that achieved MI after FSK and prorenin treatment also seemed to correspond to the intra-oocyte cAMP concentrations that were observed in response to the same treatment. Hence, these results together suggest that prorenin induces the resumption of meiosis in bovine oocytes by reducing cAMP levels within the oocytes.

Our findings confirm the postulated positive effect of the prorenin receptor in the resumption of meiosis. The present study provides insights into the function of (P)RR in an alternative RAS pathway during important reproductive events such as meiosis resumption. We also suggest that (P)RR dysregulation involved in the development of diabetes or hypertension might impair oocyte maturation, although this remains to be investigated in future studies. Therefore, the signaling pathways activated by (P)RR-bound prorenin are a new pharmacotherapeutic target to be studied in the treatment of infertility and application of reproductive biotechnologies.

In summary, prorenin induces the resumption of oocyte meiosis through the (P)RR via an AngII-independent pathway, even in the presence of follicular cells. Moreover, prorenin causes the resumption of FSK-inhibited oocyte meiosis in a manner that is independent of the follicular cells, suggesting a potential role for prorenin and/or the (P)RR in regulating intra-oocyte cAMP. In conclusion, these novel findings have identified prorenin and/or the (P)RR, which is widespread in the bovine ovary, as a potential inducer of the resumption of meiosis in oocytes.

Conflict of interest declaration

The authors declare that there are no conflicts of interest that could prejudice the impartiality of the research reported.

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Authors' contributions

Dr. Dau contributed substantially to all steps associated with the collection of the data and the preparation of the manuscript. Dr. Silva and Dr. Rosa made considerable contributions towards acquiring the data relating to the resumption of meiosis. Dr. Bastiani and Dr. Gutierrez made important contributions to the acquisition of the RT-PCR data. Dr. Ilha contributed significantly to attaining the western blot data. Dr. Comim and Dr. Gonçalves contributed substantially to the design of the research, the analysis of the data, the interpretation of the results, and the preparation of the manuscript.

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4. **ARTIGO 2**

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO:

(Pro)renin receptor is upregulated in granulosa cells following gonadotropin-releasing hormone administration in cattle

Andressa Minussi Pereira Dau, Paulo Roberto Antunes da Rosa, Joabel Tonellotto dos Santos, Juliana Germano Ferst, João Colombo, Fabio Vasconcellos Comim and Paulo Bayard Dias Gonçalves.

REPRODUCTION IN DOMESTIC ANIMALS, 2017

(Pro)renin receptor is upregulated in granulosa cells following gonadotropin-releasing hormone administration in cattle

Andressa Minussi Pereira Dau[A], Paulo Roberto Antunes da Rosa[A], Joabel Tonellotto dos Santos[A], Juliana Germano Ferst[A], João Colombo[A], Fabio Vasconcellos Comim[A, B], and Paulo Bayard Dias Gonçalves[A]

[A] Laboratory of Biotechnology and Animal Reproduction, BioRep, Federal University of Santa Maria, Roraima Avenue 1000, 97105-900 Veterinary Hospital, Santa Maria, RS, Brazil

[B] Department of Medical Clinic, Faculty of Medicine, Federal University of Santa Maria, Roraima Avenue 1000, 97105-900, Santa Maria, RS, Brazil

[D]**Corresponding author:** Paulo Bayard D. Gonçalves, Departamento de Clínica de Grandes Animais, Hospital Veterinário, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil. Email address: bayard@ufsm.br

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Running Head: (Pro)renin receptor in preovulatory granulosa cells

22 **Abstract**

23 The objective of this study was to evaluate whether prorenin induces genes related to
24 ovulation in granulosa cells through (P)RR or whether prorenin and (P)RR mRNA are
25 dependent of epidermal growth factor (EGF) signaling. In addition, we studied the profile of
26 mRNA encoding prorenin, (P)RR, and profibrotic proteins in granulosa cells during the
27 preovulatory period in cattle. Transcript for (P)RR and ADAM17 were detected in granulosa
28 cells, but no induction by luteinizing hormone (LH) with or without prorenin (P>0.05). LH
29 increased EREG mRNA in granulosa cells (P<0.05). Supplementation of culture medium with
30 three concentrations of prorenin plus LH induced AREG mRNA (P<0.05) and only 0.1 nM of
31 prorenin plus LH increased PTGS2 mRNA in granulosa cells in compared with control
32 (P<0.05). Prorenin/(P)RR did not stimulate LH-induced EREG and AREG transcript in
33 granulosa cells cultured for 6 h (P>0.05). PTGS2 mRNA was not induced by LH or prorenin
34 via (P)RR (P>0.05). EGFR pathway was not required for prorenin and (P)RR mRNA *in vitro*
35 (P>0.05). Intrafollicular injection with the EGF receptor inhibitor AG1478 did not abrogate
36 prorenin and (P)RR mRNA at 6 h post-GnRH (P>0.05). Granulosa cells were obtained from
37 bovine preovulatory follicles at 0, 3, 6, 12, and 24 h after gonadotropin-releasing hormone
38 (GnRH) administration. Prorenin mRNA and (P)RR protein increased in granulosa cells 12 h
39 post-GnRH (P<0.05). TGFβ1, PAI-I, collagen type I, and fibronectin increased 12 and 24 h
40 post-GnRH (P<0.05). Finally, prorenin/(P)RR did not induce ovulation-related genes and its
41 molecular expression was not dependent of EGFR signaling *in vitro* and *in vivo*. Importantly,
42 we found that (P)RR protein and transcripts for prorenin or profibrotic genes were increased
43 from 12 h post-GnRH, suggesting that prorenin/(P)RR play a role at later stages in ovulation
44 inducing profibrotic genes in bovine granulosa cells.

45

46 **Introduction**

47 (Pro)renin receptor ([P]RR), encoded in ATP6AP2, promotes renin-angiotensin
48 system (RAS) signaling through an alternative pathway independent of angiotensin II (AngII;
49 Nguyen et al. 2002). It is well known that binding of prorenin or renin to (P)RR stimulates
50 mitogen-activated protein (MAP) kinase ERK1 (p44) and ERK2 (p42) phosphorylation and it
51 upregulates transcription of transforming growth factor (TGF)-β, plasminogen activator
52 inhibitor (PAI)-1, type I collagen and fibronectin (profibrotic molecules) (Nguyen et al. 2002,

53 Huang et al. 2006, Ferri et al. 2011). In the Ang II dependent pathway, (P)RR activation also
54 stimulates AngII pathway, promoting the cleavage of angiotensinogen to angiotensin I
55 (Nguyen et al. 2002, Uraoka et al. 2009).
56 Prorenin exhibits higher affinity for (P)RR than for renin (Nabi et al. 2009). In ovarian
57 follicular fluid, prorenin levels increases two times more than renin levels in follicular fluid
58 after LH surge in cattle (Hagemann et al. 1994). (P)RR protein was identified in bovine ovary,
59 including granulosa cells from follicles larger than 10 mm in diameter (Dau et al. 2016). A
60 role of prorenin/(P)RR in the follicular divergence *in vivo* (Ferreira et al. 2011) and
61 resumption of meiosis in oocytes *in vitro* were described in cattle (Dau et al. 2016). Our
62 recent studies indicates that (P)RR also is involved in ovulation and progesterone synthesis
63 during luteinization.
64 The (P)RR signaling pathway has been demonstrated to be involved in epidermal growth
65 factor-like ([EGF]-like) cascade, although exist controversial data whether prorenin induces
66 epidermal growth factor receptor (EGFR) transactivation or EGFR is required for (P)RR
67 activation. Binding of prorenin to (P)RR induced EGFR phosphorylation in rat vascular
68 smooth muscle cells (Liu et al. 2011) and human embryo kidney 293 cells (Shibayama et al.
69 2013), but it was not observed in monocytes (Feldt et al. 2008). Prorenin/(P)RR also acts in
70 the dependent pathway of Ang II, which is considered essential for ovulation and an important
71 cofactor of LH for induction of the ovulatory cascade (Siqueira et al. 2012, Ferreira et al.
72 2007, Portela et al. 2011). In bovine granulosa cells *in vitro,* Ang II increased the transcripts
73 for LH-induced metalloprotease 17 (ADAM17), epiregulin (EREG), amphiregulin (AREG),
74 and prostaglandin-endoperoxide synthase (PTGS2) (Portela et al. 2011). However, whether
75 prorenin induces EGF-like cascade in granulosa cells through (P)RR in cattle need to be
76 investigated.

77 The aims of this study were to evaluate whether prorenin stimulates (EGF)-like cascade in
78 granulosa cells via (P)RR, to evaluate whether transcripts for prorenin and (P)RR require
79 EGFR transactivation, to characterize (P)RR mRNA and protein, as well as transcripts for
80 prorenin and profibrotic molecules in granulosa cells during preovulatory period in cattle.
81
82 **Materials and methods**
83 All experimental procedures with cattle were reviewed and approved by the local
84 Animal Ethics Committee of Federal University of Santa Maria (nº.115/2014) in agreement
85 with the National Council for the Control of Animal Experimentation (CONCEA; Brazilian
86 Ministry of Science, Technology, and Innovation).
87
88 *Chemicals*
89 All chemicals used in this study were obtained from Sigma-Aldrich Corporation (St
90 Louis, MO, USA), unless otherwise indicated.
91
92 *Granulosa cells recovery for in vitro experiments*
93 Approximately twenty pairs of ovaries from non-pregnant cows at different stages of
94 the estrous cycle were obtained from a local abattoir and transported to the laboratory in
95 saline solution containing penicillin (100 IU mL^{-1}) and streptomycin sulfate (50 µg mL^{-1}) at
96 30ºC. Follicles larger than 12 mm in diameter and considered healthy were selected for the
97 experiments. The follicles were considered healthy based on the presence of light yellow
98 follicular fluid, absence of corpus luteum, and by a high oestradiol (E2): progesterone (P4)
99 ratio (>2) in the fluid follicular (Ireland et al. 1980, McNatty et al. 1984). The E2 and P4
100 concentrations were measured by electrochemiluminescence in the follicular fluid pooled
101 from follicles (at the least 4) that were used for each experimental replicate with cultured

102 theca cells. Granulosa cells isolation was adapted of studies previously described (da Rosa et
103 al. 2016, Portela et al. 2011). Briefly, granulosa cells were obtained by flushing performed
104 gently with DMEM-F12 suplemented with 0.1% of bovine serum albumin (BSA), 2.5 µg mL^{-1} of amphotericin, 100 IU mL^{-1} of penicillin, 100 µg mL^{-1} of streptomycin sulfate, and 0.5 UI
106 mL^{-1} of heparin sodium salt (basic medium). The absence of contamination with theca cells
107 was verified by the lack of cytochrome P450 17A1 (CYP17A1) mRNA in theca cells, as
108 determined by qRT-PCR (Buratini *et al.* 2007). The experiments *in vitro* were performed in
109 six replicates repeated on different days.
110

111 *Granulosa cells cultures*

112 The *in vitro* model was adapted from previous studies (Portela et al. 2011, da Rosa et
113 al. 2016). Isolated granulosa cells were washed twice in basic medium by centrifugation at
114 200 x *g* for 10 min, filtered through 70-µm Nylon Mesh strainers (Fisher Scientific, Shanghai,
115 China), and seeded in 96-well tissue culture plates (Corning) at a concentration of 5.10^4 viable
116 cells per well. The number of viable cells were determined using 0.4% trypan blue (v/v). The
117 percentage of viable cells was over 90% for all replicates and it was estimated by flow
118 cytometry analysis (FACSVerse ™, BD Biosciences, Franklin Lakes, NJ, USA) using FITC-
119 Annexin V (5 µL; BD Biosciences) and propidium iodide (50 µg/mL) according to the
120 manufacturer's instructions. The cells were cultured in DMEM/F12 (Gibco Labs, Waltham,
121 MA, USA) supplemented with 1ng mL^{-1} of follicle-stimullating hormone (FSH), 10^{-7}M of
122 androstenedione, 10ng mL^{-1} of insulin, 2.5 µg mL^{-1} of transferrin, 4 ng mL^{-1} of selenium, 100
123 IU mL^{-1} of penicillin, 100 µg mL^{-1} of streptomycin sulfate, 2.5 µg mL^{-1} of amphotericin, and
124 0.1% of BSA at 38ºC in 5.0% CO_2 and saturated humidity.
125

126 *Animal procedures, intrafollicular injection, and collection of the granulosa cells*

127 Twenty cows (*Bos taurus taurus*) for the first *in vivo* experiment and forty cows for
128 the second *in vivo* experiment were submitted for the same hormonal protocol. Cows
129 exhibiting normal estrous cycles had luteolysis induced and a new follicular wave initiated
130 using a hormonal protocol, as previously described (Siqueira et al. 2012, Tonellotto dos
131 Santos et al. 2012). Briefly, 2 injections with a 12-h dosing interval of a prostaglandin F2α
132 (PGF2α) analog (cloprostenol, 250 mg, IM; Schering-Plough Animal Health, Brazil), 2 mg of
133 estradiol benzoate (EB), and an intravaginal progesterone device (1 g progesterone, DIB®;
134 Intervet, Brazil) for 9 days. The presence of preovulatory follicles (≥12 mm) was evaluated by
135 transrectal ultrasonography using an 8-MHz, linear-array transducer (AquilaVet scanner, Pie
136 Medical, Netherlands) 12 h after removal of the vaginal device. Only cows with follicles ≥12
137 mm in diameter were challenged with 100 µg of gonadorelin acetate (Profertil®, Tortuga,
138 Brazil), and thus, were treated by intrafollicular injection and ovariectomized in the first *in*
139 *vivo* experiment and only ovariectomized in specific time points during the preovulatory
140 period in the second *in vivo* experiment.

141 Intrafollicular injections were preformed immediately after GnRH administration and
142 were guided by an ultrasound (Aquila Vet, Pie Medical Equipment BV, Holanda) equipped
143 with 7.5 MHz convex probe. A double-needle system was used to treat the preovulatory
144 follicle (intrafollicular region) with AG1478. Final concentrations of AG1478 of 5 µM (Park
145 et al. 2004) were administered after estimating the follicular fluid volume, as previously
146 described (Ferreira et al. 2007). The cows were examined by transrectal ultrasonography 2 h
147 after intrafollicular treatment, and those with reductions larger than 1 mm in diameter in the
148 injected follicle were discarded from study.

149 Cows were ovariectomysed by colpotomy (Drost et al. 1992) and granulosa
150 cells were obtained as previously described (Buratini et al. 2007), deposited in cryogenic
151 tubes containing 600 µL of PBS, immediately frozen in liquid nitrogen, and stored at −80ºC

152 for subsequent mRNA and protein evaluation. The absence of granulosa cell contamination by
153 theca cells was confirmed by the lack of CYP17A1 mRNA, as determined by real time
154 polymerase chain reaction (qRT-PCR) (Buratini et al. 2007).
155
156 *Ribonucleic acid extraction, reverse transcription, and the quantitative polymerase*
157 *chain reaction*
158 Total ribonucleic acid (RNA) extraction was performed using Trizol® (Invitrogen,
159 Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA quantity and
160 purity (based on the ratios of the absorbance at 260 and 280 nm) was determined using a
161 NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Only RNAs
162 samples with an absorbance ration of >1.8 were used in the experiments. RNA integrity was
163 verified by visualization of ribosomal RNA (rRNA) in a 1.2% agarose gel. The
164 complimentary deoxyribonucleic acid (cDNA) was performed using RNA (500 ng)
165 previously treated with 0.1 U of deoxyribonuclease I (DNase I, Amplification Grade,
166 Invitrogen Life Technologies, Waltham, MA, USA) and incubated at 37ºC for 5 min, then at
167 65ºC for 10 min. Subsequently, the 15 µL of DNA-free RNA was reversed transcribed using 5
168 µL iScript™ cDNA Synthesis Kit® (Bio-Rad Laboratories, Hercules, CA), according to the
169 manufacturer's instructions.
170 qRT-PCR was conducted in a StepOnePlus™ instrument (Applied Biosystems, Foster
171 City, CA, USA) using 1µL of cDNA (25 ng) per reaction, Platinum SYBR Green qRT-PCR
172 SuperMix (Life Technologies, Carlsbad, CA, USA), and 0.2 µM of specific bovine primers.
173 The primer sequences (Table 1) were taken from the literature or designed using Primer
174 Express Software, version 3 (Life Technologies, Carlsbad, CA, USA), and the primers were
175 synthetized by Invitrogen (Waltham, Massachusetts, USA). After an initial denaturation step
176 at 95ºC for 3 min, 40 cycles of 95ºC for 15 s and 30s at 60ºC, and 30 s at 72ºC. Reactions

177 were performed in duplicate, and melting-curves were analyzed to determine the identity of
178 the products. Variability in mRNA expression levels was expressed relative to expression of
179 the reference genes GAPDH and cyclophilin (PPIA) (Ferreira et al. 2011, Siqueira et al. 2012,
180 da Rosa et al. 2016). Calculation of relative expression levels was performed as previously
181 described(Pfaffl 2001).
182
183 *Western blotting*
184 Proteins were extracted from granulosa cells using radioimmunoprecipitation assay buffer,
185 and western blotting was performed as previously described (Dau et al. 2016). The proteins
186 were boiled at 95°C for 5 min, subjected to 12% sodium dodecyl sulfate (SDS)
187 polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. After
188 blocking the membranes for 3 h in Tris-buffered saline (TBS) containing 5% skimmed milk
189 and 0.1% Tween® 20 (TBS-T), the blots were incubated overnight at 4°C with antibodies
190 against (P)RR (anti-ATP6IP2; 1:1000; ab40790; Abcam plc., Cambridge, UK), with gentle
191 agitation. Subsequently, the blots were washed 3 times for 5 min in TBS-T. The blots were
192 then incubated with a goat anti-rabbit secondary antibody (diluted 1:2,000; IgG-HRP; sc-
193 2004; Santa Cruz Biotechnology, Inc., Dallas TX, USA) for 1 h while being agitated, which
194 was followed by 3 5-min washes in TBS-T. Immunoreactivity was detected using the
195 Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in
196 accordance with the manufacturer's instructions. The images were analyzed using the
197 ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The
198 blots were incubated for 1 h at 50ºC in a western blot stripping buffer, which comprised β-
199 mercaptoethanol, 20% SDS, and 1 M Tris-HCl (pH 6.8). Then, the membranes were washed 3
200 times with TBS-T, with each wash lasting 20 min, and the membranes were re-blotted with an
201 anti-beta actin antibody (diluted 1: 5,000; control; ab8227; Abcam plc., Cambridge, UK). The

202 specificity of the (P)RR antibody was verified by antigen blocking using the human ATP6IP2
203 peptide (diluted 1: 1000; ab40790; Abcam plc., Cambridge, UK), as previously described
204 (Nostramo et al. 2015).
205
206 *Experimental design*
207
208 *Evaluation of the effect of prorenin on transcripts for epidermal growth factor-like*
209 *cascade in granulosa cells through (pro)renin receptor*
210 These experiments were designed to determine whether prorenin induces genes related
211 to ovulation in cultured granulosa cells via (P)RR. Bovine granulosa cells were incubated in
212 the absence or presence of LH (100 ng mL^{-1}, The National Hormone and Peptide Program,
213 Torrance, CA, USA) and LH plus 3 doses of prorenin (0.01, 0.1, and 1 nM) for 6 h. The LH
214 concentration and time was determined based on previous study, wherein Ang II increased
215 EREG, AREG and PTGS2 mRNA induced by 100 ng mL^{-1} of LH in bovine granulosa cells 6
216 h post-treatment (Portela et al. 2011). The prorenin concentrations used in the present study
217 were based in our previous study, wherein 0.1 nM prorenin induced the resumption of oocyte
218 meiosis (Dau et al. 2016). Transcripts for Bax, Bcl2, luteinizing hormone receptor (LHR),
219 (P)RR, ADAM17, EREG, AREG, and PTGS2 were verified in granulosa cells by qRT-PCR.
220 This experiment was performed in six replicates and repeated on different days.
221 The functional requirement of the (P)RR in EREG, AREG, and PTGS2 mRNA was
222 also evaluated after 6 h in culture using the following treatments: 1) control; 2) aliskiren
223 ([P]RR inhibitor; 0.1 µM); 3) prorenin (0.1 nM); 4) prorenin with aliskiren (0.1 nM and 0.1
224 µM, respectively); 5) LH (100 ng mL^{-1}); 6) LH with prorenin (100 ng mL^{-1} and 0.1 nM,
225 respectively); 7) LH with aliskiren (100 ng mL^{-1} and 0.1 µM, respectively); 8) LH with
226 prorenin and aliskiren (100 ng mL^{-1}, 0.1nM, and 0.1 µM, respectively). The mRNA

227 expression of EREG, AREG, and PTGS2 were verified in granulosa cells by qRT-PCR. This
228 experiment was performed in six replicates and repeated on different days.
229
230 *Investigation of the EGFR signaling requirement for (P)RR expression in preovulatory*
231 *granulosa cells in vitro and in vivo*
232 These experiments were designed to determine whether EGFR signaling is required
233 for expression of prorenin and (P)RR mRNA in cultured granulosa cells. *In vitro* experiment
234 was performed incubating bovine granulosa cells in the absence or presence of LH (100 ng
235 mL^{-1}) and LH plus 0.5 or 5 µM doses of AG1478 (an EGFR tyrosine kinase inhibitor) for 6 h.
236 Prorenin and (P)RR were verified in granulosa cells by qRT-PCR. This experiment was
237 performed in triplicate and repeated on different days. *In vivo* experiment was performed
238 using intrafollicular treatment with saline (vehicle/control; n = 4) or AG1478 (5 µM; n = 5)
239 in cows challenged with GnRH analogue (100 µg; IM). The ovary-bearing preovulatory
240 follicle from each cow was obtained by ovariectomy via colpotomy (Drost et al. 1992) at 6 h
241 after intrafollicular injection. This *in vivo* model was validated in our previous experiments,
242 wherein we observed a decrease in CYP17A1 protein expression verified in theca cells from
243 preovulatory follicles treated with AG1478 compared to control ($P<0.05$), which was
244 evaluated by western blotting. Prorenin and (P)RR were verified in granulosa cells by qRT-
245 PCR.
246
247 *Characterization of the (pro)renin receptor, prorenin, and profibrotic molecules in*
248 *granulosa cells during the preovulatory period in cattle*
249 This experiment was performed to evaluate whether (P)RR mRNA and protein or
250 transcripts for prorenin and profibrotic molecules are regulated in granulosa cells following
251 GnRH treatment. The ovary-bearing preovulatory follicle from each cow was obtained by

252 ovariectomy via colpotomy (Drost et al. 1992) at 0 (n = 4), 3 (n = 4), 6 (n = 3), 12 (n = 5), and
253 24 h (n = 4) post-GnRH administration. This *in vivo* model was adapted from previous studies
254 (Komar et al. 2001, Bridges et al. 2006) and confirmed by measuring the estradiol (E2)
255 concentration in follicular fluid from all preovulatory follicles dissected from each ovary, as
256 previously described (Tonellotto dos Santos et al. 2012). (P)RR protein levels in granulosa
257 cells were evaluated by western blot. Transcripts of *(P)RR*, prorenin, fibronectin 1 (*FN1*),
258 plasminogen activator inhibitor 1 (*PAI1*), transforming growth factor beta-1 (*TGFB1*), and
259 collagen type I in granulosa cells were detected by qRT-PCR.
260
261 *Statistical analysis*
262 Data of relative transcript and protein abundance were tested for normality using the
263 Shapiro–Wilk test and normalized when necessary. Data were analyzed by ANOVA using
264 JMP software (SAS Institute Inc., Cary, NC, USA). The effects of different treatments on
265 gene expression in granulosa cells were analyzed using a multiple-comparison test, least-
266 squares means Student's *t*-test. The significance level adopted was 5% and gene-expression
267 results are presented as the mean ± standard error of the mean.
268
269 **Results**
270 *Lack of an effect of prorenin on transcripts for epidermal growth factor-like cascade*
271 *through (pro)renin receptor in cultured granulosa cells*
272 Transcripts for Bax and Bcl2 were expressed in granulosa cells cultured for 6 h
273 without LH, with LH plus 0, 0.01, 0.1, and 1 nM prorenin, respectively (data not shown).
274 Bcl2 mRNA abundance (anti-apoptotic gene) was higher than Bax mRNA. The ratio
275 Bax/Bcl2 was not regulated by LH or prorenin in granulosa cells ($P > 0.05$). Supplementation
276 of culture medium with 1nM of prorenin plus LH increased LHR mRNA (Fig. 1*a*) in

277 granulosa cells compared to control ($P < 0.05$). LH and/or prorenin ($P > 0.05$) did not
278 stimulate transcripts for (P)RR and ADAM17 when compared control, LH plus 0, 0.01, 0.1,
279 and 1 nM prorenin, respectively (data not shown). LH increased EREG mRNA (Fig. 1*b*) in
280 granulosa cells after 6 h of culture ($P < 0.05$). Treatment with LH plus three doses of prorenin
281 (0.01, 0.1, and 1 nM) significantly increased AREG mRNA expression (Fig. 1*c*) in granulosa
282 cells cultured for 6 h compared with that observed in control cells (without LH; $P < 0.05$).
283 Only 0.1 nM of prorenin plus LH increased PTGS2 mRNA (Fig. 1*d*) in granulosa cells
284 related to control cells ($P<0.05$). The supplementation of culture medium with LH plus
285 prorenin did not increase mRNA for EREG, AREG or PTGS2 in granulosa cells compared to
286 cells treated with LH alone at 6 h of culture ($P > 0.05$).

287 The hypothesis that the (P)RR is required for EREG, AREG, and PTGS2 mRNA in
288 granulosa cells was also tested using aliskiren, which is an (P)RR inhibitor, at 0.1 µM.
289 Treatment with aliskiren plus prorenin and LH or with aliskiren plus LH did not regulate LH-
290 induced EREG (Fig. 2*a*; $P > 0.05$) and AREG (Fig. 2b; $P > 0.05$) mRNA in granulosa cells
291 cultured for 6 h. Similarly, no effect was observed on PTGS2 mRNA (Fig. 2*c*; $P > 0.05$) in
292 granulosa cells 6 h after culture with LH, prorenin and/or aliskiren. Prorenin alone did not
293 stimulate EREG (Fig. 2*a*), AREG (Fig. 2*b*) or PTGS2 (Fig. 2*c*) mRNA in granulosa cells at 6
294 h of culture.

295

296 *EGFR signaling was not required for prorenin and (P)RR expression in preovulatory*
297 *granulosa cells in vitro and in vivo*

298 The supplementation of medium culture without LH, LH plus 0, 0.5, and 5 µM
299 AG1478 did not regulate prorenin or (P)RR mRNA expression in granulosa cells cultured for
300 6 h (data not shown). Similarly, intrafollicular injection of an EGFR inhibitor (AG1478) did

301 not decrease prorenin or (P)RR mRNA in granulosa cells compared to control group at 6 h
302 post-intrafollicular injection (P > 0.05; data not shown).
303

304 *Protein for (pro)renin receptor and transcripts for prorenin and profibrotic molecules*
305 *increased in granulosa cells at later stages post-GnRH in cattle*

306 While (P)RR mRNA (Fig. 3*a*) decreased in granulosa cells at 24 h following GnRH
307 treatment (P < 0.05), (P)RR mRNA (Fig. 3*b*) increased at 24 h compared to cells obtained at
308 0h post-GnRH (P < 0.05). Prorenin mRNA (Fig. 3*c*) increased in bovine granulosa cells
309 isolated from a preovulatory follicle at 12 h post-GnRH administration compared that cells
310 obtained at 24 h (P < 0.05). Transcripts for TGFB1 (Fig. 3*d*), PAI1 (Fig. 3*e*), COL1 (Fig. 3*f*)
311 and FN1 (Fig. 3*g*) increased in granulosa cells at 12 and 24 h compared to 0 h after GnRH
312 treatment in cattle.
313

314 **Discussion**

315 The role of prorenin, independent of renin, through its receptor on ovulation, was
316 postulated in 1980's (Itskovitz et al. 1988). The results obtained in this study revealed that
317 prorenin did not stimulate LH-induced (EGF)-like cascade in granulosa cells through (P)RR
318 *in vitro.* Prorenin and (P)RR mRNA were not regulated in granulosa cells by AG1478 (EGFR
319 inhibitor) at 6 h in our culture system. Similarly, intrafollicular blocking of EGFR did not
320 abrogate prorenin or (P)RR mRNA in granulosa cells at 6 h after intrafollicular treatment and
321 GnRH administration in cows. In adition, finding from this study demonstrated a marked
322 increase in the (P)RR protein and transcripts encoding prorenin and profibrotic molecules in
323 granulosa cells from 12 h following GnRH treatment in cattle.

324 The involvement of prorenin/(P)RR with genes related to ovulation in granulosa cells
325 was proposed because binding of prorenin to (P)RR has been related to EGFR transactivation

326 (Liu et al. 2011, Shibayama et al. 2013), as well as to upregulation of PTGS2 gene expression
327 (Gonzalez et al. 2013). In addition, it is well stablished that prorenin via (P)RR acts in the
328 same signaling pathway of Ang II (Nguyen 2008), which plays a role as LH co-factor for
329 stimulate (EGF)-like cascade in bovine granulosa cells (Portela et al. 2011). Our culture
330 system was adapted from (Portela et al. 2011) and in this study, similarly, LH
331 supplementation increased partially AREG and PTGS2 mRNA at 6 h of culture. Our culture
332 system was also validated by dramatic increase in level of EREG mRNA observed in
333 response to LH treatment of the granulosa cells 6 h post-culture, which is consistent with
334 results obtained *in vivo* 6 h after hCG challenge in cattle (Sayasith et al. 2013). Moreover,
335 Bcl2 mRNA is an anti-apoptotic gene and it was detected in higher levels than Bax mRNA,
336 which is pro-apoptotic gene, suggesting absence of apoptosis induction in granulosa cells
337 cultured for 6 h (Antonsson 2001).

338 Prorenin plus LH did not induce significantly EREG, AREG and PTGS2 mRNA in
339 granulosa cells compared to cells treated only with LH, as observed for Ang II (Portela et al.
340 2011). Transcript for LHR was dramatically increased in granulosa cells when these cells
341 were treated with LH plus the higher dose of prorenin, but it did not induce the same response
342 for EREG, AREG, and PTGS2 mRNA. ADAM17 and (P)RR mRNA were detected in
343 granulosa cells submitted for all treatments, suggesting that its availability is not limiting to
344 EGFR transactivation or (P)RR activation, respectively. ADAM17 releases the ectodomains
345 of AREG and EREG and transactivates EGFR (Park et al. 2004) and its transcript is
346 transiently induced 6 to 12 h post-hCG in bovine granulosa cells *in vivo* (Sayasith and Sirois
347 2015). ADAM17 mRNA was not increased in granulosa cells by prorenin treatment at 6 h of
348 culture, as verified for Ang II (Portela et al. 2011).

349 Lack of effect of prorenin to induce (EGF)-like cascade through (P)RR was confirmed
350 treating granulosa cells with prorenin alone and/or with aliskiren plus LH. Blocking (P)RR

351 did not regulate LH-induced EREG and AREG mRNA in granulosa cells. Absence increased
352 levels of PTGS2 mRNA in granulosa cells in response for LH at 6 h post-treatment is
353 supported by previous studies *in vitro* (Portela et al. 2011) and *in vivo* (Li et al. 2007).
354 Although PTGS2 mRNA was not regulated for LH, prorenin and/or aliskiren treatment, LH
355 increased significantly AREG and EREG mRNA in granulosa cells at 6 h post-culture,
356 supporting our culture system for investigating ovulatory cascade induced by LH during
357 preovulatory period (Sayasith et al. 2013). The dose of prorenin used in this experiment was
358 chosen because LH only increased significantly PTGS2 mRNA in granulosa cells when
359 supplemented with 0.1 nM of prorenin in the first *in vitro* experiment. In addition, *in vitro*, 0.1
360 nM of prorenin induces resumption of meiosis in bovine oocytes, which is blocked by 0.1 µM
361 of aliskiren (Dau et al. 2016). Aliskiren in concentration of 0.1 µM inhibits intra- and
362 extracellular signaling of the (P)RR *in vitro* (Biswas et al. 2010, Ferri et al. 2011, Ma et al.
363 2012). Thus, in contrast to ovulatory cascade induced by Ang II plus LH in granulosa cells *in*
364 *vitro* (Portela et al. 2011), we found no evidence that prorenin activates (EGF)-like cascade in
365 granulosa cells via (P)RR. These results suggest that prorenin/(P)RR acts in signaling
366 pathway different of the AngII in bovine granulosa cells.
367 *In vitro* and *in vivo* experiment were used to evaluate whether EGFR acts upstream of
368 the (P)RR signaling pathway in granulosa cells. Prorenin and (P)RR mRNA were not
369 increased by LH at 6 h of culture and neither regulated by AG1478 *in vitro*. Similarly,
370 intrafollicular blocking of EGFR also did not regulate prorenin and (P)RR mRNA expression
371 in granulosa cells induced by GnRH, supporting our result obtained *in vitro*. The AG1478 (an
372 EGFR inhibitor) effect was observed from expected increase of CYP17A1 protein in theca
373 cells according previous study (Spicer and Stewart 1996). In addition, AG1478 blocked LH-
374 induced NPPC mRNA in granulosa cells in our models *in vitro* and *in vivo* (data submitted for

375 publication). Taken together, EGFR signaling was not required for prorenin and (P)RR
376 transcripts in granulosa cells at 6 h after LH or GnRH treatment in cattle.

377 Evidencing that prorenin/(P)RR were not involved at the beginning of the ovulatory
378 cascade and based on knowledge that LH surge induces prorenin level in follicular fluid in
379 cattle (Hagemann et al. 1994), we addressed the question of whether prorenin/(P)RR mRNA
380 are upregulated in granulosa cells by LH surge during preovulatory period. Prorenin mRNA
381 and (P)RR protein was increased in granulosa cells from 12 h post-GnRH, proposing that
382 prorenin and (P)RR are required in the final process of ovulation. Profibrotic molecules are
383 induced by binding prorenin to (P)RR (Huang et al. 2006, Nguyen et al. 2002, Ferri et al.
384 2011, Nguyen 2008) and have been related for rupture of preovulatory follicle, which occurs
385 between 24 and 32 h post-GnRH in cattle (Dow et al. 2002, Liu et al. 2013). Thus, we
386 investigated profile of transcripts encoding profibrotic molecules in the same granulosa cells
387 to determine whether it has the same expression pattern than prorenin mRNA or (P)RR
388 protein. TGFB1, PAI1, COL1, and FN1 mRNA were upregulated from 12 h after GnRH
389 administration in cattle, supporting our hypothesis. These results, although remains to be
390 confirmed, suggest that prorenin may induce profibrotic molecules in granulosa cells through
391 (P)RR to promote follicular rupture.

392 The (P)RR protein increased in granulosa cells at the end of the ovulatory process.
393 This result support increased prorenin levels in follicular fluid after LH surge (Hagemann et
394 al. 1994) and positive correlation between prorenin and progesterone levels (Hagemann et al.
395 1997), which increases dramatically in the follicular fluid at 24 h post-GnRH in cattle
396 (Fortune et al. 2009). The endogenous LH surge is expected to occur 2 h after GnRH analog
397 treatment in cows, increasing E2 in the follicular fluid within 3 h post-GnRH (Komar et al.
398 2001). The profile of E2 concentration in follicular fluid was previously determined by our
399 research group (Tonellotto dos Santos et al. 2012) and was similar to prior study (Komar et al.

400 2001), validating our *in vivo* model to verify profile of gene expression in granulosa cells
401 during preovulatory period. Moreover, the highest abundance of mRNA for PAI-1 was
402 detected in granulosa cells collected at about time of ovulation (24h), as observed in
403 periovulatory bovine follicular in preceding study (Dow et al. 2002).
404 In summary, prorenin did not induce ovulatory cascade in granulosa cells via (P)RR
405 and EGFR was not required for prorenin or (P)RR mRNA expression in granulosa cells at 6 h
406 after LH or GnRH treatment in cattle. Protein for (P)RR and transcripts for prorenin and
407 profibrotic molecules in granulosa cells were upregulated from 12 h after GnRH. Collectively,
408 these results suggest that prorenin plays a role at end of preovulatory cascade through (P)RR
409 in bovine granulosa cells.
410
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418
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549 **Figure legends**

550 **Fig. 1.** The dose-response effects of combined treatment with prorenin (0.01, 0.1, and
551 1 nM) and LH (100 ng mL^{-1}) on mRNA expression of (*a*) LHR, (*b*) EREG, (*c*) AREG, and (*d*)
552 PTGS2 in granulosa cells at 6 h post-treatment. Results are shown as the mean ± standard
553 error. The different letters indicate the statistical differences among groups ($P < 0.05$).
554 Experiment was performed in six replicates.

555 **Fig. 2.** Effect of combined treatment with prorenin (0.1), LH (100 ng mL^{-1}), and
556 aliskiren (0.1 μM) on mRNA expression of (*a*) EREG, (*b*) AREG, and (*c*) PTGS2 in
557 granulosa cells at 6 h post-treatment. Results are shown as the mean ± standard error. The
558 different letters indicate the statistical differences among groups ($P < 0.05$). Experiment was
559 performed in six replicates.

560 **Fig. 3.** Profile of (pro)renin receptor ([P]RR) (mean ± standard error) (*a*) mRNA and
561 (*b*) protein expression as well as, of transcripts for (*c*) prorenin, (*d*) transforming growth
562 factor beta 1 (TGFB1), (*e*) plasminogen activator inhibitor 1 (PAI1), (*f*) type I collagen
563 (COL1), and (*g*) fibronectin (FN1) in bovine granulosa cells isolated from preovulatory
564 follicles obtained at 0 (n = 4), 3 (n = 4), 6 (n = 3), 12 (n = 5), or 24 h (n = 4) after GnRH was
565 administered to induce an LH surge. Western blot images revealed a specific band at
566 approximately 42 kDa for the (P)RR and at 42 kDa for β-actin (ACTB). Representative blots
567 from each time point and summaries of densitometric analysis are shown. Different letters
568 indicate statistical differences observed between groups ($P < 0.05$).

569

570 **Table 1. Primers used for quantitative real-time PCR.**

Gene		Primer sequence	Reference or accession number
GAPDH	F:	GATTGTCAGCAATGCCTCCT	Ferreira *et al.* (2011b)
	R:	GGTCATAAGTCCCTCCACGA	
PPIA	F:	GGTCATCGGTCTCTTTGGAA	Ledoux *et al.* (2006)
	R:	TCCTTGATCACACGATGGAA	
(P)RR	F:	TGATGGTGAAAGGAGTGGACAA	Ferreira *et al.* (2011b)
	R:	TTTGCCACGCTGTCAAGACT	
PRORENIN	F:	GGGTGCCGTCCACCAA	NM_001206509.1
	R:	TCCGTCCCATTCTCCACATAG	
FN1	F:	TGGGACCACGCAGAACTATG	NM_001163778.1
	R:	GCGATACATGACCCCTTCGT	
PAI1	F:	CACCATCTCTGTGCCCATGAT	NM_174137.2
	R:	GGTAGGGCAATTCCAGGATGT	
TGFB1	F:	CTGAGCCAGAGGCGGCGGACTAC	NM_001166068.1
	R:	CTGTGCGAGCTAGACTTCATTTTG	
COL1	F:	CATGACCGAGACGTGTGGAA	NM_001034039.2
	R:	CAGTCCTTAAGTTCGTCGCAGAT	
ADAM17	F:	TTCATGGGACAATGCAGGTTT	XM_002691486.2
	R:	GAAGTGCCTTTCACCAGGTTTT	
AREG	F:	CCATTTTCTTGTCGAAGTTTCTTTC	Li *et al.* (2009)
	R:	TGTTTTTATTACAATCCTGCTTCGAA	
EREG	F:	ACTGCACAGCATTAGTTCAAACTGA	XM_010806226.1
	R:	TGTCCATGCAAACAGTAGCCATT	
LHR	F:	GCACAGCAAGGAGACCAAATAA	ENSBTAT00000022047
	R:	TTGGGTAAGCAGAAACCATAGTCA	
BAX	F:	TTCTGACGGCAACTTCAACT	NM_173894
	R:	CGAAGGAAGTCCAATGTCCA	
BCL2	F:	CATCGTGGCCTTCTTTGAGT	NM_001166486
	R:	CATGCTAGGGCCATACAGC	
CYP17A1	F:	CCATCAGAGAAGTGCTCCGAAT	Lagaly *et al.* (2008)
	R:	GCCAATGCTGGAGTCAATGA	
CYP19A1	F:	GTGTCCGAAGTTGTGCCTATT	Luo and Wiltbank (2006)
	R:	GGAACCTGCAGTGGGAAATGA	

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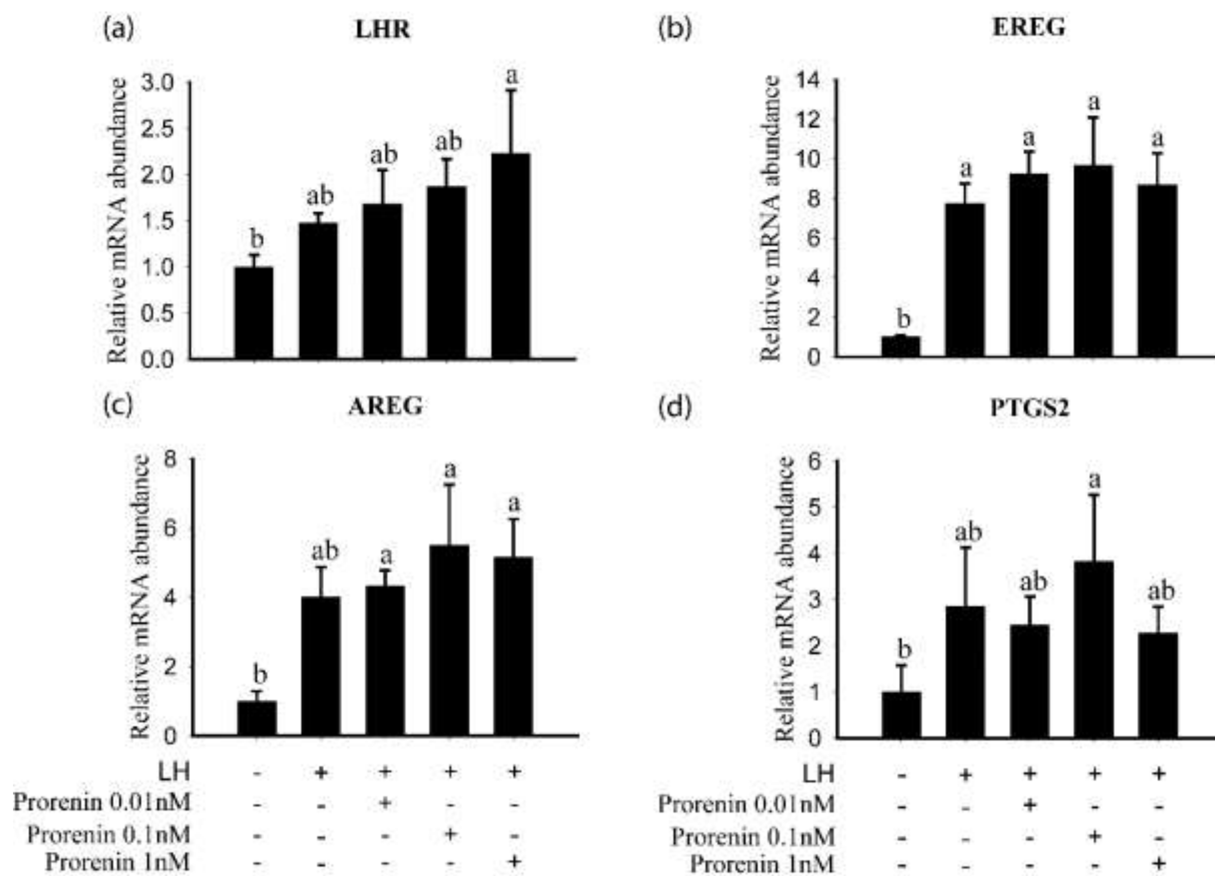
573 **Figure 1**



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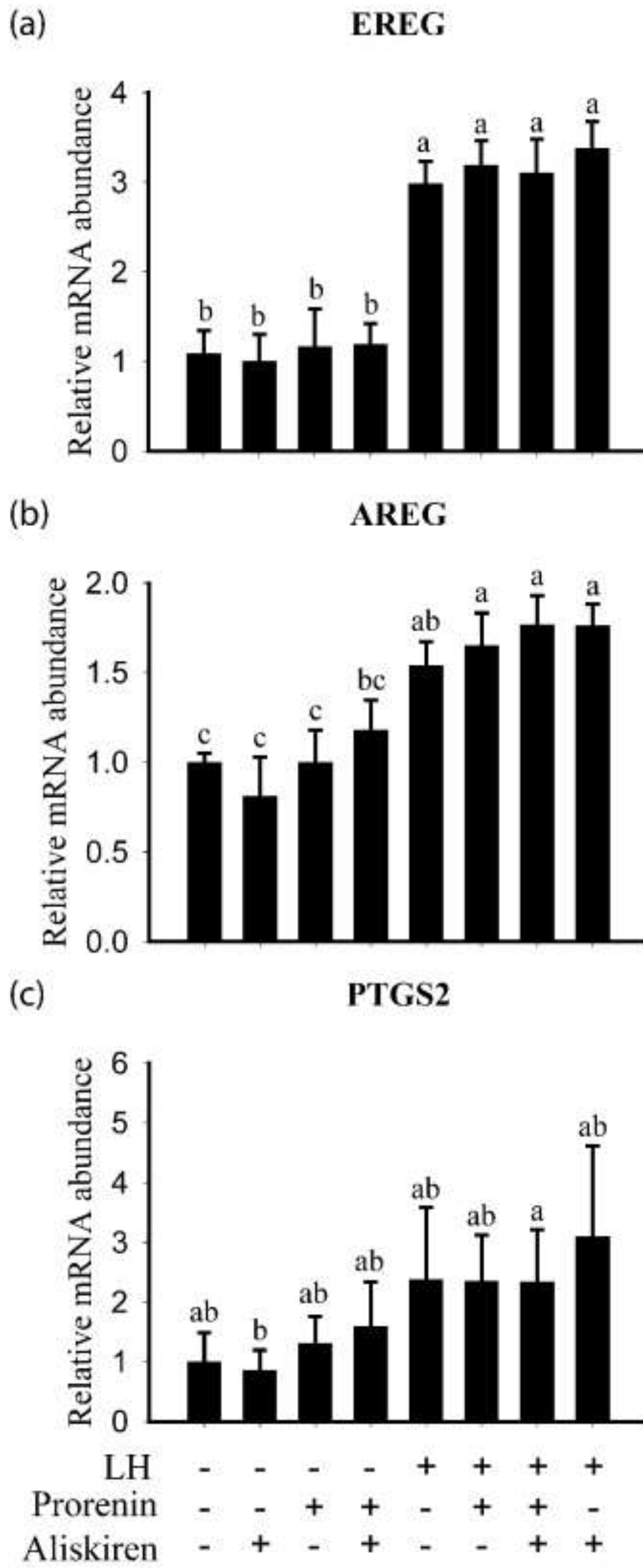
576 **Figure 2**



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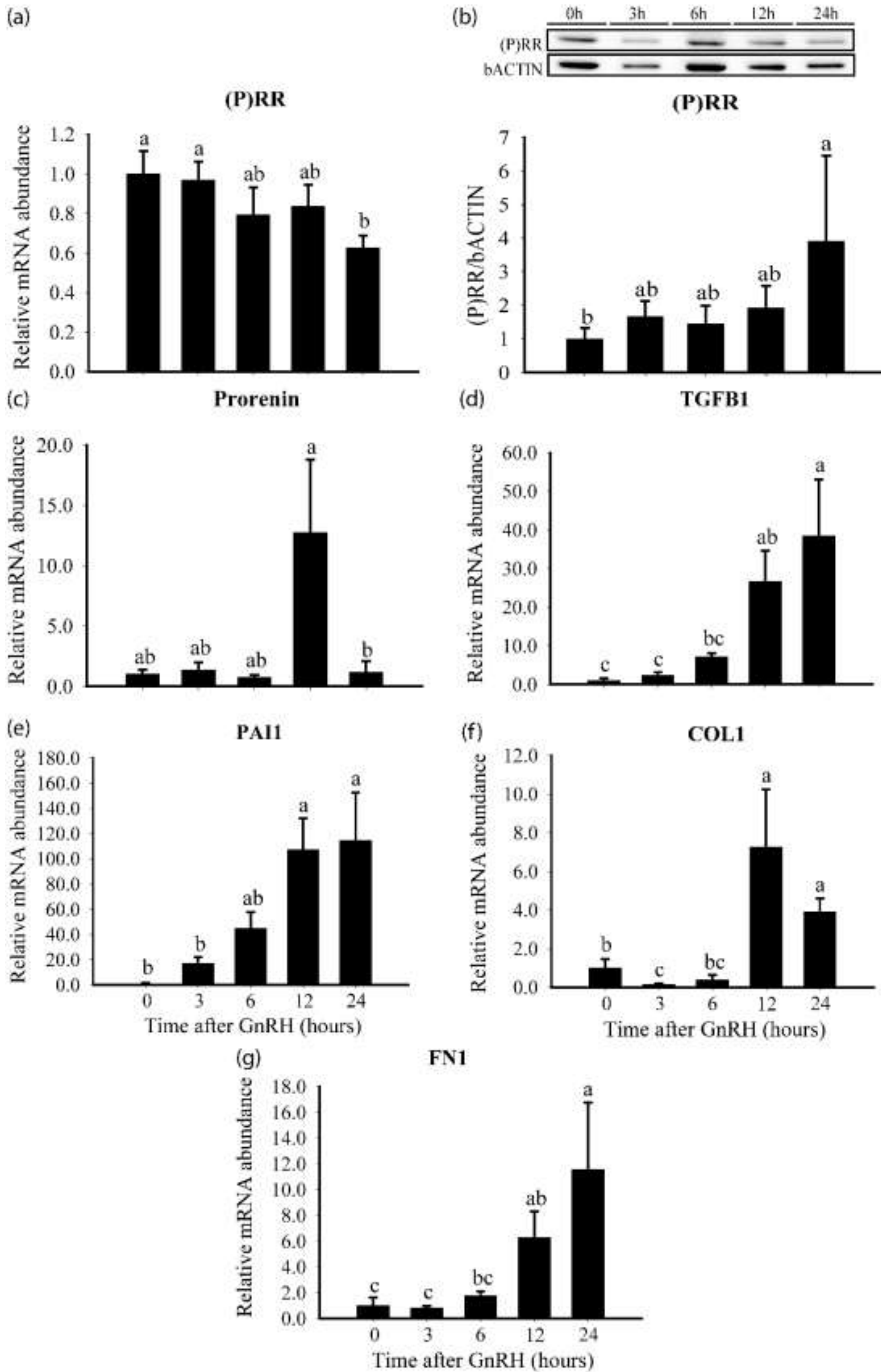
579 **Figure 3**



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5. ARTIGO 3

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

The expression of (pro)renin receptor increases after luteinizing hormone surge independently of the epidermal growth factor pathway in bovine theca cells

Andressa Minussi Pereira Dau, Melânia Lazzari Rigo, Paulo Roberto Antunes da Rosa, Joabel Tonellotto dos Santos, Juliana Germano Ferst, Renato Zanella, Osmar Damim Prestes, Fabio Vasconcellos Comim and Paulo Bayard Dias Gonçalves.

REPRODUCTION, FERTILITY AND DEVELOPMENT, 2017

582 **The expression of (pro)renin receptor increases after luteinizing hormone surge**
583 **independently of the epidermal growth factor-like pathway in bovine theca cells**

584 Andressa Minussi Pereira Dau[A], Melânia Lazzari Rigo[A], Werner Giehl Glanzner[A],
585 Paulo Roberto Antunes da Rosa[A], Joabel Tonellotto dos Santos[A], Juliana Germano Ferst[A],
586 Renato Zanella[B], Osmar Damian Prestes[B], Fabio Vasconcellos Comim[A, C], and Paulo Bayard
587 Dias Gonçalves[A, D]
588
589 [A] Laboratory of Biotechnology and Animal Reproduction, BioRep, Federal University
590 of Santa Maria, Roraima Avenue 1000, 97105-900 Veterinary Hospital, Santa Maria, RS,
591 Brazil
592 [B] Laboratory of Pesticide Residue Analysis, Federal University of Santa Maria,
593 Roraima Avenue 1000, 97105-900, Santa Maria, RS, Brazil
594 [C] Department of Medical Clinic, Faculty of Medicine, Federal University of Santa
595 Maria, Roraima Avenue 1000, 97105-900, Santa Maria, RS, Brazil
596
597 [D]**Corresponding author:** Paulo Bayard D. Gonçalves, Departamento de Clínica de
598 Grandes Animais, Hospital Veterinário, Universidade Federal de Santa Maria, 97105-900
599 Santa Maria, RS, Brazil. Email address: bayard@ufsm.br
600
601 **Keywords:** antral follicle; follicular fluid; ovulation; androgen
602
603 **Running Head:** (Pro)renin receptor in preovulatory theca cells
604

605 **Abstract**

606 The objective of this study was to evaluate the function of (pro)renin receptor ([P]RR),
607 named ATP6AP2, in ovulation and whether it increases in theca cells after luteinizing
608 hormone (LH) surge. In addition, we studied the potential effects of prorenin/(P)RR and
609 epidermal growth factor (EGF) signaling on thecal steroidogenesis. Theca cells were obtained
610 from bovine preovulatory follicles at 0, 3, 6, 12, and 24 h after gonadotropin-releasing
611 hormone (GnRH). (P)RR mRNA and protein expression increased in theca cells at 6 h post-
612 GnRH ($P<0.05$). LH treatment increased (P)RR mRNA in theca cells cultured for 6 h
613 ($P<0.05$). Two out of 6 cows (33.4%) did not ovulate after GnRH and intrafollicular treatment
614 with aliskiren ([P]RR inhibitor; $P>0.05$) compared with 66,6% (4/4) in the control group.
615 Prorenin or LH did not increase ADAM17 mRNA *in vitro* ($P>0.05$). Intrafollicular injection
616 of the EGF receptor inhibitor AG1478 did not regulate LH-induced (P)RR ($P>0.05$), but
617 increased CYP17A1 expression in theca cells ($P<0.05$). In cultured theca cells,
618 androstenedione and testosterone synthesis were not regulated by prorenin/(P)RR and/or LH.
619 Finally, prorenin did not regulate ovulation-related genes and/or thecal steroidogenesis *in*
620 *vitro*. Importantly, we found that prorenin/(P)RR inhibition partially affected ovulation and
621 that (P)RR functions as a novel target of renin-angiotensin system, independently of EGFR, in
622 preovulatory theca cells.

623

624 **Introduction**

625 In 2002, the (pro)renin receptor ([P]RR), also known as ATP6AP2, was found to
626 promote renin-angiotensin system (RAS) signaling through an alternative pathway. Binding
627 of prorenin to (P)RR induces mitogen-activated protein kinases (MAPKs) activation,
628 independent of angiotensin II (Ang II) (Uraoka *et al.* 2009). Although prorenin was thought to
629 be an inactive precursor of renin, the presence of the (P)RR in the ovary was postulated in the
630 1980s (Sealey *et al.* 1985; Glorioso *et al.* 1986; Itskovitz *et al.* 1988). A possible functional

631 role for prorenin was uncovered when the (P)RR was identified and it was shown that
632 prorenin exhibited a 2–3-fold higher affinity for (P)RR than for renin (Nguyen *et al.* 2002;
633 Nabi *et al.* 2009).

634 Prorenin levels in ovarian follicular fluid are 12 times higher than those in the blood of
635 women (Glorioso *et al.* 1986) and are increased by luteinizing hormone (LH) surge in heifers
636 (Hagemann *et al.* 1994). Prorenin is mainly secreted by theca cells in cattle (Schultze *et al.*
637 1989), where (P)RR was identified (Dau *et al.* 2016). Increased (P)RR mRNA expression in
638 the second largest follicle suggested an association with follicular dominance (Ferreira *et al.*
639 2011b), although the reason for these modifications in expression remain speculative. A role
640 of prorenin/(P)RR in the resumption of meiosis *in vitro* was also described in bovine oocytes
641 (Dau *et al.* 2016). Nevertheless, the involvement of ovarian (P)RR in other relevant biological
642 processes, such as ovulation or steroidogenesis, has not been explored.

643 The (P)RR signaling pathway has been proposed to be mediated by the epidermal growth
644 factor receptor (EGFR). In rat vascular smooth muscle cells, prorenin stimulated EGFR
645 phosphorylation and pretreatment with EGFR inhibitor impaired prorenin-induced
646 extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorilation (Liu *et al.*
647 2011). In human embryo kidney 293 cells, prorenin caused EGFR phosphorylation, which
648 were inhibited by (P)RR siRNA (Shibayama *et al.* 2013). However, prorenin did not induce
649 EGFR transactivation and EGFR inhibitor had no effect in MAPK/ERK pathway in
650 monocytes, suggesting that EGFR may act upstream of the (P)RR signaling pathway (Feldt *et*
651 *al.* 2008). Thus, the crosstalk between (P)RR and EGFR is unclear. In bovine theca cells
652 cultured *in vitro*, EGF decreases LH/cAMP-induced androstenedione synthesis (Spicer and
653 Stewart 1996). At later stages in ovulation, the mRNA levels of cytochrome P450 17A1
654 (CYP17A1) decrease in theca cells and, consequently, androstenedione/testosterone
655 production decrease in follicular fluid (Komar *et al.* 2001; Fortune *et al.* 2009). Taken

656 together, we hypothesized that EGFR regulates CYP17A1 induced by LH and it is mediated
657 by prorenin/(P)RR.

658 The aims of this study were to evaluate whether (P)RR expression increases in theca cells
659 after an LH surge, to investigate the role of (P)RR in the ovulation and whether it is related
660 with EGFR signaling, to determine whether EGFR regulates steroidogenic enzymes in theca
661 cells, and to determine the effect of prorenin/(P)RR signaling in thecal steroidogenesis.

662

663 **Materials and methods**

664 All experimental procedures with cattle were reviewed and approved by the local
665 Animal Ethics Committee of Federal University of Santa Maria (nº.115/2014) in agreement
666 with the National Council for the Control of Animal Experimentation (CONCEA; Brazilian
667 Ministry of Science, Technology, and Innovation).

668

669 *Experimental design*

670 *Characterization of the (pro)renin receptor in theca cells during the preovulatory*
671 *period*

672 To evaluate whether (P)RR increased in theca cells after GnRH treatment, the ovary-
673 bearing preovulatory follicle was randomly isolated by ovariectomy via colpotomy (Drost *et*
674 *al.* 1992) from individual cows at 0 (n = 3), 3 (n = 3), 6 (n = 3), 12 (n = 3), and 24 h (n = 4)
675 post-GnRH administration. This *in vivo* model was adapted from previous studies (Komar *et*
676 *al.* 2001; Bridges *et al.* 2006) and confirmed by measuring the estradiol (E2) concentration in
677 follicular fluid from all preovulatory follicles dissected from each ovary, as previously
678 described (Tonellotto dos Santos *et al.* 2012). The transcript and protein levels of (P)RR were
679 evaluated in theca cells by qRT-PCR and western blotting, respectively.

680

681 *Evaluation of the effect of LH on (pro)renin receptor mRNA expression in theca cells*

682 This experiment was designed to determine whether LH induces (P)RR mRNA
683 expression in cultured theca cells. Bovine theca cells were incubated in basal culture medium
684 alone or with LH (The National Hormone and Peptide Program, Torrance, CA, USA; 100 ng
685 mL^{-1}) for 6 h. The time was determined based on the result of the first experiment. Expression
686 of the (P)RR transcript was evaluated by qRT-PCR. This experiment was performed in
687 triplicate and repeated on different days.
688
689 *Examining the roles of the (pro)renin receptor on ovulation*
690 The preovulatory follicle from each cow challenged with GnRH was treated with PBS
691 (vehicle/control; n = 4) or aliskiren (10 μM; n = 6), which is a direct renin inhibitor and a
692 potent inhibitor of the receptor-bound renin or prorenin (Biswas *et al.* 2010). The
693 concentration of aliskiren used in this experiment was enough to block prorenin effect in the
694 resumption of meiosis in bovine oocytes (Dau *et al.* 2016). We monitored ovulation at 24, 48,
695 and 72 h after intrafollicular treatment by ultrasound, as previously described (Ferreira *et al.*
696 2007). Briefly, ovulation was identified by the disappearance of the preovulatory follicle
697 between 2 consecutives evaluations and corpus luteum formation. These findings were
698 confirmed by measuring plasma P4 concentrations (>2 ng mL^{-1}) obtained from jugular vein at
699 6 days post-follicular treatment and measured by electrochemiluminescence.
700
701 *Examination of the effect of prorenin on ADAM17, AREG, and EREG mRNA*
702 *expression in theca cells*
703 The effect of prorenin on the transcription of genes involved in ovulation was
704 evaluated in the absence or presence of LH (100 ng mL^{-1}) and LH plus 3 doses of prorenin
705 (0.01, 0.1, and 1 nM) for 24 h. The time was determined based on previous studies, wherein
706 AREG and ADAM17 mRNA were increased in bovine theca cells 24 h following GnRH

707 injection in cows (Li *et al.* 2009). The prorenin concentrations used in the present study were
708 based in our previous studies, wherein 0.1 nM prorenin induced the resumption of oocyte
709 meiosis via the Ang II-independent pathway (Dau *et al.* 2016). The concentration of LH also
710 was established by previous studies (Stewart *et al.* 1995). ADAM17, AREG, and EREG
711 mRNA were verified in theca cells by qRT-PCR. This experiment was performed in triplicate
712 and repeated on different days.

713

714 *Investigation of the EGFR requirement for LH-induced (P)RR expression in*
715 *preovulatory theca cells in vivo*

716 Cows were randomly treated intrafollicularly with saline (vehicle/control; n = 4) or
717 AG1478 (an EGFR tyrosine kinase inhibitor; 5 µM; n = 5) after challenge with GnRH. The
718 ovary-bearing preovulatory follicle from each cow was obtained by ovariectomy via
719 colpotomy (Drost *et al.* 1992) at 6 h after intrafollicular injection. (P)RR and CYP17A1
720 protein expression were verified in theca cells by western blotting and the levels of E2, P4,
721 androstenedione, and testosterone were measured in the follicular fluid by
722 electrochemiluminescence.

723

724 *Examination of prorenin and (pro)renin receptor on thecal steroidogenesis*

725 The effect of prorenin on LH-induced steroidogenesis was evaluated in the absence or
726 presence of LH (100 ng mL^{-1}) and LH plus 3 doses of prorenin (0.01, 0.1, and 1 nM) for 24 h.
727 The functional requirement of the (P)RR in theca cell steroidogenesis was also evaluated after
728 24 h in culture using the following treatments: 1) without LH; 2) LH (100 ng mL^{-1}); 3)
729 prorenin (0.1 nM); 4) prorenin with aliskiren ([P]RR inhibitor 0.1 µM); 5) LH with prorenin;
730 and 6) LH with prorenin and aliskiren. The concentration of aliskiren (0.1 µM) and LH (100
731 ng mL^{-1}) were established by previous studies (Stewart *et al.* 1995; Dau *et al.* 2016). The

732 mRNA expression of STAR, CYP11A1, HSD3B2, and CYP17A1 were verified in theca cells
733 by qRT-PCR. Androstenedione and testosterone secretion were evaluated in the culture
734 medium by UHPLC-MS/MS. This experiment was performed in triplicate and repeated on
735 different days.
736
737 *Chemicals*
738 All chemicals used in this study were purchased from Sigma-Aldrich Corporation (St
739 Louis, MO, USA), unless otherwise indicated.
740
741 *Animal procedures, intrafollicular injection, and collection of the theca cells*
742 Cows (*Bos taurus*) exhibiting normal estrous cycles had luteolysis induced and a new
743 follicular wave initiated using a hormonal protocol, as previously described (Siqueira *et al.*
744 2012; Tonellotto dos Santos *et al.* 2012). Briefly, 2 injections with a 12-h dosing interval of a
745 prostaglandin F2α (PGF2α) analog (cloprostenol, 250 mg, IM; Schering-Plough Animal
746 Health, Brazil), 2 mg of estradiol benzoate (EB), and an intravaginal progesterone device (1 g
747 progesterone, DIB®; Intervet, Brazil) for 9 days. The presence of preovulatory follicles (≥12
748 mm) was evaluated by transrectal ultrasonography using an 8-MHz, linear-array transducer
749 (AquilaVet scanner, Pie Medical, Netherlands) 12 h after removal of the vaginal device. Only
750 cows with follicles ≥12 mm in diameter were challenged with 100 μg of gonadorelin acetate
751 (Profertil®, Tortuga, Brazil), and thus, were treated by intrafollicular injection and/or
752 ovariectomized.
753 Intrafollicular injections were guided by an ultrasound (Aquila Vet, Pie Medical
754 Equipment BV, Holanda) equipped with 7.5 MHz convex probe. A double-needle system was
755 used to treat the preovulatory follicle (intrafollicular region) with aliskiren or AG1478. Final
756 concentrations of aliskiren of 10 μM or AG1478 of 5 μM were administered after estimating
757 the follicular fluid volume, as previously described (Ferreira *et al.* 2007; Ferreira *et al.*

758 2011a). The aliskiren and AG1478 concentrations used in this study were established
759 previously (Park *et al.* 2004; Dau *et al.* 2016). The cows were examined by transrectal
760 ultrasonography 2 h after intrafollicular treatment, and those with reductions larger than 1 mm
761 in diameter in the injected follicle were discarded from study.

762 Theca cells were obtained at specific time points during the preovulatory
763 period. Theca cells were dissected from the stromal tissue as previously described (Buratini *et*
764 *al.* 2007), deposited in cryogenic tubes containing 600 µL of PBS, immediately frozen in
765 liquid nitrogen, and stored at −80ºC for subsequent mRNA and protein evaluation. The
766 absence of theca cell contamination by granulosa cells was confirmed by the lack of
767 aromatase (CYP19A1) mRNA, as determined by real time polymerase chain reaction (qRT-
768 PCR; (Buratini *et al.* 2007).

769

770 *Theca cell recovery for in vitro experiments*

771 Pairs of ovaries (n=15) from non-pregnant cows at different stages of the estrous cycle
772 were obtained from a local abattoir and transported to the laboratory in saline solution
773 containing penicillin (100 IU mL^{-1}) and streptomycin sulfate (50 µg mL^{-1}) at 30ºC. Follicles
774 larger than 10 mm in diameter and considered healthy were selected for the experiments. The
775 follicles were considered healthy based on the presence of light yellow follicular fluid, corpus
776 luteum (<1 cm or absent), and by E2: progesterone (P4) ratio higher than two in the fluid
777 follicular (Ireland *et al.* 1980; McNatty *et al.* 1984). Theca cell isolation was performed as
778 previously described (Stewart *et al.* 1995; Comim *et al.* 2013). Briefly, follicles were
779 dissected from the ovary and hemisectioned, after which the internal walls of the follicles
780 were rinsed in PBS to eliminate granulosa cells. The theca cell layer was peeled away from
781 the surrounding stroma and digested in 1 mg mL^{-1} of collagenase solution for 1 h at 37ºC. The
782 absence of contamination with granulosa cells was verified by the lack of aromatase

783 (CYP19A1) mRNA in theca cells, as determined by qRT-PCR (Buratini *et al.* 2007). The E2
784 and P4 concentrations were measured by electrochemiluminescence in the follicular fluid
785 pooled from follicles (n = 4) that were used for each experimental replicate with cultured
786 theca cells. *In vitro* experiments were performed in triplicate.

787

788 *Theca cells cultures*

789 The *in vitro* model was adapted from previous studies (Stewart *et al.* 1995; Comim *et*
790 *al.* 2013). After enzymatic digestion, the theca cells from four follicles per replicate were
791 washed in culture media, seeded in 60-mm plates, and cultured for 48 h in DMEM/F12
792 (Gibco Labs, Waltham, MA, USA) supplemented with 1 µg mL^{-1} of transferrin, 1 ng mL^{-1} of
793 selenium, 100 IU mL^{-1} of penicillin, 50 µg mL^{-1} of streptomycin sulfate, 2.5 µg mL^{-1} of
794 amphotericin, and 10% fetal bovine serum (FBS) at 38.5ºC in 5.0% CO_2 and saturated
795 humidity. At the end of the culture period, the cells were trypsinized (0.25% trypsin) at 37ºC
796 for 5 min, and seeded in 96-well plates (Corning) at a concentration of 3×10^4 viable
797 cells/well under the same culture conditions for 24 h. Cell viability was determined by
798 staining with 0.4% Trypan Blue. Subsequently, the cells were washed twice and cultured in
799 150 µL of the same basic medium, although without FBS, and supplemented with 100 ng mL^{-}
800 ^{1} of insulin and different treatments, as indicated.

801

802 *Ribonucleic acid extraction, reverse transcription, and the quantitative polymerase*
803 *chain reaction*

804 Total ribonucleic acid (RNA) extraction was performed using Trizol® (Invitrogen,
805 Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA quantity and
806 purity (based on the ratios of the absorbance at 260 and 280 nm) was determined using a
807 NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Only RNAs

808 samples with an absorbance ration of >1.8 were used in the experiments. RNA integrity was
809 verified by visualization of ribosomal RNA (rRNA) in a 1.2% agarose gel. The
810 complimentary deoxyribonucleic acid (cDNA) was performed using RNA (500 ng)
811 previously treated with 0.1 U of deoxyribonuclease I (DNase I, Amplification Grade,
812 Invitrogen Life Technologies, Waltham, MA, USA) and incubated at 37ºC for 5 min, then at
813 65ºC for 10 min. Subsequently, the 15 µL of DNA-free RNA was reversed transcribed using 5
814 µL iScript™ cDNA Synthesis Kit® (Bio-Rad Laboratories, Hercules, CA), according to the
815 manufacturer's instructions.

816 qRT-PCR was conducted in a CFX384 Touch™ ® instrument (BIO-RAD) using 2µL of
817 cDNA (3.125 ng) per reaction, GoTaq® qPCR Master Mix (Promega, Madison, WI, USA),
818 and 0.2 µM of specific bovine primers. The primer sequences (Table 1) were taken from the
819 literature or designed using Primer Express Software, version 3 (Life Technologies, Carlsbad,
820 CA, USA), and the primers were synthetized by Invitrogen (Waltham, Massachusetts, USA).
821 After an initial denaturation step at 95ºC for 3 min, 40 cycles of 95ºC for 10 s and 1 min at
822 60ºC, and melting curve from 65ºC to 95ºC by 0.5 ºC for 5s were carried out to amplify each
823 transcript. Reactions were performed in duplicate, and melting-curves were analyzed to
824 determine the identity of the products. Variability in mRNA expression levels was expressed
825 relative to expression of the reference genes GAPDH and cyclophilin (PPIA) (Ferreira *et al.*
826 2011a; Ferreira *et al.* 2011b; Siqueira *et al.* 2012). qRT-PCR assays were validated using
827 standard curve and reactions with efficiency between 90 and 110% and coefficient of
828 determination (R^2) greater than 0.98 were considered optimized. Calculation of relative
829 expression levels was performed as previously described (Pfaffl 2001).

830

831 *Western blotting*

832 Proteins were extracted from theca cells using radioimmunoprecipitation assay buffer, and
833 western blotting was performed as previously described (Dau *et al.* 2016). The proteins were
834 boiled at 95°C for 5 min, subjected to 12% sodium dodecyl sulfate (SDS) polyacrylamide gel
835 electrophoresis, and transferred onto nitrocellulose membranes. After blocking the
836 membranes for 3 h in Tris-buffered saline (TBS) containing 5% skimmed milk and 0.1%
837 Tween® 20 (TBS-T), the blots were incubated overnight at 4°C with antibodies against (P)RR
838 (anti-ATP6IP2; 1:1000; ab40790; Abcam plc., Cambridge, UK) and CYP17A1 (anti-
839 cytochrome P450 17A1; 1:100; ab80206; Abcam plc., Cambridge, UK), with gentle agitation.
840 Subsequently, the blots were washed 3 times for 5 min in TBS-T. The blots were then
841 incubated with a goat anti-rabbit secondary antibody (diluted 1:2,000; IgG-HRP; sc-2004;
842 Santa Cruz Biotechnology, Inc., Dallas TX, USA) for 1 h while being agitated, which was
843 followed by 3 5-min washes in TBS-T. Immunoreactivity was detected using the Clarity™
844 Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in accordance with
845 the manufacturer's instructions. The images were analyzed using the ChemiDoc™ XRS+
846 imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blots were incubated
847 for 1 h at 50ºC in a western blot stripping buffer, which comprised β-mercaptoethanol, 20%
848 SDS, and 1 M Tris-HCl (pH 6.8). Then, the membranes were washed 3 times with TBS-T,
849 with each wash lasting 20 min, and the membranes were re-blotted with an anti-beta actin
850 antibody (diluted 1: 5,000; control; ab8227; Abcam plc., Cambridge, UK). The specificity of
851 the (P)RR antibody was verified by antigen blocking using the human ATP6IP2 peptide
852 (diluted 1: 1000; ab40790; Abcam plc., Cambridge, UK), as previously described (Nostramo
853 *et al.* 2015).
854
855 *Androstenedione and testosterone measurements by Ultra High-Performance Liquid*
856 *Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS)*

857 The identification and quantification of androstenedione and testosterone in the culture
858 medium were performed by Ultra High-Performance Liquid Chromatography-Tandem Mass
859 Spectrometry (UHPLC-MS/MS), as previously described (Rigo *et al.* 2015). Briefly, the
860 samples were diluted 1: 5 in acetonitrile and injected in the UHPLC-MS/MS system. An
861 analytical solution (internal pattern) for androstenedione and testosterone was added to the
862 mixture (to reach a final concentration of 50 ng L^{-1}) and was injected again in the system. The
863 linearity and the detection limits of each analyte were determined using analyte solutions with
864 concentration ranging from 50 to 5000 ng L^{-1}. The spectrophotometer was operated in
865 selected reaction monitoring (SRM) mode with 2 transitions for each analyte. The ionization
866 mode used was electrospray ionization in positive mode for androstenedione and testosterone,
867 with a column oven temperature of 40ºC, a pressure of 15,000 psi, a capillary of 2.8 kV, a
868 desolvation temperature of 500ºC, a gas flow rate of 800 L h^{-1}, a collision gas (argon) flow
869 rate of 0.15 mL min^{-1}, and a source temperature of approximately 150ºC. The mobile phase
870 consisted of aqueous solution as solvent A and 0.05% ammonium hydroxide plus methanol as
871 solvent B. A boiling gradient was used with a flow rate of 0.150 mL min^{-1}, an injection
872 volume of 10 µL, and a total running time of 5 min. The limit of detection (LOD) was 0.003
873 µg L^{-1} and limit for quantification (LOQ) was 0.01 µg L^{-1} for testosterone and
874 androstenedione.
875
876 *Estradiol, progesterone, androstenedione, and testosterone measurements by*
877 *electrochemiluminescence immunoassay*
878 The identification and quantification of E2 (coefficient of variation [CV] 1.5%;
879 sensitivity 0.0118 ng mL^{-1}), P4 (CV 1.23%; sensitivity 0.21 ng mL^{-1}), androstenedione (CV
880 6.2%; sensitivity 0.3 ng mL^{-1}) and testosterone (CV 2.7%; sensitivity 0.1 ng mL^{-1}) in the
881 follicular fluid were performed by electrochemiluminescence immunoassay (ADVIA

Centaur® P4 assay; Bayer Diagnostics, Tarrytown, NY, USA) in accordance with the manufacturer's protocol.

Statistical analysis

The ovulation rates following different treatments were statistically analyzed using the Categorical Data Analysis Procedures (CATMOD) procedure for categorical data modeling. The gene-expression and hormonal-synthesis data were tested for normality using the Shapiro–Wilk test, and normalized when necessary. The treatment effect in gene-expression and hormonal-synthesis were assessed by paired Student's *t*-test. Multiple-comparisons between times or treatments were analyzed using least-squares means. Statistical analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA), and the significance level adopted was 5%. Ovulation data are presented as percentages and gene-expression and homonal-synthesis results are presented as the mean ± standard error of the mean.

Results

(Pro)renin receptor mRNA and protein expression increased in theca cells after treating cows with GnRH

(P)RR mRNA (Fig. 1*a*) and protein (Fig. 1*b*) levels increased dramatically in bovine theca cells isolated from preovulatory follicles at 6 h post-GnRH administration *in vivo* ($P < 0.05$). While (P)RR transcript levels were not different at other timepoints after GnRH administration (0, 3, 12, and 24 h; $P > 0.05$), the relative abundance of (P)RR protein levels were constant between 0 and 3 h, representing higher expression relative to that observed after 12 and 24 h ($P < 0.05$).

907 *LH induces (P)RR mRNA expression in cultured theca cells*

908 Based on results showing that the (P)RR mRNA and protein expression levels were
909 elevated at 6 h after GnRH treatment, we examined whether LH could stimulate (P)RR
910 transcription in theca cells cultured for 6 h. The LH increased (P)RR mRNA compared with
911 control group ($P < 0.05$; Fig. 2).

912

913 *The (pro)renin receptor was not essential for bovine ovulation*

914 Blocking the intrafollicular (pro)renin receptor in the preovulatory follicle did not
915 completely impair ovulation. Two out of 6 cows (33.4%) did not ovulate, while all cows from
916 the control group underwent ovulation (4 out of 4).

917

918 *Effects of prorenin on ADAM17, AREG, and EREG mRNA in theca cells in vitro*

919 The metalloproteinase ADAM17 mRNA was not affected by prorenin or LH in theca
920 cells cultured for 24 h, compared to control group ($P > 0.05$; data not shown). The levels of
921 AREG and EREG mRNA were very low or undetectable in theca cells.

922

923 *EGFR signaling was not required for LH-induced (P)RR expression, but regulated*
924 *CYP17A1 expression in preovulatory theca cells in vivo*

925 The intrafollicular injection of an EGFR inhibitor (AG1478) did not decrease LH-
926 induced (P)RR protein expression in theca cells compared to control group at 6 h post-
927 intrafollicular injection (Fig. 3*a*). However, intrafollicular injection with AG1478 increased
928 CYP17A1 protein expression in theca cells relative to that observed with saline treatment at 6
929 h after intrafollicular injection (Fig. 3*b*). Consequently, androstenedione (Fig. 3*c*) and
930 testosterone (Fig. 3*d*) tended to increase in the follicular fluid ($P < 0.1$). The control and

931 AG1478 groups did not differ in terms of E2 levels in the follicular fluid (Fig. 3*e*), but P4
932 (Fig. 3*f*) appeared to decrease following AG1478 treatment ($P < 0.1$).
933
934 *Lack of an effect of prorenin on steroidogenesis in cultured theca cells*
935 Two in vitro experiments were designed to verify the effect of prorenin/(P)RR on
936 steroidogenesis in theca cells. Supplementation with LH and prorenin at concentrations of
937 0.01, 0.1, and 1 nM in the culture medium did not increase STAR (Fig. 4*a*), CYP11A1 (Fig.
938 4*b*), or HSD3B2 (Fig. 4*c*) mRNA expression compared to that observed in theca cells cultured
939 for 24 h in the presence or absence of LH ($P > 0.05$). Treatment with LH plus 0.1 nM of
940 prorenin significantly decreased CYP17A1 mRNA expression in theca cells cultured for 24 h,
941 compared with that observed in control cells (without LH; Fig. 4*d*). However,
942 androstenedione (Fig. 4*e*) and testosterone (Fig. 4*f*) synthesis were not regulated by prorenin
943 and/or LH in theca cells cultured for 24 h.
944 The hypothesis that the (P)RR is required for steroidogenesis in theca cells was also
945 tested using aliskiren, which is an (P)RR inhibitor, at 0.1 µM. Treatment with aliskiren plus
946 prorenin or with prorenin plus LH did not regulate the mRNA expression of STAR or the
947 steroidogenic enzymes CYP11A1, HDS3B2, or CYP17A1 between treatments ($P > 0.05$; data
948 not shown). Similarly, no effect was observed on androstenedione and testosterone production
949 (data not shown).
950
951 **Discussion**
952 The results obtained in this study demonstrated that (P)RR transcription and protein
953 production increased in theca cells following the gonadotropin surge in cattle challenged with
954 GnRH. The (P)RR transcript was also induced by LH in cultured theca cells. The
955 intrafollicular blocking of (P)RR impaired 2 out of 6 GnRH-induced ovulations. However,

956 prorenin did not induce an increase in ADAM17 mRNA in theca cells, and LH-induced
957 (P)RR expression was not regulated by an EGF-like pathway. Despite the marked changes of
958 (P)RR expression in theca cells in response to LH, a role of prorenin in regulating
959 steroidogenesis was not observed. In addition, this study provides the first *in vivo*
960 confirmation that CYP17A1 expression may be regulated through EGFR signaling after an
961 LH surge in bovine theca cells.

962 In the present study, (P)RR mRNA and protein expression were markedly increased in
963 theca cells 6 h after GnRH injection in cattle. These results support the observation of
964 increased prorenin levels in follicular fluid after LH surge (Glorioso *et al.* 1986; Hagemann *et*
965 *al.* 1994). The temporal changes expected to occur after GnRH analog treatment in cows are
966 well established: an endogenous LH surge within 2 h; an immediate increase in E2 (3 h), and
967 a second, larger increase in P4 levels in the follicular fluid at 24 h (Komar *et al.* 2001; Bridges
968 *et al.* 2006; Fortune *et al.* 2009; Tonellotto dos Santos *et al.* 2012). In temporal studies
969 performed in woman, prorenin seems to be induced by LH and E2, and may play a role in P4
970 synthesis (Sealey *et al.* 1985) (Itskovitz *et al.* 1987), supporting our data obtained at different
971 time points during the preovulatory period in cattle.

972 The (P)RR acts through of both Ang II-dependent and -independent pathways
973 (Nguyen and Contrepas 2008). Recently, we identified a role for prorenin/(P)RR in the
974 resumption of meiosis in bovine oocytes (Dau *et al.* 2016), as observed for Ang II (Barreta *et*
975 *al.* 2008). However, intrafollicular (P)RR blocking in the preovulatory follicle did not
976 significantly compromise the rate of ovulation, as evidenced by injecting Ang II antagonists
977 into ovulatory follicles (Ferreira *et al.* 2007). This finding may be explained by the presence
978 of active Ang II receptors (AGTR1 and AGTR2), which seems to have been supported by the
979 lack of (P)RR signaling, which was blocked by aliskiren. The intra- and extracellular
980 signaling of the (P)RR is inhibited by aliskiren (Biswas *et al.* 2010; Ferri *et al.* 2011; Ma *et al.*

981 2012), which can also efficiently block the resumption of meiosis in bovine oocytes induced
982 by prorenin (Dau *et al.* 2016).

983 The involvement of the (P)RR in the EGF-like pathway was investigated as a potential
984 mechanism to clarify why 2 out of 6 cows did not ovulate, in contrast to control cows
985 showing 100% ovulation. The EGFR pathway is a critical mediator of the LH surge during
986 the induction of ovulation-related genes in follicular cells (Park *et al.* 2004), and a previous
987 report showed that prorenin binding to the (P)RR promotes ERK1/2 phosphorylation via
988 EGFR signaling in rat vascular smooth muscle cells (Liu *et al.* 2011) and in human embryo
989 kidney 293 cells (Daud *et al.* 1990). Moreover, the transcripts of genes related to the EGF-like
990 pathway, such as AREG and ADAM17, were increased in theca cells 24 h post-GnRH
991 administration in cattle (Li *et al.* 2009). Thus, we hypothesized that prorenin may act as a co-
992 factor of LH to induce ADAM17, AREG, and EREG expression in theca cells. However, LH
993 and/or prorenin did not stimulate changes in the abundance of ADAM17 mRNA in theca cells
994 cultured for 24 h. ADAM17 plays an important role in preovulatory follicles, releasing the
995 ectodomains of AREG and EREG and transactivating the EGFR (Park *et al.* 2004). Therefore,
996 the constant presence of ADAM17 mRNA suggests that its availability is not limiting to
997 EGFR transactivation. Nevertheless, the AREG and EREG transcripts were expressed at very
998 low or undetectable levels in cultured theca cells, as also observed in theca cells from bovine
999 preovulatory follicles 6 h post-GnRH (Sayasith *et al.* 2013). Thus, we considered that EGFR
1000 may act upstream of the (P)RR signaling pathway in theca cells.

1001 The intrafollicular blocking of EGFR did not regulate (P)RR protein expression in
1002 theca cells induced by GnRH, suggesting that the peak of gonadotropins may have induced
1003 (P)RR independently of the EGFR pathway. However, the present study evidenced the role of
1004 EGFR in ovarian steroidogenesis in cattle. Intrafollicular blocking of the EGFR pathway
1005 increased CYP17A1 expression in theca cells challenged with GnRH and tended to increase

1006 androstenedione and testosterone levels in follicular fluid. These data supported the results
1007 obtained from bovine theca cell culture, wherein the supplementation of EGF in the culture
1008 medium decreased androstenedione production induced by LH and IGF-I (Spicer and Stewart
1009 1996). Moreover, the production of androstenedione in follicular fluid and CYP17A1 mRNA
1010 in theca cells decreased 6 h after cows were treated with GnRH injection (Komar *et al.* 2001).
1011 Thus, we confirmed EGFR as a potentially new modulator of the steroidogenic enzyme
1012 CYP17A1 in theca cells after GnRH. Alternatively, in our study, when EGFR was blocked,
1013 the P4 levels appeared to decrease in the follicular fluid, supporting the increase observed in
1014 conversion into androgens. In addition, P4 levels are increased in the follicular fluid at 6 h
1015 post-GnRH treatment (Fortune *et al.* 2009). Therefore, EGFR also may be required for P4
1016 synthesis induced by gonadotropins.

1017 The (P)RR did not appear to be involved in regulating the expression of genes related to
1018 ovulation in theca cells. Thus, the (P)RR may be induced by LH to modulate thecal
1019 steroidogenesis. Many factors may account for these findings, considering that the (P)RR-
1020 bound prorenin or renin stimulates Ang II production by to increase cleavage of AngI and
1021 actives the ERK1/2 pathway (Nguyen 2008; Uraoka *et al.* 2009). One consideration is that
1022 Ang II can increase hCG-induced testosterone synthesis in rabbit theca cells cultured *in vitro*
1023 (Feral *et al.* 1995). In addition, ERK1/2 phosphorylation is required for androgen synthesis by
1024 bovine theca cells (Tajima *et al.* 2005). However, this was not confirmed in the present study.
1025 Only LH (100 ng mL^{-1}) plus 0.1 nM prorenin regulated CYP17A1 mRNA in theca cells after
1026 24 h of culture, which was consistent with the depletion of CYP17A1 mRNA in theca cells in
1027 cattle at 24 h post-GnRH administration (Dieleman *et al.* 1983; Voss and Fortune 1993;
1028 Komar *et al.* 2001). The absence of the role of prorenin/(P)RR on thecal steroidogenesis in
1029 our culture system was confirmed using prorenin and/or aliskiren, which did not affect the

1030 transcription of steroidogenic enzymes and the synthesis of androstenedione and testosterone
1031 by theca cells.

1032 The present study provides insights into the function of the (P)RR as an
1033 alternative RAS pathway in the ovulatory process. Interestingly, this system and EGF
1034 signaling are involved in polycystic ovarian syndrome (PCOS), a common endocrine disorder
1035 of women that is characterized by increased androgen synthesis (Palumbo *et al.* 1993;
1036 Poretsky *et al.* 1999; Armanini *et al.* 2012; Alphan *et al.* 2013; Arefi *et al.* 2013). Thus, our
1037 results suggest that EGFR dysregulation may be involved in the development and/or in
1038 maintaining PCOS in woman, although this remains to be explored in future studies. In
1039 addition, signaling pathways activated by (P)RR-bound prorenin are new
1040 pharmacotherapeutic targets to be studied for reproductive disorder.

1041 High expression of (P)RR mRNA and protein in theca cells determined in this study
1042 occurred before the Ang II levels plateaued in follicular fluid, as described previously by our
1043 research group (Siqueira *et al.* 2012). The (P)RR transcript was induced by LH in theca cells,
1044 as observed for AGTR2 mRNA encoding the Ang II receptor (Siqueira *et al.* 2012).
1045 Intrafollicular injection with a (P)RR antagonist revealed that (P)RR was partially required for
1046 ovulation. This finding highlight the requirement for alternative signaling pathways beyond
1047 (P)RR in the ovulation process, which may be limited to Ang II production. It is well known
1048 that Ang II plays a critical role in ovulation in cattle (Ferreira *et al.* 2007). In our culture
1049 system, prorenin neither affected the regulation of ovulation-related genes nor thecal
1050 steroidogenesis. Despite the fact that EGFR regulates the CYP17A1 protein in theca cells
1051 from preovulatory follicles, EGFR did not mediate (P)RR induction post-GnRH treatment. In
1052 conclusion, LH induced (P)RR in theca cells and (P)RR appeared to be an alternative target
1053 for RAS to impact the ovulation process, independently of the EGFR pathway. In addition, it
1054 was observed that EGFR is a potential regulator of CYP17A1 in theca cells in cattle.

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1056 **Acknowledgments**

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1232 **Figure legends**

1233 **Fig. 1.** Profile of (pro)renin receptor ([P]RR) (mean ± standard error) (*a*) mRNA and
1234 (*b*) protein expression in bovine theca cells isolated from preovulatory follicles obtained at 0
1235 (n = 3), 3 (n = 3), 6 (n = 3), 12 (n = 3), or 24 h (n = 4) after GnRH was administered to induce
1236 an LH surge. Western blot images revealed a specific band at approximately 42 kDa for the
1237 (P)RR and at 42 kDa for β-actin (ACTB). Representative blots from each time point and
1238 summaries of densitometric analysis are shown. The different letters indicate statistical
1239 differences observed between groups ($P < 0.05$).

1240 **Fig. 2.** Effect of LH (100 ng mL^{-1}) on (P)RR mRNA expression in theca cells cultured
1241 *in vitro* for 6 h. Different letters indicate statistical differences observed between groups ($P <$
1242 0.05). Experiment was performed in triplicate.

1243 **Fig. 3.** Effect of intrafollicular EGFR blocking using AG1478 (an EGFR tyrosine
1244 kinase inhibitor; n = 5; 5 μM) or saline (control; n = 4) on (*a*) (P)RR and (*b*) CYP17A1
1245 protein expression in theca cells, and the levels of (*c*) androstenedione, (*d*) testosterone, (*e*)
1246 estradiol, and (*f*) progesterone in the follicular fluid of cows that received intramuscular
1247 injection of GnRH, at 6 h post-treatment. Western blot images revealed a specific band at
1248 approximately 42 kDa for the (P)RR, at 60 kDa for CYP17A1, and at 42 kDa for β-
1249 actin(ACTB). Representative blots from each group and a summary of the densitometric
1250 analysis are shown. Different letters indicate the statistical differences between groups ($P <$
1251 0.05).

1252 **Fig. 4.** The dose-response effects of combined treatment with prorenin (0.01, 0.1, and
1253 1 nM) and LH (100 ng mL^{-1}) on mRNA expression of (*a*) STAR, (*b*) CYP11A1, (*c*) HSD3B2,
1254 and (*d*) CYP17A1 in theca cells, and on (*e*) androstenedione and (*f*) testosterone secretion in
1255 the culture medium, at 24 h post-treatment. The results are shown as the mean ± standard
1256 error. Different letters indicate the statistical differences among groups ($P < 0.05$).
1257 Experiment was performed in triplicate.

1258 **Table 1 Information about primers used in the expression analysis of candidate**

1259 **mRNAs**

1260 F, forward primer; R, reverse primer

Gene		Primer sequence	Reference or accession number
GAPDH	F:	GATTGTCAGCAATGCCTCCT	Ferreira *et al.* (2011b)
	R:	GGTCATAAGTCCCTCCACGA	
PPIA	F:	GGTCATCGGTCTCTTTGGAA	Ledoux *et al.* (2006)
	R:	TCCTTGATCACACGATGGAA	
(P)RR	F:	TGATGGTGAAAGGAGTGGACAA	Ferreira *et al.* (2011b)
	R:	TTTGCCACGCTGTCAAGACT	
ADAM17	F:	TTCATGGGACAATGCAGGTTT	XM_002691486.2
	R:	GAAGTGCCTTTCACCAGGTTTT	
AREG	F:	CCATTTTCTTGTCGAAGTTTCTTTC	Li *et al.* (2009)
	R:	TGTTTTTATTACAATCCTGCTTCGAA	
EREG	F:	ACTGCACAGCATTAGTTCAAACTGA	XM_010806226.1
	R:	TGTCCATGCAAACAGTAGCCATT	
STAR	F:	CCCAGCAGAAGGGTGTCATC	Buratini *et al.* (2005)
	R:	TGCGAGAGGACCTGGTTGAT	
HSD3B2	F:	GCCCAACTCCTACAGGGAGAT	Orisaka *et al.* (2006)
	R:	TTCAGAGCCCACCCATTAGCT	
CYP11A1	F:	CTTGCACCTTTCTGGCTAGG	Orisaka *et al.* (2006)
	R:	AAGGGGAAGAGGTAGGGTGA	
CYP17A1	F:	CCATCAGAGAAGTGCTCCGAAT	Lagaly *et al.* (2008)
	R:	GCCAATGCTGGAGTCAATGA	
CYP19A1	F:	GTGTCCGAAGTTGTGCCTATT	Luo and Wiltbank (2006)
	R:	GGAACCTGCAGTGGGAAATGA	

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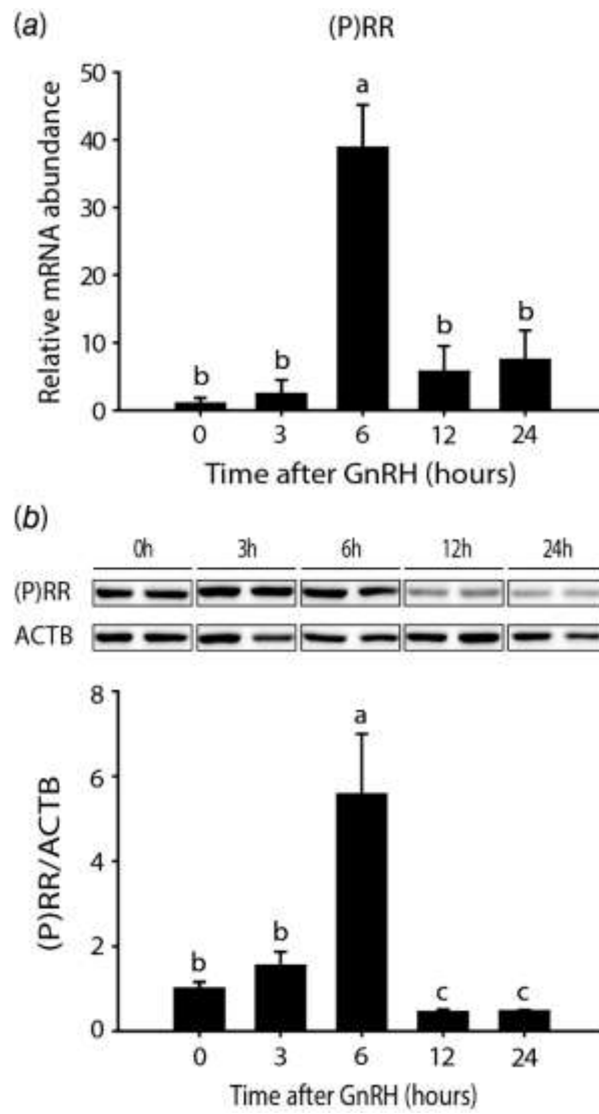
1272 **Figure 1**



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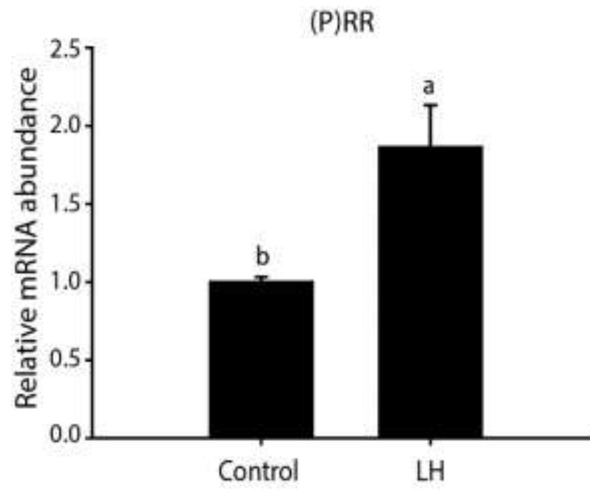
1275 **Figure 2**



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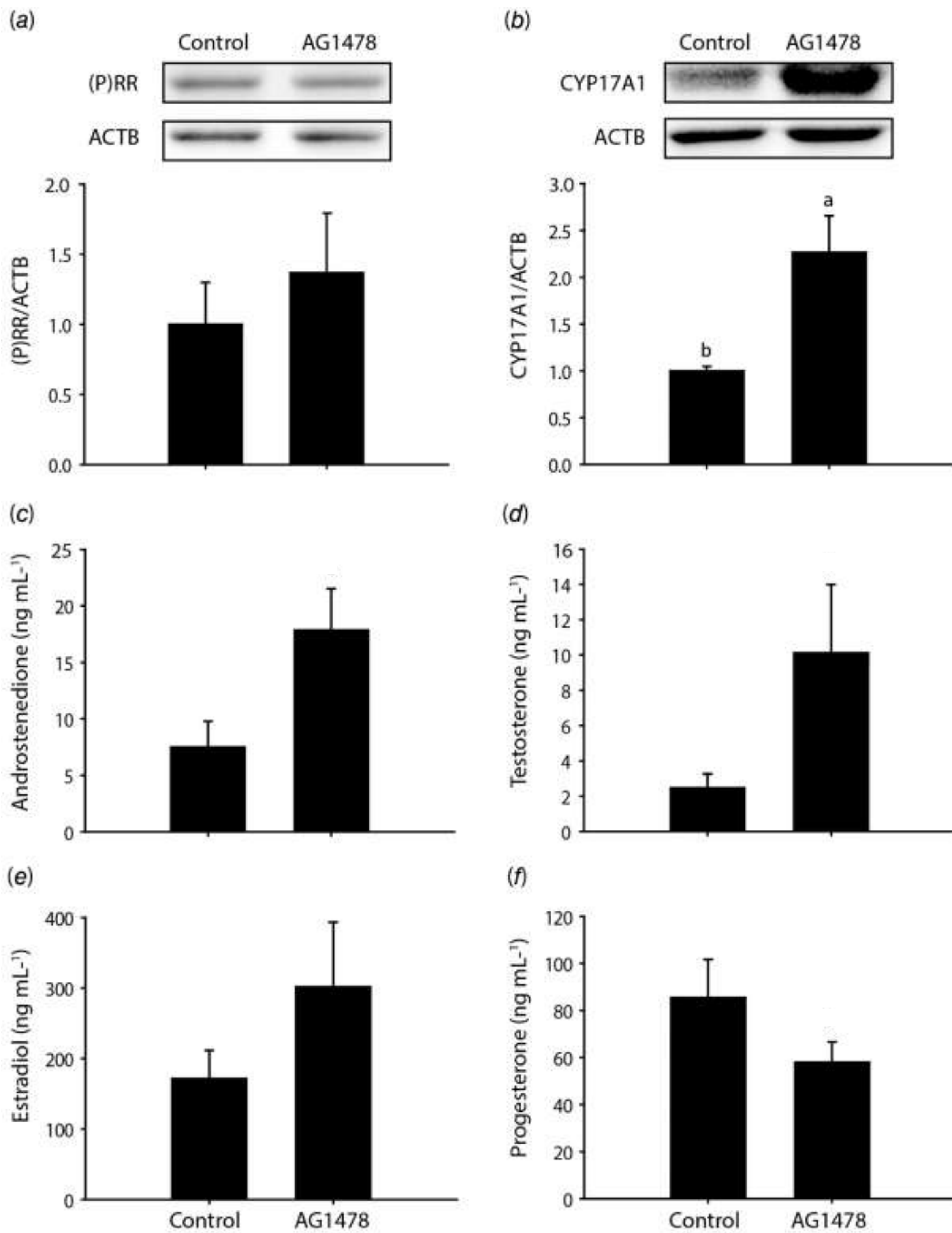
1278 **Figure 3**



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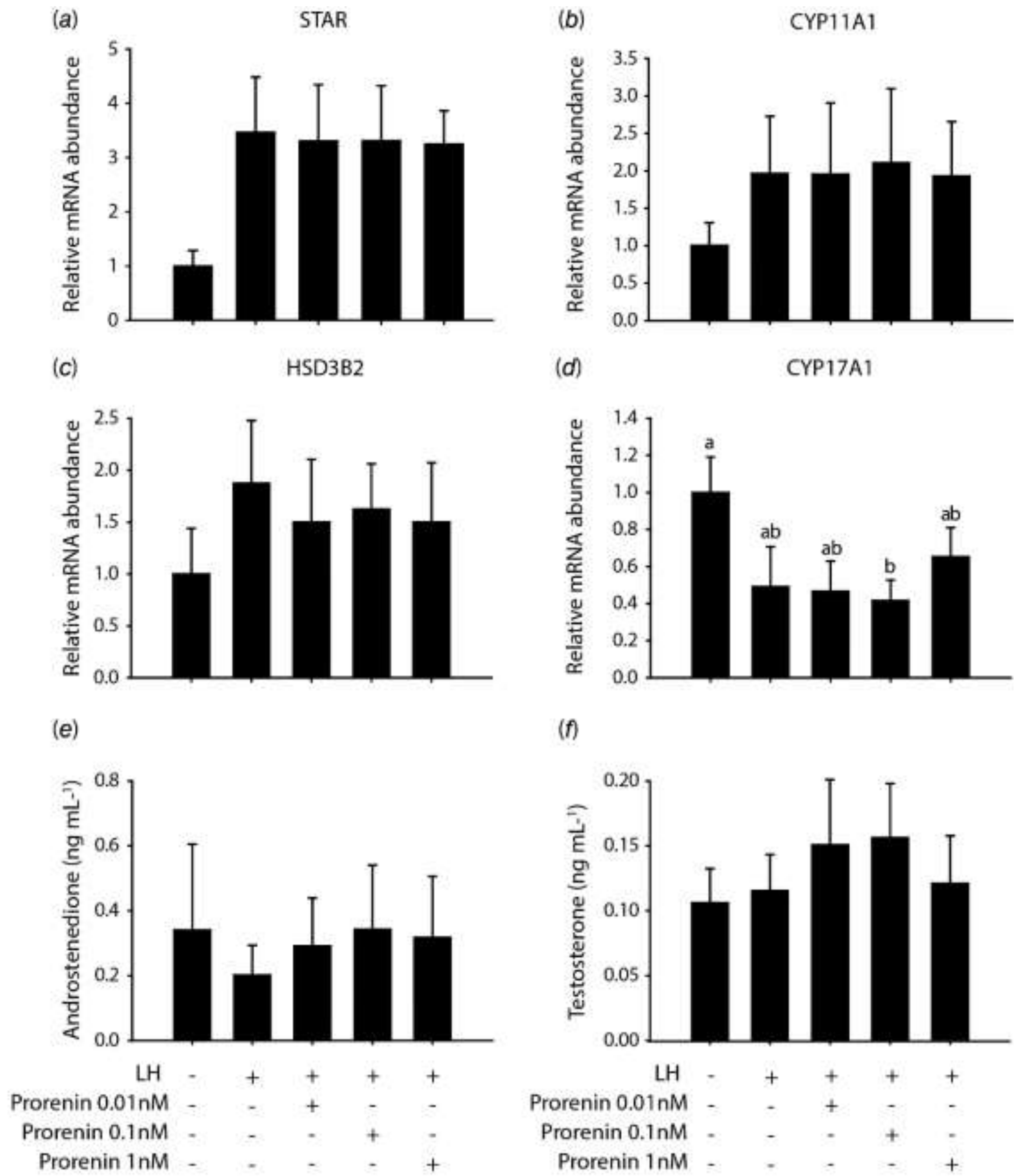
1281 **Figure 4**



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6. ARTIGO 4

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

Prorenin induces progesterone synthesis via (pro)renin receptor in cattle

Andressa Minussi Pereira Dau, Paulo Roberto Antunes da Rosa, Joabel Tonellotto dos Santos, Juliana Germano Ferst, Mariana Priotto de Macedo, Monique Rovani, Fabio Vasconcellos Comim and Paulo Bayard Dias Gonçalves.

REPRODUCTION, 2017

1283 **Prorenin induces progesterone synthesis via (pro)renin receptor in cattle**

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1285 Andressa Minussi Pereira Dau[1], Paulo Roberto Antunes da Rosa[1], Joabel Tonellotto
1286 dos Santos[1], Juliana Germano Ferst[1], Mariana Priotto de Macedo[1], Monique Rovani[1], Fabio
1287 Vasconcellos Comim[1,2], and Paulo Bayard Dias Gonçalves[1]

1288

1289 [1]Laboratory of Biotechnology and Animal Reproduction, BioRep, Federal University
1290 of Santa Maria, Roraima Avenue 1000, 97105-900 Veterinary Hospital, Santa Maria, RS,
1291 Brazil.

1292 [2]Department of Medical Clinic, Faculty of Medicine, Federal University of Santa
1293 Maria, Roraima Avenue 1000, 97105-900, Santa Maria, RS, Brazil.

1294

1295 **Correspondance should be addressed to** P B D Gonçalves, Departamento de Clínica
1296 de Grandes Animais, Hospital Veterinário, Universidade Federal University of Santa Maria,
1297 97105-900 Santa Maria, RS, Brazil. Email address: bayard@ufsm.br

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1299 **Key words:** luteinization, corpus luteum, cattle.

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1301 **Short title:** Prorenin induces progesterone synthesis

1302

1303 **Abstract**

1304 Despite the importance of the renin-angiotensin-aldosterone system in mammalian
1305 reproduction, the role of the (pro)renin receptor, (P)RR, in the corpus luteum remains
1306 speculative. The aim of this study was to evaluate molecular profiles of the prorenin, (P)RR,
1307 and profibrotic proteins during luteinization and luteolysis. Additionally, we investigated the
1308 effect of prorenin in promoting progesterone synthesis through (P)RR, and whether it
1309 occurred via mitogen-activated protein kinase and/or epidermal growth factor (EGF) signaling
1310 pathways. We report a remarkable increase in the expression of prorenin and (P)RR mRNA *in*
1311 *vivo* in luteal tissue on day 10 compared to day 5 of the estrous cycle ($P < 0.05$). Prorenin and
1312 (P)RR remained present during luteolysis and profibrotic genes increased significantly after
1313 prostaglandin treatment. Intrafollicular treatment with a (P)RR inhibitor, aliskiren, decreased
1314 serum progesterone levels on day 6 after addition of gonadotropin-releasing hormone to cows
1315 that ovulated ($P < 0.05$). Prorenin induced progesterone synthesis via (P)RR in cultured luteal
1316 tissue, even in the presence of saralasin, an angiotensin receptor antagonist. However,
1317 synthesis was abolished by AG1478, an EGF receptor tyrosine kinase inhibitor ($P < 0.05$).
1318 Prorenin induced phosphorylation of the mitogen-activated protein kinase ERK1 in cultured
1319 luteal tissue ($P < 0.05$) and progesterone synthesis was partially reduced by blocking ERK
1320 activation with PD0325901. In summary, we report for the first time that prorenin promotes
1321 progesterone synthesis by acting through (P)RR, through a process that, at least in cultured
1322 bovine luteal tissue, is mediated by ERK1/2 and EGF receptor pathways.

1323

1324 **Introduction**

1325 The (pro)renin receptor, (P)RR, has been hypothesized to be an important modulator
1326 of the renin-angiotensin-aldosterone system (RAS) in ovarian cells, such as oocytes, theca,
1327 and granulosa cells (Ferreira *et al.* 2011b, Dau *et al.* 2016). Emerging evidence supports the

1328 existence of a functional RAS in the corpus luteum (CL) of mammals. Nevertheless, the role
1329 of a (P)RR-dependent system in the CL during luteinization and/or luteolysis remains unclear.

1330 The effect of renin-independent prorenin on progesterone (P4) synthesis was initially
1331 proposed in the 80's (Sealey *et al.*, 1986). Luteinizing hormone-induced prorenin was found
1332 to stimulate P4 synthesis (Sealey *et al.* 1985, Itskovitz *et al.* 1987). In superovulated heifers,
1333 prorenin levels in follicular fluid positively correlated with P4 concentration (Hagemann *et al.*
1334 1994). However, only in 2002, was the effect of prorenin revealed to depend on (P)RR in
1335 human fetal mesangial cell line (Nguyen *et al.* 2002).

1336 Prorenin has high affinity for (P)RR (Nabi *et al.* 2009), which is widespread in bovine
1337 ovaries (Dau *et al.* 2016). In addition, (P)RR seems to play a role in follicular divergence in
1338 cattle (Ferreira *et al.* 2011b), resumption of meiosis in cattle (Dau *et al.* 2016), and in early
1339 gestation placentae in women (Pringle *et al.* 2011).

1340 Binding of prorenin to (P)RR stimulates the angiotensin II (Ang II) pathway,
1341 promoting the cleavage of angiotensinogen to angiotensin I (Nguyen *et al.* 2002, Uraoka *et al.*
1342 2009). Moreover, (P)RR activation mediates epidermal growth factor receptor (EGFR)
1343 transactivation, as well as mitogen-activated protein kinases (MAPKs) ERK1 (p44) and
1344 ERK2 (p42) phosphorylation in rat vascular smooth muscle cells (Liu *et al.* 2011) and human
1345 embryonic kidney (HEK) 293 cells (Shibayama *et al.* 2013). Profibrotic genes are also
1346 upregulated by phosphorylation of ERK1/2 following prorenin binding to (P)RR on
1347 mesangial and aortic smooth muscle cells (Nguyen *et al.* 2002, Huang *et al.* 2006, Ferri *et al.*
1348 2011). The prorenin/(P)RR pathway in non-reproductive cells has been related to luteinization
1349 or luteolysis in the CL. In bovines, Ang II induces P4 synthesis in the early CL, while
1350 inhibiting P4 release from mid-cycle CL (Hayashi & Miyamoto 1999, Kobayashi *et al.* 2001).
1351 EGFR and ERK1/2 signaling pathways have also been related to CL maturation and P4
1352 synthesis (Murray *et al.* 1993, Pan *et al.* 2014). Profibrotic molecules have been implicated in

1353 luteal regression (Casey *et al.* 2005, Kliem *et al.* 2007, Hou *et al.* 2008, Maroni & Davis
1354 2012).
1355 The aim of this study was to evaluate the molecular expression profile of prorenin,
1356 (P)RR and profibrotic genes in luteal tissue during luteinization and luteolysis. Additionally,
1357 we investigated the role of (P)RR in P4 synthesis in luteal cells and its relationships with
1358 EGFR and/or ERK1/2 signaling.
1359
1360 **Materials and methods**
1361 Experimental procedures with cattle were reviewed and approved by the local Animal
1362 Ethics Committee of the Federal University of Santa Maria (nr. 115/2014) in agreement with
1363 the National Council for the Control of Animal Experimentation (CONCEA), Brazilian
1364 Ministry of Science, Technology, and Innovation.
1365
1366 ***Chemicals***
1367 All chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA),
1368 unless otherwise indicated.
1369
1370 ***Animal procedures, intrafollicular injection, and CL collection***
1371 Thirty cows of European breed (*Bos taurus taurus*), aged 4–8 years, were supplied by
1372 Lion Farm. The animals were kept in their native field with free access to water and feed.
1373 Cows exhibiting normal estrous cycle were synchronized with an intramuscular injection of
1374 sodic cloprostenol (500 µg, PGF2A; Intervet, São Paulo, Brazil). Following estrus detection,
1375 ovulation was monitored by ultrasonography. The animals were randomly ovariectomized on
1376 days 5 (n = 4) and 10 (n = 5) after ovulation. Another group of cows with a CL of 10 days (0
1377 h) after estrus observation received 500 µg PGF2A by intramuscular injection; the animals

1378 were then randomly ovariectomized after 12 (n = 3), 24 (n = 4), and 48 h (n = 4). This *in vivo*
1379 model was adapted from previous protocols (Shirasuna *et al.* 2008, Shirasuna *et al.* 2012a)
1380 and confirmed by P4 serum measurements. CL samples were obtained at specific stages of the
1381 estrous cycle, dissected from the stromal tissue as previously described (Shirasuna *et al.*
1382 2008), deposited in cryogenic tubes, frozen immediately in liquid nitrogen, and stored at -80
1383 °C for mRNA and protein evaluation.

1384 Luteolysis was induced in 20 cows (4-8 years of age) supplied by Tiger Farm and kept
1385 in their native field with free access to water and feed. A new follicular wave was initiated
1386 using a previously described hormonal protocol (Tonellotto dos Santos *et al.* 2012, Siqueira *et*
1387 *al.* 2013). Briefly, cows were given two intramuscular (IM) injections of 250 mg PGF2A at
1388 12-h intervals and one IM injection of 2 mg estradiol benzoate. Additionally, an intravaginal
1389 P4 device (1 g P4, DIB®; Intervet) was removed on day 9. The presence of preovulatory
1390 follicles (≥ 12 mm in diameter) was evaluated by transrectal ultrasonography using an 8-MHz
1391 linear-array transducer (AquilaVet scanner; Pie Medical Equipment BV, Maastricht,
1392 Netherlands) 12 h after removal of the intravaginal P4 device on day 0. Only cows with
1393 follicles ≥ 12 mm in diameter were treated with 100 µg of gonadorelin acetate (Profertil®;
1394 Tortuga, Brazil), and were subjected to intrafollicular injection guided by ultrasound
1395 (AquilaVet) using a 7.5-MHz convex probe. A double-needle system was used to inject
1396 aliskiren and/or vehicle (phosphate-buffered saline, PBS) in the preovulatory follicle. The
1397 final intrafollicular concentration of aliskiren (10 µmol/L) was calculated based on estimates
1398 of the follicular fluid volume (Ferreira *et al.* 2007, Ferreira *et al.* 2011a) and was established
1399 by previous studies (Park *et al.* 2004, Dau *et al.* 2016). The cows were examined by
1400 transrectal ultrasonography 2 h after intrafollicular treatment; those with a reduction of > 1
1401 mm in the diameter of the injected follicles were discarded.

1402

1403 ***Recovery of luteal tissue for in vitro experiments***

1404 Cow ovaries were obtained from a local abattoir, and transported to the laboratory in
1405 saline solution containing penicillin (100 IU/mL) and streptomycin sulfate (50 µg/mL) at 4
1406 °C. CL samples with a diameter of 10–20 mm, showing a bloody surface and pink to tan or
1407 orange color were selected. Specimens were defined as between early II (days 5–6 after
1408 ovulation) and mid (days 8–12 after ovulation) luteal stage according to previously described
1409 criteria (Ireland *et al.* 1980, Miyamoto *et al.* 2000). CL (n = 5/per replicate) were dissected
1410 from the ovaries and luteal slices were digested in Dulbecco's modified Eagle's medium
1411 (DMEM)/F12 (Gibco, Grand Island, NY, USA) containing 1 mg/mL collagenase for 1 h at 37
1412 °C.
1413
1414 ***CL cultures***

1415 Following enzymatic digestion, the mixed luteal cells were washed three times in
1416 culture medium, filtered through 70-µm Nylon Mesh strainers (Fisher Scientific, Shanghai,
1417 China) and seeded in 90-mm plates. Cells were cultured in DMEM/F12 supplemented with
1418 100 IU/mL penicillin, 50 µg/mL streptomycin sulfate, and 10% fetal bovine serum (FBS; v/v)
1419 at 37 °C in an atmosphere of 5% CO_2 (v/v) and saturated humidity for five days. Medium was
1420 changed every two days. At the end of the culture period, cells were trypsinized, seeded in 96-
1421 well plates (Corning, Corning, NY, USA) at a concentration of 3×10^4 viable cells/well, and
1422 cultured for 12 h under the same conditions as above. Cell viability was determined by
1423 staining with 0.4% trypan blue (v/v) and flow cytometry analysis (FACSVerse™, BD
1424 Biosciences, Franklin Lakes, NJ, USA) using FITC-Annexin V (5 µL; BD Biosciences) and
1425 propidium iodide (50 µg/mL) according to the manufacturer's instructions. Viability was over
1426 85% for all replicates. Prior to treatment, cells were washed twice and pre-equilibrated with
1427 200 µL of FBS-free medium containing 0.1% bovine serum albumin (v/v) for 2 h.

1428

1429 ***RNA extraction, reverse transcription, and quantitative real-time polymerase chain***
1430 ***reaction (qRT-PCR)***

1431 Total RNA extraction was performed using TRIzol® (Invitrogen, Carlsbad, CA, USA)
1432 in accordance with the manufacturer's instructions. Quantity and purity of RNA were verified
1433 with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) based
1434 on the ratios of absorbance at 260 nm and 280 nm. Only RNA samples with a purity > 1.8
1435 were used. RNA integrity was verified by visualization of ribosomal RNA (rRNA) on a 1.2%
1436 agarose gel (w/v). Complementary DNA (cDNA) was synthesized starting from RNA (500
1437 ng) pretreated with 0.1 U deoxyribonuclease I (amplification grade, Invitrogen) and incubated
1438 at 37 °C for 5 min, followed by incubation at 65 °C for 10 min. Subsequently, DNA-free
1439 RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit® (Bio-Rad
1440 Laboratories, Hercules, CA, USA) in accordance with the manufacturer's instructions.

1441 qRT-PCR was conducted in a CFX384 Touch™ ® instrument (Bio-Rad Laboratories)
1442 using the GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) and specific bovine
1443 primers (0.2 µmol/L; Table 1). Primer sequences were based on published data or designed
1444 using Primer Express version 3 software (Invitrogen), and synthesized by Invitrogen.
1445 Transcripts were amplified following an initial denaturation step at 95 °C for 3 min, 40 cycles
1446 at 95 °C for 10 s and 60 °C for 1 min, and a melting curve from 65 °C to 95 °C with 5-s steps
1447 of 0.5 °C. Reactions were performed in duplicate, and melting curves were analyzed to
1448 determine the product's identity. Variations in mRNA levels were normalized to the mean of
1449 the reference genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and cyclophilin
1450 (*PPIA*) (Casey *et al.* 2005, Hou *et al.* 2008). Relative expression was calculated as previously
1451 described (Pfaffl 2001).

1452

1453 ***Western blotting***

1454 Proteins from luteal tissue were extracted using a radioimmunoprecipitation assay
1455 buffer and western blots were performed as previously described (Dau *et al.* 2016). Proteins
1456 were boiled at 95 °C for 5 min, subjected to 12% sodium dodecyl sulfate (SDS; w/v)
1457 polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes. After
1458 blocking for 3 h using 5% skim milk in Tris-buffered saline (TBS; w/v) containing 0.1%
1459 Tween® 20 (TBS-T; v/v), membranes were incubated overnight with antibodies against (P)RR
1460 (1:1000, ab40790; Abcam plc, Cambridge, UK) and phospho-ERK1/2 (1:2000, #4370; Cell
1461 Signaling, Danvers, MA, USA) at 4 °C with gentle stirring. Subsequently, membranes were
1462 washed three times for 10 min each in TBS-T, incubated with a goat anti-rabbit horseradish
1463 peroxidase-conjugated secondary antibody (1:2000, sc-2004; Santa Cruz Biotechnology Inc.,
1464 Dallas TX, USA) for 1 h with agitation, and followed by three washes of 10 min each in TBS-
1465 T. Immunoreactivity was detected using the Clarity™ Western ECL Substrate (Bio-Rad
1466 Laboratories) in accordance with the manufacturer's instructions. Images were analyzed using
1467 the ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories). Membranes were incubated
1468 for 1 h at 50 °C with a western blot stripping buffer containing β-mercaptoethanol, 20% SDS
1469 (w/v), and 1 mol/L Tris-HCl, at pH 6.8, washed three times for 20 min each in TBS-T, and
1470 probed again with anti-ERK1/2 (1:1000, #4695; Cell Signaling) and/or anti-β-actin antibodies
1471 (1:5000, ab8227; Abcam plc). The specificity of the (P)RR (ATP6IP2) antibody was verified
1472 by antigen blocking using human ATP6IP2 peptide (1:1000, ab40790; Abcam plc) as
1473 described previously (Nostramo *et al.* 2015).

1474

1475 ***P4 measurements***

1476 Identification and quantification of P4 in culture medium and serum were performed
1477 using an electrochemiluminescence immunoassay (ADVIA Centaur® P4 assay; Bayer

1478 Diagnostics, Tarrytown, NY, USA) in accordance with the manufacturer's protocol.
1479 Sensitivity was 0.21 ng/mL and intra-assay and inter-assay coefficients of variation were 2.5
1480 and 3.1%, respectively.
1481

1482 ***Experimental design***

1483 *In vivo* experiments

1484 *Molecular profiles of (P)RR, prorenin, and profibrotic genes in the CL during*
1485 *luteinization and luteolysis*

1486 Ovaries with the CL in a specific luteal stage were randomly isolated by colpotomy-
1487 based ovariectomy (Drost *et al.* 1992) from individual cows on days 5 (n = 4) and 10 (n = 5)
1488 after ovulation, and at 12 (n = 3), 24 (n = 4), and 48 h (n = 4) post-PGF2A treatment. (P)RR
1489 protein levels in luteal tissue were assessed by western blot. Transcripts of *(P)RR*, prorenin,
1490 fibronectin 1 (*FN1*), plasminogen activator inhibitor 1 (*PAI1*), transforming growth factor
1491 beta-1 (*TGFB1*), and collagen type I in luteal tissue were evaluated by qRT-PCR.
1492

1493 *Effect of intrafollicular blocking of (P)RR on P4 synthesis during luteinization*

1494 The preovulatory follicle from each cow treated with gonadotropin-releasing hormone
1495 (GnRH) was injected with PBS (vehicle/control; n = 4) or 10 µmol/L aliskiren (n = 6), a
1496 potent renin and (P)RR inhibitor (Biswas *et al.* 2010). Ovulation was monitored by ultrasound
1497 at 24, 48, and 72 h after intrafollicular treatment as previously described (Ferreira *et al.* 2007).
1498 Briefly, ovulation was identified by disappearance of the preovulatory follicle between two
1499 consecutives ultrasound evaluations and CL formation. Blood was collected from the jugular
1500 vein on days 6 and 8 after follicular treatment and serum P4 was measured by
1501 electrochemiluminescence.
1502

1503 *In vitro* experiments

1504 *Effect of prorenin and (P)RR on P4 synthesis and luteal steroidogenesis*

1505 The effect of prorenin in steroidogenesis and P4 synthesis was evaluated after treating
1506 luteal tissue with three doses of prorenin (0.1, 1, and 10 nmol/L) for 4 h. (P)RR (*ATP6AP2*),
1507 steroidogenic acute regulatory protein (*STAR*), cytochrome P450 family 11 subfamily A
1508 member 1 (*CYP11A1*), and progesterone reductase (*HSD3B2*) transcripts were quantified by
1509 qRT-PCR. P4 in culture medium was evaluated by an electrochemiluminescence
1510 immunoassay. Functional requirements for (P)RR in P4 synthesis and its Ang II-independent
1511 effect were evaluated after subjecting luteal tissue to the following 4-h treatments: 1) control;
1512 2) 1 µmol/L Ang II; 3) 1 µmol/L Ang II plus 10 µmol/L saralasin (a nonspecific Ang II
1513 receptor antagonist); 4) 1 nmol/L prorenin; 5) 1 nmol/L prorenin plus 10 µmol/L aliskiren;
1514 and 6) 1 nmol/L prorenin plus 10 µmol/L saralasin. Cells were pretreated with saralasin or
1515 aliskiren for 1 h prior to Ang II or prorenin treatment. Concentrations of Ang II, saralasin,
1516 prorenin, and aliskiren were based on previous reports (Uraoka *et al.* 2009, Dau *et al.* 2016).
1517 Experiments were performed in quadruplicate and repeated on different days.
1518

1519 *Examination of P4 induction by the prorenin pathway in luteal cells*

1520 The effect of prorenin on ERK1/2 phosphorylation *in vitro* was evaluated by treating
1521 luteal tissue with three doses of prorenin (0.1, 1, and 10 nmol/L) for 20 min. ERK1/2
1522 activation was evaluated by western blot. Experiments were performed in quadruplicate and
1523 repeated on different days.

1524 Functional requirements for ERK1/2 and EGF receptor pathways in P4 synthesis were
1525 evaluated after subjecting luteal tissue to the following 4-h treatments: 1) control; 2) 1 nmol/L
1526 prorenin; 3) 1 nmol/L prorenin plus 1 µmol/L PD0325901 (a MAPK/ERK kinase inhibitor);
1527 and 4) 1 nmol/L prorenin plus 5 µmol/L AG1478 (an EGFR inhibitor). Cells were pretreated

1528 with the inhibitors for 1 h prior to prorenin treatment. P4 levels in culture medium were
1529 evaluated by an electrochemiluminescence immunoassay. PD0325901 and AG1478
1530 concentrations were based on previous reports (Park *et al.* 2004, Yao *et al.* 2016).
1531 Experiments were performed in quadruplicate and repeated on different days.
1532

1533 ***Statistical analysis***

1534 Gene expression and hormonal synthesis data were tested for normality using the
1535 Shapiro-Wilk test, and normalized when necessary. Differences between groups were
1536 analyzed using a multiple comparison least-squares means (LSMEANS) Student's *t*-test. Data
1537 from P4 measurements following different treatments and on different days were compared
1538 by two-way analysis of variance (ANOVA). Analyses were performed using SAS statistical
1539 software (SAS Institute Inc., Cary, NC, USA), and a significance level of 5% was used. Gene
1540 expression results are presented as means ± standard errors of the means.
1541

1542 **Results**

1543 ***Prorenin and (P)RR mRNA increase in CL on day 10 of the estrous cycle, and***
1544 ***transcripts of profibrotic genes increase after treatment with PGF2A***

1545 *(P)RR* mRNA increased dramatically in bovine luteal tissue obtained on day 10 (120 h
1546 after PGF2A treatment) compared to day 5 of the estrous cycle (0 h after PGF2A treatment; P
1547 < 0.05), but was not affected by the amount of PGF2A (Fig. 1A). (P)RR was detected in CL
1548 on days 5 and 10 after ovulation and at 12, 24, and 48 h post-PGF2A treatment, but did not
1549 change significantly between different stages or in response to PGF2A (Fig. 1B). Prorenin
1550 transcripts in the CL were also higher on day 10 than on day 5 of the estrous cycle (P < 0.05;
1551 Fig. 1C), and remained constant 12, 24, and 48 h (P > 0.05) after PGF2A treatment.

1552 Expression of *FN1*, *PAI1*, and *TGFB1* was induced in luteal tissue following PGF2A
1553 treatment. Whereas *FN1* mRNA in the CL (Fig. 1D) increased 24 h after PGF2A addition,
1554 *PAI1* (Fig. 1E) and *TGFB1* (Fig. 1F) increased already after 12 h. The transcripts of
1555 profibrotic molecules did not change. Collagen type I mRNA was not affected by PGF2A
1556 treatment at -120, 0, 12, 24, and 48 h (data not shown).
1557

1558 ***(P)RR is required in preovulatory follicles for P4 synthesis on day 6 post-GnRH***

1559 Intrafollicular inhibition of (P)RR impaired P4 synthesis on day 6 after GnRH
1560 treatment in ovulated cows ($P < 0.05$; Fig. 2). Concentration of P4 on day 8 post-GnRH did
1561 not differ between aliskiren-treated and control animals ($P > 0.05$). The diameter of treated
1562 follicles was approximately 14 mm in both groups. Two out of six aliskiren-treated cows did
1563 not ovulate, whereas all cows from the control group (4/4) underwent ovulation. Cows that
1564 did not ovulate were discarded from P4 analysis.
1565

1566 ***Prorenin induces P4 synthesis via (P)RR in cultured luteal cells***

1567 *ATP6AP2* , *STAR*, *CYP11A1*, and *HSD3B2* transcripts were detected after culturing
1568 luteal tissue for 4 h with 0, 0.1, 1, and 10 nmol/L prorenin (data not shown). However, none
1569 of the transcripts appeared to respond to prorenin levels. Prorenin (1 nmol/L) increased P4
1570 levels in the culture medium of luteal tissue ($P < 0.05$; Fig. 3A), yet this effect was abolished
1571 by aliskiren ($P < 0.05$; Fig. 3B). Saralasin pretreatment (Fig. 3B) inhibited Ang II-induced (P
1572 < 0.05) but not prorenin-induced P4 synthesis ($P > 0.05$).
1573

1574 ***Prorenin induces P4 synthesis in luteal tissue via ERK1/2 activation and EGFR***
1575 ***transactivation in vitro***

1576 A 10 nmol/L concentration of prorenin was sufficient to induce ERK1/2
1577 phosphorylation in luteal tissue after 20 min of culture, compared to control cells ($P < 0.05$;
1578 Fig. 4A). Pretreatment with 1 µmol/L of the MAP kinase inhibitor PD0325901 did not
1579 completely prevent the stimulatory effect of prorenin on P4 secretion ($P < 0.05$; Fig. 4B). In
1580 contrast, the EGFR inhibitor AG1478 blocked prorenin-induced P4 release in bovine luteal
1581 tissue cultured for 4 h ($P > 0.05$).
1582

1583 **Discussion**

1584 For over 20 years, it has been suggested that a receptor mediating prorenin activities
1585 independently of renin was important for reproductive events, including ovulation,
1586 steroidogenesis, and CL maturation (Itskovitz *et al.* 1988). Results obtained in the present
1587 study provide an additional insight into the role of prorenin/(P)RR in P4 synthesis by bovine
1588 luteal cells: 1) prorenin and (P)RR mRNA increased in the CL from day 10 of the estrous
1589 cycle; 2) (P)RR was required in preovulatory follicles for P4 release during luteinization; 3)
1590 binding of prorenin to (P)RR induced P4 synthesis in luteal cells. Additionally, ERK1/2
1591 phosphorylation and EGFR transactivation mediated prorenin-induced P4 secretion in luteal
1592 tissue.

1593 High P4 levels have been detected in the bovine CL from 6 to 10 days post-ovulation
1594 (Rekawiecki *et al.* 2010), coinciding here with increased prorenin and (P)RR transcription.
1595 These results suggest that prorenin may play a role in P4 synthesis (Sealey *et al.* 1985,
1596 Itskovitz *et al.* 1987, Hagemann *et al.* 1994). On the contrary, profibrotic molecules
1597 upregulated by ERK1/2 phosphorylation following prorenin binding to (P)RR in non-
1598 reproductive cells (Nguyen *et al.* 2002, Huang *et al.* 2006, Nguyen 2008, Ferri *et al.* 2011) did
1599 not increase in the CL during luteinization in cattle.

1600 In this study, a 12-h PGF2A treatment stimulated *FN1*, *PAI1*, and *TGFB1* expression
1601 in the bovine CL. Similar results on *TGFB1* and *PAI1* in PGF2A-treated cows were reported
1602 previously (Kliem *et al.* 2007, Hou *et al.* 2008), further supporting our *in vivo* model. In cows,
1603 luteolysis is induced between 30 min and 2 h after PGF2A administration (Shirasuna *et al.*
1604 2012a, Shirasuna *et al.* 2012b); it depends on a decrease in P4 and the degeneration of luteal
1605 tissue (McGuire *et al.* 1994, Pate 1994, Niswender *et al.* 2000). Supplementation with TGFB1
1606 lowers P4 secretion in bovine luteal cell cultures and stimulates profibrotic activities in luteal
1607 fibroblasts during CL structural regression (Hou *et al.* 2008, Maroni & Davis 2012). To the
1608 best of our knowledge, this is the first time that a quantitative *FN1* mRNA profile is
1609 demonstrated during luteolysis in cattle. Transcripts for components of collagen type I are
1610 usually upregulated in regressed luteal tissue in cattle (Casey *et al.* 2005). In contrast, here,
1611 collagen type I mRNA expression was not induced by PGF2A. Interestingly, prorenin/(P)RR
1612 transcripts and (P)RR protein were still detected in luteal tissue during CL regression in cattle.
1613 Taken together, stimulation of profibrotic genes by prorenin/(P)RR during bovine luteolysis
1614 cannot be ruled out.

1615 The involvement of prorenin was more evident during luteinization than during
1616 luteolysis in our first experiment. Moreover, the effect of prorenin/(P)RR on P4 synthesis in
1617 luteal cells was confirmed *in vivo* and *in vitro.* Use of aliskiren to block intrafollicular (P)RR
1618 in the preovulatory follicle lowered P4 serum levels on day 6 post-treatment in cows that
1619 ovulated. This result demonstrates that (P)RR in the preovulatory follicle is required for P4
1620 synthesis during luteinization. *In vitro*, prorenin binding to (P)RR also upregulated P4
1621 production by luteal cells.

1622 *STAR*, *CYP11A1*, and *HSD3B2* transcripts were detected in luteal tissue cultured for 4
1623 h after PGF2A treatment. STAR, CYP11A1, and HSD3B2 are steroidogenic luteal cell
1624 markers critical for P4 synthesis (Tsai & Wiltbank 1998, Atli *et al.* 2012, Maroni & Davis

1625 2012). Briefly, STAR transports cholesterol into the mitochondria, CYP11A1 transforms
1626 cholesterol into pregnenolone, and HSD3B2 converts pregnenolone to P4 (Rekawiecki *et al.*
1627 2008). Downregulation of *STAR* and *HSD3B2* and inhibition of steroidogenic pathways by
1628 PGF2A appears to decrease the release of P4 in bovine CL (Tsai & Wiltbank 1998, Atli *et al.*
1629 2012). Thus, the availability of STAR, CYP11A1, and HSD3B2 signals the presence of
1630 undifferentiated luteal cells in our culture system and allows for P4 synthesis.

1631 (P)RR mRNA was detected in luteal tissue cultured for 4 h and prorenin treatment (1
1632 nmol/L) increased P4 levels in the culture medium. This effect was blocked by aliskiren,
1633 indicating that (P)RR-bound prorenin stimulated P4 release by luteal cells *in vitro.* Aliskiren
1634 inhibits ERK1/2 phosphorylation and Ang I production from angiotensinogen, which is
1635 induced by (P)RR binding to prorenin or renin (Biswas *et al.* 2010, Ferri *et al.* 2011, Ma *et al.*
1636 2012). Our observation is in line with evidence showing that Ang II stimulates P4 secretion in
1637 the early CL (Kobayashi *et al.* 2001). To examine whether prorenin induced P4 through an
1638 Ang II-independent pathway, luteal tissue were pretreated with saralasin and prorenin.
1639 Saralasin (10 μmol/L) did not prevent prorenin-dependent resumption of meiosis in bovine
1640 oocytes, confirming that the role of prorenin was independent of the Ang II pathway (Dau *et*
1641 *al.* 2016). In this study, saralasin did not completely block prorenin-induced P4 production in
1642 luteal cells, although it abolished Ang II-induced P4 synthesis. These results suggest that
1643 prorenin induces P4 secretion in luteal cells through both Ang II-dependent and -independent
1644 pathways.

1645 ERK1/2 activation in granulosa cells is required for P4 release during luteinization
1646 (Fan *et al.* 2009, Pan *et al.* 2014). In endothelial cells, 1 nmol/L prorenin is sufficient to
1647 induce ERK phosphorylation in a dose-dependent manner (Uraoka *et al.* 2009). In the present
1648 study, 10 nmol/L prorenin activated ERK1/2 intracellular signaling in bovine luteal tissue.
1649 The involvement of MAPKs in prorenin-induced P4 synthesis was investigated using a

1650 MAPK/ERK kinase inhibitor *in vitro*. Pretreatment with 1 µmol/L PD0325901 did not inhibit
1651 completely prorenin-induced P4 secretion, even though 0.5–3 µmol/L PD0325901 was shown
1652 to be sufficient to inhibit ERK1/2 phosphorylation in cancer cell lines (Yao *et al.* 2016).
1653 These results suggest that prorenin stimulates P4 synthesis via ERK1/2 and support the
1654 possible involvement of other signaling pathways such as the Ang II pathway.

1655 EGFR has also been demonstrated to mediate prorenin/(P)RR function (Liu *et al.*
1656 2011, Shibayama *et al.* 2013). Prorenin has been shown to induce EGFR transactivation in
1657 HEK 293 cells (Shibayama *et al.* 2013) and pretreatment with AG1478 in rat vascular smooth
1658 muscle cells abrogated prorenin-induced ERK1/2 phosphorylation (Liu *et al.* 2011). Here,
1659 AG1478 blocked prorenin-induced P4 synthesis in luteal cells. The cross-talk between EGFR
1660 and ERK1/2 in prorenin-induced P4 release requires further investigation. Nevertheless,
1661 EGFR transactivation may be an important mediator of prorenin-dependent P4 secretion in
1662 the bovine CL. This result supports a role for EGF/EGFR in P4 synthesis, as already
1663 demonstrated in mice (Hsieh *et al.* 2011, Pan *et al.* 2014) and ovine models (Murray *et al.*
1664 1993).

1665 The present study provides insights into the function of (P)RR as an alternative RAS
1666 pathway responsible for P4 synthesis. RAS is studied largely in connection with diabetes and
1667 hypertension, which are often related to infertility (Amaral *et al.* 2008, Hutcheon *et al.* 2011,
1668 Nartita *et al.* 2016). Interestingly, (P)RR function has been reported in human placenta during
1669 early gestation (Pringle *et al.* 2011). Secretion of P4 is critical for regulating the estrous cycle,
1670 early embryonic development, and maintaining pregnancy (Niswender *et al.* 2000). Thus,
1671 (P)RR may be an important pharmacotherapeutic target in reproductive disorders.

1672 In summary, the high expression of prorenin and (P)RR mRNA in the bovine CL
1673 coincides with elevated P4 levels in cattle (Rekawiecki *et al.* 2010). Our results indicate that
1674 PGF2A in CL stimulates the expression of profibrotic molecules, which are upregulated by

1675 prorenin/(P)RR (Ferri *et al.* 2011). Prorenin induces the release of P4 in cultured luteal tissue.
1676 (P)RR is required for P4 secretion *in vitro* and *in vivo*. This appears to be mediated by
1677 ERK1/2 and EGFR transactivation in our culture system (Fig. 5). In conclusion, (P)RR plays
1678 a role in P4 synthesis in bovine luteal cells, and ERK1/2 and EGFR are potential mediators of
1679 prorenin-induced P4 secretion in cattle.

1680

1681 **Declaration of interest**

1682 The authors declare that there is no conflict of interest that could be perceived as
1683 prejudicing the impartiality of the reported research.

1684

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1690

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1696

1697 **References**

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1870 **Figure legends**

1871 **Figure 1** mRNA and protein expression levels in bovine corpus luteum (CL). (A)
1872 mRNA and (B) protein profiles of (pro)renin receptor, (P)RR. mRNA profiles of (C)
1873 *PRORENIN*, (D) fibronectin (*FN1*), (E) plasminogen activator inhibitor 1 (*PAI1*), and (F)
1874 transforming growth factor beta-1 (*TGFB1*) in bovine CL samples isolated from ovaries at -
1875 120 h (day 5 of estrous cycle; n = 4), 0 (day 10 of estrous cycle; n = 5), 12 (n = 3), 24 (n = 4),
1876 and 48 h (n = 4) after PGF2A treatment. Results are shown as mean ± standard error. Western
1877 blot images revealed a specific band at approximately 42 kDa for (P)RR and at 42 kDa for β-
1878 actin. Representative blots from each time point and a summary of the densitometric analysis
1879 are shown. Different letters indicate statistical differences between groups ($P < 0.05$).

1880 **Figure 2** Effect of intrafollicular aliskiren on progesterone levels of cows challenged
1881 with gonadotropin-releasing hormone (GnRH). Serum progesterone (ng/mL) on day 6 and 8
1882 after intramuscular injection of GnRH and intrafollicular injection of 10^{-5} mol/L aliskiren (n =
1883 4) or phosphate-buffered saline (control; n = 4) in cows that ovulated. Results are shown as
1884 mean ± standard error. Different letters indicate statistical differences between groups ($P <$
1885 0.05).

1886 **Figure 3** Effect of prorenin on progesterone levels via (P)RR in cultured luteal tissue.
1887 Dose-response effect of (A) prorenin, and (B) different combinations of compounds, such as 1
1888 µmol/L angiotensin II plus 10 µmol/L saralasin, 1 nmol/L prorenin plus 10 µmol/L aliskiren,
1889 or 1 nmol/L prorenin plus 10 µmol/L saralasin, on progesterone (ng/mL) in the culture
1890 medium of luteal tissue after 4 h of treatment. Results are shown as mean ± standard error.
1891 Different letters indicate statistical differences between groups ($P < 0.05$). Experiments were
1892 performed in quadruplicate.

1893 **Figure 4** Role of mitogen-activated protein kinase (MAPK) and epidermal growth
1894 factor receptor (EGFR) pathway on progesterone levels induced by prorenin. (A) Dose-

1895 response effect of prorenin on MAPK ERK1/2 phosphorylation in luteal tissue after 20 min of
1896 treatment. (B) Effect of 1 nmol/L prorenin plus 1 μmol/L PD0325901 (MAPK/ERK kinase
1897 inhibitor) or 1 nmol/L prorenin plus 5 μmol/L AG1478 (epidermal growth factor receptor
1898 inhibitor) on the level of progesterone (ng/mL) in the culture medium after 4 h of treatment.
1899 Western blot images revealed specific bands of approximately 44 and 42 kDa for the
1900 phosphorylated and non-phosphorylated ERK1/2, respectively. Representative blots from
1901 each group and a summary of the densitometric analysis are displayed. Results are shown as
1902 mean ± standard error. Different letters indicate statistical differences between groups ($P <$
1903 0.05). Experiments were performed in quadruplicate.

1904 **Figure 5** Proposed model of the (pro)renin receptor in the regulatory mechanism of
1905 progesterone synthesis in luteal cells.

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1907 **Table 1. Information about primers used in the expression analysis of candidate mRNAs**

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Gene		Primer sequence	Reference or accession number
GAPDH	F:	GATTGTCAGCAATGCCTCCT	Ferreira *et al.* (2011b)
	R:	GGTCATAAGTCCCTCCACGA	
PPIA	F:	GGTCATCGGTCTCTTTGGAA	Ledoux *et al.* (2006)
	R:	TCCTTGATCACACGATGGAA	
ATP6AP2	F:	TGATGGTGAAAGGAGTGGACAA	Ferreira *et al.* (2011b)
	R:	TTTGCCACGCTGTCAAGACT	
PRORENIN	F:	GGGTGCCGTCCACCAA	NM_001206509.1
	R:	TCCGTCCCATTCTCCACATAG	
FN1	F:	TGGGACCACGCAGAACTATG	NM_001163778.1
	R:	GCGATACATGACCCCTTCGT	
PAI1	F:	CACCATCTCTGTGCCCATGAT	NM_174137.2
	R:	GGTAGGGCAATTCCAGGATGT	
TGFB1	F:	CTGAGCCAGAGGCGGCGGACTAC	NM_001166068.1
	R:	CTGTGCGAGCTAGACTTCATTTTG	
COL1	F:	CATGACCGAGACGTGTGGAA	NM_001034039.2
	R:	CAGTCCTTAAGTTCGTCGCAGAT	
STAR	F:	CCCAGCAGAAGGGTGTCATC	Buratini *et al.* (2005)
	R:	TGCGAGAGGACCTGGTTGAT	
HSD3B2	F:	GCCCAACTCCTACAGGGAGAT	Orisaka *et al.* (2006)
	R:	TTCAGAGCCCACCCATTAGCT	
CYP11A1	F:	CTTGCACCTTTCTGGCTAGG	Orisaka *et al.* (2006)
	R:	AAGGGGAAGAGGTAGGGTGA	

1909 F, forward primer; R, reverse primer;

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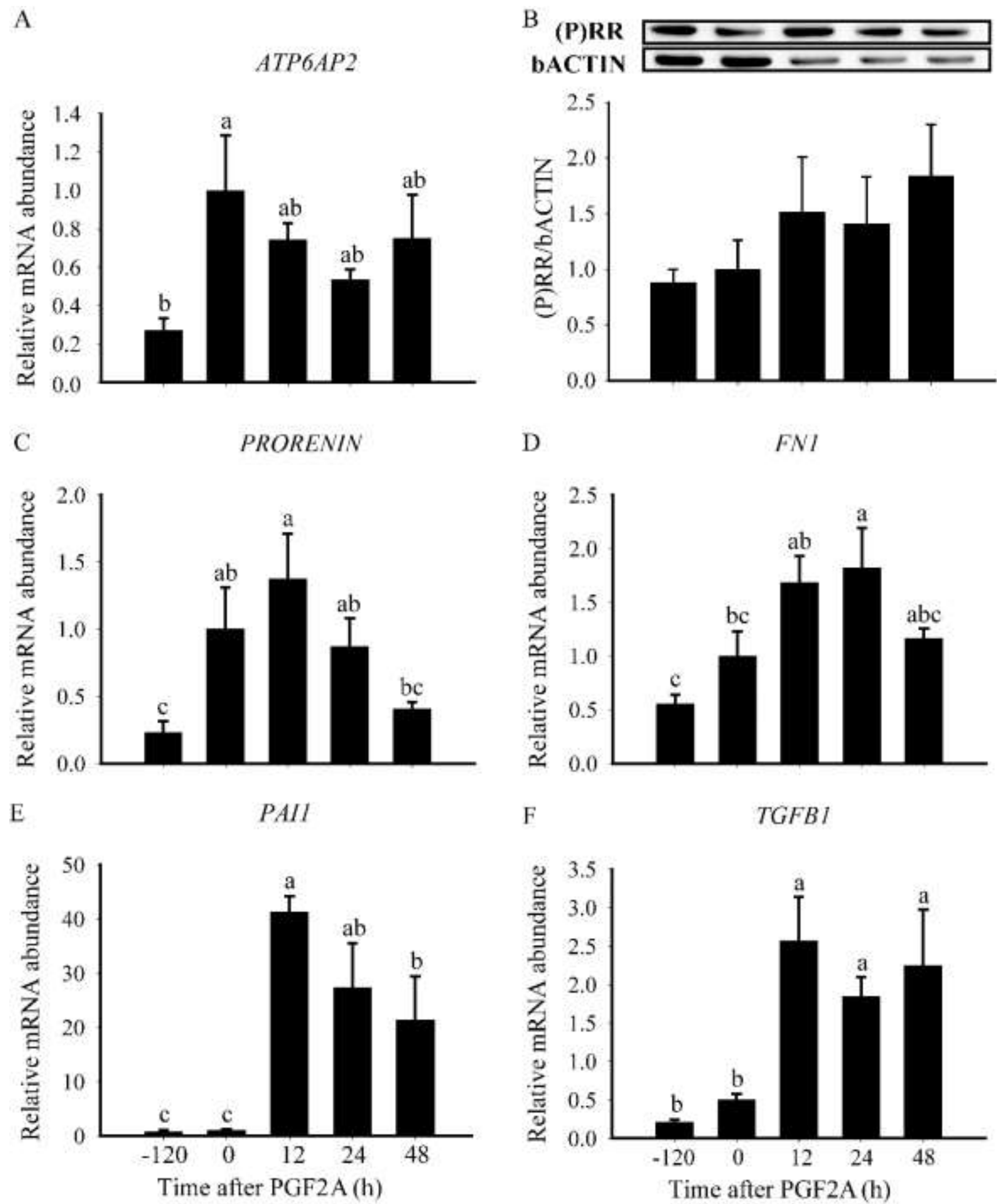
1921 **Figure 1**



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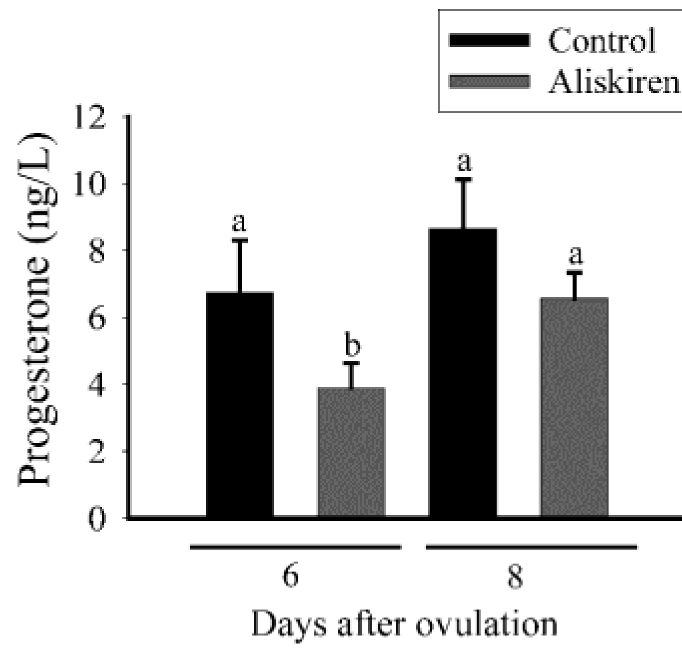
1924 **Figure 2**



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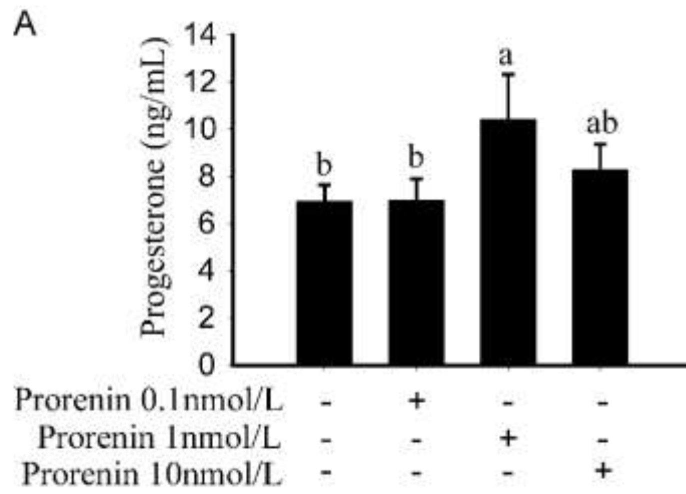
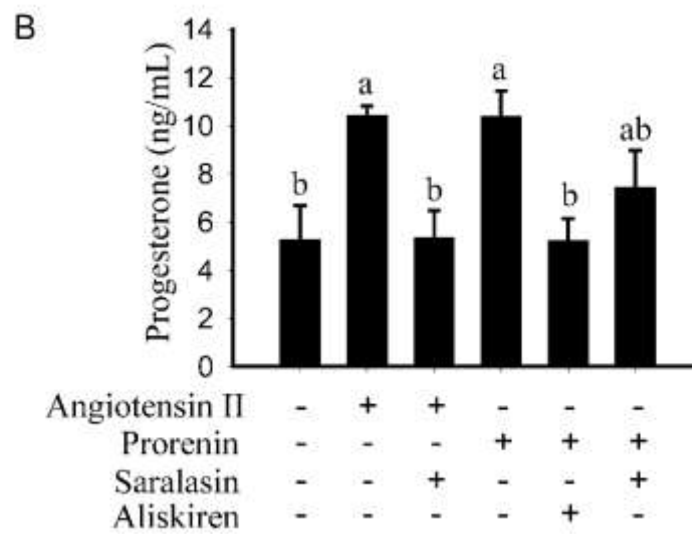
1927 **Figure 3**





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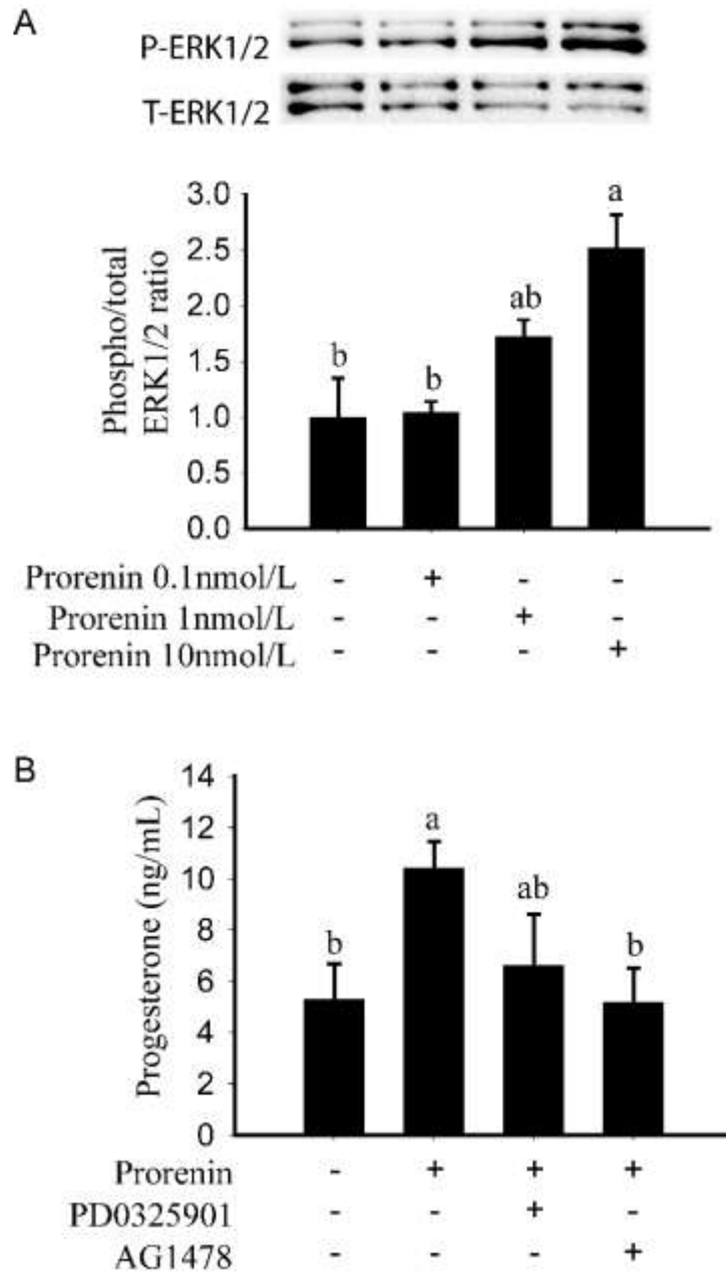
1930 **Figure 4**



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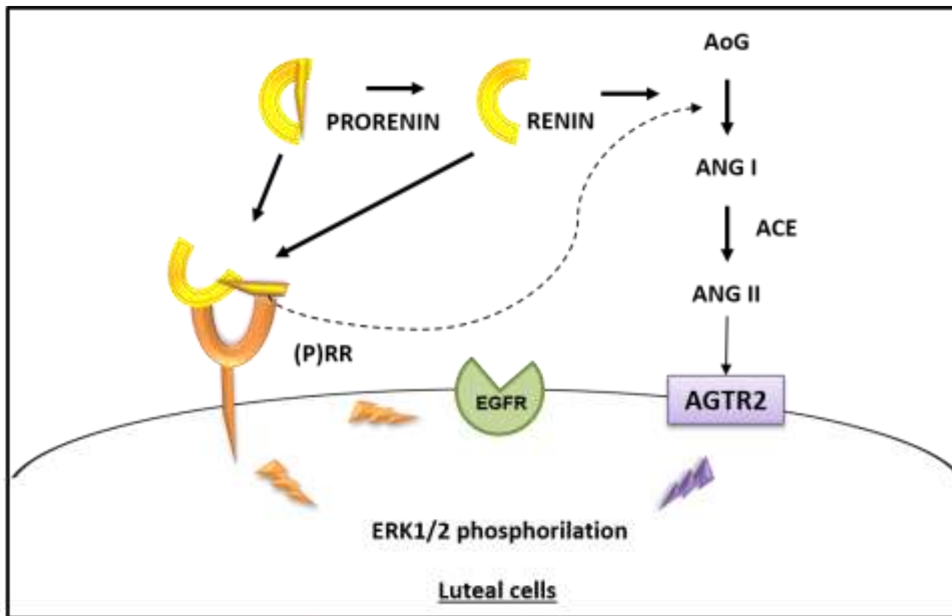
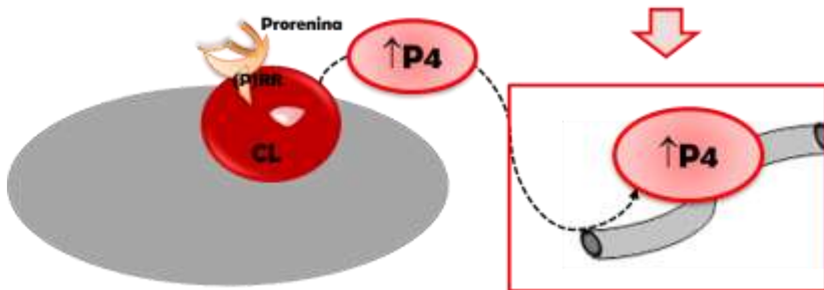
1933 **Figure 5**





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7. DISCUSSÃO

A ovulação e, consequente, luteinização das células foliculares ocorre durante o período peri-ovulatório, o qual compreende desde o pico de gonadotrofinas até o aumento da síntese de progesterona. Ao longo dos anos, nosso grupo de pesquisa tem evidenciado a participação do RAS nesses mecanismos celulares decorrentes do pico de LH em bovinos. Buscando preencher lacunas existentes sobre o papel da pró-renina nas células foliculares durante o período peri-ovulatório, conduzimos modelos experimentais *in vivo* e *in vitro,* muitos dos quais foram amplamente utilizados pelo nosso grupo de pesquisa para elucidar o envolvimento de Ang II neste mesmo período (FERREIRA *et al.*, 2007; PORTELA *et al.*, 2011; TONELLOTTO DOS SANTOS *et al.*, 2012; SIQUEIRA *et al.*, 2013).

No reinicio da meiose oocitária, evidenciamos o papel da prorenina via (P)RR utilizando o modelo *in vitro* de co-cultivo de complexo cumulus-oócito e metades foliculares (DAU *et al.*, 2016; GIOMETTI *et al.* 2005; BARRETA *et al.,* 2008). Ainda, confirmamos o efeito de prorenina no reinicio da meiose de oócitos mesmo na ausência de metades foliculares modelo na presença de forscolina (DAU *et al.*, 2016). O cultivo de células da granulosa foi adaptado do modelo de estudo que propõe o papel da Ang II como co-fator de LH para ativar a cascata pré-ovulatória de fatores de crescimento semelhantes ao EGF (PORTELA *et al.*, 2011). Embora nosso sistema tenha apresentado resultados similares ao estudo supracitado quando as células foram tratadas com LH, a pró-renina testada em três doses diferentes não estimulou os genes regulados por LH nas células da granulosa. A nossa hipótese, embora não tenha sido confirmada, foi fundamentada nos dados de Ang II nas células da granulosa e, ainda, reforçada pela transativação de EGFR e aumento de RNAm para PTGS2 em resposta a ligação de pró-renina ao seu receptor em células não reprodutivas (LIU *et al.*, 2011; PORTELA *et al.*, 2011; GONZALEZ *et al.*, 2013; SHIBAYAMA *et al.*, 2013). Dessa forma, avaliamos se EGFR poderia ser necessário nas células da granulosa para expressão de RNAm para pró-renina e (P)RR através do mesmo modelo de estudo *in vitro,* entretanto, suplementando o meio com LH e AG1478. Nenhuma regulação foi observada para os genes avaliados nas células da granulosa em resposta ao LH ou inibidor de EGFR após 6 horas de cultivo.

O perfil de expressão molecular de pró-renina, (P)RR e genes prófibróticos nas células da teca e da granulosa foi estabelecido através da avaliação das células obtidas em momentos específicos após o tratamento de vacas com GnRH. Este modelo de experimento *in vivo* também foi utilizado, em estudos prévios do nosso laboratório, para caracterizar o RAS nas

células da teca e da granulosa (SIQUEIRA, C. S. *et al.*, 2012; TONELLOTTO DOS SANTOS *et al.*, 2012). Observamos nos nossos resultados que, enquanto o (P)RR é estimulado após o pico de LH no início do processo ovulatório nas células da teca, o mesmo é aumentado mais próximo da ruptura folicular nas células da granulosa. O (P)RR aumentou nas células da granulosa nas 24 horas após GnRH, como também foi observado quanto a expressão de RNAm para AOG nas mesmas células e níveis de Ang II no fluido folicular em estudos prévios (SIQUEIRA, C. S. *et al.*, 2012). Com base nestes resultados, podemos sugerir que o (P)RR nas células da granulosa está envolvido com a produção de Ang II intrafolicular e na ovulação através do estimulo dos genes prófibróticos. Entretanto, essa proposta ainda necessita ser confirmada através de estudos futuros. Nas células da teca, o (P)RR aumenta 6 horas após GnRH, mesmo momento em que a abundância de RNAm para AGTR2 é detectada na sua menor quantidade no mesmo tipo celular (SIQUEIRA, C. S. *et al.*, 2012), sugerindo uma participação da via independete de Ang II, através de (P)RR, nas células da teca neste mesmo período.

Buscando determinar se EGFR intrafolicular é necessário para a expressão gênica de (P)RR, induzido por LH, nas células da teca de bovinos, utilizamos um modelo *in vivo* através de injeção intrafolicular com AG1478 (inibidor de EGFR) e induzimos o pico de gonadotrofinas com GnRH intramuscular em vacas pré-sincronizadas. A expressão de (P)RR foi independente de EGFR nas células da teca 6 horas após tratamento. Ainda, confirmamos nossos resultados obtidos *in vitro* nas células da granulosa, em que o RNAm de pró-renina e (P)RR não foram regulados pelo inibidor de EGFR. O efeito de AG1478 intrafolicular foi validado pelo aumento na quantidade de CYP17A1 detectada por western blot nas células da teca tratadas com AG1478, o qual confirma o papel de EGFR na regulação de CYP17A1 que ocorre neste período pré-ovulatório (SPICER e STEWART, 1996; KOMAR *et al.*, 2001).

O estimulo de LH sobre a expressão de (P)RR nas células da teca foi confirmado *in vitro* utilizando um modelo de cultivo das células da teca adaptado de prévios estudos (STEWART *et al.*, 1995; COMIM *et al.*, 2013). A função de (P)RR induzido por LH nas células da teca foi investigado na esteroidogênese através deste mesmo modelo de cultivo. Entretanto, não observamos nenhuma evidência de regulação na esteroidogênese nas células da teca submetidas ao nosso sistema de cultivo. Resultados similares foram obtidos quando as células da teca foram tratadas com Ang II (RIGO *et al.*, 2015).

A avaliação da função do (P)RR intrafolicular para ovulação foi determinado utilizando um modelo *in vivo* de injeção intrafolicular com alisquireno administrada imediatamente após GnRH (IM) e observando a ovulação por ultrassom 24, 48 e 72 h após

tratamento, o que foi confirmado pela avaliação de P4 plasmática dia 6 e dia 8 após GnRH. Embora não observamos regulação na taxa de ovulação por (P)RR, como observado para Ang II (FERREIRA *et al.*, 2011), evidenciamos uma redução na síntese de progesterona em resposta ao tratamento intrafolicular com alisquireno. O resultado sobre o papel de (P)RR sobre a taxa de ovulação, apesar de prejudicado pelo baixo número de animais disponível para realização deste experimento, pode ser explicado pela disponibilidade de AGTR2, o qual pode ter suprido a ausência da via independente da Ang II [(P)RR]. Os dados referentes a regulação da produção de P4 por alisquireno intrafolicular, confirmam a correlação positiva entre pró-renina e P4 no fluido folicular, demonstrada em estudos realizados na década de 80 (ITSKOVITZ *et al.*, 1987; ITSKOVITZ *et al.*, 1988; HAGEMANN *et al.*, 1997).

A participação de pró-renina/(P)RR nas células do corpo lúteo bovino promovendo a síntese de P4 foi evidenciada pela alta expressão de RNAm para pró-renina e (P)RR identificada no corpo lúteo de vacas coletados no dia 10 do ciclo estral comparado ao dia 5, o que foi confirmado *in vitro* através de cultivo de corpo lúteo. No nosso sistema de cultivo, Ang II induziu a síntese de P4, como observado em estudo prévio (KOBAYASHI *et al.*, 2001). O efeito de pró-renina via (P)RR observado nas células lúteas *in vitro* parece ser tanto na via independente de Ang II, quanto na via dependente da Ang II, uma vez que saralasina não bloqueou completamente a síntese de P4 induzida por pró-renina.

Pró-renina estimulou a fosforilação de ERK1/2 nas células de corpo lúteo bovino *in vitro,* assim como observado em células endoteliais (URAOKA *et al.*, 2009). O mecanismo pelo qual a pró-renina induz a síntese de progesterona foi investigado no mesmo sistema de cultivo utilizando a suplementação do meio de cultivo com pró-renina e um bloqueador para fosforilação de ERK1/2 (PD0325901) ou um inibidor de EGFR (AG1478). De acordo com nossos resultados, observamos que pró-renina induz a síntese de P4 via ERK1/2, entretanto, sugerimos a existência de outras vias de sinalização, como a via dependente da Ang II ou de EGFR. AG1478, por sua vez, bloqueou completamente o efeito de pró-renina na síntese de P4. Nossos resultados suportam a participação de EGFR e ERK1/2 na síntese de progesterona observado em outros estudos (MURRAY *et al.*, 1993; FAN *et al.*, 2009; HSIEH *et al.*, 2011; PAN *et al.*, 2014).

A combinação de experimentos *in vitro* e *in vivo* proporcionaram a elucidação de função e mecanismos envolvidos com o gene alvo de estudo, como o (P)RR. Apesar de não utilizarmos curto RNA de interferência para bloquear o (P)RR, o uso de fármacos antagonistas e inibidores auxiliaram para evidenciarmos possíveis vias de ação da pró-renina e seu efeito através de (P)RR nas células foliculares durante o período peri-ovulatório de

bovinos. Nossos resultados preenchem algumas lacunas existentes desde a década de 80 (ITSKOVITZ *et al.*, 1988) sobre a participação de pró-renina via seu receptor nos eventos reprodutivos desencadeados por LH.

8. CONCLUSÃO

Os resultados do presente estudo permitem concluirmos que (P)RR participa do início do período pré-ovulatório, sendo estimulado nas células da teca nas primeiras horas após o pico de LH e atuando no reinicio da meiose oocitária em bovinos. Nas células da granulosa, (P)RR aumenta próximo da ovulação. Logo, os efeitos de (P)RR parecem ser independentes de EGFR nas células da teca e da granulosa após pico de LH. Entretanto, a função de (P)RR nas células da teca e da granulosa durante o período pré-ovulatório ainda precisa ser esclarecida. No corpo lúteo, o (P)RR possui um importante papel na síntese de P4 e sugerimos o envolvimento de EGFR e ERK1/2 neste processo.

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