UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

AÇÃO DA ANGIOTENSINA II NA REGULAÇÃO DA DOMINÂNCIA FOLICULAR EM BOVINOS

TESE DE DOUTORADO

Rogério Ferreira

Santa Maria, RS, Brasil

2010

AÇÃO DA ANGIOTENSINA II NA REGULAÇÃO DA DOMINÂNCIA FOLICULAR EM BOVINOS

por

Rogério Ferreira

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da 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, PhD

Santa Maria, RS, Brasil

2010

F383a Ferreira, Rogério
Ação da angiotensina II na regulação da dominância folicular em bovinos / por Rogério Ferreira. – 2010.
78 f. ; il. ; 30 cm

Orientador: Paulo Bayard Dias Gonçalves
Coorientador: Chistopher Price
Tese (doutorado) – Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós-Graduação em Medicina Veterinária, RS, 2010

1. Medicina veterinária 2. RAS 3. Divergência folicular 4. Desvio folicular 5. Esteroidogênese 6. Granulosa 7. Bovinos I. Gonçalves, Paulo Bayard Dias II. Price, Chistopher III. Título.

CDU 619:636.2.082

Ficha catalográfica elaborada por Cláudia Terezinha Branco Gallotti – CRB 10/1109
Biblioteca Central UFSM

Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-Graduação em Medicina Veterinária

A Comissão Examinadora, abaixo assinada,
aprova a Tese de Doutorado

AÇÃO DA ANGIOTENSINA II NA REGULAÇÃO DA DOMINÂNCIA FOLICULAR EM BOVINOS

elaborada por
Rogério Ferreira

como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

COMISSÃO EXAMINADORA:

Paulo Bayard Dias Gonçalves, PhD
(Presidente/Orientador)

Adelina Martha dos Reis, Dra. (UFMG)

Marlon Nadal Maciel, Dr. (UFSM)

Luiz Ernani Henkes, Dr. (UNIPAMPA)

Vilceu Bordignon, PhD (McGill University)

Santa Maria, 02 de março de 2010.

AGRADECIMENTOS

Agradeço:

À família pelo suporte incondicional;

Ao meu orientador, Paulo Bayard Dias Gonçalves, pela amizade, ensinamentos e exemplo de dedicação à pesquisa;

Aos companheiros de experimentos, risadas, e muito trabalho: Bernardo, Joabel, Marcos e Monique;

Aos meus irmãos do 31, Bito, Bernardo e Endrigo, pela amizade e excelente convivência;

Ao meu co-orientador, Christopher Price, pelos ensinamentos e por disponibilizar seu laboratório para realizar parte do meu doutorado;

A toda equipe do laboratório BioRep pela alegria e companheirismo;

Aos colaboradores do laboratório BioRep: Fazenda do Leão, Vinicius de Oliveira, Walter Ferreira e José Manoel Ferreira. Sem eles, não seria possível a realização do presente trabalho;

À CAPES pela concessão da bolsa de estudos e ao CNPq pela concessão da bolsa de estágio no exterior e apoio financeiro à pesquisa;

Enfim, a todos que contribuíram e torceram por mim.

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

AÇÃO DA ANGIOTENSINA II NA REGULAÇÃO DA DOMINÂNCIA FOLICULAR EM BOVINOS

Autor: Rogério Ferreira
Orientador: Paulo Bayard Dias Gonçalves
Data e Local da Defesa: Santa Maria, 02 de março de 2010.

Os objetivos do presente trabalho foram determinar a concentração de angiotensina II (AngII) no fluido folicular, caracterizar a expressão dos genes do sistema renina-angiotensina (RAS) nas células foliculares e verificar o papel da AngII na onda folicular, utilizando um modelo in vivo de injeção intrafolicular e in vitro de cultivo de células da granulosa. A concentração de AngII no fluido folicular aumentou no folículo de maior diâmetro, durante e após a divergência folicular. A administração intrafolicular de saralasina, um bloqueador competitivo dos receptores de AngII, inibiu o crescimento folicular em todas as vacas injetadas (4/4), demonstrando que a AngII é essencial para o crescimento de folículos de 7-8mm de diâmetro. Contudo, a injeção de AngII não afetou o crescimento folicular, sugerindo que os folículos contém AngII suficiente para o seu desenvolvimento. Em um outro experimento, a administração sistêmica de FSH reverteu o efeito inibitório da saralasina, sugerindo que a AngII é indispensável para o desenvolvimento folicular após o período de dependência ao FSH. A injeção, no segundo maior folículo, de AngII ou CGP42122 (agonista AGTR2) preveniu a regressão esperada do folículo subordinado, demonstrando que a AngII desempenha um papel fundamental na seleção do folículo dominante. Para determinar o mecanismo de atresia induzido por saralasina, o folículo dominante de cada vaca foi injetado com saralasina ou solução salina e os animais foram ovariectomizados após 24 horas. A inibição de AngII diminuiu a concentração de estradiol no fluido folicular e a abundância de mRNA que codifica para os genes aromatase (CYP19), 3β HSD, LHr, Serpin E2 e ciclina D2 nas células da granulosa. Além disso, em cultivo primário de células da granulosa, a AngII, somente na presença do FSH, induziu um aumento na expressão de aromatase. Em resumo, os resultados demonstram que a sinalização da AngII é essencial para o crescimento folicular, regulando genes envolvidos com a proliferação (ciclina D2) e diferenciação (LHr, aromatase, 3βHSD) das células da granulosa os quais são necessários para o desenvolvimento do folículo dominante. Em conclusão, os resultados sugerem que a AngII está envolvida no desenvolvimento e dominância folicular em bovinos.

Palavras-chaves: divergência folicular; fatores foliculares; granulosa; esteroidogênese.

ABSTRACT

Tese de doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

THE ROLE OF ANGIOTENSIN II ON FOLLICULAR DOMINANCE OF BOVINE

Autor: Rogério Ferreira
Orientador: Paulo Bayard Dias Gonçalves
Data e Local da Defesa: Santa Maria, 02 de março de 2010.

The objectives of the present study was to determine the concentration of angiotensin II (AngII) in follicular fluid, to characterize the expression of renin-angiotensin system (RAS) genes in follicular cells and to verify the role of AngII in the follicular wave, using an in vivo model with intrafollicular injection and in vitro model with granulosa cells culture. AngII concentration in follicular fluid increased on dominant follicle during and after deviation. Saralasin inhibited follicular growth in all treated cows (4/4), suggesting that AngII is essential for follicular growth in 7-8mm follicles. However, intrafollicular injection of AngII affected neither follicular growth nor the pattern of follicular dynamics, which were similar to control cows. These results imply that bovine ovarian follicles contain sufficient AngII for follicle development. In another experiment, saralasin inhibitory effect was overcome by systemic FSH supplementation, suggesting that AngII is essential to follicular growth during FSH-low dependence stages. The injection of AngII or angiotensin receptor type 2 (AGTR2) agonist in second largest follicle prevented the expected atresia of subordinate follicle and the treated follicle grew at the same rate as the dominant during 24h. To understand why a single intrafollicular injection of AngII antagonist induces follicular atresia, dominant follicle was injected with saralasin or saline and the cows were ovariectomized 24h later. The inhibition of AngII action decreased estradiol concentration in follicular fluid and abundance of mRNA encoding aromatase (CYP19), 3β-hydroxysteroid dehydrogenase (3βHSD), LH receptor, SerpinE2 and cyclinD2 in granulosa cells. On granulosa cell culture, AngII increased CYP19 expression just in the presence of FSH. Taken together, these results show that AngII is essential for follicular growth, and plays important role in regulating genes involved in granulosa cell proliferation (cyclinD2) and differentiation (LHr, aromatase, 3βHSD), which are necessary for development of the dominant follicle. In conclusion, these data suggest that AngII signaling is involved in follicle growth and dominance in cattle.

Key words: follicular deviation; intrafollicular factors; granulosa cells; steroidogenesis.

LISTA DE FIGURAS

CAPÍTULO 1

CAPÍTULO 2

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

SUMÁRIO

1. INTRODUÇÃO

Em espécies monovulares, a fase de divergência folicular é caracterizada por uma diminuição nos níveis plasmáticos de FSH e diminuição da capacidade esteroidogênica dos folículos subordinados com consequente início do processo de atresia (Ginther *et al.*, 1996). O crescimento do folículo dominante durante esta fase está associado com um aumento na concentração folicular de estradiol (Mihm *et al.*, 2000), diminuição na dependência de FSH (Mihm *et al.*, 2006) associado a um aumento na expressão do gene para o receptor de LH (Beg *et al.*, 2001) nas células da granulosa. É observado também uma regulação nos fatores envolvidos nos eventos de proliferação e resistência à apoptose nas células da teca e granulosa (Mihm *et al.*, 2008). Além disso, fatores locais, atuando de maneira autócrina/parácrina são capazes de suportar o crescimento folicular, modulando funções básicas como esteroidogênese, proliferação e diferenciação celular (Mihm *et al.*, 2000; Fortune *et al.*, 2001; Pierre *et al.*, 2005; Knight e Glister, 2006; Miyoshi *et al.*, 2007; Gasperin *et al.*, 2008; Juengel *et al.*, 2009).

Participando da busca pelo conhecimento dos fatores locais que atuam no desenvolvimento folicular, nossa equipe iniciou um estudo investigando o papel da angiotensina II (AngII) como um fator local envolvido no crescimento, assim como, na regulação da seleção e divergência folicular. Em bovinos, além das funções bem estabelecidas em termos de regulação da pressão arterial e angiogênese, a AngII possui um papel indispensável para que ocorra ovulação (Ferreira *et al.*, 2007) e a maturação nuclear de oócitos (Giometti *et al.*, 2005; Barreta *et al.*, 2008). Resultados recentes sugerem uma regulação dos receptores tipo 2 de angiotensina (AT2 ou AGTR2) durante o crescimento folicular. Os níveis de mRNA do receptor AGTR2 são significativamente mais elevados em células da granulosa de folículos estrogênicos em comparação aos não-estrogênicos e a quantidade de mRNA de receptor AGTR2 apresentou uma correlação positiva com as concentrações de estradiol no fluido folicular. Esses dados suportam a hipótese de uma função da AngII no controle do crescimento folicular via receptor AGTR2 e uma interação com outros fatores locais envolvidos no crescimento folicular (Portela *et al.*, 2008). Quanto ao efeito da AngII durante o crescimento folicular, estudos foram realizados somente em ratos. Nesta espécie, a AngII induz apoptose nas células da granulosa e com isso atresia de folículos antrais (Speth *et al.*, 1999). No entanto, os resultados recentes do nosso grupo sugerem um efeito positivo da AngII durante o crescimento folicular de bovinos. Além disso, em coelhas,

a perfusão ovariana de IGF aumentou a produção ovariana de AngII (Yoshimura *et al.*, 1996a). Portanto, o presente trabalho foi desenvolvido com o objetivo de caracterizar o perfil de AngII durante desenvolvimento folicular, assim como testar a hipótese de que a AngII participa no desenvolvimento folicular de não roedores. O entendimento dos mecanismos que regulam o crescimento e dinâmica folicular permite novas abordagens no tratamento da infertilidade em diferentes espécies e ainda possibilita um maior controle sobre a função reprodutiva.

2. REVISÃO BIBLIOGRÁFICA

2.1. Crescimento folicular antral

O desenvolvimento folicular de bovinos é primariamente coordenado por gonadotrofinas hipofisiárias. O hormônio folículo estimulante (FSH) possui um papel chave na regulação da emergência das ondas foliculares e manutenção do crescimento dos folículos durante o período inicial de desenvolvimento (antes da divergência folicular). Em cada onda folicular, o período inicial é caracterizado por uma fase de desenvolvimento comum, onde os folículos crescem em diâmetro em resposta ao aumento dos níveis séricos de FSH (Adams *et al.*, 1992). Em espécies monovulares, o período de seleção folicular resulta na diminuição do número de folículos em crescimento até o completo estabelecimento da dominância, onde geralmente é observado o crescimento de somente um folículo. Esta diminuição do número de folículos em crescimento se deve por um aumento na capacidade secretória de estradiol pelos folículos em desenvolvimento e com isso uma regulação na secreção de FSH (Price e Webb, 1988; Mihm *et al.*, 2000). O aumento das concentrações séricas de estradiol diminuem a expressão e a estabilidade do gene que codifica a subunidade beta do FSH (Roche, 1996), fazendo com isso uma diminuição dos níveis plasmáticos desse hormônio. Está bem estabelecido que a divergência folicular, a qual é caracterizada por uma diferença na taxa de crescimento entre o futuro folículo dominante e seus subordinados, ocorre concomitante com a diminuição dos níveis plasmáticos de FSH (Ginther *et al.*, 1996). Durante essa fase, a falta de estímulo gonadotrófico para manutenção da esteroidogênese folicular faz com os folículos da mesma onda entrem em atresia, com exceção do folículo dominante que adquire uma "independência" ao FSH, o que permite que este folículo continue o seu crescimento mesmo na ausência desta gonadotrofina. O papel da expressão de receptores de LH nas células da granulosa para o estabelecimento da dominância é tema de contradição há bastante tempo. Inicialmente, se acreditava que a independência ao FSH era determinada diretamente pela aquisição de receptores de LH nas células da granulosa (Ginther *et al.*, 1999; Ginther *et al.*, 2001b). No entanto, EVANS & FORTUNE (1997) demonstraram através de hibridização *in situ* níveis indetectáveis de LHr nas células da granulosa durante a divergência folicular. O mesmo grupo caracterizou a participação de fatores locais que suportam o crescimento folicular durante essa fase de baixos níveis séricos de FSH (Fortune *et al.*, 2001; Rivera e Fortune, 2001; Fortune *et al.*, 2004). Dentre esses fatores, a participação do IGF no

crescimento folicular de bovinos, assim como suas proteínas de ligação (IGFBPs) e proteases específicas, está bem estabelecida (Spicer e Aad, 2007). Estes resultados suportam a hipótese de que a seleção folicular não é mediada, e sim uma causa, da aquisição de receptores de LH na granulosa. Mais recentemente, tornou-se evidente que outros fatores produzidos localmente atuam em um controle autócrino/parácrino da foliculogênese, desempenhando um papel essencial na modulação do crescimento de folículos e potencialização do efeito das gonadotrofinas (Mihm *et al.*, 2000; Fortune *et al.*, 2001; Pierre *et al.*, 2005; Knight e Glister, 2006; Miyoshi *et al.*, 2007; Gasperin *et al.*, 2008; Juengel *et al.*, 2009).

Após o período de divergência, o folículo dominante passa por um processo de diferenciação, principalmente na camada das células da granulosa. Esse processo é caracterizado por um aumento na capacidade mitótica e esteroidogênica do folículo dominante. Este fato se deve por um aumento na expressão de genes, nas células da granulosa, que codificam receptores para gonadotrofinas (FSHr e LHr; Evans e Fortune, 1997), enzimas esteroidogênicas chave (aromatase e 3ßHSD; Evans e Fortune, 1997; Irving-Rodgers *et al.*, 2003) e genes relacionados com remodelamento da matriz extracelular (SerpinE2; Bédard *et al.*, 2003), proliferação celular (ciclina D2; Sicinski *et al.*, 1996) e proteção contra apoptose (XIAP, GADD45b, etc; Li *et al.*, 1998; Sheikh *et al.*, 2000; De Smaele *et al.*, 2001).

2.1.1. Esteroidogênese folicular

Está bem estabelecido que folículos morfologicamente saudáveis apresentam uma concentração mais elevada de estradiol e mais reduzida de progesterona, quando em comparação com folículos atrésicos. Além disso, pequenos folículos antrais apresentam uma baixa concentração de estradiol no fluido folicular, a qual aumenta em folículos saudáveis ao longo do crescimento. No momento em que o folículo dominante atinge seu diâmetro máximo, a concentração de estradiol no fluido folicular cai drasticamente, fazendo com que o folículo entre em atresia (Price *et al.*, 1995).

O crescimento folicular e esteroidogênese são dependentes das ações coordenadas do FSH, LH e seus receptores. O modelo bem aceito e estabelecido para o crescimento folicular e esteroidogênese é o denominado “duas células/duas gonadotrofinas” (Fortune e Quirk, 1988). De acordo com este modelo as células foliculares atuam de maneira coordenada para a

produção do estradiol 17ß, sendo que as células da granulosa atuam sob o estímulo do FSH e as da teca do LH.

Folículos pré-antrais expressam FSHr; no entanto, não possuem outros elementos chaves para secreção de estradiol até a formação da camada de células da teca. Em folículos secundários e antrais iniciais, as células da teca iniciam a expressão de LHr, P450 side-chain cleavage (P450scc), 17α-hidroxilase (P450 17-OH) e 3β hidroxi-esteróide desidrogenase (3β-HSD), tornando-se hábil, desta forma, a secretar progesterona e andrógenos. No momento em que os folículos são recrutados em uma onda folicular, em conseqüência da maior responsividade ao FSH, as células da granulosa passam a expressar P450scc e aromatase (P450arom ou CYP19) e, portanto estão hábeis a sintetizar pregnolona e de converter androstenediona em estrona. Após o recrutamento, as células da teca começam a expressar a enzima StAR (steroidogenic acute regulatory protein), e portanto completando sua capacidade esteroidogênica. A medida que os folículos crescem e um se torna dominante, as células da granulosa começam a expressar 3β-HSD e também aumentam a expressão das enzimas aromatase, nas células da granulosa, e StAR, na teca (para revisão Bao e Garverick, 1998).

2.2. Angiotensina II

A angiotensina II (AngII) tem uma importante função na regulação da pressão sanguínea sistêmica e homeostase dos fluidos. O precursor do RAS, angiotensinogênio, é clivado pela renina na ligação leucina-leucina para formar o decapeptídeo angiotensina I (Clauser *et al.*, 1989; Palumbo *et al.*). A enzima conversora de angiotensina (ECA) cliva a Ang I formando a AngII. Além dessa conhecida via de produção da AngII, há evidências da presença do sistema renina-angiotensina (RAS) ovariano, incluindo a presença de componentes do RAS no ovário e mRNA de angiotensinogênio e pró-renina (Ohkubo *et al.*, 1986; Kim *et al.*, 1987). A AngII, octapeptídeo ativo do RAS, está presente em altas concentrações nos ovários de mamíferos (Husain *et al.*, 1987; Lightman *et al.*, 1987; Lightman *et al.*, 1988; Palumbo *et al.*, 1989), o que sugere uma função ovariana.

A presença de componentes do RAS em diversos tecidos, incluindo o ovário, gerou um novo conceito de RAS “local” ou “tecidual”. Além disso, a regulação destes sistemas locais é independente do controle sistêmico. Estes sistemas locais atuam de maneira autócrina/parácrina com diferentes funções dependendo do tecido (Phillips e Sumners, 1998; Kim e Iwao, 2000).

Há muitos fatores que evidenciam uma produção ovariana de AngII. Animais tratados com hCG demonstram concentrações mais altas de AngII no fluido folicular em comparação ao plasma, sugerindo produção local desse peptídeo (Yoshimura *et al.*, 1994). Husain et al. (1987) detectaram altos níveis ovarianos de AngII em animais nefrectomizados bilateralmente. Alguns trabalhos demonstram um aumento de AngII no fluido folicular após o pico ovulatório de gonadotrofinas. Yoshimura et al. (1994) verificaram um aumento na secreção folicular de AngII após a administração de hCG em ovários de coelhas perfundidos *in vitro*. Este aumento parece estar relacionado com o aumento da atividade intrafolicular da renina. Em bovinos, foi demonstrado *in vivo*, através de um sistema de microdiálise, um aumento nas concentrações de AngII no fluido folicular após o pico de LH (Acosta *et al.*, 2000).

A ativação da renina necessita da clivagem de um segmento da pró-renina (Do *et al.*, 1987) e parece ocorrer somente nos rins, uma vez que aquela não é detectada em animais nefrectomizados bilateralmente (Sealey *et al.*, 1977). A pró-renina é produzida e secretada principalmente pelos rins. Entretanto, há outras fontes de pró-renina, pois é detectada em machos e fêmeas que sofreram nefrectomia bilateral (Sealey *et al.*, 1977). As concentrações de pró-renina no fluido folicular são 100 vezes maiores que as concentrações plasmáticas (Glorioso *et al.*, 1986; Sealey *et al.*, 1986), e estão relacionadas com o número de folículos pré-ovulatórios (Itskovitz *et al.*, 1987), sugerindo uma produção local.

Formas incompletas de pró-renina produzidas pela ação de peptidases têm demonstrado uma atividade "semelhante à renina" (Shinagawa *et al.*, 1992). Mulheres com ciclo menstrual normal apresentam atividade semelhante à renina mais elevada no fluido folicular do que no plasma, sugerindo uma produção local desta proteína. Em um modelo *in vitro* usando ovários perfundidos de coelhas, a atividade semelhante à renina aumenta 2 a 4 horas após exposição ao hCG (Yoshimura *et al.*, 1994), sugerindo que as gonadotrofinas desempenham uma função importante na regulação da atividade de renina.

Mais recentemente, foi demonstrado que a pró-renina pode sofrer uma ativação catalítica ou não catalítica (Nguyen *et al.*, 2002; Suzuki *et al.*, 2003). Na ativação proteolítica, o pró-peptídeo é clivado por enzimas renais, incluindo pró-convertase 1 e catepsinas. Já a ativação não proteolítica é caracterizada por uma alteração conformacional e reversível, originando a renina (Suzuki *et al.*, 2003). O *(pro)renin receptor* (receptor de renina e pró-renina; (P)RR) além de ligar na renina e pró-renina e realizar a transdução da sinalização intracelular, ativa a pró-renina pela indução de uma modificação conformacional na molécula da pró-renina (Nguyen *et al.*, 2002; Nabi *et al.*, 2006; Batenburg *et al.*, 2007). A pró-renina de

ratos não é ativada pelo (P)RR humano, no entanto se liga e induz a transdução do sinal deste receptor (Kaneshiro *et al.*, 2007). Um estudo demonstrou que os níveis de AngII em ratos transgênicos com um aumento na expressão de (P)RR humano permanecem inalterados. No entanto, estes animais demonstraram um aumento nos níveis de aldosterona sérica e um aumento na ciclooxigenase 2 na córtex renal (Kaneshiro *et al.*, 2007). Estes resultados suportam o novo conceito de efeito de pró-renina independente de angiotensina. Além disso, algumas proteínas como a proteína de ligação de renina (RnBP) interagem com a renina inibindo sua ação *in vivo* (Takahashi *et al.*, 1992).

2.2.1. Receptores de angiotensina II

Com base nas diferentes propriedades farmacológicas e bioquímicas, os receptores de AngII foram classificados em dois subtipos (Chiu *et al.*, 1989; Whitebread *et al.*, 1989). O receptor tipo 1 (AT1 ou AGTR1) tem sido demonstrado mediando funções bem conhecidas da AngII como contração de musculatura lisa, síntese e secreção de aldosterona e angiogênese. Já o receptor tipo 2 (AT2 ou AGTR2) é responsável por efeitos opostos ao receptor AGTR1 e por mediar funções reprodutivas como esteroidogênese, maturação de oócitos e ovulação em algumas espécies (Yoshimura *et al.*, 1996b; Ferreira *et al.*, 2007).

Os receptores de AngII fazem parte da família de receptores com 7 domínios transmembrana ligados a proteína G. Mais recentemente foi demonstrado que esses receptores possuem a capacidade de não só se ligar a heterodímeros da proteina G, mas também a outras proteínas, incluindo proteínas solúveis. A denominada "proteína associada ao receptor AGTR1" (ATRAP) possui 3 domínios transmembrana e atua negativamente na sinalização via AGTR1, regulando a internalização destes receptores (Cui *et al.*, 2000; Lopez-Ilasaca *et al.*, 2003). Esta proteína está co-localizada com os receptores AGTR1 em túbulos renais de camundongos (Tsurumi *et al.*, 2006). Além disso, em culturas de cardiomiócitos, o aumento na expressão de ATRAP induzido por vetor viral determinou uma diminuição da presença do receptor AGTR1 na superfície celular, assim como uma diminuição da hipertrofia cardíaca mediada por AngII (Tanaka *et al.*, 2005).

Da mesma forma que no AGTR1, os receptores do tipo AGTR2 também possuem a capacidade de se ligar a proteínas. A "proteína de interação aos receptores AGTR2" (ATIP) parece atuar inibindo a proliferação celular (Nouet *et al.*, 2004) e estimulando a diferenciação celular e remodelamento tecidual mediada por AGTR2 (Li *et al.*, 2007; Min *et al.*, 2008).

Outra proteína, denominada “proteína de ligação aos receptores AGTR2” (ATBP), atua como uma proteína de membrana associada ao aparelho de golgi,regulando o transporte do AGTR2 para membrana celular (Wruck *et al.*, 2005).

2.2.2. Funções reprodutivas da angiotensina II

Os receptores da AngII foram descritos nas células da teca e granulosa de ratas (Husain *et al.*, 1987), coelhas (Yoshimura *et al.*, 1996b) e vacas (Portela *et al.*, 2008); e em macacas principalmente nas células da teca (Aguilera *et al.*, 1989). Em bovinos, a presença de receptores de AngII nas células foliculares (Schauser *et al.*, 2001; Berisha *et al.*, 2002; Portela *et al.*, 2008) e o aumento nas concentrações de AngII após o pico de LH (Acosta *et al.*, 2000) sugerem uma atividade biológica desse peptídeo nesta espécie. Recentemente, nosso grupo demonstrou que a AngII atua como mediador na ovulação induzida por gonadotrofinas em bovinos (Ferreira *et al.*, 2007). A aplicação intrafolicular de 100 μM de saralasina (inibidor dos receptores de AngII) bloqueou a ovulação somente antes do estro, portanto, antes do pico de LH (14.3% e 83.3% das vacas ovularam nos grupos saralasina e controle, respectivamente). O pico ovulatório de LH ocorre cerca de uma hora após o início do estro (Saumande e Humblot, 2005) e a AngII aumenta no fluido folicular após este acontecimento. A saralasina foi capaz de bloquear a ovulação somente quando administrada no momento e 6 horas após o tratamento com análogo do GnRH, mas não quando este inibidor foi administrado 12 horas após o GnRH (Ferreira *et al.*, 2007). Embora as concentrações de AngII permaneçam elevadas durante todo o processo de ovulação (Acosta *et al.*, 2000), nossos resultados demonstraram que a AngII desempenha uma função essencial somente no início deste evento.

Para determinar qual subtipo de receptor de AngII está envolvido na ovulação induzida por LH, uma injeção intrafolicular de losartan (antagonista dos receptores AGTR1), PD123,319 (antagonista AGTR2), losartan+PD123,319 ou solução salina foi realizada no momento em que as vacas foram desafiadas com análogo de GnRH. A ovulação foi inibida pela aplicação de PD123,319 e losartan+PD123,319, mas não pela aplicação de losartan ou solução salina. Portanto, a injeção intrafolicular do antagonista AGTR2 (PD123,319) bloqueou a ovulação independente da presença do antagonista do receptor AGTR1 (losartan), mostrando que somente o receptor AGTR2 desempenha uma função indispensável no processo de ovulação (Ferreira *et al.*, 2007). Além disso, foi observado uma maior

concentração de receptores AGTR2 em folículos pré-ovulatórios (Schauser *et al.*, 2001) e uma correlação positiva entre a produção de estrógeno pelas células da granulosa e a expressão de AGTR2 (Portela *et al.*, 2008), sugerindo uma participação deste receptor nos estádios finais de crescimento e ovulação destes folículos. Em cultivo de células da granulosa, a administração de FSH, IGF-I ou BMP-7 aumenta a secreção de estradiol e a expressão do receptor AGTR2 (Portela *et al.*, 2008). Esses dados suportam a hipótese de uma função da AngII no controle do crescimento folicular via receptor AGTR2 e uma interação com outros fatores locais envolvidos no crescimento folicular.

Em bovinos, a AngII apresenta um efeito positivo sobre o reinício da meiose, o qual foi demonstrado utilizando um modelo in vitro com co-cultivo de oócitos e células foliculares (Giometti *et al.*, 2005; Stefanello *et al.*, 2006). Com o nosso modelo in vivo de injeção intrafolicular, foi demonstrado que a AngII é requerida para o reinício da meiose induzido pelo pico ovulatório de LH em bovinos. A administração intrafolicular de saralasina bloqueou o reinício da meiose induzido pelo pico ovulatório de LH. Além disso, a inibição não seletiva da COX-1 e 2 inibiu o reinício da meiose induzido pela AngII no sistema de co-cultivo *in vitro* de oócitos e metades foliculares (Barreta *et al.*, 2008). Esses resultados evidenciam uma importante participação da AngII nos dois eventos finais do crescimento folicular (maturação de oócito e ovulação) culminando com a liberação de um oócito maturo e competente para a fecundação e desenvolvimento embrionário.

3. CAPÍTULO 1

ANGIOTENSIN II SIGNALING PROMOTES FOLLICLE GROWTH AND DOMINANCE IN CATTLE

Rogério Ferreira, Bernardo Gasperin, Monique Rovani, Joabel Santos, Marcos Barreta, Rodrigo Bohrer, Christopher Price, Paulo Bayard Gonçalves

ENDOCRINOLOGY, 2010

Angiotensin II signaling promotes follicle growth and dominance in cattle.

Rogério Ferreira[1,2], Bernardo Gasperin[1], Monique Rovani[1], Joabel Santos[1], Marcos Barreta[1], Rodrigo Bohrer[1], Christopher Price[2], Paulo Bayard Gonçalves[1]

[1]*Laboratório de Biotecnologia e Reprodução Animal, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil.*

[2]*Centre de Recherche en Reproduction Animale, Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, QC, Canada.*

Running Title: Angiotensin II and follicular growth.

Key Words: follicular deviation; intrafollicular factors; granulosa cells; steroidogenesis.

Corresponding author and reprint requests:

Paulo Bayard Dias Gonçalves

e-mail: bayard@biorep.ufsm.br

Address: Universidade Federal de Santa Maria,

Avenida Roraima, nº 1000, Prédio 97, sala 416.

Postal code 97105-900

Santa Maria, RS, Brazil

Tel: +55 (55) 32208752

Fax: +55 (55) 32208484

This work was funded by CNPq Brazil and NSERC Canada.

Disclosure statement: The authors of this manuscript have nothing to declare.

Abstract

It is generally understood that angiotensin (AngII) promotes follicle atresia in rats, although recent data suggested that this may not be true in cattle. In this study, we aimed to determine in vivo whether AngII alters follicle development in cattle, using intrafollicular injection of AngII or antagonist into the growing dominant follicle or the second largest subordinate follicle. Injection of saralasin, an AngII antagonist, into the growing dominant follicle inhibited follicular growth and this inhibitory effect was overcome by systemic FSH supplementation. Injection of AngII into the dominant follicle did not affect follicular growth, whereas injection of AngII into the second largest follicle prevented the expected atresia of this subordinate follicle and the treated follicle grew at the same rate as the dominant follicle for the following 24h. Inhibition of AngII action in the dominant follicle decreased estradiol concentrations in follicular fluid and the abundance of mRNA encoding aromatase, 3β-hydroxysteroid dehydrogenase, LH receptor and cyclinD2 in granulosa cells, with minimal effects on theca cells. The effect of AngII on aromatase mRNA levels was confirmed using an in vitro granulosa cell culture system. In conclusion, these data suggest that AngII signaling promotes follicle growth in cattle, and does so by regulating genes involved in estradiol secretion and granulosa cell proliferation and differentiation.

Introduction

Ovarian function in mammals is primarily orchestrated by endocrine factors, mainly gonadotropins (FSH and LH), their receptors (FSHR and LHR) and ovarian steroids. It is now well established that follicle growth occurs in waves, and that the growth of a cohort of follicles is stimulated by a transient increase in FSH. In single-ovulating species, as FSH levels decline one follicle is selected to continue growing, while the remainder of the cohort regresses (1). The differential expression of several genes involved in survival and prevention of apoptosis in granulosa and theca cells, including that of LHR and members of the IGF1 family, allows the dominant follicle to become "FSH independent" and to continue its growth during the nadir of FSH secretion (2). It has also become clear that locally-produced paracrine factors play important roles in ovarian function, including members of the IGF and TGFβ families (3-5) and the renin-angiotensin system (6, 7).

An active renin-angiotensin system (RAS) is well described within the ovary. Angiotensin II (AngII) is the most potent peptide of the RAS, and acts through type 1 (AGTR1 or AT1) and type 2 receptors (AGTR2 or AT2). While the AGTR1 receptor mediates a number of well-known AngII effects on smooth muscle contraction, aldosterone secretion and blood pressure regulation, the AGTR2 receptor mediates the effects of AngII in the ovary (6-8). AngII is most known for its pivotal roles in ovulation and oocyte maturation (7-10).

Several lines of evidence in rats and rabbits also suggest that AngII alters the fate of the growing follicle, driving it toward atresia; the type 2 receptor occurs on atretic but not healthy granulosa cells (11-13), receptor binding increased with the induction of granulosa apoptosis in vitro (14), and treatment of granulosa cells with AngII induced DNA fragmentation and decreased estradiol secretion (15, 16). This, however, may not be true in other species. We reported that granulosa cell expression of AGTR2 is higher in healthy than in atretic follicles in

cattle, and is stimulated by FSH (6). This intriguing observation suggests that AngII may promote follicle development in cattle.

The objective of this study was to test the hypothesis that AngII promotes follicular development in cattle. We employed a model of intrafollicular injection of the dominant or future subordinate follicle with AngII agonist or antagonist in vivo to evaluate the direct impact of AngII on follicle growth. A further objective was to gain insight into mechanisms involved in AngII action.

Materials and Methods

Animals and sequential ultrasound scanning

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee (ACUC nº 23081.009594/2007-41). Cycling, non-lactating, mature cows of predominantly Angus and Hereford breeds, with a body condition score of 3 or 4 (scale from 1-thin to 5-obese) were used in this study. To induce a new follicular wave, all follicles larger than 5 mm were ablated via transvaginal ultrasound-guided follicular aspiration (17) using an ultrasound equipped with a convex 7.5 MHz transducer attached to a biopsy guide and a scanner (Aquila Vet Scanner; Pie Medical Equipment BV, Maastricht, The Netherlands). On the day of follicular ablation, all cows were given two doses of a PGF2α analogue (cloprostenol, 250 μg; Schering-Plough Animal Health, Brazil) im, 12h apart.

Follicular growth was monitored daily through transvaginal ultrasonography by a single operator. During each evaluation, all follicles larger than 5mm were recorded using 3 to 5 virtual slices of the ovary allowing a three-dimensional localization of follicles and the monitoring of individual follicles during the wave. Follicular growth rate was defined as the change in follicular diameter per 24h.

Experimental design

The first experiment was conducted to evaluate the effect of AngII or AngII antagonist on follicular growth at the expected time of follicle deviation. A new follicular wave was induced and follicular growth was monitored by ultrasound as described above. When the largest follicle reached 7 to 8 mm, AngII (final concentration of 10 μM), saralasin (AngII receptor antagonist; 10 μM) or saline was injected into the largest follicle (n=4/group). When injected follicles reached the ovulatory size (12 mm), a systemic injection of GnRH-analog (gonadorelin, 100 μg i.m.; Tortuga, Brazil) was performed to induce ovulation.

In a second series of experiments, the second largest, potentially subordinate follicle was injected with AngII (10 μM; n=5), AGTR2-receptor agonist (10 μM; CGP42112A, Sigma; n=4) or saline (n=4) when the difference between the largest and second largest follicle reached 1 mm. The mean follicular sizes of the largest and second largest follicles at the time of injection were 8.0±0.2 and 7.0±0.2 mm, respectively. Follicle growth was followed by ultrasonography for 72 h.

To evaluate whether FSH alters the follicle response to AngII antagonist, cows were treated with saralasin with or without FSH (Pluset, Laboratórios Calier, Brazil). A new follicular wave was induced and, when the largest follicle reached 6 - 7 mm diameter, cows from all groups received a systemic injection of FSH. Twelve hours later, the largest follicle from each cow was injected with saline (n=2) or saralasin (groups SAR, n=2, and SAR+FSH, n=3). The cows from SAR+FSH group received more 5 systemic injections of FSH (10 IU) every 12 h thereafter for 60 h.

The mechanisms of AngII action were explored with in vivo and in vitro models. For the in vivo approach, the dominant follicle was injected with saralasin or saline and the cows were ovariectomized 24h later. The follicular fluid was aspirated to determine steroid concentrations, and granulosa and theca cells were recovered to measure gene expression. In vitro, granulosa cells were cultured in serum-free medium with AngII (0, 0.1 or 10 μM). After treatment, the cells were recovered in Trizol for RNA extraction.

Ultrasound-guided intrafollicular injection and ovariectomy

The intrafollicular injections were performed with a double-needle system and guided by ultrasound as previously described (7). Briefly, under epidural anesthesia the ovary was manipulated so that the needle penetrates the follicle via penetration of the ovarian stroma at the base of the follicle. The needle path to the injected follicle contained ovarian stroma and no additional antral follicles or corpus luteum. When the ovary and follicle of interest were in position, the outer needle was advanced until the image of its tip became visible on the screen, approximately 1 mm from the follicle. At this moment, a second operator advanced the inner needle until the image of the needle tip was visible inside the follicle. Swirling of the fluid entering the follicle indicated that the injection was successful. The dose of each treatment (described in Results) was calculated based on the volume of follicular fluid in order to obtain the desired final concentration inside the follicle. The follicular fluid volume was estimated by the linear regression equation $V=-685.1+120.7D$, where V corresponds to the estimated follicular volume and D to the diameter of the follicle to be injected (7). Cows were excluded from the experiment if the injected follicle had a reduction in diameter larger than 1 mm within 2 hours of injection, which is evidence of follicle leakage.

After treatment, ovaries were harvested by colpotomy (18) for RNA extraction. Ovaries were collected 24 hours following treatments, follicular cells were recovered as previously described (19) and stored in RNAlater (Qiagen, Mississauga, ON, Canada) at -80 C. Cross-contamination of theca and granulosa cells was tested by PCR detection of the mRNAs that encode cytochrome P450 aromatase (CYP19A1) and 17a-hydroxylase (CYP17A1) respectively. Granulosa cells that expressed CYP17A1 and theca cells that expressed CYP19A1 were discarded (20).

Granulosa cell culture

The serum-free, nonluteinizing granulosa cell culture system was based on that described by Gutiérrez et al. (21) with slight modifications (22). All materials were obtained from Invitrogen Life Technologies (Burlington, Ontario, Canada) except where otherwise stated. Briefly, follicles were dissected from ovaries of abattoir origin and those with obvious signs of atresia (avascular theca or debris in antrum) were discarded. Cells from 6-8mm follicles were harvested by repeatedly passing bisected follicle walls through a pipette and filtered through 150 mesh stainless steel strainer (Sigma). Granulosa cells were washed twice by centrifugation at 200 g for 20 min each, and suspended in supplemented α-MEM containing 1 ng/ml FSH (19). Cell viability was estimated with 0.4% Trypan Blue stain. Cells were seeded into 24-well tissue culture plates (Sarstedt, Montreal, Quebec, Canada) at a density of $0.5x10^6$ viable cells per well in 1ml medium. Cultures were maintained at 37 C in 5% CO2 in air for 4 d, with 70% of medium being replaced every 2 d. On day 4, cells were treated with AngII (0, 0.1 or 10 μM) for 6 h. At the end of culture, the medium was recovered for estradiol measurement and the cells collected in Trizol for extraction of total RNA.

Nucleic Acid Extraction and Real-Time RT-PCR

Total RNA was extracted using Trizol (theca cells) or silica based protocol (granulosa cells; Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions and was quantified by absorbance at 260 nm. Total RNA (1 mg) was first treated with 0.2 U DNase (Invitrogen) at 37 C for 5 minutes to digest any contaminating DNA, followed by heating to 65 C for 3 minutes. The RNA was reverse transcribed (RT) in the presence of 1 μM oligo(dT), primer, 4 U Omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, ON, Canada), 0.5 μM dideoxynucleotide triphosphate (dNTP) mix, and 10 U RNase Inhibitor (Invitrogen) in a volume of 20 μL at 37 C for 1 hour. The reaction was terminated by incubation at 93 C for 5 minutes.

Real-time polymerase chain reaction (PCR) was conducted in an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) and bovine-specific primers for aromatase (CYP19), 3β-hydroxysteroid dehydrogenase (3βHSD), LHR, serine protease inhibitor E2 (SerpinE2), cyclinD2, steroidogenic acute regulatory protein (StAR), 17β-hydroxysteroid dehydrogenase (17βHSD), FSHR, growth arrest and DNA damage inducible protein 45b (GADD45b) and X-linked inhibitor of apoptosis protein (XIAP) (Table 1). Common thermal cycling parameters (3 minutes at 95 C, 40 cycles of 15 seconds at 95 C, 30 seconds at 60 C, and 30 seconds at 72 C) were used to amplify each transcript. Samples were run in duplicate and melting-curve analyses were performed to verify product identity. Data were normalized to a calibrator sample and relative values calculated with correction for amplification efficiency (23). Cyclophilin was the housekeeping gene. Bovine-specific primers were taken from the literature or designed using Primer Express Software v3.0 (Applied Biosystems) and synthesized by Invitrogen.

Steroid assay

Estradiol was measured in follicular fluid and in conditioned medium in duplicate as described (24) without solvent extraction. Intra- and interassay coefficients of variation were 3.1 and 4.9%, respectively. Progesterone was measured in follicular fluid in duplicate as described (25) with mean intra- and interassay coefficients of variation of 4.7 and 11%, respectively. The sensitivity of these assays was 10 and 4 pg per tube for estradiol and progesterone, respectively.

Statistical analysis

The assessment of treatment effects on follicular dynamics was performed as repeated measures data and analyzed using the MIXED procedure with a repeated measure statement. Main effects of treatment group, day and their interaction were determined. Differences between

follicular sizes at a specific time point were compared between groups using estimates. Other continuous data were submitted to ANOVA using the General Linear Models (GLM) and multi-comparison between groups was performed by least square means. The differences between dominant and subordinate follicle was accessed by paired Student's t test using cow as subject. Data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. All analyses were performed using SAS software package (SAS Institute Inc., Cary, NC). Results are presented as means ± standard error of the mean. A $P<0.05$ was considered statistically significant.

Results

AngII and follicular dynamics

The first experiment was conducted to assess the role AngII plays in follicular development by injecting AngII or antagonist directly into the growing dominant follicle. Follicles receiving saline or AngII continued to grow after injection (Figure 1A) and ovulated after systemic injection of GnRH-analog. However, the intrafollicular injection of saralasin inhibited follicular growth in all treated cows ($P<0.01$, Fig 1A). All cows treated with saralasin had subsequent development of a new follicular wave (Figure 1B).

As the injection of AngII did not alter the growth of healthy dominant follicles, we assessed the effect of AngII on the second largest, future subordinate follicle. AngII delayed the expected regression of the subordinate follicle, which continued to grow at a rate similar to the dominant follicle for 24h (Figure 2). After 24h, the injected follicle stopped growing and regressed (data not shown). Injection of the AT2-specific agonist CGP42112A had a similar effect (Figure 2).

FSH drives follicle growth during the development of the follicle wave, therefore we assessed if the follicle regression induced by AngII blockade can be reversed by FSH. As before, intrafollicular injection of saralasin induced regression of the dominant follicle, but

supplementation with FSH overcame this effect ($P<0.05$; Figure 3). All cows (3/3) ovulated at 120h after treatment of saralasin plus FSH whereas those treated with saralasin without FSH did not ovulate (Figure 3B).

Mechanism of action of AngII

To identify target genes of AngII action, follicles were recovered 24 h after a single intrafollicular injection of saralasin. Compared with saline-injected follicles, saralasin induced follicular regression and decreased the estradiol:progesterone ratio in follicular fluid (Figure 4). Inhibition of AngII action decreased the abundance of mRNA encoding CYP19, 3βHSD, LHR, SerpinE2 and cyclinD2 in granulosa cells, but did not alter abundance of StAR, 17βHSD, FSHR GADD45b, XIAP or AGTR2 mRNAs (Figure 5). In contrast, in theca cells the inhibition of AngII decreased the expression of AGTR2 but not the expression of genes encoding steroidogenic enzymes or LHR (not shown).

As inhibition of AngII signaling mostly affected granulosa cells, we assessed the direct effect of AngII on granulosa cells in serum-free culture. AngII increased CYP19 mRNA abundance and there was no effect of AngII on 3βHSD or 17βHSD mRNA levels ($P>0.05$; Figure 6).

Discussion

In rats, it is established that AngII stimulates follicle atresia (14-16), whereas in cattle the pattern of AGTR2 expression suggests that AngII may stimulate follicle growth (6). The present study provides the first evidence that AngII promotes follicle growth, and thus represents a major departure from the aforementioned studies in rodents. Our significant findings are that intrafollicular injection of AngII antagonist inhibited growth of the dominant follicle, and that

injection of AngII or AGTR2 agonist into the future subordinate follicle prevented the expected regression of this follicle at deviation.

Follicles injected with saline reached ovulatory size and ovulated after challenge with GnRH agonist, demonstrating that the intrafollicular injection did not affect the future of the follicle, as previously demonstrated by us and others (7, 26, 27). Intrafollicular injection of saralasin, an AngII antagonist, inhibited follicular growth in all treated cows, suggesting that AngII signaling is essential for follicular growth in 7 to 8mm follicles. However, intrafollicular injection of AngII in the future dominant follicle affected neither follicular growth nor the pattern of follicular dynamics. These results imply that dominant follicles contain sufficient AngII for follicle development. The dominant follicle is characterized by high free-IGF1 content that permits its continued growth at a time when FSH concentrations are declining (28). In rabbits, IGF1 increased ovarian AngII production and in cattle IGF1 increased AGTR2 mRNA and protein (6); therefore one mechanism by which IGF1 acts to maintain follicle growth may be through increased activity of the local RAS. To our knowledge, there are no studies reporting AngII concentrations in follicular fluid during follicular development, although the LH surge increased preovulatory AngII secretion in cattle (29).

If our hypothesis is correct, increased AngII should promote growth of subordinate follicles otherwise destined for regression during the decline in systemic FSH concentrations. The second largest follicle is the future subordinate follicle, and regresses as the dominant follicle grows; this occurred in saline-injected future subordinate follicles. In support of our hypothesis, intrafollicular injection of AngII prevented regression of this follicle. An AGTR2-specific agonist had the same effect, confirming previous studies identifying the type 2 receptor as the mediator of AngII action in the ovary (6-9, 30). The ‘rescue’ from atresia of the future subordinate follicle was relatively short-lived, as the injected follicle started to regress from 48 h after injection. It is possible that AngII diffuses from or is metabolized within the follicle within this time period, and decreases to ineffective concentrations. Repeated injections may be required to maintain effective

intrafollicular levels of AngII, but this would be difficult to do as repeated puncture is likely to have deleterious effects on the follicle.

The above experiments were performed around the time of deviation, when FSH concentrations are declining and the dominant follicle becomes 'FSH-independent' (1, 31). It would be of interest to assess the effects of AngII before deviation during the growth of the cohort under FSH stimulation, but such experiments are complicated by the larger number of smaller follicles – tracking individual follicles becomes difficult. Therefore we mimicked these conditions once the two largest follicles were detectable by augmenting serum FSH concentrations by systemic injections (32). Under these conditions, AngII blockade did not inhibit growth of the injected follicle. Collectively, these data suggest that AngII is not necessary for early development of the follicle cohort driven by FSH, but is essential for continued development under the low-FSH environment occurring post-deviation.

To determine the targets of AngII action, granulosa and theca cells were collected from follicles 24 h after AngII inhibition by saralasin, which is the time we observed the first change in diameter of saralasin-treated follicles. Saralasin caused minor changes in gene expression in theca cells, but resulted in major inhibition of key genes in granulosa cells. The marked inhibition of CYP19, LHR and 3βHSD are consistent with regression of the subordinate follicle (5, 33, 34). Saralasin also decreased cyclinD2 mRNA abundance. CyclinD2 regulates cell proliferation by controlling the G1 to S transition and is regulated by FSH and estradiol (35-37). Saralasin did not alter FSHR mRNA levels, which may be in part because the reduction in FSHR mRNA in granulosa cells during follicle regression occurs later than changes of other transcripts (33). It is noteworthy that saralasin did not affect mRNA encoding proteins associated with cell damage and atresia, GADD45B and XIAP (38-40). It has been demonstrated that GADD45B mRNA levels differ between dominant and subordinate follicles in cattle (5) and is associated with granulosa cell death in vitro (41). It is possible that changes in GADD45B expression is a relatively late event in atresia, and was therefore not detected at 24 h after AngII inhibition.

Collectively these data suggest that AngII regulates granulosa cell growth/differentiation but has little direct effect on atresia.

We extended these findings by assessing the short-term response of estrogenic bovine granulosa cells to AngII in vitro. A low dose of AngII increased CYP19 mRNA abundance after the 6 h treatment period, but did not affect abundance of mRNA encoding 3βHSD or 17βHSD. The increase in CYP19 mRNA by AngII is consistent with the inhibition of this mRNA observed in vivo after saralasin injection and with the stimulatory effect of AngII on growth of the subordinate follicle. The lack of effect of AngII on 3βHSD mRNA levels in vitro is likely due in part to the relatively short period of exposure in vitro (6 h) compared to the in vivo experiment with saralasin (24 h), and suggests that CYP19 is a principal target of AngII action in the follicle.

In summary, the present data point to a critical role for AngII in promoting the development of the post-deviation dominant follicle, which stands in contrast to the inhibitory effects previously reported in rats. The present data suggest that AngII enhances CYP19 expression and estradiol secretion, and ensures differentiation and development of the dominant follicle. Conversely, the follicle in which local AngII production decreases would experience a reduction in CYP19 mRNA levels and reduced estradiol secretion, the consequence of which would be a compromise in granulosa cell differentiation leading to follicle atresia.

Acknowledgements

The authors would like to thank the Fazenda do Leão, Dr. José Manoel Ferreira and Mr Walter N Ferreira for providing the animals used in this work. We are very grateful to Dr. Vinicius de Oliveira for providing animal facilities. We also would like to thank Dr. Cláudio Alves Pimentel, Dr. Alfredo Antoniazzi and Dr. Gustavo Zamberlam for technical assistance.

References

1. **Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K** 1996 Selection of the dominant follicle in cattle. Biol Reprod 55:1187-1194
2. **Mihm M, Baker PJ, Ireland JLH, Smith GW, Coussens PM, Evans ACO, Ireland JJ** 2006 Molecular Evidence That Growth of Dominant Follicles Involves a Reduction in Follicle-Stimulating Hormone Dependence and an Increase in Luteinizing Hormone Dependence in Cattle. Biol Reprod 74:1051-1059
3. **Knight PG, Glister C** 2006 TGF-ß superfamily members and ovarian follicle development. Reproduction 132:191-206
4. **Fortune JE, Rivera GM, Yang MY** 2004 Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. Anim Reprod Sci 82-83:109-126
5. **Mihm M, Baker PJ, Fleming LM, Monteiro AM, O'Shaughnessy PJ** 2008 Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. Reproduction 135:253-265
6. **Portela VM, Goncalves PBD, Veiga AM, Nicola E, Buratini J, Jr., Price CA** 2008 Regulation of Angiotensin Type 2 Receptor in Bovine Granulosa Cells. Endocrinology 149:5004-5011
7. **Ferreira R, Oliveira JF, Fernandes R, Moraes JF, Gonçalves PB** 2007 The role of angiotensin II in the early stages of bovine ovulation. Reproduction 134:713-719
8. **Yoshimura Y, Karube M, Aoki H, Oda T, Koyama N, Nagai A, Akimoto Y, Hirano H, Nakamura Y** 1996 Angiotensin II induces ovulation and oocyte maturation in rabbit ovaries via the AT2 receptor subtype. Endocrinology 137:1204-1211
9. **Giometti IC, Bertagnolli AC, Ornes RC, da Costa LFS, Carambula SF, Reis AM, de Oliveira JFC, Emanuelli IP, Gonçalves PBD** 2005 Angiotensin II reverses the inhibitory action produced by theca cells on bovine oocyte nuclear maturation. Theriogenology 63:1014-1025
10. **Barreta MH, Oliveira JFC, Ferreira R, Antoniazzi AQ, Gasperin BG, Sandri LR, Goncalves PBD** 2008 Evidence that the effect of angiotensin II on bovine oocyte nuclear maturation is mediated by prostaglandins E2 and F2α. Reproduction 136:733-740
11. **de Gooyer TE, Skinner SL, Wlodek ME, Kelly DJ, Wilkinson-Berka JL** 2004 Angiotensin II influences ovarian follicle development in the transgenic (mRen-2)27 and Sprague-Dawley rat. J Endocrinol 180:311-324
12. **Pucell AG, Hodges JC, Sen I, Bumpus FM, Husain A** 1991 Biochemical properties of the ovarian granulosa cell type 2-angiotensin II receptor. Endocrinology 128:1947-1959
13. **Daud AI, Bumpus FM, Husain A** 1988 Evidence for selective expression of angiotensin II receptors on atretic follicles in the rat ovary: an autoradiographic study. Endocrinology 122:2727-2734
14. **Tanaka M, Ohnishi J, Ozawa Y, Sugimoto M, Usuki S, Naruse M, Murakami K, Miyazaki H** 1995 Characterization of Angiotensin II Receptor Type 2 during

Differentiation and Apoptosis of Rat Ovarian Cultured Granulosa Cells. Biochem Biophys Res Commun 207:593-598

15. **Kotani E, Sugimoto M, Kamata H, Fujii N, Saitoh M, Usuki S, Kubo T, Song K, Miyazaki M, Murakami K, Miyazaki H** 1999 Biological roles of angiotensin II via its type 2 receptor during rat follicle atresia. Am J Physiol Endocrinol Metab 276:E25-33
16. **Feral C, LeGall S, Leymarie P** 1995 Angiotensin II modulates steroidogenesis in granulosa and theca in the rabbit ovary: Its possible involvement in atresia. Eur J Endocrinol 133:747-753
17. **Bergfelt DR, Lightfoot KC, Adams GP** 1994 Ovarian dynamics following ultrasound-guided transvaginal follicle ablation in cyclic heifers. Theriogenology 41:161-161
18. **Drost MD, Savio JD, Barros CM, Badinga L, Thatcher WW** 1992 Ovariectomy by colpotomy in the cow. J Am Vet Med Assoc 200:337-342
19. **Buratini J, Jr., Pinto MGL, Castilho AC, Amorim RL, Giometti IC, Portela VM, Nicola ES, Price CA** 2007 Expression and Function of Fibroblast Growth Factor 10 and Its Receptor, Fibroblast Growth Factor Receptor 2B, in Bovine Follicles. Biol Reprod 77:743-750
20. **Buratini J, Jr., Teixeira AB, Costa IB, Glapinski VF, Pinto MGL, Giometti IC, Barros CM, Cao M, Nicola ES, Price CA** 2005 Expression of fibroblast growth factor-8 and regulation of cognate receptors, fibroblast growth factor receptor-3c and -4, in bovine antral follicles. Reproduction 130:343-350
21. **Gutierrez CG, Campbell BK, Webb R** 1997 Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle- stimulating hormone, and morphological characteristics. Biol Reprod 56:608-616
22. **Silva JM, Price CA** 2000 Effect of Follicle-Stimulating Hormone on Steroid Secretion and Messenger Ribonucleic Acids Encoding Cytochromes P450 Aromatase and Cholesterol Side-Chain Cleavage in Bovine Granulosa Cells In Vitro. Biol Reprod 62:186-191
23. **Pfaffl MW** 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45
24. **Belanger A, Couture J, Caron S, Roy R** 1990 Determination of nonconjugated and conjugated steroid levels in plasma and prostate after separation on C-18 columns. Ann N Y Acad Sci 595:251-259
25. **Lafrance M, Goff AK** 1985 Effect of pregnancy on oxytocin-induced release of prostaglandin F2 alpha in heifers. Biol Reprod 33:1113-1119
26. **Kot K, Gibbons JR, Ginther OJ** 1995 A technique for intrafollicular injection in cattle: Effects of hCG. Theriogenology 44:41-50
27. **Ginther OJ, Bergfelt DR, Beg MA, Meira C, Kot K** 2004 In Vivo Effects of an Intrafollicular Injection of Insulin-Like Growth Factor 1 on the Mechanism of Follicle Deviation in Heifers and Mares. Biol Reprod 70:99-105
28. **Rivera GM, Fortune JE** 2003 Proteolysis of insulin-like growth factor binding proteins -4 and -5 in bovine follicular fluid: implications for ovarian follicular selection and dominance. Endocrinology 144:2977-2987

29. **Acosta TJ, Ozawa T, Kobayashi S, Hayashi K, Ohtani M, Kraetzl WD, Sato K, Schams D, Miyamoto A** 2000 Periovulatory Changes in the Local Release of Vasoactive Peptides, Prostaglandin F2α, and Steroid Hormones from Bovine Mature Follicles In Vivo. Biol Reprod 63:1253-1261
30. **Kuji N, Sueoka K, Miyazaki T, Tanaka M, Oda T, Kobayashi T, Yoshimura Y** 1996 Involvement of angiotensin II in the process of gonadotropin-induced ovulation in rabbits. Biol Reprod 55:984-991
31. **Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ** 1992 Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. J Reprod Fertil 94:177-188
32. **Rivera GM, Fortune JE** 2001 Development of codominant follicles in cattle is associated with a follicle-stimulating hormone-dependent insulin-like growth factor binding protein-4 protease. Biol Reprod 65:112-118
33. **Bao B, Garverick HA** 1998 Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. J Anim Sci 76:1903-1921
34. **Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS** 1995 Expression of messenger ribonucleic acid encoding cytochrome P450 side-chain cleavage, cytochrome p450 17 alpha-hydroxylase, and cytochrome P450 aromatase in bovine follicles during the first follicular wave. Endocrinology 136:981-989
35. **Robker RL, Richards JS** 1998 Hormone-Induced Proliferation and Differentiation of Granulosa Cells: A Coordinated Balance of the Cell Cycle Regulators Cyclin D2 and p27Kip1. Mol Endocrinol 12:924-940
36. **Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, Elledge SJ, Weinberg RA** 1996 Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature 384:470-474
37. **Quirk SM, Cowan RG, Harman RM** 2006 The susceptibility of granulosa cells to apoptosis is influenced by oestradiol and the cell cycle. J Endocrinol 189:441-453
38. **Li J, Kim J-M, Liston P, Li M, Miyazaki T, Mackenzie AE, Korneluk RG, Tsang BK** 1998 Expression of Inhibitor of Apoptosis Proteins (IAPs) in Rat Granulosa Cells during Ovarian Follicular Development and Atresia. Endocrinology 139:1321-1328
39. **De Smaele E, Zazzeroni F, Papa S, Nguyen DU, Jin R, Jones J, Cong R, Franzoso G** 2001 Induction of gadd45ß by NF-κB downregulates pro-apoptotic JNK signalling. Nature 414:308-313
40. **Sheikh MS, Hollander MC, Fornace AJ** 2000 Role of Gadd45 in apoptosis. Biochem Pharmacol 59:43-45
41. **Portela VM, Zamberlam G, Price CA** 2010 Cell plating density alters the ratio of estrogenic to progestagenic enzyme gene expression in cultured granulosa cells. Fertil Steril 93:2050-2055
42. **Hamel M, Vanselow J, Nicola ES, Price CA** 2005 Androstenedione increases cytochrome P450 aromatase messenger ribonucleic acid transcripts in nonluteinizing bovine granulosa cells. Mol Reprod Dev 70:175-183

43. **Ledoux S, Campos DB, Lopes FL, Dobias-Goff M, Palin MF, Murphy BD** 2006 Adiponectin induces periovulatory changes in ovarian follicular cells. Endocrinology 147:5178-5186
44. **Lagaly DV, Aad PY, Grado-Ahuir JA, Hulsey LB, Spicer LJ** 2008 Role of adiponectin in regulating ovarian theca and granulosa cell function. Mol Cell Endocrinol 284:38-45
45. **Luo W, Wiltbank MC** 2006 Distinct Regulation by Steroids of Messenger RNAs for FSHR and CYP19A1 in Bovine Granulosa Cells. Biol Reprod 75:217-225
46. **Orisaka M, Mizutani T, Tajima K, Orisaka S, Shukunami K-i, Miyamoto K, Kotsuji F** 2006 Effects of ovarian theca cells on granulosa cell differentiation during gonadotropin-independent follicular growth in cattle. Mol Reprod Dev 73:737-744
47. **Bédard J, Brûlé S, Price CA, Silversides DW, Lussier JG** 2003 Serine protease inhibitor-E2 (SERPINE2) is differentially expressed in granulosa cells of dominant follicle in cattle. Mol Reprod Dev 64:152-165
48. **Boelhauve M, Sinowatz F, Wolf E, Paula-Lopes FF** 2005 Maturation of Bovine Oocytes in the Presence of Leptin Improves Development and Reduces Apoptosis of In Vitro-Produced Blastocysts. Biol Reprod 73:737-744

Table 1 - Primers used for expression analysis of candidate genes. Primer sequences and concentration used to amplify each product are shown for each gene investigated.

Gene		Sequence	Size (bp)	Conc. (µM)	Reference or accession nº
CYP19	F	CTGAAGCAACAGGAGTCCTAAATGTACA	289	200	(42)
	R	AATGAGGGGCCCAATTCCCAGA		300	
AGTR2	F	GACCTGGCACTTCCTTTTGC	100	200	XM_001249373.1
	R	GGAGCTTCTGCTGGAACCTATTC		200	
CyclinD2	F	TGCCCCAGTGCTCCTACTTC	482	200	(5)
	R	CGGGTACATGGCAAACTTGA		200	
Cyclophilin	F	GGTCATCGGTCTCTTTGGAA	117	200	(43)
	R	TCCTTGATCACACGATGGAA		200	
CYP17	F	CCATCAGAGAAGTGCTCCGAAT	80	200	(44)
	R	GCCAATGCTGGAGTCAATGA		200	
FSHr	F	AGCCCCTTGTCACAACTCTATGTC	105	200	(45)
	R	GTTCCTCACCGTGAGGTAGATGT		200	
GADD45B	F	TACGAGTCGGCCAAGCTGAT	81	200	(5)
	R	GTCCTCCTCTTCCTCGTCGAT		200	
HSD17b1	F	TGTGGTACTCATTACCGGCTGTT	100	200	NM_001102365.1
	R	CAGCGTGGCATACACTTTGAA		200	
HSD3b	F	GCCCAACTCCTACAGGGAGAT	135	200	(46)
	R	TTCAGAGCCCACCCATTAGCT		200	
LHCGr	F	GCACAGCAAGGAGACCAAATAA	100	200	NM_174381.1
	R	TTGGGTAAGCAGAAACCATAGTCA		200	
SerpinE2	F	TCCGTGACGTTGCCCTCTGTG	555	200	(47)
	R	CCGTGATCTCCACAAACCCTT		200	
StAR	F	CCCAGCAGAAGGGTGTCATC	157	200	(46)
	R	TGCGAGAGGACCTGGTTGAT		200	
XIAP	F	GAAGCACGGATCATTACATTTGG	89	200	(48)
	R	CCTTCACCTAAAGCATAAAATCCAG		200	

F, Forward primer; R, Reverse primer; Conc., primer concentration used for gene amplification. Gene names are provided in the text.

Figure Legends

Figure 1

In vivo effect of AngII or AngII-inhibitor (SAR) treatment on bovine follicular growth (A). Panel B shows regression of a saralasin-injected follicle in a representative cow. A new follicular wave was induced and when the largest follicle reached 7 to 8mm, AngII (10 µM; n=4), saralasin (AngII receptor antagonist; 10 µM; n=4) or saline (n=4) was intrafollicularly injected in a single follicle per cow. Main effects of treatment group, day and their interaction were determined by

MIXED procedure with repeated measure and differences between follicular size at a specific time point were compared between groups using estimates (a≠b: $P<0.05$).

Figure 2

Follicular growth of largest (1LF) and second largest follicle (2LF) for 24 hours after follicular injection of AngII (10 μM; n=5), CGP42122 (AGTR2 receptor agonist; 10 μM; n=4) or saline (n=4) in the 2LF. At follicular deviation, the second largest follicle was injected with AngII, CGP42112A or saline to verify whether this peptide has a pivotal role on follicular dominance. Asterisk (*) indicates statistical difference between largest and second largest follicle accessed by paired Student's T test using cow as subject.

Figure 3

Effect of systemic FSH on AngII-antagonist treated follicle. Saralasin (AngII receptor antagonist; 10 μM) was injected and the cows were treated or not with systemic FSH (10 I.U. every 12h for 96h). The figure shows twenty-four hour follicular growth rate (A), ovulation rate (B) and follicular dynamics (C) after intrafollicular injection of saralasin (SAR; AngII blocker; n=2), SAR plus systemic FSH (12/12h; i.m.; n=3) or saline (n=2). Asterisk (*) indicates statistical difference from control group (SALINE; $P<0.05$). Statistical analysis was not performed in Panel B as the difference in ovulation rate was 0 vs. 100%. In Panel C, main effects of treatment group, day and their interaction were determined by MIXED procedure with repeated measure and differences between follicular size at a specific time point were compared between groups using estimates (a≠b: $P<0.05$).

Figure 4

Effect of in vivo treatment with saralasin (AngII inhibitor) on follicular size (A) and estradiol:progesterone ratio in follicular fluid (B). A single 7-8 mm follicle was injected with

505 saralasin (SAR) or saline and the cows were ovariectomized 24h later. Asterisk (*) indicates
506 statistical difference from control group (SALINE; $P<0.05$).

507 **Figure 5**

508 Effect of in vivo treatment with saralasin (AngII inhibitor) on gene expression in granulosa cells.
509 A single 7-8 mm follicle was injected with saralasin (SAR) or saline and the cows were
510 ovariectomized 24h later. Asterisk (*) indicates statistical difference from control group
511 (SALINE; $P<0.05$). The results are represented as fold of increase of gene expression in control
512 group. 3βHSD, 3β-hydroxysteroid dehydrogenase; GADD45b, growth arrest and DNA damage
513 inducible; XIAP, X-linked inhibitor of apoptosis protein.

514 **Figure 6**

515 In vitro effect of three doses of AngII (0, 0.1 or 10 μM) on aromatase, 3βHSD and 17βHSD
516 mRNA in granulosa cells. After 4 days of culture, granulosa cells were submitted to the
517 treatments for 6 hours and recovered to study gene expression. Bars with no common letters are
518 significantly different ($P<0.05$).

519

Figure 1

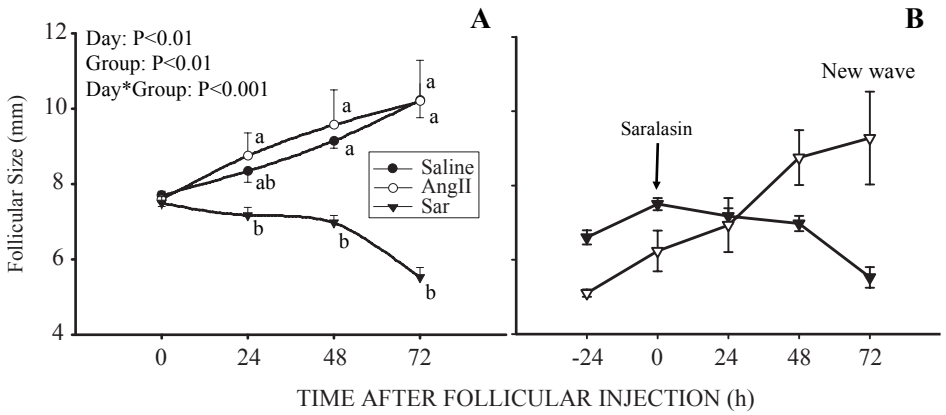

A
B
Day: P<0.01
Group: P<0.01
Day*Group: P<0.001
Saline
AngII
Sar
Saralasin
New wave
Follicular Size (mm)
TIME AFTER FOLLICULAR INJECTION (h)

Figure 2

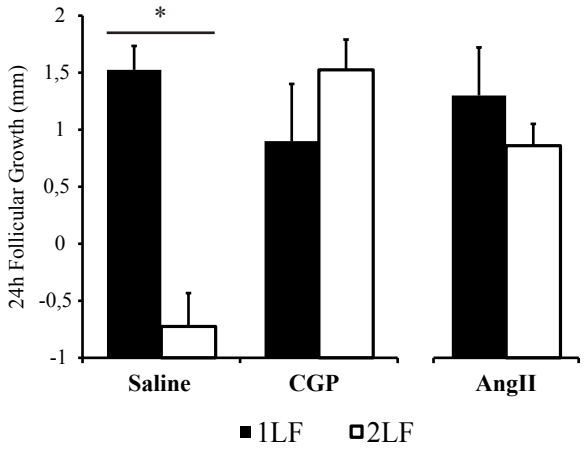

*
2
1,5
1
0,5
0
-0,5
-1
24h Follicular Growth (mm)
Saline
CGP
AngII
1LF
2LF

Figure 3

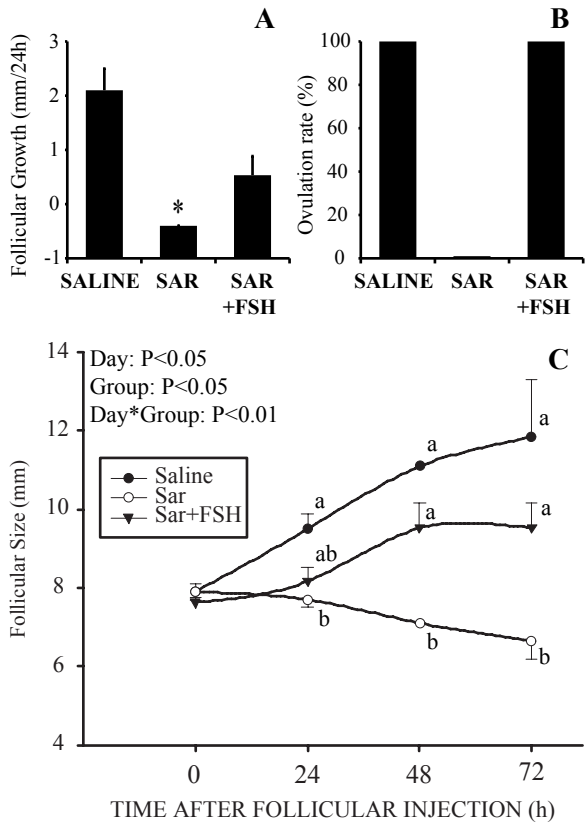

A
B
C
Follicular Growth (mm/24h)
Ovulation rate (%)
SALINE
SAR
SAR +FSH
Day: P<0.05
Group: P<0.05
Day*Group: P<0.01
Saline
Sar
Sar+FSH
Follicular Size (mm)
TIME AFTER FOLLICULAR INJECTION (h)

Figure 4

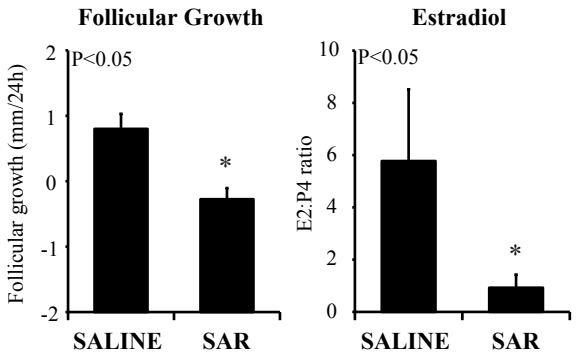

Follicular Growth
Estradiol
P<0.05
P<0.05
Follicular growth (mm/24h)
E2:P4 ratio
2
1
0
-1
-2
10
8
6
4
2
0
*
*
SALINE
SAR
SALINE
SAR

Figure 5

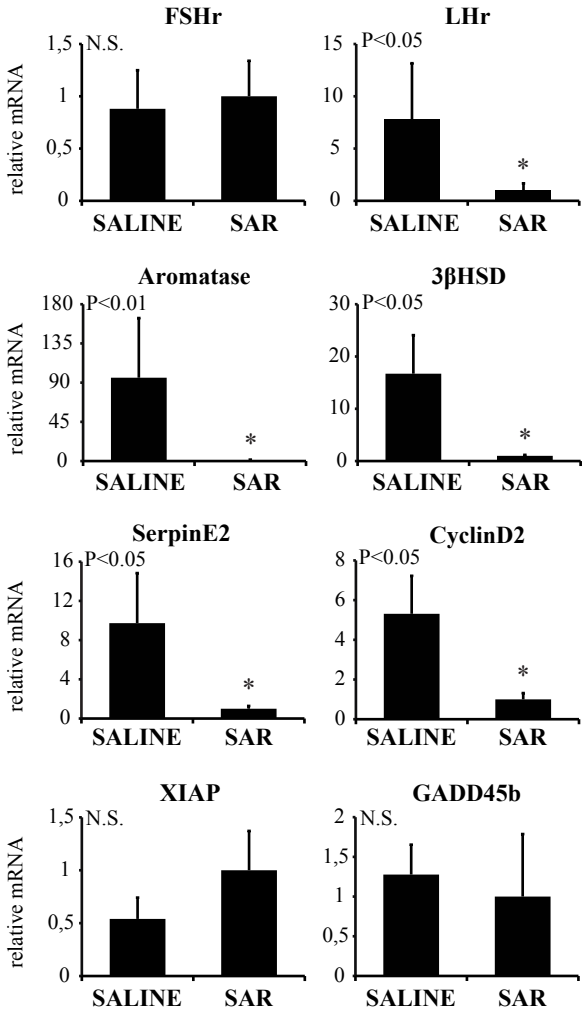
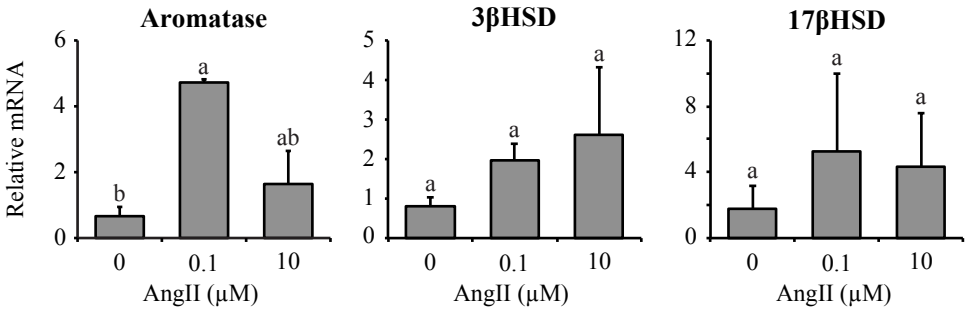

FSHr
LHr
Aromatase
3βHSD
SerpinE2
CyclinD2
XIAP
GADD45b
relative mRNA
N.S.
P<0.05
P<0.01
SALINE
SAR
*

Figure 6



4. CAPÍTULO 2

TRABALHO ENVIADO PARA PUBLICAÇÃO:

Angiotensin II profile and mRNA encoding RAS proteins during bovine follicular wave

Rogério Ferreira, Bernardo Gasperin, Joabel Santos, Monique Rovani, Robson Santos, Karina Gutierrez, João Francisco Oliveira, Adelina Reis, Paulo Bayard Gonçalves

JOURNAL OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM, 2010

Angiotensin II profile and mRNA encoding RAS proteins during bovine follicular wave

Rogério Ferreira[1], Bernardo Gasperin[1], Joabel Santos[1], Monique Rovani[1], Robson Santos[2], Karina Gutierrez[1], João Francisco Oliveira[1], Adelina Reis[2], Paulo Bayard Gonçalves[1,3]

[1] Laboratory of Biotechnology and Animal Reproduction - BioRep, Federal University of Santa Maria, Santa Maria, RS, Brazil.

[2] Department of Physiology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

Short Title: RAS profile in bovine follicular wave

Footnotes

[3]Correspondences: Paulo Bayard Gonçalves, Departamento de Clínica de Grandes Animais, Hospital Veterinário, Universidade Federal de Santa Maria, Postal code 97105-900, Santa Maria, RS, Brazil, Phone: 55-55-3220-8484 or 55-55-3220-8752 and Fax: 55-55-3220-8484. E-mail: bayard@biorep.ufsm.br

Abstract

We previously demonstrated important roles of angiotensin II (AngII) in regulating ovarian follicle development, ovulation and oocyte meiotic resumption. The objective of present study was to characterize the AngII profile and mRNA encoding RAS proteins in bovine follicular wave. Cows were ovariectomized when the size between the largest (F1) and second largest follicle (F2) was not statistically different (day 2), slightly different (day 3) or markedly different (day 4). AngII was measured in follicular fluid and mRNA abundance of genes encoding angiotensin converting enzyme (ACE), (pro)renin receptor and renin binding protein (RnBP) were evaluated in follicular cells from F1 and F2. AngII levels increased at the expected time of follicular deviation in F1 but did not significantly change in F2. However, the expression of genes encoding ACE, (pro)renin receptor and RnBP was not regulated in F1 but was upregulated during or after follicular deviation in F2. Moreover, RnBP gene expression increased when the F1 was treated with estrogen receptor-antagonist *in vivo*. In conclusion, AngII concentration increased in the follicular fluid of the dominant follicle during and after deviation and further supports our recent finding that a local RAS is present in the ovary regulating follicular dominance.

Keywords: angiotensin II, ACE, RnBP, (pro)renin receptor, follicular growth

Introduction

The renin-angiotensin system (RAS) is well known for its systemic control that regulates blood pressure and fluid homeostasis. According to the systemic overview, angiotensinogen is expressed by the liver and is cleaved by renin, enzyme secreted by the kidneys, to produce the decapeptide angiotensin I (AngI). AngI is cleaved by angiotensin converting enzyme (ACE), largely present in endothelial cells (1), to form angiotensin II (AngII), more powerful and active

peptide of the RAS. However, the presence of the RAS components in specific tissues, such as in the ovarian follicle, takes on a new concept of "local" or "tissue" renin angiotensin systems. Moreover, the regulation of local system is independent of systemic control. These local renin-angiotensin systems act as an autocrine/paracrine factor, with a different role on heart, vessels, kidney, brain and endocrine glands (2, 3).

Prorenin is the precursor of renin and has been assumed to be an inactive precursor form (4). Renin is activated on kidneys and is not detected in nephrectomized animals (5). However, AngII concentration in follicular fluid remains unaffected in bilaterally nephrectomized rats (6). More recently, however, it was demonstrated that prorenin can have a proteolytic or non-proteolytic activation (7, 8). In the proteolitic activation, the propeptide is removed by various renal processing enzymes, including proconvertase 1 and cathepsin B. The non-proteolytic activation is reversible, characterized by an unfolding of the propeptide from the enzymatic cleft (8). The (pro)renin receptor ((P)RR) not only binds renin and prorenin, but also activates prorenin by inducing a conformational change in the prorenin molecule (7, 9, 10). Interestingly, rat prorenin that is not activated by human (pro)renin receptor (h(P)RR) binds and induces signaling through this receptor (11). The plasma and tissue angiotensin levels were unaltered in transgenic rats that overexpress the h(P)RR. However, these animals displayed increased levels of aldosterone in blood plasma and cyclooxygenase-2 in the renal cortex (11). These results are in agreement with the concept that (P)RR induces angiotensin-independent effects. Also, proteins that interact with renin, such as a renin binding protein (RnBP), appears to inhibit renin *in vivo* (RnBP; 12). However, the physiological role of RnBP and the relationship between RnBP and renin metabolism, and the tissue-specific regulation of RnBP gene expression are not yet

60 understood. Moreover, for our knowledge, the presence of (P)RR and RnBP has not been demonstrated in mammalian ovary.

In addition to the well-known AngII effects on smooth muscle contraction, aldosterone secretion and blood pressure regulation, our group has demonstrated that this peptide has a pivotal role on the ovulation (13) and oocyte maturation (14). However, the profile of RAS
65 components had not been demonstrated during follicular wave development and can be helpful to understand the mechanisms involved in dominant follicle selection of monovular species. Cattle provide an excellent model for studying the role of local factors on control of follicle development, since follicular wave can be accurately monitored on a day-to-day basis by ultrasonography *in vivo* (15-17) and follicular environment can be easily modified by ultrasound-
70 guided intrafollicular injection (13, 18). In the present study, we characterized the expression of elements of new concept of local RAS, as (P)RR and RnBP, during follicular development. We have also induced follicular atresia by intrafollicular injection of estradiol-receptor inhibitor to test the regulation of local RAS during health/atresia transition.

Materials and Methods

75 *Experiment 1: Angiotensin II and mRNA encoding RAS proteins during bovine follicular wave*

Thirty-six weaned beef cows (predominantly Hereford and Aberdeen-Angus), with an average body condition score of 3 (1–5, emaciated to obese) were used in this study. Cows were given two doses of a PGF2α analogue (cloprostenol, 125 μg; Schering-Plough Animal Health, Brazil) intramuscularly (im), 12h apart. Animals were observed in estrus within 3–5 days after
80 PGF2α, and the experiment was performed during the first follicular wave of the estrous cycle. Ovaries were then examined once a day by transrectal ultrasonography, using an 8 MHz linear-array transducer (Aquila Vet scanner, Pie Medical, Netherlands) and all follicles larger than

5mm were drafted using 3 to 5 virtual slices of the ovary allowing a three-dimensional localization of follicles and monitoring individual follicles during follicular wave (19). The day of the follicular emergence was designated as Day 0 of the wave and was retrospectively identified as the last day on which the dominant follicle was 4 or 5 mm in diameter (20). Cows were randomly assigned to be ovariectomized by colpotomy on days 2, 3 or 4 of the follicular wave (4 cows for each day) to recover the largest and second largest follicle from each cow. This approach allowed to investigate the RAS components and follicular fluid content before, during and after follicular divergence.

Experiment 2: mRNA encoding RAS proteins during initial atresia

To further demonstrate that RAS proteins mRNA expression is upregulated during initial atresia, twenty Bos taurus taurus adult cyclic cows (as previously described) were synchronized with a progesterone releasing intravaginal device (1 g progesterone, DIB – Intervet Schering Plough), an injection of 2 mg estradiol benzoate im (Genix, Anápolis, Brazil) and two injections of 250 μg sodium cloprostenol im (twelve hours apart; Ciosin - Intervet Schering Plough) being all treatments performed at the same time on day 0. Four days after, the progesterone devices were removed and ovaries were daily monitored until the largest follicle of the growing cohort reached the diameter of 7-8 mm. At this moment, it was performed an intrafollicular injection of fulvestrant (selective estrogen receptor antagonist) in a final concentration of 100 μM or saline (based on a previous dose-response experiment; data not shown). Cows were ovariectomized 12 (n=3/group) or 24 hours (n=4/group) after intrafollicular injection. Intrafollicular injections were performed as previously described (13).

Follicles

After ovariectomy, follicular fluid, granulosa and theca cells were recovered from F1 and F2 (experiment 1) and from fulvestrant or saline treated follicles (experiment 2) and stored at -80ºC. Follicular fluid estradiol levels from all follicles were determined by ELISA following the manufacturer's instructions (Cayman Biochemical). Cross-contamination of theca and granulosa cells was tested by RT-PCR to detect cytochrome P450 aromatase (CYP19A1) and 17α-hydroxylase (CYP17A1) mRNA. Granulosa cells that expressed CYP17A1 and theca cells that expressed CYP19A1 were discarded (21).

Follicular fluid from F1 and F2 (experiment 1) was recovered to measure AngII and stored in the presence of the following protease inhibitors: 10^{-5} M phenylmethylsul-fonylfluoride, 10^{-5} M pepstatin A, 10^{-5} M EDTA, 10^{-5} M p-hydroxymercuribenzoate, and $9x10^{-4}$ M orthophenanthroline, all purchased from Sigma-Aldrich Corp. AngII was measured as described by (22).

Nucleic Acid Extraction and Real-Time RT-PCR

Total RNA was extracted using Trizol (theca cells) or silica-based protocol (granulosa cells; Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions and was quantified by absorbance at 260 nm. Total RNA (1 µg) was first treated with 0.2 U DNase (Invitrogen) at 37°C for 5 minutes to digest any contaminating DNA, followed by heating to 65°C for 3 minutes. The RNA was reverse transcribed (RT) in the presence of 1 µM oligo(dT) primer, 4 U Omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, ON, Canada), 0.5 µM dideoxynucleotide triphosphate (dNTP) mix, and 10 U RNase Inhibitor (Invitrogen) in a volume of 20 µL at 37°C for 1 hour. The reaction was terminated by incubation at 93°C for 5 minutes.

Real-time polymerase chain reaction (PCR) was conducted in a Step One Plus instrument (Applied Biosystems, Foster City, CA) with Platinum SYBR Green qPCR SuperMix (Invitrogen) and bovine-specific primers (Table 1). Common thermal cycling parameters (3 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C) were used to amplify each transcript. Melting-curve analyses were performed to verify product identity. Samples were run in duplicate and were expressed relative to GAPDH as housekeeping gene. The relative quantification of gene expression across treatments was evaluated using the ddCT method (23). Briefly, the dCT is calculated as the difference between the CT of the investigated gene and the CT of GAPDH in each sample. The ddCT of each investigated gene is calculated as the difference between the dCT in each treated sample and the dCT of the sample with lower gene expression (higher dCT). The fold change in relative mRNA concentrations was calculated using the formula 2^{-ddCT}. Bovine-specific primers (Table 1) were taken from literature or designed using Primer Express Software v3.0 (Applied Biosystems) and synthesized by Invitrogen.

Statistical analysis

The differences on continuous data between dominant and subordinate follicle was accessed by paired Student's T test using cow as subject. The regulation of AngII and mRNA encoding RAS proteins was analyzed by ANOVA and multi-comparison between days or groups was performed by least square means. Data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. All analyses were performed using JMP software (SAS Institute Inc., Cary, NC) and a $P<0.05$ was considered statistically significant. Data are presented as means ± sem.

Results

Ovarian follicle model

The cows were ovariectomized on days 2, 3 and 4 of the first wave of follicular development. This experimental design allowed to recover follicles when the follicular size of the largest and second largest was not different (day 2; $P>0.05$), slightly different (day 3; $P<0.05$) or markedly different (day 4; $P<0.01$; Figure 1; 17). The mRNA abundance levels of CYP19 in granulosa cells increased in dominant and decreased in subordinate follicles during development (Figure 1). These results confirm that the ovaries obtained at days 2, 3 and 4 of the first follicular wave were, respectively, before, during and after follicular deviation. Samples were discarded when cross-contaminations between theca and granulosa cells were detected or the amount of follicular fluid or extracted RNA was insufficient to be processed. When one follicle was discarded, the data of both follicles (largest and second largest) from de same cow was excluded from the statistical analysis.

Follicular fluid AngII concentration

The concentration of AngII was measured in follicular fluid to test the hypothesis that AngII is differentially regulated in dominant and subordinate follicles during follicular wave. The AngII concentrations increased in follicular fluid during deviation only in the dominant follicle (Figure 2). In the second largest follicle, AngII concentration did not significantly change throughout follicular wave and the deviation was very high (data not shown).

RAS components gene expression

The results provide evidences that ACE, (P)RR and RnBP are differentially regulated in granulosa cells of second largest follicle during follicular wave development (Figure 3). The

mRNA expression of (P)RR was upregulated in granulosa cells at the expected moment of follicular deviation while RnBP mRNA increased during and after deviation process. Nevertheless, ACE mRNA expression upregulation was only observed after expected moment of follicular deviation, when the subordinate follicle undergo atresia (Figure 3). On theca cells, there was no regulation of ACE, (P)RR or RnBP gene expression during follicular growth nor between dominant and subordinate follicle (data not shown).

The mRNA encoding RAS proteins was further assessed after intrafollicular injection of fulvestrant to induce atresia. We have previously confirmed that intrafollicular injection of fulvestrant (100 μM) decreased CYP19A1 gene expression and induced follicular atresia from 12 hours after treatment. The ACE and (P)RR (at 12 and 24 h) and RnBP (at 12 h after intrafollicular injection) mRNA expression in granulosa cells did not differ between fulvestrant and saline treated follicles. However, RnBP mRNA expression was upregulated in fulvestrant treated follicles at 24 h after intrafollicular injection (Figure 4; $P<0.05$).

Discussion

We used a well-established experimental model proposed by Rivera et al. (24), and found that the concentration of AngII in follicular fluid of the dominant follicle increased at the expected time of follicular deviation. There is evidence that AngII is involved in the mechanism of follicular deviation in cattle. Recently, we found that AngII is required for dominance and follicle development when FSH levels are low during cow follicular wave (after follicular deviation, 25). It is well known that the concentration of AngII in follicular fluid increases after LH surge in the bovine (26); however, AngII concentration had not been measured during follicular wave development in mammals.

The expression of genes codifying for ACE, (P)RR and RnBP was upregulated in the second largest follicle during and after follicular divergence. Berisha et al. (27) observed an upregulation of ACE gene in the highest steroidogenic follicles (with diameter higher than 12 mm) in ovarian follicles from abattoir. However, Daud et al. (28) observed low ACE levels in preovulatory follicles and suggested a role for ACE in follicular atresia. Moreover, in ovariectomized rats, the replacement of estrogen reduced ACE activity in aorta and kidney tissue and plasma (29). These results together suggest that estrogen secretion by a dominant follicle can suppress ACE expression in dominant follicle.

We observed that ACE gene is upregulated but did not result in a concomitant increase of AngII levels in the follicular fluid of the second largest follicle. Captropril, an ACE inhibitor, did not inhibit hCG-induced ovulation in rabbit perfused ovaries (28). In contrast, treatment with saralasin, an AT1 and AT2 blocker, is able to inhibit ovulation and oocyte maturation in rabbits (30) and cattle (13, 14). There are some possible reasons for the discrepant results between captopril and saralasin, which may also explain the upregulation of ACE without increasing in AngII. For example, ACE inhibitors have other effects, including prevention of the hydrolysis of bradykinin (31, 32) and clearance of Ang1-7 (33). Alternative enzymes have been demonstrated to be able to cleave AngI into AngII, such as members of plasminogen activator family (34), cathepsin D and chymase (35), giving rise to the possibility of an alternative pathways to produce AngII in ovarian follicular cells. The perfusion of isolated rabbit ovaries with IGF-1, an important local factor that controls follicular dominance, increases follicular growth and intrafollicular plasminogen activator activity (36). Moreover, the same authors stimulated *in vitro* both follicular growth and the intrafollicular AngII content using streptokinase, an exogenous PA.

The present results provide, to our knowledge, the first direct evidence of differential regulation of local RAS components during follicular deviation in the bovine ovary. However, our hypothesis that (P)RR is regulating the AngII production in ovarian follicle during follicular deviation was not confirmed. The regulatory pattern of AngII was different from that observed for (P)RR in the dominant follicle. Many factors may account for these differences. One is that the (P)RR system seems to have at least two different functions. One is angiotensin-independent that (P)RR induces an intracellular signal and a downstream effect. Another is an angiotensin-dependent function related to the increased catalytic activity of receptor-bound (pro)renin (37). Estradiol seems to affect negatively renin activity in follicular fluid (29) and a high pro-renin like activity was observed in atretic follicles (38). Renin and ACE was demonstrated in granulosa and thecal cells of antral follicles in cattle (39), which may explain the presence of AngII but do not explain the differential regulatory pattern of AngII in dominant follicle. Therefore, on the basis of the actual knowledge, we cannot speculate about the biological function of the upregulation of (P)RR mRNA in subordinate follicles.

Renin binding protein is a protein that binds to renin and inhibits its activity. It can be found as a complex with renin called high molecular weight renin (40) and as a single protein (41). In the present study, RnBP was highly expressed in subordinated follicle and increased expression during follicular deviation. This result was further confirmed when we assessed RnBP mRNA expression in *in vivo* derived follicles 24 h after intrafollicular treatment with selective estrogen receptor antagonist fulvestrant. In rat, the tissue distribution of RnBP mRNA was similar to that of renin mRNA and was highly expressed in the ovary (42). Moreover, the same authors suggested a regulation of RnBP gene expression by estradiol and intravenous injection of the RnBP into rats resulted in a rapid and strong inhibition of plasma renin activity,

which persisted at least for 2 h. However, knockout of the gene encoding for this protein in mice did not show any effect on RAS activity or blood pressure (43). Therefore, more studies are necessary to understand the role of RnBP in the control of ovarian renin activity and AngII production.

We presented here a regulatory pattern of AngII and mRNA expression of local RAS enzymes throughout follicular wave development in cattle. AngII concentration increased in dominant follicle during and after follicular deviation, which supports our recent finding that AngII is required for follicular development when the levels of FSH decrease during deviation. Using an *in vivo* model, we found that the expression of ACE, RnBP and (P)RR mRNA are upregulated in the second largest follicle during and after follicular deviation and that intrafollicular injection of estradiol receptor antagonist upregulates RnBP mRNA expression, suggesting an interaction between estradiol and RAS system in bovine follicle. In conclusion, our findings support the hypothesis that a local RAS is present in the ovary regulating follicular dominance in cattle.

Acknowledgments

The authors would like to thank the Fazenda do Leão, Dr. José Manoel Ferreira and Dr. Vinicius de Oliveira for providing the animals and facilities. This study was supported by Brazilian Council of Scientific and Technological Development (CNPq # 502074/2008-6).

References

1. Peach MJ. Renin-angiotensin system: biochemistry and mechanisms of action. Physiol Rev. 1977 Apr;57(2):313-70.

2. Phillips MI, Sumners C. Angiotensin II in central nervous system physiology. Regul Pept. 1998 Nov 30;78(1-3):1-11.

3. Kim S, Iwao H. Molecular and Cellular Mechanisms of Angiotensin II-Mediated Cardiovascular and Renal Diseases. Pharmacol Rev. 2000 March 1, 2000;52(1):11-34.

4. Do YS, Shinagawa T, Tam H, Inagami T, Hsueh WA. Characterization of pure human renal renin. Evidence for a subunit structure. J Biol Chem. 1987;262(3):1037-43.

5. Sealey JE, White RP, Laragh JH, Rubin AL. Plasma prorenin and renin in anephric patients. Circul Res. 1977;41(4):17-21.

6. Husain A, Bumpus FM, Silva PD, Speth RC. Localization of Angiotensin II Receptors in Ovarian Follicles and the Identification of Angiotensin II in Rat Ovaries. Proceedings of the National Academy of Sciences of the United States of America. 1987;84(8):2489-93.

7. Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T, Sraer JD. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. J Clin Invest. 2002 Jun;109(11):1417-27.

8. Suzuki F, Hayakawa M, Nakagawa T, Nasir UM, Ebihara A, Iwasawa A, et al. Human prorenin has "gate and handle" regions for its non-proteolytic activation. J Biol Chem. 2003 Jun 20;278(25):22217-22.

9. Nabi AH, Kageshima A, Uddin MN, Nakagawa T, Park EY, Suzuki F. Binding properties of rat prorenin and renin to the recombinant rat renin/prorenin receptor prepared by a baculovirus expression system. Int J Mol Med. 2006 Sep;18(3):483-8.

10. Batenburg WW, Krop M, Garrelds IM, de Vries R, de Bruin RJ, Burckle CA, et al. Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. J Hypertens. 2007 Dec;25(12):2441-53.

11. Kaneshiro Y, Ichihara A, Sakoda M, Takemitsu T, Nabi AH, Uddin MN, et al. Slowly progressive, angiotensin II-independent glomerulosclerosis in human (pro)renin receptor-transgenic rats. J Am Soc Nephrol. 2007 Jun;18(6):1789-95.

12. Takahashi S, Inoue H, Miyake Y. The human gene for renin-binding protein. J Biol Chem. 1992 Jun 25;267(18):13007-13.

13. Ferreira R, Oliveira JF, Fernandes R, Moraes JF, Gonçalves PB. The role of angiotensin II in the early stages of bovine ovulation. Reproduction. 2007 November 1, 2007;134(5):713-9.

14. Barreta MH, Oliveira JFC, Ferreira R, Antoniazzi AQ, Gasperin BG, Sandri LR, et al. Evidence that the effect of angiotensin II on bovine oocyte nuclear maturation is mediated by prostaglandins E2 and F2α. Reproduction. 2008 December 1, 2008;136(6):733-40.

15. Savio JD, Keenan L, Boland MP, Roche JF. Pattern of growth of dominant follicles during the oestrous cycle of heifers. Journal of Reproduction and Fertility. 1988;83(2):663-71.

16. Ginther OJ, Kastelic JP, Knopf L. Composition and characteristics of follicular waves during the bovine estrous cycle. Anim Reprod Sci. 1989;20(3):187-200.

17. Evans ACO, Fortune JE. Selection of the Dominant Follicle in Cattle Occurs in the Absence of Differences in the Expression of Messenger Ribonucleic Acid for Gonadotropin Receptors. Endocrinology. 1997;138(7):2963-71.

18. Kot K, Gibbons JR, Ginther OJ. A technique for intrafollicular injection in cattle: Effects of hCG. Theriogenology. 1995;44(1):41-50.

19. Jaiswal RS, Singh J, Adams GP. Developmental pattern of small antral follicles in the bovine ovary. Biol Reprod. 2004 Oct;71(4):1244-51.

20. Rivera GM, Fortune JE. Development of codominant follicles in cattle is associated with a follicle-stimulating hormone-dependent insulin-like growth factor binding protein-4 protease. Biol Reprod. 2001;65(1):112-8.

21. Buratini J, Jr., Teixeira AB, Costa IB, Glapinski VF, Pinto MGL, Giometti IC, et al. Expression of fibroblast growth factor-8 and regulation of cognate receptors, fibroblast growth factor receptor-3c and -4, in bovine antral follicles. Reproduction. 2005;130(3):343-50.

22. Costa APR, Fagundes-Moura CR, Pereira VM, Silva LF, Vieira MA, Santos RAS, et al. Angiotensin-(1-7): A Novel Peptide in the Ovary. Endocrinology. 2003;144(5):1942-8.

23. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. Methods. 2001;25(4):402-8.

24. Rivera GM, Chandrasekher YA, Evans AC, Giudice LC, Fortune JE. A potential role for insulin-like growth factor binding protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle. Biol Reprod. 2001;65(1):102-11.

25. Ferreira R, Gasperin B, Bohrer RC, Rovani MT, Barreta M, Santos JE, et al., editors. The Role of Angiotensin II in Bovine Follicular Growth. 41th Annual Meeting of the Society for the Study of Reproduction; 2008; 41th Annual Meeting of the Society for the Study of Reproduction: Society for the Study of Reproduction; 2008.

26. Acosta TJ, Ozawa T, Kobayashi S, Hayashi K, Ohtani M, Kraetzl WD, et al. Periovulatory Changes in the Local Release of Vasoactive Peptides, Prostaglandin F2α, and Steroid Hormones from Bovine Mature Follicles In Vivo. Biol Reprod. 2000;63(5):1253-61.

27. Berisha B, Schams D, Miyamoto A. The mRNA Expression of Angiotensin and Endothelin System Members in Bovine Ovarian Follicles During Final Follicular Growth. The Journal of reproduction and development. 2002;48(6):573-82.

28. Daud AI, Bumpus FM, Husain A. Characterization of angiotensin I-converting enzyme (ACE)-containing follicles in the rat ovary during the estrous cycle and effects of ACE inhibitor on ovulation. Endocrinology. 1990;126(6):2927-35.

29. Brosnihan KB, Senanayake PS, Li P, Ferrario CM. Bi-directional actions of estrogen on the renin-angiotensin system. Braz J Med Biol Res. 1999 Apr;32(4):373-81.

30. Yoshimura Y, Karube M, Koyama N, Shiokawa S, Nanno T, Nakamura Y. Angiotensin II directly induces follicle rupture and oocyte maturation in the rabbit. FEBS Lett. 1992;307(3):305-8.

31. Gainer JV, Morrow JD, Loveland A, King DJ, Brown NJ. Effect of bradykinin-receptor blockade on the response to angiotensin-converting-enzyme inhibitor in normotensive and hypertensive subjects. N Engl J Med. 1998 Oct 29;339(18):1285-92.

32. Campbell DJ. The kallikrein-kinin system in humans. Clin Exp Pharmacol Physiol. 2001 Dec;28(12):1060-5.

33. Yamada K, Iyer SN, Chappell MC, Ganten D, Ferrario CM. Converting enzyme determines plasma clearance of angiotensin-(1-7). Hypertension. 1998 Sep;32(3):496-502.

34. Peterson MC, Morioka N, Zhu C, Ryan JW, LeMaire WJ. Angiotensin-converting enzyme inhibitors have no effect on ovulation and ovarian steroidogenesis in the perfused rat ovary. Reprod Toxicol. 1993;7(2):131-5.

345 35. Paul M, Poyan Mehr A, Kreutz R. Physiology of Local Renin-Angiotensin Systems. Physiol Rev. 2006 July 1, 2006;86(3):747-803.

36. Yoshimura Y, Aoki N, Sueoka K, Miyazaki T, Kuji N, Tanaka M, et al. Interactions between Insulin-like Growth Factor-I (IGF-I) and the Renin-Angiotensin System in Follicular Growth and Ovulation. J Clin Invest. 1996;98(2):308-16.

350 37. Nguyen G, Danser AH. Prorenin and (pro)renin receptor: a review of available data from in vitro studies and experimental models in rodents. Exp Physiol. 2008 May;93(5):557-63.

38. Schultze D, Brunswig B, Mukhopadhyay AK. Renin and prorenin-like activities in bovine ovarian follicles. Endocrinology. 1989 Mar;124(3):1389-98.

39. Schauser KH, Nielsen AH, Winther H, Dantzer V, Poulsen K. Localization of the Renin-
355 Angiotensin System in the Bovine Ovary: Cyclic Variation of the Angiotensin II Receptor Expression. Biol Reprod. 2001;65(6):1672-80.

40. Takahashi S, Ohsawa T, Miura R, Miyake Y. Purification of high molecular weight (HMW) renin from porcine kidney and direct evidence that the HMW renin is a complex of renin with renin binding protein (RnBP). J Biochem. 1983 Jan;93(1):265-74.

360 41. Takahashi S, Ohsawa T, Miura R, Miyake Y. Purification and characterization of renin binding protein (RnBP) from porcine kidney. J Biochem. 1983 Jun;93(6):1583-94.

42. Tada M, Takahashi S, Miyano M, Miyake Y. Tissue-specific regulation of renin-binding protein gene expression in rats. J Biochem. 1992 Aug;112(2):175-82.

43. Schmitz C, Gotthardt M, Hinderlich S, Leheste JR, Gross V, Vorum H, et al. Normal
365 blood pressure and plasma renin activity in mice lacking the renin-binding protein, a cellular renin inhibitor. J Biol Chem. 2000 May 19;275(20):15357-62.

44. Luo W, Wiltbank MC. Distinct Regulation by Steroids of Messenger RNAs for FSHR and CYP19A1 in Bovine Granulosa Cells. Biol Reprod. 2006;75(2):217-25.

370

370 Table 1 - Primers used in the expression analysis of candidate genes. Primer sequences and concentrations used to amplify each product are described.

Gene		Sequence	Conc. (μM)	Reference or accession nº
ACE	F	ACTCCTGGAGGTCCATGTACGA	200	AJ309016.1
	R	ACGTAGGCGTGCAGGTTCAG	200	
Aromatase	F	GTGTCCGAAGTTGTGCCTATT	300	(44)
	R	GGAACCTGCAGTGGGAAATGA	300	
CYP17	F	GAATGCCTTTGCCCTGTTCA	200	(21)
	R	CGCGTTTGAACACAACCCTT	200	
GAPDH	F	GATTGTCAGCAATGCCTCCT	200	NM_001034034.1
	R	GGTCATAAGTCCCTCCACGA	200	
(pro)renin receptor	F	TGATGGTGAAAGGAGTGGACAA	200	ENSBTAT00000023668
	R	TTTGCCACGCTGTCAAGACT	200	
RnBP	F	GGCAGGACATGGAGAAGGAA	200	NM_001046223.1
	R	TGGGAATGATCCAGCCAGAA	200	

F, Forward primer; R, Reverse primer; Conc., primer concentration used for gene amplification.

Figure 1. Follicular diameter and granulosa cells aromatase (CYP19) relative mRNA abundance of largest (black bar) and second largest (open bar) follicle (mean±s.e.m.) collected at days 2 (n=4), 3 (n=4) or 4 (n=4) of the first follicular wave of a cycle. Asterisk (* or **) indicates statistical difference between largest and second largest follicle accessed by paired Student's T test using cow as subject. * p<0,05; ** p<0,001.

Figure 2. Angiotensin II (AngII) in follicular fluid of the largest follicle from cows ovariectomized at days 2 (n=2), 3 (n=4) or 4 (n=4) of the first follicular wave of a cycle. Bars with no common letter are different (a≠b, P < 0.05).

Figure 3. Expression of renin-angiotensin system related genes in granulosa cells during follicular development. Granulosa cells were recovered from largest (black bar) and second largest (open bar) follicle (mean±s.e.m.) collected at days 2 (n=3), 3 (n=4) or 4 (n=4) of the first follicular wave of a cycle. Asterisk (* or **) indicates statistical difference between largest and second largest follicle accessed by paired Student's T test using cow as subject. * p<0.05; ** p<0.001. ACE, angiotensin converting enzyme. RnBP, renin binding protein.

Figure 4. Expression of renin-angiotensin system related genes in granulosa cells 12 or 24 h after intrafollicular selective estrogen receptor antagonist (fulvestrant) treatment. Granulosa cells were recovered from saline (black bar) and fulvestrant (open bar) treated follicles 12 (n=3/group) or 24h (n=4/group) after intrafollicular injection (mean±s.e.m.). Asterisk (*) indicates statistical difference between groups (p<0.05). ACE, angiotensin converting enzyme. RnBP, renin binding protein.

Figure 1

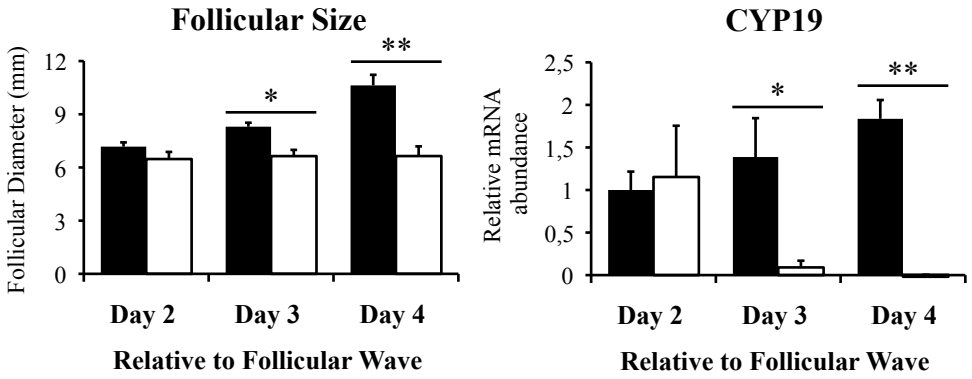

Follicular Size
Follicular Diameter (mm)
12
9
6
3
0
*
**
Day 2
Day 3
Day 4
Relative to Follicular Wave
CYP19
Relative mRNA abundance
2,5
2
1,5
1
0,5
0
*
**
Day 2
Day 3
Day 4
Relative to Follicular Wave

Figure 2

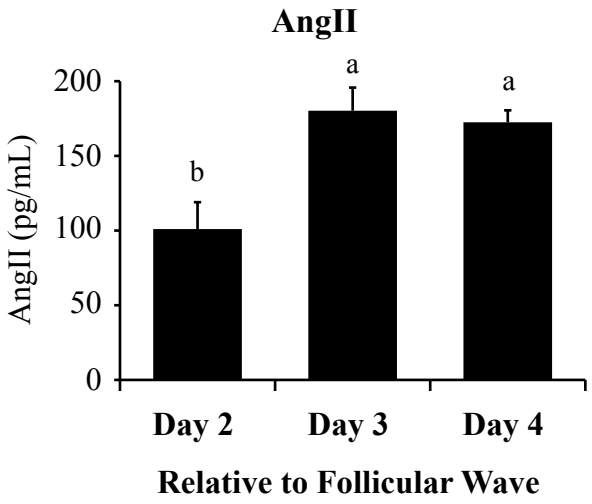

AngII
200
150
100
50
0
AngII (pg/mL)
a
a
b
Day 2
Day 3
Day 4
Relative to Follicular Wave

Figure 3

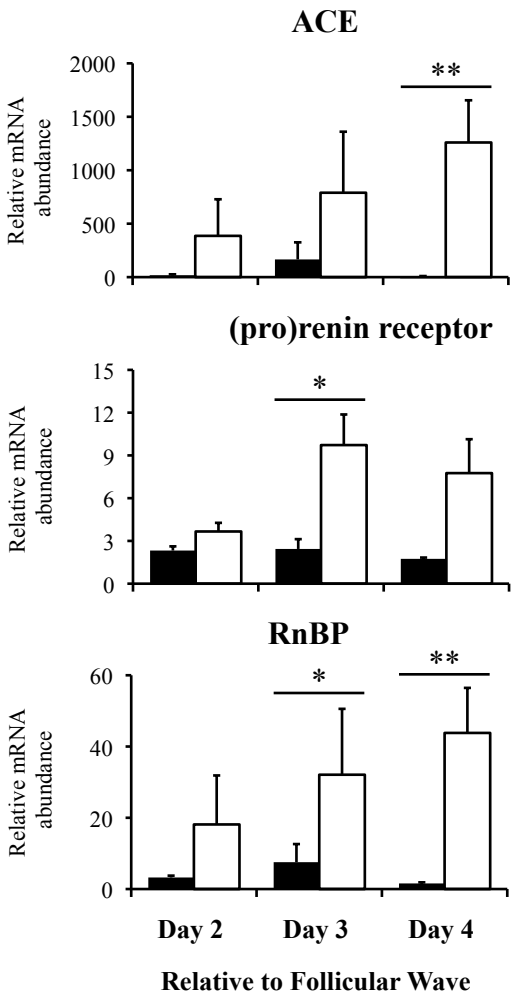
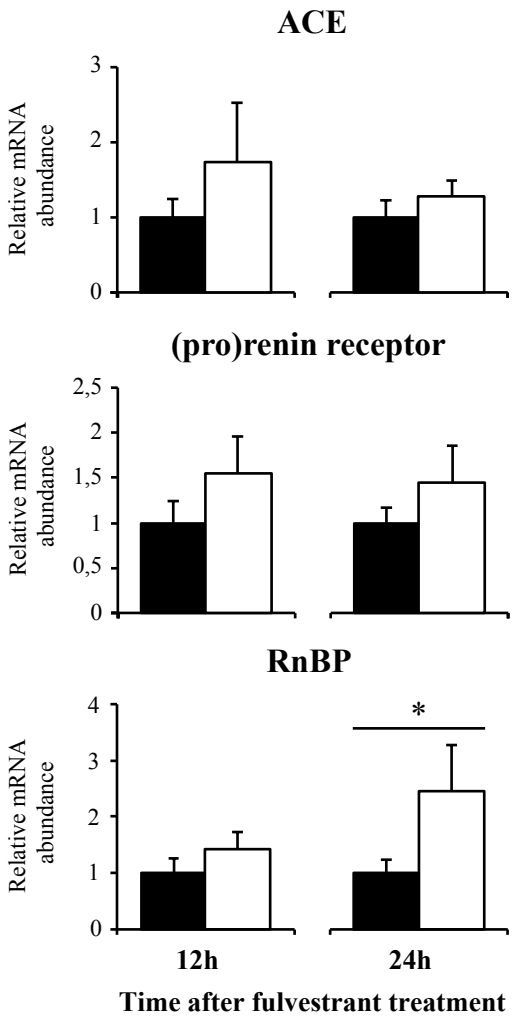

ACE
Relative mRNA abundance
2000
1500
1000
500
0
**
(pro)renin receptor
Relative mRNA abundance
15
12
9
6
3
0
*
RnBP
Relative mRNA abundance
60
40
20
0
*
**
Day 2
Day 3
Day 4
Relative to Follicular Wave

Figure 4


ACE
Relative mRNA abundance
3
2
1
0
(pro)renin receptor
Relative mRNA abundance
2,5
2
1,5
1
0,5
0
RnBP
Relative mRNA abundance
4
3
2
1
0
*
12h
24h
Time after fulvestrant treatment

5. DISCUSSÃO

Com o avanço nas técnicas de dosagem hormonal e o uso da ultra-sonografia na medicina veterinária, no final da década de 80 e na década de 90, propiciaram um melhor entendimento sobre a dinâmica de crescimento folicular em mamíferos. Com base nesses achados, se determinou que o crescimento folicular ocorre em ondas foliculares (Sirois e Fortune, 1988) em consequência de uma regulação do eixo hipotalâmico-hipofisiário-gonadal (Price e Webb, 1988). Dentro deste contexto, ficou bem determinado que a emergência de uma nova onda de crescimento folicular ocorre em consequência do aumento dos níveis de FSH (Adams *et al.*, 1992). O período em que o estradiol 17ß produzido pelos folículos em crescimento é capaz de diminuir a secreção de FSH pela adenohipófise, fazendo com que a maioria dos folículos em crescimento iniciem o processo de atresia, é denominado divergência folicular (Ginther *et al.*, 1996; Ginther *et al.*, 1999). Neste momento, o folículo capaz de continuar o seu crescimento, mesmo em baixos níveis de FSH, será o futuro folículo dominante (Ginther *et al.*, 2001a). Uma das características bem determinadas para o estabelecimento da dominância folicular é a aquisição de receptores de LH nas células da granulosa (Beg *et al.*, 2001). No entanto, um estudo demonstrou que a seleção do folículo dominante ocorre na ausência de um aumento de expressão de receptores de LH nas células da granulosa (Evans e Fortune, 1997). Além disso, alguns autores têm revisado a presença de fatores locais que conferem ao futuro folículo dominante a característica de continuar o seu crescimento mesmo em baixos níveis séricos de FSH (para revisão Fortune *et al.*, 2001). Além disso, um trabalho mais recente (Mihm *et al.*, 2008) utilizando uma abordagem de microarranjo, caracterizou diversos fatores que são diferentemente expressos entre folículos dominantes e subordinados. Esses fatores locais atuam de maneira autócrina/parácrina atuando em funções indispensáveis para o desenvolvimento folicular, incluindo esteroidogênese, regulação do ciclo celular, diferenciação e proteção contra apoptose.

Uma vez que o angiotensinogênio após expresso deve sofrer clivagens sucessivas para formar angiotensina I e AngII, estudos com abordagem que utilizam expressão gênica ou microarranjos não contemplam variações no RAS ou na concentração folicular de AngII. Este fato justifica a carência de resultados em relação a regulação do RAS ovariano e também das funções da angiotensina durante o crescimento folicular. Dentro do panorama supracitado, nosso grupo começou investigar o papel da AngII no crescimento folicular de bovinos, especialmente durante o período de divergência folicular, período pelo qual é caracterizado

por uma maior participação de fatores locais. Uma vez que os receptores AGTR2 parecem ser expressos diferentemente em folículos dominantes e subordinados, além de serem regulados por FSH e fatores locais (Portela *et al.*, 2008), estabelecemos a hipótese de que a AngII também é regulada durante o desenvolvimento folicular e atua em funções básicas para a manutenção do futuro folículo dominante.

O presente estudo foi baseado em três metodologias para caracterizar a função e regulação da AngII no desenvolvimento folicular: 1) recuperação de fluido e células foliculares em períodos em que não é observada diferença morfológica entre os dois maiores folículos da onda (dia 2 relativo ao início da onda); ou que é observada uma discreta (dia 3) ou marcada (dia 4) diferença entre os dois maiores folículos e avaliação dos constituintes do RAS. 2) administração de agonista ou antagonista e avaliação do crescimento folicular e expressão de genes relacionados com a esteroidogênese e desenvolvimento folicular. 3) administração de AngII em um cultivo não luteinizante de células da granulosa. Com essas metodologias, nossos principais achados foram: 1) a concentração de AngII aumenta no fluido folicular do futuro folículo dominante no momento esperado para a divergência folicular; 2) o crescimento folicular é totalmente bloqueado quando os receptores AGTR1 e AGTR2 são inibidos em folículos entre 7 e 8mm; 3) a injeção folicular de AngII ou agonista AGTR2 no segundo maior folículo na onda é capaz de prevenir a regressão esperada para o período de divergência folicular; 4) a AngII parece atuar no crescimento folicular regulando a expressão de genes responsáveis pela proliferação e diferenciação das células da granulosa.

No presente trabalho, foram apresentados alguns resultados de regulação de enzimas do sistema renina-angiotensina durante o desenvolvimento folicular. Foi observado um aumento na concentração folicular de AngII durante e após o momento esperado para a divergência folicular. Esses resultados suportam os nossos achados de participação da AngII, principalmente, durante os estádios de crescimento folicular que não são dependentes de altas concentrações de FSH, ou seja, após o período de divergência folicular. Utilizado-se o mesmo modelo *in vivo*, foi observado um aumento na abundância de mRNA que codifica para as proteínas ECA, RnBP e receptor de (pro)renina no segundo maior folículo após a divergência folicular. A regulação de ECA e do receptor de (pro)renina parecem estar relacionados com o aumento de atividade de renina descrita em folículos atrésicos, mas não justificam os nossos achados de elevação AngII no fluido folicular do folículo dominante. Portanto, mais estudos são necessários para entendermos a regulação destas enzimas dentro do novo contexto de sistemas locais de produção de AngII.

Os resultados de funcionalidade sugerem fortemente que a AngII promove o crescimento e dominância folicular em bovinos. Um melhor entendimento dos mecanismos envolvidos na foliculogênese possibilita um maior controle sobre essa função fisiológica. Pode ainda, servir como base de ferramentas para melhor explorar o potencial reprodutivo de fêmeas bovinas e obtenção de melhores resultados na utilização de biotécnicas da reprodução, seja com fins comerciais ou de preservação das espécies.

6. CONCLUSÃO

Os resultados do presente estudo nos permitem concluir que há uma regulação na concentração folicular de AngII durante o desenvolvimento folicular e que a AngII é um peptídeo indispensável para o desenvolvimento folicular, especialmente após o período de dependência de FSH. O fato de agonistas de AngII resgatarem o folículo subordinado do processo de atresia inicial permite inferir que a AngII possui um papel chave na manutenção do crescimento/diferenciação folicular pós-divergência. Além disso, os resultados demonstram que a sinalização da AngII é essencial para o crescimento folicular, regulando genes envolvidos com a proliferação (ciclina D2) e diferenciação (LHr, aromatase, 3βHSD) das células da granulosa, os quais são necessários para o desenvolvimento do folículo dominante, sendo mediados, provavelmente, pela ativação do receptor AGTR2. Em conjunto, os resultados sugerem fortemente que a AngII promove o crescimento e dominância folicular em bovinos.

7. REFERÊNCIAS

Acosta, T. J., *et al.* Periovulatory Changes in the Local Release of Vasoactive Peptides, Prostaglandin F2α, and Steroid Hormones from Bovine Mature Follicles In Vivo. **Biology of Reproduction**, v.63, n.5, p.1253-1261. 2000.

Adams, G. P., *et al.* Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. **Journal of Reproduction and Fertility**, v.94, n.1, p.177-88. 1992.

Aguilera, G., *et al.* Angiotensin II receptors in the gonads. **American Journal of Hypertension**, v.2, n.5, p.395-402. 1989.

Bao, B. e H. A. Garverick. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. **Journal of Animal Science**, v.76, n.7, p.1903-21. 1998.

Barreta, M. H., *et al.* Evidence that the effect of angiotensin II on bovine oocyte nuclear maturation is mediated by prostaglandins E2 and F2α. **Reproduction**, v.136, n.6, p.733-740. 2008.

Batenburg, W. W., *et al.* Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. **Journal of Hypertension**, v.25, n.12, p.2441-53. 2007.

Bédard, J., *et al.* Serine protease inhibitor-E2 (SERPINE2) is differentially expressed in granulosa cells of dominant follicle in cattle. **Molecular Reproduction and Development**, v.64, n.2, p.152-165. 2003.

Beg, M. A., *et al.* Follicular-Fluid Factors and Granulosa-Cell Gene Expression Associated with Follicle Deviation in Cattle. **Biology of Reproduction**, v.64, n.2, p.432-441. 2001.

Berisha, B., *et al.* The mRNA Expression of Angiotensin and Endothelin System Members in Bovine Ovarian Follicles During Final Follicular Growth. **The Journal of reproduction and development**, v.48, n.6, p.573-582. 2002.

Chiu, A. T., *et al.* Identification of angiotensin II receptor subtypes. **Biochemical and Biophysical Research Communications**, v.165, n.1, p.196-203. 1989.

Clauser, E., *et al.* Regulation of angiotensinogen gene. **American Journal of Hypertension**, v.2, n.5, p.403-410. 1989.

Cui, T., *et al.* ATRAP, novel AT1 receptor associated protein, enhances internalization of AT1 receptor and inhibits vascular smooth muscle cell growth. **Biochemical and Biophysical Research Communications**, v.279, n.3, p.938-41. 2000.

De Smaele, E.*, et al.* Induction of gadd45ß by NF-κB downregulates pro-apoptotic JNK signalling. **Nature**, v.414, n.6861, p.308-313. 2001.

Do, Y. S.*, et al.* Characterization of pure human renal renin. Evidence for a subunit structure. **Journal of Biological Chemistry**, v.262, n.3, p.1037-1043. 1987.

Evans, A. C. O. e J. E. Fortune. Selection of the Dominant Follicle in Cattle Occurs in the Absence of Differences in the Expression of Messenger Ribonucleic Acid for Gonadotropin Receptors. **Endocrinology**, v.138, n.7, p.2963-2971. 1997.

Ferreira, R.*, et al.* The role of angiotensin II in the early stages of bovine ovulation. **Reproduction**, v.134, n.5, p.713-719. 2007.

Fortune, J. E. e S. M. Quirk. Regulation of steroidogenesis in bovine preovulatory follicles. **Journal of Animal Science**, v.66, n.Supplement_2, p.1-8. 1988.

Fortune, J. E.*, et al.* Differentiation of dominant versus subordinate follicles in cattle. **Biology of Reproduction**, v.65, n.3, p.648-654. 2001.

Fortune, J. E.*, et al.* Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. **Animal Reproduction Science**, v.82-83, p.109-126. 2004.

Gasperin, B.*, et al.* Dominant follicle growth is interrupted by intrafollicular injection of FGF10 in cattle. **II International Symposium on Animal Biology of Reproduction**. São Paulo, Brasil: Colégio Brasileiro de Reprodução Animal, 2008. p.203.

Ginther, O. J.*, et al.* Follicle selection in monovular species. **Biology of Reproduction**, v.65, n.3, p.638-647. 2001a.

Ginther, O. J.*, et al.* Follicle selection in cattle: role of luteinizing hormone. **Biology of Reproduction**, v.64, n.1, p.197-205. 2001b.

Ginther, O. J.*, et al.* Selection of the dominant follicle in cattle: establishment of follicle deviation in less than 8 hours through depression of fsh concentrations. **Theriogenology**, v.52, n.6, p.1079-1093. 1999.

Ginther, O. J.*, et al.* Selection of the dominant follicle in cattle. **Biology of Reproduction**, v.55, n.6, p.1187-1194. 1996.

Giometti, I. C.*, et al.* Angiotensin II reverses the inhibitory action produced by theca cells on bovine oocyte nuclear maturation. **Theriogenology**, v.63, n.4, p.1014-1025. 2005.

Glorioso, N.*, et al.* Prorenin in high concentrations in human ovarian follicular fluid. **Science**, v.233, n.4771, p.1422-1424. 1986.

Husain, A.*, et al.* Localization of Angiotensin II Receptors in Ovarian Follicles and the Identification of Angiotensin II in Rat Ovaries. **Proceedings of the National Academy of Sciences of the United States of America**, v.84, n.8, p.2489-2493. 1987.

Irving-Rodgers, H. F.*, et al.* Cholesterol Side-Chain Cleavage Cytochrome P450 and 3ß-Hydroxysteroid Dehydrogenase Expression and the Concentrations of Steroid Hormones in the Follicular Fluids of Different Phenotypes of Healthy and Atretic Bovine Ovarian Follicles. **Biology of Reproduction**, v.69, n.6, p.2022-2028. 2003.

Itskovitz, J.*, et al.* Plasma Prorenin Response to Human Chorionic Gonadotropin in Ovarian-Hyperstimulated Women: Correlation with the Number of Ovarian Follicles and Steroid Hormone Concentrations. **Proceedings of the National Academy of Sciences of the United States of America**, v.84, n.20, p.7285-7289. 1987.

Juengel, J. L.*, et al.* Effects of active immunization against growth differentiation factor 9 and/or bone morphogenetic protein 15 on ovarian function in cattle. **Reproduction**, v.138, n.1, p.107-14. 2009.

Kaneshiro, Y.*, et al.* Slowly progressive, angiotensin II-independent glomerulosclerosis in human (pro)renin receptor-transgenic rats. **Journal of the American Society of Nephrology**, v.18, n.6, p.1789-95. 2007.

Kim, S. e H. Iwao. Molecular and Cellular Mechanisms of Angiotensin II-Mediated Cardiovascular and Renal Diseases. **Pharmacological Reviews**, v.52, n.1, p.11-34. 2000.

Kim, S. J.*, et al.* Identification of renin and renin messenger RNA sequence in rat ovary and uterus. **Biochemical and Biophysical Research Communications**, v.142, n.1, p.169-175. 1987.

Knight, P. G. e C. Glister. TGF-ß superfamily members and ovarian follicle development. **Reproduction**, v.132, n.2, p.191-206. 2006.

Li, J.*, et al.* Expression of Inhibitor of Apoptosis Proteins (IAPs) in Rat Granulosa Cells during Ovarian Follicular Development and Atresia. **Endocrinology**, v.139, n.3, p.1321-1328. 1998.

Li, J. M.*, et al.* Angiotensin II-induced neural differentiation via angiotensin II type 2 (AT2) receptor-MMS2 cascade involving interaction between AT2 receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1. **Molecular Endocrinology**, v.21, n.2, p.499-511. 2007.

Lightman, A.*, et al.* Immunocytochemical localization of angiotensin II immunoreactivity and demonstration of angiotensin II binding in the rat. **American Journal of Obstetrics and Gynecology**, v.159, n.2, p.526-530. 1988.

Lightman, A.*, et al.* The ovarian renin-angiotensin system: renin-like activity and angiotensin II/III immunoreactivity in gonadotropin-stimulated. **American Journal of Obstetrics and Gynecology**, v.156, n.4, p.808-816. 1987.

Lopez-Ilasaca, M.*, et al.* The angiotensin II type I receptor-associated protein, ATRAP, is a transmembrane protein and a modulator of angiotensin II signaling. **Molecular Biology of the Cell**, v.14, n.12, p.5038-50. 2003.

Mihm, M.*, et al.* Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers. **Biology of Reproduction**, v.63, n.3, p.811-9. 2000.

Mihm, M.*, et al.* Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. **Reproduction**, v.135, n.2, p.253-265. 2008.

Mihm, M.*, et al.* Molecular Evidence That Growth of Dominant Follicles Involves a Reduction in Follicle-Stimulating Hormone Dependence and an Increase in Luteinizing Hormone Dependence in Cattle. **Biology of Reproduction**, v.74, n.6, p.1051-1059. 2006.

Min, L. J.*, et al.* Angiotensin II type 2 receptor deletion enhances vascular senescence by methyl methanesulfonate sensitive 2 inhibition. **Hypertension**, v.51, n.5, p.1339-44. 2008.

Miyoshi, T.*, et al.* Differential Regulation of Steroidogenesis by Bone Morphogenetic Proteins in Granulosa Cells: Involvement of Extracellularly Regulated Kinase Signaling and Oocyte Actions in Follicle-Stimulating Hormone-Induced Estrogen Production. **Endocrinology**, v.148, n.1, p.337-345. 2007.

Nabi, A. H.*, et al.* Binding properties of rat prorenin and renin to the recombinant rat renin/prorenin receptor prepared by a baculovirus expression system. **International Journal of Molecular Medicine**, v.18, n.3, p.483-8. 2006.

Nguyen, G.*, et al.* Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. **Journal of Clinical Investigation**, v.109, n.11, p.1417-27. 2002.

Nouet, S.*, et al.* Trans-inactivation of receptor tyrosine kinases by novel angiotensin II AT2 receptor-interacting protein, ATIP. **Journal of Biological Chemistry**, v.279, n.28, p.28989-97. 2004.

Ohkubo, H.*, et al.* Tissue distribution of rat angiotensinogen mRNA and structural analysis of its heterogeneity. **Journal of Biological Chemistry**, v.261, n.1, p.319-323. 1986.

Palumbo, A.*, et al.* Immunohistochemical localization of renin and angiotensin II in human ovaries. **American Journal of Obstetrics and Gynecology**, v.160, n.1, p.8-14. 1989.

Phillips, M. I. e C. Sumners. Angiotensin II in central nervous system physiology. **Regulatory Peptides**, v.78, n.1-3, p.1-11. 1998.

Pierre, A.*, et al.* Bone Morphogenetic Protein 5 Expression in the Rat Ovary: Biological Effects on Granulosa Cell Proliferation and Steroidogenesis. **Biology of Reproduction**, v.73, n.6, p.1102-1108. 2005.

Portela, V. M.*, et al.* Regulation of Angiotensin Type 2 Receptor in Bovine Granulosa Cells. **Endocrinology**, v.149, n.10, p.5004-5011. 2008.

Price, C. A.*, et al.* Comparison of hormonal and histological changes during follicular growth, as measured by ultrasonography, in cattle. **Journal of Reproduction and Fertility**, v.103, n.1, p.63-8. 1995.

Price, C. A. e R. Webb. Steroid control of gonadotropin secretion and ovarian function in heifers. **Endocrinology**, v.122, n.5, p.2222-31. 1988.

Rivera, G. M. e J. E. Fortune. Development of codominant follicles in cattle is associated with a follicle-stimulating hormone-dependent insulin-like growth factor binding protein-4 protease. **Biology of Reproduction**, v.65, n.1, p.112-118. 2001.

Roche, J. F. Control and regulation of folliculogenesis--a symposium in perspective. **Reviews of Reproduction**, v.1, n.1, p.19-27. 1996.

Saumande, J. e P. Humblot. The variability in the interval between estrus and ovulation in cattle and its determinants. **Animal Reproduction Science**, v.85, n.3-4, p.171-182. 2005.

Schauser, K. H.*, et al.* Localization of the Renin-Angiotensin System in the Bovine Ovary: Cyclic Variation of the Angiotensin II Receptor Expression. **Biology of Reproduction**, v.65, n.6, p.1672-1680. 2001.

Sealey, J. E.*, et al.* Prorenin as a reproductive hormone. New form of the renin system. **The American Journal of Medicine**, v.81, n.6, p.1041-1046. 1986.

Sealey, J. E.*, et al.* Plasma prorenin and renin in anephric patients. **Circulation Research**, v.41, n.4, p.17-21. 1977.

Sheikh, M. S.*, et al.* Role of Gadd45 in apoptosis. **Biochemical Pharmacology**, v.59, n.1, p.43-45. 2000.

Shinagawa, T.*, et al.* Purification and characterization of human truncated prorenin. **Biochemistry**, v.31, n.10, p.2758-2764. 1992.

Sicinski, P.*, et al.* Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. **Nature**, v.384, n.6608, p.470-4. 1996.

Sirois, J. e J. E. Fortune. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. **Biology of Reproduction**, v.39, n.2, p.308-317. 1988.

Speth, R. C.*, et al.* Angiotensin II: a reproductive hormone too? **Regulatory Peptides**, v.79, n.1, p.25-40. 1999.

Spicer, L. J. e P. Y. Aad. Insulin-like growth factor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: role of follicle-stimulating hormone and IGF2 receptor. **Biology of Reproduction**, v.77, n.1, p.18-27. 2007.

Stefanello, J. R.*, et al.* Effect of angiotensin II with follicle cells and insulin-like growth factor-I or insulin on bovine oocyte maturation and embryo development. **Theriogenology**, v.66, n.9, p.2068-2076. 2006.

Suzuki, F.*, et al.* Human prorenin has "gate and handle" regions for its non-proteolytic activation. **Journal of Biological Chemistry**, v.278, n.25, p.22217-22. 2003.

Takahashi, S.*, et al.* The human gene for renin-binding protein. **Journal of Biological Chemistry**, v.267, n.18, p.13007-13. 1992.

Tanaka, Y.*, et al.* The novel angiotensin II type 1 receptor (AT1R)-associated protein ATRAP downregulates AT1R and ameliorates cardiomyocyte hypertrophy. **FEBS Letters**, v.579, n.7, p.1579-86. 2005.

Tsurumi, Y.*, et al.* Interacting molecule of AT1 receptor, ATRAP, is colocalized with AT1 receptor in the mouse renal tubules. **Kidney International**, v.69, n.3, p.488-94. 2006.

Whitebread, S.*, et al.* Preliminary biochemical characterization of two angiotensin II receptor subtypes. **Biochemical and Biophysical Research Communications**, v.163, n.1, p.284-291. 1989.

Wruck, C. J.*, et al.* Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated Golgi protein. **Arteriosclerosis, Thrombosis, and Vascular Biology**, v.25, n.1, p.57-64. 2005.

Yoshimura, Y.*, et al.* Interactions between Insulin-like Growth Factor-I (IGF-I) and the Renin-Angiotensin System in Follicular Growth and Ovulation. **Journal of Clinical Investigation**, v.98, n.2, p.308-316. 1996a.

Yoshimura, Y.*, et al.* Angiotensin II induces ovulation and oocyte maturation in rabbit ovaries via the AT2 receptor subtype. **Endocrinology**, v.137, n.4, p.1204-1211. 1996b.

Yoshimura, Y.*, et al.* Gonadotropin stimulates ovarian renin-angiotensin system in the rabbit. **Journal of Clinical Investigation**, v.93, p.180-187. 1994.