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REGULAÇÃO DA EXPRESSÃO DO RECEPTOR AT2 E EFEITO DA ANGIOTENSINA II SOBRE A EXPRESSÃO DE GENES ENVOLVIDOS NO DESENVOLVIMENTO FOLICULAR E OVULAÇÃO EM CÉLULAS DA GRANULOSA DE BOVINOS

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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por

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elaborada por
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como requisito parcial para obtenção do grau de
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LISTA DE ABREVIATURAS

AGTR1	Receptor de angiotensina tipo 1
AGTR2	Receptor de angiotensina tipo 2
AngII	Angiotensina II
AP	Ativador do plasminogênio
BMP	Proteína morfogenética óssea
FGF	Fatores de crescimento fribroblástico
FSH	Hormônio folículo estimulante
GnRH	Hormônio liberador de gonadotrofinas
H2AFZ	Histona H2
IAP	Inibidor do ativador do plasminogênio
IGF	Fator de crescimento semelhante a insulina
LH	Hormônio luteinizante
MEC	Matriz extracelular
MMP	Metelo proteínases de matriz
PN-1	Proteína nexin 1
Ptgs	Prostaglandina Endoperoxidase Sintetase 2
RME	Remodelamento da matriz extracelular
TGF	Fator de crescimento transformante

RESUMO

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REGULAÇÃO DA EXPRESSÃO DO RECEPTOR AT2 E EFEITO DA ANGIOTENSINA II SOBRE A EXPRESSÃO DE GENES ENVOLVIDOS NO DESENVOLVIMENTO FOLICULAR E OVULAÇÃO EM CÉLULAS DA GRANULOSA DE BOVINOS

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Data e Local da Defesa: Santa Maria, 27 de setembro de 2007.

O objetivo deste trabalho foi estabelecer o controle de expressão dos receptores de angiotencina II (AngII) e determinar a ação fisiológica da AngII em células da granulosa (CG) cultivadas *in vitro*. Os receptores de AngII tipo 1 (AGTR1) e tipo 2 (AGTR2) foram localizados em folículos de bovinos de diferentes tamanhos. Verificou-se que as CG de bovinos provenientes de folículos entre 2- 5 mm e cultivadas com FSH, IGF-1, BMP-7 apresentaram aumento na expressão do receptor AGTR2 ($P<0,05$) em relação ao grupo controle (GC), bem como aumento da secreção de estradiol (E2; $P<0,05$). Em contraste, as CG tratadas com 10 ng/ml de FGF-2 ou 10 e 100 ng/ml de FGF-7 e FGF-10 apresentaram uma redução na secreção de E2 ($P<0,05$), porém somente os grupos FGF-7 e 10 nas doses 10 e 100 ng/ml reduziram ($P<0,05$) a expressão do receptor AGTR2. Para os dois experimentos, não houve diferença na expressão do receptor AGTR1 entre o GC e os grupos tratados. As CG e células da teca (CT) foram coletadas de ovários provenientes de abatedouro para extração de RNA e o fluido folicular para dosagem de E2 e progesterona P4. Esses folículos foram classificados como dominantes (FD; P4<100ng/ml e E2>100ng/ml) e atrésicos (FA; P4>40ng/ml). A expressão dos receptores AGTR1 e AGTR2 foi mensurada por RT-PCR. Não houve diferença na expressão do receptor AGTR1 entre FD e FA. No entanto, o receptor AGTR2 apresentou um aumento ($P<0,05$) na expressão em CG de FD em relação a FA. A expressão do receptor AGTR1 se manteve constante em CG e CT de FD e FA. Para determinar os afeitos da AngII através da ativação de seus receptores CG foram cultivadas em meio livre de soro com FSH e/ou AngII. A AngII não apresentou efeito na secreção de E2 ou P4, mas inibiu ($P<0.05$) mRNA e proteína para a protease nexin-1 (PN-1). Considerando a redução de expressão da PN-1 envolvida no controle do remodelamento da matriz extracelular (RME), é possível especular um efeito de AngII sobre RME durante o desenvolvimento folicular. Em um terceiro experimento, as CG de folículos grandes (>10mm) foram cultivadas durante 6h, 12h e 24h na presença de Ang (10-5) com ou sem LH (100ng/ml). A combinação de AngII e LH aumentou significativamente ($P<0,05$) a expressão de mRNA e proteína para COX-2, ativador do plasminogênio tipo U e T, bem como para PN-1. Entretanto, AngII ou LH não aumentaram a expressão de COX-2. O aumento da expressão destes gene indica uma função de AngII no processo de ovulação através das CG. Em um segundo momento, verificou-se através de qual receptor a AngII atua para controlar a expressão desses genes. As CG foram cultivadas por 6h com LH e/ou AngII com ou sem inibidores específicos para o receptor AGTR1 (losartan) e AGTR2 (PD123,319). Os resultados demonstraram que a presença do inibidor de AGTR2 bloqueou o efeito da associação de LH e AngII em relação ao grupo controle ($P<0.05$), demonstrado que a

ação da AngII é mediada pelo receptor AGTR2 em CG. Em conclusão, o receptor AGTR2 está presente nas células da granulosa de bovinos e o mRNA para o receptor AGTR2 é regulado durante o crescimento folicular. Além disso, a expressão do mRNA e a tradução da proteína para o AGTR2 são reguladas por FSH, IGF-1, BMP-7, FGF-7 e FGF-10 em CG de bovinos cultivadas *in vitro*. Os dados também sugerem que AngII regula a proteína PN-1 em CG e age como um co-fator fisiológico necessário para a ovulação.

Palavras-chave: angiotensina II, remodelamento da matriz extra celular, desenvolvimento folicular, células da granulosa.

ABSTRACT

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

REGULATION OF AT2 RECEPTORS IN BOVINE GRANULOSA CELLS, AND EFFECTS OF ANGIOTENSIN II ON GENES INVOLVED IN FOLLICLE DEVELOPMENT AND OVULATION

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The objective of this study was to investigate the factors controling the expression of angiotensin II (AngII) receptors and to determine the physiological role of AngII in granulosa cells. The AGTR2 receptor was localized in granulosa (and theca) cells from follicles of different sizes. Bovine ovaries were collected at a local abattoir and small follicles (2-5mm) were isolated for harvesting granulosa cells. The cells were cultured in free medium serum in non-luteinizing conditions without FSH (control group) or with graded doses of FSH or IGF1. In other cultures, cells were cultured with or without IGF1 and bone morphogenetic protein-7 (BMP-7), and fibroblast growth factor-2 (FGF-2). Treatment with FSH, IGF1 and BMP-7 increased ($P<0.05$) estradiol secretion and AGTR2 mRNA expression relative to control cultures. In contrast, none of these treatments affected AGTR1 receptor expression. Addition of FGF-2 significantly decreased estradiol secretion but did not affect AGTR1 or AGTR2 expression. Cells were cultured with FSH plus graded doses of FGF-7 or FGF-10, and the effects of these factors on AGTR2 protein levels were measured by Western blot. AGTR2 protein levels decreased in the groups treated with FGF-7 (10 and 100ng/ml) and FGF-10 (all concentrations; $P<0.05$), and estradiol secretion was significantly inhibited by the highest dose of each FGF ($P<0.05$). Bovine follicles greater than 5 mm diameter were dissected and granulosa and theca cells were separated for RNA extraction, and follicle fluid assayed for estradiol (E2) and progesterone (P) content. Non-atretic follicles (P less than 100ng/ml) were classed as estrogenic (E2 greater than 100ng/ml) or non-estrogenic (E2 less than 40ng/ml). There were no differences in AGTR1 receptor expression in theca and granulosa cells between estrogenic and non-estrogenic follicles. Likewise, there were no changes in AGTR2 receptor expression in theca cells with follicle state. However, AGTR2 receptor mRNA levels were significantly higher in granulosa cells of estrogenic compared to non-estrogenic follicles ($P<0.01$), and AGTR2 receptor mRNA was correlated with E2 concentrations in follicular fluid. To determine the physiological consequences of AT activation in granulosa cells, cells from small (2-5mm) bovine follicles were cultured in serum-free medium with FSH ± AngII. The addition of AngII had no effect on estradiol or progesterone secretion, but significantly inhibited protease nexin-1 (PN-1) mRNA levels and protein secretion ($P<0.05$). PN-1 is an inhibitor of proteases involved in extracellular matrix remodeling and follicle rupture. Bovine granulosa cells from large (>10 mm) follicles were cultured for 6h, 12h and 24h with LH (100ng/ml) or AngII, with or without angiotensin receptor blocker (losartan for AGTR1 and PD123,319 for AGTR2). These cells expressed Ptgs2 under basal culture conditions, which was not upregulated by either LH or AngII alone. However, LH and AngII in combination significantly enhanced Ptgs2 ($P<0.05$) mRNA and protein accumulation. Similarly,

expression of the proteolytic enzymes uPA and tPA, and their inhibitor, PN-1, were upregulated by the combination of LH and AngII but not by either factor alone. The addition of AGTR blockers inhibited the effect of AngII. In conclusion, AGTR2 receptor is present in granulosa bovine cells, and mRNA and protein are regulated by FSH, IGF-1, BMP-7, FGF-7 and FGF-10 in bovine granulosa cells in vitro, AGTR2 but not AGTR1 receptor mRNA levels are regulated during follicular growth in cattle, and that AngII regulates granulosa PN-1 secretion. These data suggest that AngII is a physiological co-factor necessary for the expression of genes in granulosa cells that are critical for ovulation.

Key words: angiotensin II, matrix extracelular remodeling, follicular development, granulosa cells.

1. INTRODUÇÃO

Em bovinos crescimento dos folículos ovarianos inicia-se com o recrutamento dos folículos primordiais e início da fase antral levando um grande número de folículos a crescer (FORTUNE et al., 2000). O futuro folículo pré-ovulatório passa por diversas fases de crescimento denominadas de seleção, divergência e dominância (GINTHER et al., 1996; FORTUNE et al., 2001) sendo que no final da ultima onda de crescimento um folículo culmina com o processo ovolatório, liberação de um oócito maduro e início da formação lútea a partir das células foliculares (MURPHY, 2004). Entretanto a maioria dos folículos degeneram durante a fase de formação de antro (MARKSTRÖM et al., 2002). Embora se conheça elemento que estão envolvidos no recrutamento, divergência e ovulação folicular este mecanismo ainda não está bem esclarecido.

Durante o crescimento folicular os folículos crescem em relação ao seu tamanho até 400 vezes entre o estádio pré-antral e pré-ovulátorio (LUSSIER et al., 1987). A área de superfície de um folículo pré-ovulatório dobra 19 vezes comparado com folículos primordiais (RODGERS et al., 1999). Também durante o desenvolvimento folicular intenso aumento da lâmina basal e mudanças na composição folicular bem como da matriz extracelular (MEC). As mudanças que ocorrem na parede folicular durante o crescimento do folículo são relatadas como efeito de uma cascata de enzimas proteolíticas reguladas pelos ativadores do plasminogênio (AP) e seus inibidores.

Os AP são proteases que convertem um grande volume de plasminogênio extracelular em plasmina, levando a degradação dos componentes da MEC, bem como ativando as metaloproteinases de matriz (MMP).

Neste sistema, especialmente em células da granulosa, também estão envolvidas proteínas reguladoras como a proteína nexin-1 (PN-1). A expressão desta proteína é durante o crescimento folicular é pouco entendida. Porém foi demonstrado que a expressão de PN-1 mRNA em células da granulosa de bovinos apresenta-se alta em folículos dominantes quando comparada com folículos pequenos (BÉDARD et al., 2003). A AngII que é um peptídeo envolvido na regulação de pressão sanguínea e tem seus receptores distribuídos em diferentes tecidos do organismo, regula negativamente a proteína PN-1 e o inibidor do AP tipo 1 em células de Schwann (BLEUEL et al., 1995).

O crescimento e desenvolvimento folicular é composto por processos que são ativados por gonadotrofina, sinais extra ovarianos e fatores intra ovarianos. O FSH é

essencial para o desenvolvimento de folículos primários e dominantes. Também se sabe que outro elemento com o IGF-1, BMPs, FGFs e EGF estão envolvidos.

2. REVISÃO BIBLIOGRÁFICA

Estudos sobre os fatores que atuam na diferenciação e no desenvolvimento folicular vêm sendo realizados há muitos anos, evidenciando que elementos como IGF e suas proteínas de ligação, níveis de estrógeno e progesterona, FSH, LH e seus receptores, atuam de forma crucial para o recrutamento, desenvolvimento e dominância folicular. No entanto, esses hormônios não são os únicos elementos envolvidos no desenvolvimento folicular; outros candidatos já conhecidos, como o ativador do plasminogênio (AP) e a angiotensina (Ang) II, podem estar envolvidos diretamente no crescimento folicular ou através do remodelamento da matriz extracelular (RME) regulando os elementos que controlam esse processo.

2.1 Angiotensina II e seus receptores

A AngII é um octapeptídeo derivado das porções terminais da AngI, e é um hormônio ativo do sistema renina-angiotensina que tem uma ação bastante conhecida na vasoconstrição arterial, angiogênese e síntese de aldosterona (HUSAIN et al., 1987; YOSHIMURA et al., 1994a; ACOSTA et al., 2000a). Baseado na presença de receptores nas células foliculares em diversas espécies, alguns autores sugerem que a AngII também desempenhe um papel na regulação da função ovariana (HUSAIN et al., 1987; AGUILERA et al., 1989; BRUNSWIG-SPICKENHEIER & MUKHOPADHYAY, 1992a; ACOSTA et al., 1999; SCHAUSER et al., 2001a). Em folículos pré-antrais de suínos, a AngII tem importante ação na formação do antro, proliferação celular e secreção de esteróides (SHUTTLEWORTH et al., 2002). A presença de receptores para a AngII já foi descrita nas células da teca e granulosa em ratas, coelhas (YOSHIMURA et al., 1996), e nas células da teca em vacas e macacas (AGUILERA et al., 1989; ACOSTA et al., 1999). Baseado em suas diferenças farmacológicas e funcionais, os receptores de AngII são classificados em dois tipos: AGTR1(também conhecido como AT1), responsável pela maioria dos efeitos conhecidos da AngII, como vasoconstrição arterial, angiogênese e secreção de aldosterona; e AGTR2 (també conhecido como AT2), relacionado com efeitos relacionados, principalmente induzindo apoptose e mediando funções reprodutivas (CHIU et al., 1989; WHITEBREAD et al., 1989).

A expressão do mRNA para o receptor AGTR1 e AGTR2 tem sido demonstrada em ovários de camundongos, coelhos, suínos e humanos (KONISHI et al., 1994; YOSHIMURA et al., 1996; KOTANI et al., 1999; LI et al., 2004). Em folículos de ratos o receptor AGTR2 está presente em células da granulosa e teca interna de folículos atrésicos (KOTANI et al., 1999; DE GOOYER et al., 2004). Em ovários de coelhos os dois tipos de receptores de AngII estão presentes tanto na célula da granulosa quanto na teca, porém somente o AGTR1 está presente em células da teca de folículos pré-ovulatórios (ACOSTA et al., 1999). Em ovários de camundongos, alguns estudos têm demonstrado que existe um predomínio da expressão do AGTR2 sobre AGTR1 sugerindo uma função mais efetiva para este receptor (BRUNSWIG-SPICKENHEIER & MUKHOPADHYAY, 1992b). No folículo bovinos, a expressão do AGTR2 é descrita somente nas células da teca, indicando que os mecanismos que envolvem os efeitos da AngII são via o receptor AGTR1 e não AGTR2 nesta espécie (ACOSTA et al., 1999; SCHAUSER et al., 2001b).

No ovário, a atividade de AngII tem sido descrita em algumas espécies e com diferentes ações. Em coelhas, sua atividade foi relacionada à maturação do oócito, ovulação e esteroidogênese (YOSHIMURA et al., 1992; YOSHIMURA et al., 1993; FÉRAL et al., 1995; TANAKA et al., 1995; HAYASHI et al., 2000). Em bovinos, a atividade de AngII está relacionada com o crescimento folicular, com a reversão da inibição maturação nuclear *in vitro* causada por células foliculares e com a esteroidogênese (NIELSEN et al., 1994; ACOSTA et al., 2000a; GIOMETTI et al., 2005b). Através da técnica de microdiálise, foi mostrado que a AngII, juntamente com outros peptídeos, também tem um papel vascular na ovulação, formação e regressão do corpo lúteo em bovinos (ACOSTA et al., 2000a). No entanto, outros autores têm demonstrado que além da função vascular, a AngII tem papel indispensável na ovulação através da produção de prostaglandinas induzidas por gonadotrofinas (YOSHIMURA et al., 1992; KUJI et al., 1996).

Algumas evidências sugerem que o sistema renina-angiotensina tem um efeito importante no processo de ovulação em bovinos. Estudos *in vitro* demonstraram que a AngII atua como um intermediário na ovulação induzida por gonadotrofinas em coelhas, ratas e vacas (KUO et al., 1991; YOSHIMURA et al., 1992; PETERSON et al., 1993; KOTANI et al., 1999; FERREIRA et al., 2007). Em bovinos, a presença de receptores para AngII nas células foliculares e o aumento nas concentrações de AngII no fluido folicular após a liberação pré-ovulatória de gonadotrofinas, sugerem uma

atividade biológica deste peptídeo também nessa espécie (BRUNSWIG-SPICKENHEIER & MUKHOPADHYAY, 1992a; ACOSTA et al., 2000b; SCHAUSER et al., 2001a). Recentemente, nosso grupo demonstrou que a AngII participa da maturação nuclear de oócitos bovinos, evento síncrono, porém independente da ovulação, e que aplicação de bloqueadores dos receptores de AngII em bovinos resulta no bloqueio da ovulação nesta espécie (GIOMETTI et al., 2005a; FERREIRA et al., 2007).

2.2 Desenvolvimento folicular

Até a formação do antro, os folículos são ativados pela ação de fatores intrínsecos e estão comprometidos a crescer (ou entrar em atresia), não podendo voltar ao estádio quiescente. Na maioria das espécies, somente a partir de folículos secundários e, em algumas espécies, de folículos primários, há uma dependência de FSH para o seu crescimento. Após a formação do antro, um grupo de folículos cresce por ação, principalmente, de baixos níveis de gonadotrofinas. Eles possuem receptores para FSH nas células da granulosa e receptores para LH nas células da teca (MARTIN et al., 1988; GINTHER et al., 1989; EVANS & FORTUNE, 1997). À medida que os folículos se desenvolvem, aumenta a resposta a gonadotrofinas, até atingirem aproximadamente 4-5 mm, quando passam a ser dependentes de elevados níveis de gonadotrofinas. Nesse estádio, os folículos dobram de tamanho a cada quatro dias até que um começa a se diferenciar, resultando em seleção do futuro folículo dominante e divergência folicular (momento em que o folículo dominante continua seu crescimento e o segundo maior folículo inicia o processo de regressão (EVANS & FORTUNE, 1997; FORTUNE et al., 2001).

A seleção e dominância folicular ocorrem por regulação endócrina, autócrina e parácrina. O conhecimento atual permite estabelecer que inibina, ativina, fator de crescimento semelhante à insulina I (IGF-I) e suas proteínas de ligação (IGFBP) estão envolvidas e atuam diretamente nas células da teca e granulosa, modulando o desenvolvimento folicular e a esteroidogênese (MAZERBOURG et al., 2001). O crescimento do folículo dominante e o incremento da produção de estradiol (E2) e inibina estão acompanhados pela diminuição nos níveis de ativina e IGFBP, simultaneamente com o aumento nas concentrações de IGF-I livre. No entanto, não há um aumento nos níveis totais de IGF-I. Há evidências de que a inibina e IGFBPs de

baixo peso molecular (IGFBP-2, -4 e -5) têm efeito negativo na ação das gonadotrofinas no folículo e que os diferentes padrões de secreção de FSH e LH regulam esses fatores na seleção, dominância e atresia do folículo dominante. A diminuição nas concentrações foliculares dessas IGFBPs, com o conseqüente aumento de IGF-I livre, é importante à dominância folicular e é conseqüência do nível de expressão gênica, degradação por atividade proteolítica ou por ambos os fatores (RIVERA & FORTUNE, 2003).

2.3 Desenvolvimento folicular e o remodelamento da matriz extracelular

Existem evidências que o AP pode estar envolvido no desenvolvimento de pequenos folículos antrais (CAO et al., 2004). A expressão do AP é variável durante o desenvolvimento folicular possuindo um inibidor específico nas células da granulosa PN-1 (CAO et al., 2006a). Em células endoteliais a AngII tem se mostrado um potente estimulador do inibidor do AP tipo 1 (SKURK et al., 2001). Já em células de Schwann a AngII tem demonstrado regular negativamente a expressão de SERPINE2 (BLEUEL et al., 1995), que é também um inibidor do AP e que no ovário esta presente em células da granulosa de ratos e bovinos (HAGGLUND et al., 1996; HASAN et al., 2002; BÉDARD et al., 2003).

A parede folicular é constituída de uma matriz rica em colágeno, onde, geralmente, ocorre a ação de enzimas proteolíticas que atuam no tecido conjuntivo no momento da ovulação. O AP e as MMPs parecem serem responsáveis por mecanismos celulares e pelo RME (CAMPBELL et al., 1987). Ativadores do plasminogênio são serinas que convertem o plasminogênio zimógeno extracelular em plasmina, uma protease ativa que degrada componentes da MEC (BLASI et al., 1987).

Para ocorrer o crescimento e desenvolvimento folicular, é necessário que ocorra não somente a liberação de gonadotrofinas e fatores de crescimento, é preciso que ocorra no folículo, desde o recrutamento até a ovulação, um extenso remodelamento tecidual, possibilitando a proliferação e diferenciação celular (SMITH et al., 1999). O remodelamento de tecido envolve várias proteases e uma cascata de enzimas, inclusive metaloproteinase de matriz e o AP (MURPHY et al., 1999). Concomitantemente, o AP ativa o plasminogênio e, conseqüentemente, a plasmina, que está envolvida na migração celular e estimula as contrações ovarianas, as quais facilitam a ruptura folicular. A hipótese de que as prostaglandinas estariam envolvidas no processo da ovulação implica

no seguinte mecanismo: AngII, prostaglandinas, E1 e E2 estimulariam a síntese da enzima ativadora do plasminogênio que forma plasmina nas células da granulosa, imediatamente antes, próximo a ovulação a plasmina ocasionaria o enfraquecimento da parede folicular e posteriormente levaria a sua ruptura (ALWACHI et al., 1981; ACOSTA et al., 1999). A AngII aumentada concentração folicular pré-ovulatória de $PGF_2\alpha$ (ACOSTA et al., 1999; ACOSTA et al., 2000a), produzida pelas células da granulosa e estimularia a síntese de enzimas como a colagenase e a elastase, nas células foliculares.

Células da granulosa de ratas, *in vivo*, são capazes de sintetizar quantidades crescentes da enzima AP, à medida que o momento da ovulação se aproxima. As células da granulosa podem ser estimuladas a sintetizar o AP mediante gonadotrofinas, porém o FSH parece ser mais ativo que o LH (CAO et al., 2004; CAO et al., 2006b). Tanto a PGE1, PGE2 como os análogos estáveis do AMPc estimulam a síntese desta enzima nas células da granulosa. Entre os elementos responsáveis pelo RME temos o AP, uma proteína que converte o plasminogênio extracelular em plasmina, uma protease ativa que degrada componentes da matriz extracelular. Duas formas de AP foram descritas em mamíferos: o AP tecidual (APt) e o urocinase (APu) (MACCHIONE et al., 2000). Esses dois elementos são produtos de dois genes, *Plat* e *Plau*, respectivamente. O tipo de AP produzido entre as espécies é célula-específica no rato (CANIPARI et al., 1987; GALWAY et al., 1989; POLITIS et al., 1990). As células da granulosa secretam predominantemente APt em folículos pré-ovulatórios de bovinos, RNAm de APt foi detectado principalmente em células da granulosa, considerando que RNAm de APu foi detectado em células da granulosa e da teca (DOW et al., 2002a). Um mecanismo para a regulação e ativação do plasminogênio tem sido sugerido através da produção de inibidores do AP (IAP) e PN-1, controlados por fatores de crescimento e FSH em células foliculares (CAO et al., 2004). Os três IAP são IAP-1, IAP-2 e a protease nexin - 1 (PN-1) (KRUITHOF, 1988; ROBERTS et al., 1995), expressos pelos genes *SERPINE1*, *SERPINEB2* e *SERPINE2* (SILVERMAN et al., 2001). Em roedores e bovinos, o gene que codifica IAP-1 é expresso, predominantemente, nas células intersticiais da teca (LIU et al., 1997; DOW et al., 2002b). Já o gene que codifica para o IAP-2 é expresso na teca de ratos tratados com hCG e em células da granulosa de ovários humanos estimulados por hCG (LEONARDSSON et al., 1995). Em contraste, o gene que codifica PN-1 é expresso fortemente em células da granulosa de ratos e bovinos (HAGGLUND et al., 1996; BÉDARD et al., 2003).

É amplamente aceito que o AP é importante durante o processo de ovulação e durante a degradação proteolítica da parede de folículo. No período final do desenvolvimento folicular, quando o folículo torna-se pré-ovulatório em ratos e primatas, ocorre nas células de granulosa um concomitante aumento da expressão do gene *Plat* que codifica para APt. O aumento da atividade do APt inicia o processo proteolítico que degrada a parede do folículo pré-ovulatório (PENG et al., 1993; LIU, 2004). Esta mesma degradação da MEC e remodelamento tecidual que ocorre na ovulação também é importante para o crescimento e desenvolvimento de folículos pequenos (LI et al., 1997b). Como os folículos bovinos sempre aumentam em tamanho várias vezes desde o estádio de pré-antral até pré-ovulatório, é necessário que ocorram modificações na MEC para que as células se diferenciem ao longo do crescimento. Estudos em ratos mostraram que APu é o AP predominante em folículos pequenos e em crescimento, enquanto que APt é predominante em folículos pré-ovulatórios (KARAKJI & TSANG, 1995; LI et al., 1997a). Não está evidente como a atividade do AP, regulada através de inibidores, interfere nas fases de desenvolvimento folicular, porém se sabe que a AngII é um potente estimulador em outros tecidos do gene que codifica para o IAP-1 (CHEN & FEENER, 2004), porém sabe-se é um elemento importante para o desenvolvimento de folículos pré-antrais (SHUTTLEWORTH et al., 2002). Em ratos, a expressão de SERPINE1 é baixa em folículos pequenos em crescimento aumentando em folículos diferenciados (LI et al., 1997a), o que ocorre semelhantemente durante a ovulação. Expressão de SERPINE2 não foi evidenciada em folículos bovinos pré-ovulatórios, mesmo antes do pico pré-ovilatório de GnRH (DOW et al., 2002a). Curiosamente, o gene SERPINE2 é altamente expresso em folículos pequenos de ratos (HAGGLUND et al., 1996; HASAN et al., 2002), e em folículos pré-antrais e antrais durante o crescimento em bovinos (BÉDARD et al., 2003). O PN-1 parece ser o principal inibidor de AP expresso em células da granulosa de folículos em crescimento.

2.4 Fatores de crescimento (BMPs e FGFs)

As proteínas morfogenéticas ósseas (BMPs) são membros da família do fator de crescimento transformante beta (TGFβ) são responsáveis por vários sinais extracelulares envolvidos com o crescimento, diferenciação e apoptose de vários tipos celulares. As BMPs já foram descritas como expressas nas células da teca, granulosa e

oócito de diversas espécies (ELVIN et al., 1999; SHIMASAKI et al., 1999; ELVIN et al., 2000). Também já foi demonstrado o envolvimento do BMP-4, 6 e 7 na regulação da esteroidogênese bem como a presença de seus receptores nas células da teca e granulosa (GLISTER et al., 2004; CAO et al., 2006c).

Já os fatores de crescimento fribroblásticos (FGFs), especialmente o FGF básico, estimula a expressão do APt e a atividade desta enzima em células da granulosa de ratos cultivadas *in vitro* em tempos e doses dependentes, sugerindo um efeito intra-ovariano induzido pelo APt (LAPOLT et al., 1990). Células da granulosa de bovinos cultivadas com FGF básico, apresentam um aumento na ploliferação celular e na expressão da proteína inibidora de metaloproteinases (HOSHI et al., 1995). Além disso, a expressão de FGF-3 e 4 em células da teca e granulosa de bovinos é modulada durante o desenvolvimento folicular pelos níveis de FSH (BURATINI et al., 2005). Recentemente, foi evidenciado que células da granulosa cultivadas com FGF-7 e 10 inibem a secreção de estradiol nas células da granulosa cultivadas *in vitro,* podendo ser um importante marcador da diferenciação celular (BURATINI et al., 2007).

2.5 Papel da Ang II na maturação de oócitos

Os oócitos permanecem em estádio de vesícula germinativa (VG) durante o desenvolvimento folicular até próximo da ovulação. *In vitro,* os oócitos reiniciam a meiose espontaneamente e progridem até o estádio de metáfase II (MII) quando são removidos de seus folículos e cultivados sob condições adequadas. Entretanto, células da teca (mas não da granulosa) são capazes de manter oócitos bovinos em estádio de VG quando cultivados *in vitro* (RICHARD & SIRARD, 1996; GIOMETTI et al., 2005b). Por outro lado, *in vivo,* a maturação meiótica dos oócitos bovinos ocorre dentro do folículo, levantando ao questionamento a respeito do envolvimento de sinal(is) positivo(s) que induzam o reinício da meiose.

Quando oócitos bovinos são cultivados diretamente com AngII ou saralasina (antagonista dos receptores de AngII), não há efeito na maturação. Entretanto, em oócitos cultivados com células foliculares, a AngII reverte o efeito inibitório das células da teca, promovendo a maturação nuclear do oócito bovino, inibindo ou estimulando a produção de algumas substâncias pelas células da teca [30]. Em folículos bovinos, receptores de AngII foram detectados principalmente nas células da teca e em menor quantidade nas células da granulosa (BRUNSWIG-SPICKENHEIER &

MUKHOPADHYAY, 1992a; ACOSTA et al., 1999; SCHAUSER et al., 2001a). Isso explica o porquê do efeito da AngII ocorrer somente quando as células foliculares estavam presentes no sistema de cultivo de oócitos. A AngII também reverteu a inibição da maturação nuclear *in vitro* causada pela presença de células foliculares. No entanto, parece que a AngII é um dos fatores positivos envolvidos na maturação de oócitos bovinos.

2.6 Papel da Angiotensina II na Ovulação e Desenvolvimento Folicular

Evidências sugerem que o sistema reniana Angiotensina tem um importante papel no processo de ovulação em bovinos. Estudos *in vitro* têm demonstrado que a AngII atua como mediador na ovulação induzida por gonadotrofinas em coelhas (KUO et al., 1991; YOSHIMURA et al., 1992) e ratas (PETERSON et al., 1993). Em bovinos, a presença de receptores de AngII nas células foliculares (BRUNSWIG-SPICKENHEIER & MUKHOPADHYAY, 1992a; ACOSTA et al., 1999; SCHAUSER et al., 2001a) e o aumento nas concentrações de AngII após o pico de LH (ACOSTA et al., 2000a) sugerem uma atividade biológica deste peptídeo nessa espécie. Recentemente, nosso grupo demonstrou que a AngII atua como mediador na ovulação induzida por gonadotrofinas em bovinos (FERREIRA et al., 2007). Foi adaptado um modelo *in vivo* o qual permite estudar o papel da AngII na ovulação, injetando antagonistas dos receptores de AngII em folículos pré-ovulatórios. Esse modelo *in vivo,* possibilita estudos em diversas áreas da reprodução sem alterar o crescimento folicular e a fisiologia da ovulação.

Nesses estudos, as vacas receberam injeções intrafoliculares de acordo com cada tratamento quando os folículos atingiram um diâmetro mínimo de 12 mm, e foram desafiadas com uma aplicação IM de análogo do GnRH. A aplicação intrafolicular de 100 mM 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)(FERREIRA et al., 2007). O pico ovulatório de LH ocorre cerca de uma hora após o início do estro e a AngII aumenta no fluído folicular após este evento (ACOSTA et al., 2000b). Uma vez que a AngII se liga aos seus receptores e inicia o mecanismo de ovulação, não há efeito da saralasina na taxa de ovulação, o que explica porquê a inibição da AngII não bloqueia a ovulação após o início do estro. Baseado nesses resultados, outro experimento foi conduzido para

determinar o momento em que a AngII desempenha seu papel na ovulação. A saralasina bloqueou a ovulação somente quando aplicada no momento e 6 horas após o tratamento com análogo do GnRH, mas não quando este inibidor foi administrado 12 horas após o GnRH. As concentrações de AngII permanecem elevadas durante todo o processo de ovulação (ACOSTA et al., 2000a). Entretanto, resultados *in vivo* demonstraram que a AngII desempenha uma função fundamental somente no início do mecanismo de ovulação em bovinos (FERREIRA et al., 2007). Yoshimura et al. (YOSHIMURA et al., 1992) induziram a ovulação com AngII na ausência de gonadotrofinas em um modelo utilizando ovários de coelhas perfundidos *in vitro*. Esses dados mostram que a AngII participa como iniciador do processo de ovulação induzido por gonadotrofinas. Em ovários de coelhas perfundidos, as concentrações de AngII no fluído folicular aumentam quatro horas após exposição a gonadotrofinas. Isso provavelmente ocorre devido a um aumento na atividade intrafolicular de renina (YOSHIMURA et al., 1994b).

Essas descobertas estão de acordo com nossos resultados, nos quais foi observado um bloqueio parcial da ovulação quando a AngII foi inibida 6 horas após a injeção de análogo do GnRH. 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. Foi observado aumento na expressão de receptor AGTR2 em folículos pré-ovulatórios, sugerindo uma participação deste receptor nos estádios finais de crescimento e ovulação destes folículos (SCHAUSER et al., 2001a). Tem sido demonstrado que a AngII aumenta as concentrações de prostaglandina em folículos pré-ovulatórios e que a ovulação é inibida quando receptores de AngII são bloqueados (PELLICER et al., 1988; KUJI et al., 1996; ACOSTA et al., 1998; FERREIRA et al., 2007). A Ang II pode atuar diminuindo a secreção de IAP (BROWN et al., 2000; PORTELA et al., 2006), portanto melhorando o remodelamento da matriz extracelular. Há evidência de que o AP pode desempenhar funções no desenvolvimento de pequenos

folículos antrais. A expressão do AP altera-se durante o crescimento folicular de bovinos, assim como ocorre com a expressão de um inibidor específico do AP das células da granulosa, a protease nexin-1 (PN-1; (CAO et al., 2004).

3. CAPÍTULO 1

REGULATION OF ANGIOTENSIN TYPE 2 RECEPTOR IN BOVINE GRANULOSA CELLS IN VITRO

Valério M Portela, Paulo BD Gonçalves, Angela M Veiga, Edmir Nicola, Gustavo Zamberlam , José Buratini, Jr., Christopher A Price.

REGULATION OF ANGIOTENSIN TYPE 2 RECEPTOR IN BOVINE GRANULOSA CELLS IN VITRO

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Key words: angiotensin receptor, granulosa cell, follicle

Running head: Angiotensin receptors in bovine follicles

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Abstract

Angiotensin II (AngII) is best known for its role in blood pressure regulation, but it also has documented actions in the reproductive system. There are two AngII receptors, type 1 (AGTR1) and type 2 (AGTR2). AGTR2 mediates the non-cardiovascular effects of AngII, including induction of ovulation. In cattle, expression of AGTR2 mRNA is reported to occur only in theca cells, whereas granulosa cells also express AGTR2 in rodents. The objectives of the present study were to determine if AngII receptors are expressed in granulosa cells and whether expression is regulated by gonadotropins and growth factors. RT-PCR demonstrated AGTR1 and AGTR2 mRNA in both granulosa and theca cells of bovine follicles. AGTR1 expression did not differ between healthy and atretic follicles, whereas granulosa but not theca AGTR2 expression was lower in atretic compared to healthy follicles. The presence of AGTR2 protein in bovine follicles was confirmed by immunohistochemistry. Granulosa cells were cultured in free serum medium, and treatment with FSH, IGF1 and BMP-7 increased ($P<0.05$) estradiol secretion and AGTR2 mRNA and protein levels relative to control cultures. In contrast, none of these treatments affected AGTR1 receptor expression. FGF-7 and FGF-10 inhibited estradiol secretion and AGTR2 protein levels. In conclusion, AGTR2 receptor is present in granulosa bovine cells, and mRNA and protein are regulated by FSH, IGF-1, BMP-7, FGF-7 and FGF-10 in bovine granulosa cells in vitro.

Introduction

The renin-angiotensin system (RAS) consists of the enzyme renin that converts angiotensinogen to angiotensin I (AngI). Angiotensin converting enzyme (ACE) then cleaves the decapeptide AngI to the octapeptide AngII, which is considered to be the major bioactive peptide of the RAS (reviewed in [1]). Local RAS have been described in several organ systems, including the ovary [1]. To date, most data point to a role for AngII in the induction of ovulation. AngII infusion induced ovulation in perfused rabbit ovaries and AngII antagonists inhibited ovulation in rabbits and rats [2-5]. AngII stimulated prostaglandin (PG) secretion from rabbit ovaries [2, 4] and bovine preovulatory follicles [6, 7]; this may be the mechanism through which AngII induces ovulation, as mice deficient in PG synthase 2 fail to ovulate [8].

AngII acts through two distinct transmembrane receptors. The effects of AngII on blood pressure regulation are mediated through the type 1 receptor (AGTR1, also known as AT1) whereas other actions are generally mediated through the type 2 receptor (AGTR2, also known as AT2) [1]. There appears to be considerable species differences in the function and location of AngII receptors in the ovary. In rabbits, receptors are mostly AGTR2 and expressed in granulosa cells of preovulatory follicles, consistent with the role of AngII in ovulation [2]. In rats, AGTR2 is the predominant receptor and is mostly expressed in granulosa cells, but only in atretic follicles [9-11]. This suggests that AngII is involved in the process of atresia, which is supported by in vitro data. Culturing rat granulosa cells without FSH induced atresia and apoptosis, and markedly increased AngII binding to AGTR2 [12], and AngII inhibited estradiol secretion from cultured rat and rabbit granulosa cells [13, 14]. In cattle, only theca cells contain AngII binding sites, predominantly AGTR2 [15, 16] although AGTR1 is also expressed [6]. The role of AngII in bovine follicle growth/atresia is unknown.

There may be a role for the RAS in follicle atresia in cattle as atretic follicles contain 4 – 5 times more prorenin that healthy follicles [17]. As bovine granulosa cells do not express AGTR2, this is likely to involve AngII signaling through the theca cell layer, in contrast to the mechanism in rats in which AngII signals through granulosa cells. Therefore, the objectives of the present study were 1) to measure AGTR1 and AGTR2 expression in bovine granulosa and theca cells in atretic and nonatretic follicles, and 2) to determine whether AGTR1 and AGTR2 expression are regulated.

Materials and methods

Follicles

Ovaries were obtained from an abattoir and transported to the laboratory in saline on ice. Follicles greater than 6 mm in diameter were dissected from the ovaries, and follicular fluid was aspirated, centrifuged and frozen for steroid assay. The antral cavity was flushed repeatedly with cold saline and granulosa cells recovered by centrifugation at 1200 g for 1 minute, and pooled with the follicular fluid pellet. The remaining granulosa cells adhering to the follicle wall were removed by gently scraping with a blunt Pasteur pipette, and the theca layer removed with forceps and washed in saline by passing repeatedly through a 1mL syringe. The samples were collected into Trizol (Invitrogen; São Paulo, Brazil) and homogenized with a Polytron. Total RNA was extracted immediately according to the Trizol protocol.

Follicles were grouped by estradiol:progesterone (E:P) ratio of >1 (healthy) or <1 (atretic) as described [18]. Cross-contamination of theca and granulosa cells was tested by detection of mRNA encoding cytochromes P450 aromatase (*Cyp19*) and 17α-hydroxylase (*Cyp17*) in each sample by PCR [19].

Granulosa cell culture

The granulosa cell culture system was based on that described by [20] with slight modifications [21]. All materials were obtained from Invitrogen Life Technologies (Burlington, ON, Canada) except where otherwise stated. Briefly, bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir, and were transported to the laboratory in PBS at 35°C containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (1 μg/ml). Cells were collected from small (2-5 mm diameter), medium (6-8 mm) or large (>8mm) follicles by repeatedly passing bisected follicle walls through a pipette, and were washed twice by centrifugation at 219 × g for 20 min each, and suspended in α-MEM containing Hepes (20 mM), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma-Aldrich Canada, Oakville, ON, Canada), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1.1 mM), androstenedione (10^{-7} M at start of culture, and 10^{-6} M at each medium change) and insulin (10 ng/ml). Cell viability was estimated with 0.4% Trypan Blue Stain. Cells were seeded into 24-well tissue culture plates (Sarstedt, Montreal, QC) at a density of 1 x 10^6 viable cells per well in 1 ml medium. Cultures were maintained at 37°C in 5% CO_2 in air for 6 days, with 700 μl medium being replaced every 2 days.

To assess hormone regulation of AGTR1 and AGTR2 expression, cells from small follicles were cultured with graded doses of FSH or insulin-like growth factor 1 (IGF1). In separate cultures, cells were cultured with IGF1 (10 ng/ml) with or without bone morphogenetic protein-7 (BMP7; 50 ng/ml), epidermal growth factor (EGF; 10 ng/ml) or fibroblast growth factor-2 (FGF-2; 10 ng/ml). These treatments have been demonstrated to alter estradiol secretion in this model [22]. We then tested the effect of graded doses of FGF-7 or FGF-10, as these factors have also been shown to alter

estradiol secretion from granulosa cells [23] [24]. Medium samples were collected on day 6, and stored at -20°C until steroid assay. For PCR assay, cells were lysed in Trizol for extraction of RNA and DNA. To measure AGTR2 protein, cells were collected into TES buffer (10mM Tris HCl, 1 mM EDTA, 250mM sucrose) containing a protease inhibitor cocktail (Complete mini, Roche) for Western blotting.

Nucleic acid extraction & semi-quantitative RT-PCR

Total RNA and DNA were extracted using Trizol according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm. Total RNA (1 μg) was first treated with 1 U DNase (Promega, Madison, WI) at 37°C for 30 min to digest any contaminating DNA, followed by adding 1 μl of EDTA stop buffer at 65°C for 10 min. The RNA was reverse transcribed in the presence of 1 mM oligo(dT) primer and 4 U Omniscript RTase (Omniscript RT Kit, Qiagen, Mississauga, ON), 0.25 mM dideoxy-nucleotide triphosphate (dNTP) mix, and 19.33 U RNase Inhibitor (Amersham Biosciences, Baie D'Urfé, QC) in a volume of 20 μl at 42°C for 2 h. The reaction was terminated by incubation at 93°C for 5 min.

Bovine-specific primers were used for amplifying *AGTR1* (sense: 5'-AAATACATTCCCCCAAAGGC -3', antisense: 5'- TGTGGCTTTGCTTTGTTGAG -3', [6]) and *AGTR2* (sense: 5`- TTTGGCTACTCTTCCTCTCTGG -3', antisense: 5'-CATACTTCTCAGGTGGGAAAGC -3', [6]). Variability in mRNA amounts was assessed by amplifying cyclophilin A (*PPIA*) [25] or histone H2AFZ (*H2AFZ*; sense: 5'-AGCGTATTACCCCTGGTCAC -3', antisense: 5'-CCAGGCATCCTTTAGACAGT -3'). An aliquot (2 μl) of the cDNA template was amplified by PCR using 0.25 μl (2.5 U) Taq Polymerase (Amersham Pharmacia Biotech Inc., Oakville, ON, Canada) in a 20-μl PCR buffer (Amersham Pharmacia

Biotech Inc.) containing 0.1 mM dNTP mix, and 0.2 μM specific primers. After an initial denaturation step for 3 min at 94°C, target cDNA was amplified with a denaturation step at 94°C for 30 sec (*AGTR1* & *AGTR2*) or 45 sec (*H2AFZ*), annealing for 45 sec at 60°C (*AGTR1, PPIA*), 64°C (*AGTR2*) or at 58°C (*H2AFZ*) for 30 sec, and elongation at 72°C for 1 min. All reactions were terminated with a final elongation at 72°C for 5 min. Semiquantitative RT-PCR was validated for each gene product. Reactions were performed for 28 cycles for *AGTR1* and 30 cycles for *AGTR2* and *H2AFZ.*

The PCR products were separated on 2 % agarose gels with 0.001% ethidium bromide, and visualized under UV light. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was expressed relative to H2a mRNA abundance.

Western Blot

AGTR2 protein abundance in cell lysates was analyzed by Western blot. Samples were subjected to electrophoresis in 12% denaturing polyacrylamide gels and electrotransferred onto nitrocellulose membranes. After blocking for 1 h in TTBS plus 0.5% skim milk, blots were incubated with 1:5000 rabbit anti-sheep AGTR2 (cat. 19134; Abcam Inc, Cambridge, MA, USA) for 2 h with agitation, followed by three washes with 0.2% TTBS. The blots were then incubated with 1:10000 anti-rabbit-HRP (Amersham, Oakville, ON, Canada) for 1 h with agitation, followed by three washes with 0.2% TTBS. Finally, the blots were incubated in Immobilon Western Chemiluminescent HRP (Millipore, Billerica, USA) for 5 min and were exposed to X-ray film for image analysis.

Immunohistochemistry

To confirm AGTR2 protein localization in bovine follicles in vivo we performed immunohistochemistry with the same antibody used in Western blotting. Ovaries were collected at a local abattoir and fixed in 4% paraformaldehyde, 0.25% picric acid in 0.1 M phosphate buffer, pH 7.3. After deparaffinization and antigen retrieval (boiling in 0.01M sodium citrate and 0.1 M citric acid for 5 min), sections were blocked in 10% normal goat serum for 1 h and then AGTR2 antibody (1:300) was applied for 18 h at 4 C. After the primary antibody, slides were washed in PBS and then incubated with biotinylated second antibody for 45 min. After two 5-min washes in PBS, a complex of avidin-peroxidase was applied for 45 min. Positive reactions were revealed by NovaRed staining. Sections were then washed in distilled water and lightly counterstained with hematoxylin for 10s. Control sections were subjected to the same procedure, except that dilute rabbit serum replaced the first antibody.

Further sections were examined by immunofluorescence, performed as above but using Cy3-conjugated second antibody (Jackson ImmunoResearch,West Grove, PA) and counterstaining with DAPI. Negative controls were performed in the absence of primary antibody.

Steroid assay

Estradiol was measured in follicular fluid and conditioned medium in duplicate as described [26], without solvent extraction. Intra- and inter-assay coefficients of variation were 8.5% and 6.3%, respectively. Progesterone was measured in duplicate as described [27] with mean intra- and inter-assay coefficients of variation were 7.2% and 18%, respectively. The sensitivity of these assays were 10 pg and 4 pg per tube for

estradiol and progesterone, equivalent to 0.3 and 20 ng/μg protein, respectively. Steroid concentrations in culture medium were corrected for cell number by expressing per unit mass of total DNA.

Statistical Analysis

Data that did not follow a normal distribution (Shapiro-Wilk test) were transformed to logarithms. Homogeneity of variance was tested with O'Brien and Brown-Forsythe tests. Analysis of data was performed with JMP software (SAS Institute) with treatment or follicle group as main effect and culture replicate (where appropriate) as a random variable in the F-test. For gene expression data, housekeeping gene abundance was included as a covariate in the main effects model. Differences between means were tested with the Tukey-Kramer HSD test. Data are presented as means ± SEM.

Results

AGTR1 and AGTR2 were first surveyed in theca and in granulosa cells from healthy and atretic follicles of abattoir origin. Healthy follicles contained 1027 ± 529 and 29 ± 3 ng/ml estradiol and progesterone, respectively, and atretic follicles contained 3 ± 1 and 37 ± 7 ng/ml, respectively. Relative *AGTR1* and *AGTR2* mRNA abundance in theca cells was not different between healthy and atretic follicles (Fig 1). In granulosa cells, *AGTR2* mRNA abundance was significantly higher in healthy compared with atretic follicles, whereas *AGTR1* mRNA levels did not differ (Fig 1). AGTR2 protein was detected by immunohistochemistry in theca and granulosa cells of antral follicles of different sizes (Fig. 2). Strong staining was also observed in the wall of blood vessels, but staining was weak or absent in ovarian stroma or in sections processed without

AGTR2 antibody. Staining was specific for AGTR2, as the same antibody recognized a single band of the correct size in Western blot (Fig 2).

The above data suggest that *AGTR2* expression in granulosa cells may be regulated during follicle development, therefore we employed a non-luteinizing granulosa cell culture model to examine the regulation of *AGTR2* expression. When cells from small, medium and large follicles were cultured for 6 days in serum-free medium supplemented with 1 ng/ml FSH, the cells from large follicles expressed greater abundance of *AGTR2* mRNA compared with cells from small follicles, but at levels that did not vary with time in culture (Fig 3). FSH caused a dose-dependent increase in estradiol secretion and *AGTR2* mRNA abundance (Fig 4). Doses higher than 1 ng/ml did not further alter mRNA levels (not shown). *AGTR1* mRNA abundance was not affected by FSH (Fig 4).

IGF1 caused a dose-dependent increase in estradiol secretion and granulosa *AGTR2* mRNA abundance (Fig. 5), but had no effect on *AGTR1* expression (not shown). Treatment of IGF1-stimulated cells with BMP-7 increased estradiol secretion and *AGTR2* mRNA expression relative to control cultures, and did not affect *AGTR1* expression (Fig 6).

To determine if changes in *AGTR2* mRNA are reflected by changes in receptor protein, cultures were performed for Western blot analysis. Cellular AGTR2 protein abundance was significantly increased in the presence of FSH (1ng/ml) or BMP7 (50ng/ml) ($P<0.05$, Fig 7). FGF-2 did not alter AGTR2 protein levels in FSH-stimulated cells at the dose used but did inhibit estradiol secretion, whereas FGF-7 and FGF-10 decreased AGTR2 protein and estradiol secretion ($P<0.01$; Fig 8).

Discussion

In rats, AngII is suspected to play a role in follicular atresia based on reports that AGTR2 is expressed only in the granulosa cells of atretic follicles and that AngII inhibits granulosa cell estradiol secretion [11, 14]. In cattle however, AGTR2 was reported only in theca cells [16], suggesting alternative roles for AngII in ruminants. This study shows that in fact AGTR2 is expressed in bovine granulosa cells, that granulosa *AGTR2* gene expression is decreased in atretic compared to healthy follicles, and that granulosa *AGTR2* gene expression is stimulated by gonadotropic hormones. These data argue that AGTR2 signaling may support follicle health and growth in cattle rather than be involved in atresia.

Using RT-PCR, we detected *AGTR1* and *AGTR2* mRNA in both theca and granulosa cells. Previous studies investigating localization of AngII receptors in the bovine follicle reported ^{125}I-AngII binding predominantly or exclusively to the theca cell layer [15, 16]. These ligand binding studies may not have been sensitive enough to detect AngII binding in granulosa cells, as no AngII binding was detected in ovarian blood vessels [16], a tissue rich in AngII receptors [28]. As PCR is more sensitive and prone to artifacts, we verified AGTR2 localization by immunohistochemistry and showed protein in both thecal and granulosa layers, in agreement with the PCR data. Blood vessels within the ovarian stroma also stained positive for AGTR2, consistent with studies in sheep uterus [29]. *AGTR1* mRNA abundance in theca or granulosa did not differ between healthy and atretic follicles, in agreement with data from bovine theca cells [6], but *AGTR2* mRNA abundance was significantly downregulated in granulosa cells of atretic follicles. This is in contrast to studies with rats that show significant upregulation of AGTR2 in granulosa cells of atretic follicles [9-11]. This discrepancy may reflect a physiological difference between species, or the comparison

of *in vivo* rat studies with bovine follicles of abattoir origin and thus unknown stage of the estrous cycle.

As *AGTR2* mRNA levels were affected by follicle health in granulosa, not theca cells. We used a serum-free granulosa cell culture system to investigate the regulation of *AGTR2* expression. In this culture system, FSH and IGF1 stimulate estradiol secretion and expression of genes encoding steroidogenic enzymes [21, 30, 31] and, as demonstrated here, also stimulate *AGTR2* but not *AGTR1* expression. These results are in contrast with those from rats showing that FSH decreased AngII binding to granulosa cells in culture [12, 32], but in agreement with studies showing upregulation of AGTR2 by IGF1 in a rat fibroblast cell line [33] and rat vascular smooth muscle cells [34]. BMP7, another growth factor that stimulates estradiol secretion in this cell model [22], also increased *AGTR2* mRNA levels. We are not aware of any other reports describing the effects of BMP7 on *AGTR2* expression.

The stimulatory effects of FSH, IGF1 and BMP7 on *AGTR2* expression were reflected by similar stimulation of AGTR2 protein levels. This coupling between mRNA and protein suggests that receptor protein levels are regulated transcriptionally, although the role of transcription rate and mRNA stability remains to be determined. Bearing in mind that the total cell receptor content does not necessarily reflect the number of receptors available at the cell surface, it would appear that endocrine/paracrine agents that affect *AGTR2* mRNA levels are likely to have a impact on AGTR2 signaling. To assess further the role of growth factors, we tested the effect on cell AGTR2 content of three FGFs that have been shown to inhibit estradiol secretion from bovine granulosa cells, FGF-2 [35], FGF-7 [23] and FGF-10 [24]. FGF-2 did not alter AGTR2 protein levels in granulosa cells, whereas this growth factor has been reported to inhibit AGTR2 expression in rat fibroblast and pheochromocytoma cell

lines [36]. However, FGF-7 and FGF-10 both inhibited AGTR2 protein levels at doses that decreased estradiol secretion. FGF-2 and FGF-7/10 likely have different affects on cells as they do not activate the same receptor. There are 5 known FGF receptor (FGFR) genes and at least 3 of these undergo alternative splicing to give rise to functionally distinct receptors. FGF-2 activates FGFR2c, FGFR3c and FGFR4, whereas FGF7 and FGF10 activate predominantly FGFR2b [37, 38]. We are not aware of any other reports of FGF regulation of angiotensin receptors.

In summary, the present data show that bovine granulosa cells express AGTR2 mRNA and protein, and that receptor levels are greater in larger, healthy follicles, in contrast to the situation in rats where AGTR2 is expressed only in atretic follicles. In addition, we demonstrate growth factor regulation of AGTR2 expression such that endocrine/paracrine factors that increase estradiol secretion also increase AGTR2 expression, and vice versa. Therefore we conclude that AGTR2 may play a role in maintaining follicle health and viability in cattle.

Acknowledgements

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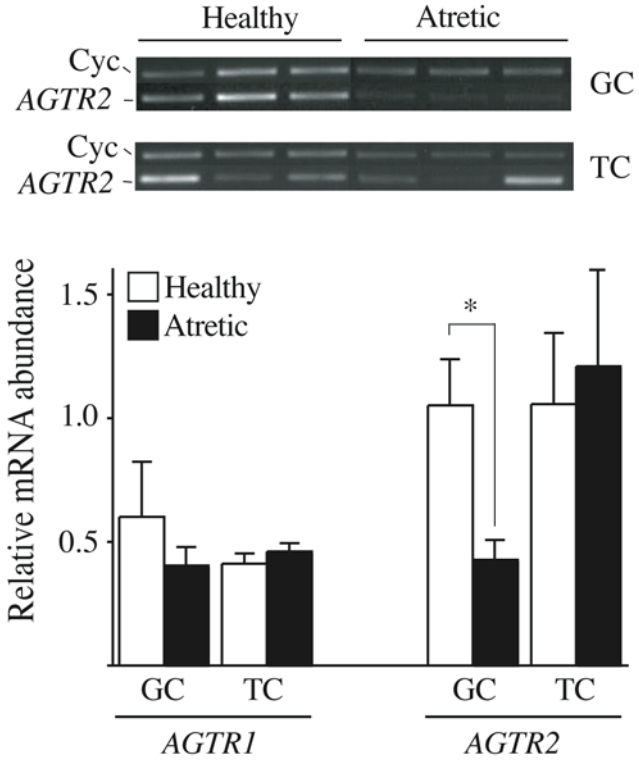

Figure 1. Expression of *AGTR1* and *AGTR2* in theca (TC) and in granulosa cells (GC) from follicles classified as healthy (E:P >1; n=9) or atretic (E:P<1; n=9). Gene expression was measured by semiquantitative RT-PCR and expressed relative to the housekeeping gene *PPIA*. The representative ethidium bromide-stained gel shows *PPIA* and *AGTR2* amplicons from three healthy and three atretic follicles. Data are means ± SEM. * P<0.05.

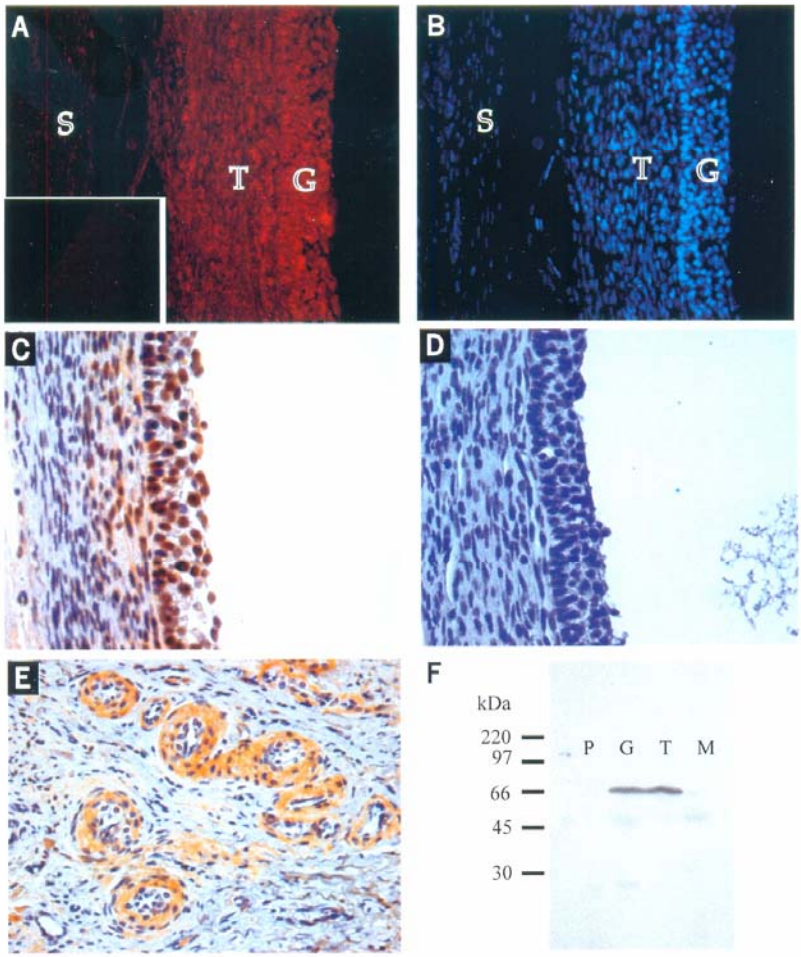

Figure 2. Immunohistochemical localization of AGTR2 in bovine ovary. Immunofluorescent detection of AGTR2 in the follicle wall (A), and DAPI staining in the same follicle (B). The inset in (A) is an adjacent section processed in the absence of AGTR2 antibody and shows weak background fluorescence. Colorimetric immunostaining (C) confirms the localization observed by immunofluorescence, and absence of staining in the negative control (D). Ovarian blood vessels stained strongly for AGTR2 (E). Western blot with the same antibody demonstrating a specific bands in granulosa and theca cell lysates, but not in pancreas or myometrium lysates. G, granulosa; T, theca; M, myometrium; P, pancreas; S, stroma. Original magnification X400.

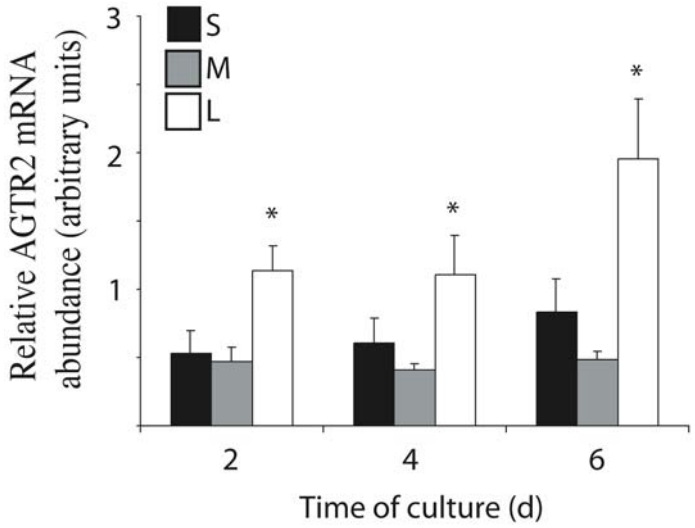

Figure 3. Expression of *AGTR1* and *AGTR2* mRNA in bovine granulosa cells cultured in serum-free medium. Cells were derived from small (S; 2-5mm diameter), medium (M; 6-8mm) or large (L; >8mm) follicles and cultured for up to 6 days. Gene expression was measured by semiquantitative RT-PCR and expressed relative to the housekeeping gene *H2AFZ*. Within time of culture, asterisks indicate significant differences between follicle size groups ($P<0.05$). Data are means ± SEM of three independent culture replicates.

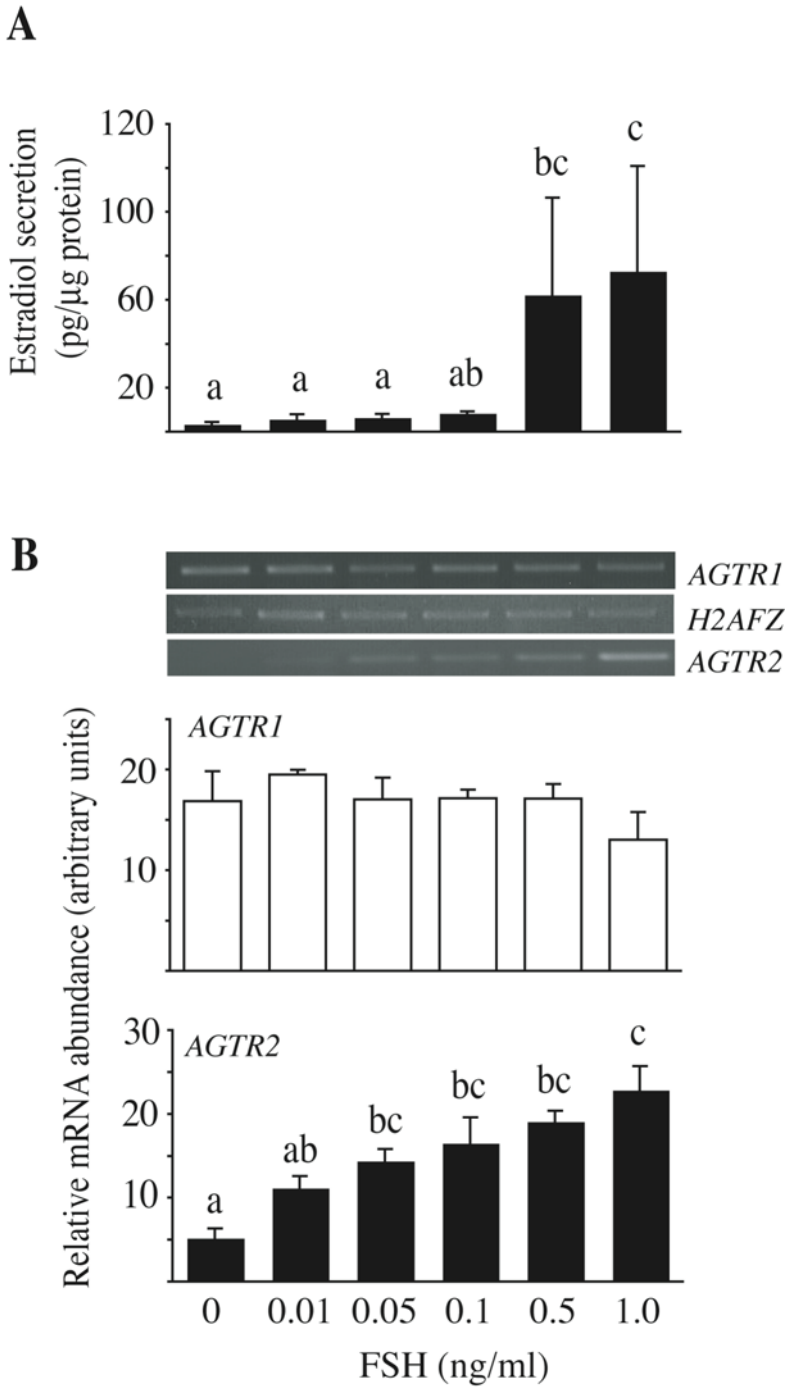

Figure 4. Effect of FSH on **A**) estradiol secretion and **B**) *AGTR1* and *AGTR2* mRNA expression in bovine granulosa cells *in vitro*. Cells were cultured in serum-free medium for 6 days with the stated doses of FSH. Gene expression was measured by semiquantitative RT-PCR and a representative gel is shown. Bars with different letters are significantly different ($P<0.05$). Data are means ± SEM of three independent culture replicates.

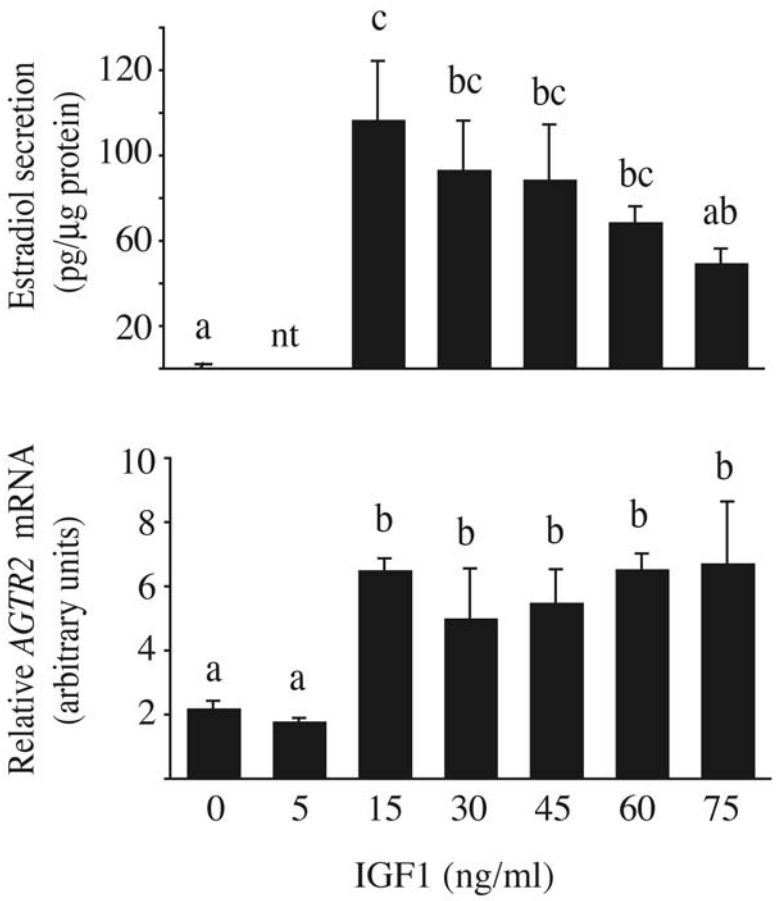

Figure 5. Effect of IGF on estradiol secretion and *AGTR2* mRNA expression in bovine granulosa cells *in vitro*. Cells were cultured in serum-free medium for 6 days with the stated doses of FSH. Gene expression was measured by semiquantitative RT-PCR. Bars with different letters are significantly different ($P<0.05$). Data are means ± SEM of three independent culture replicates. nt, not tested.

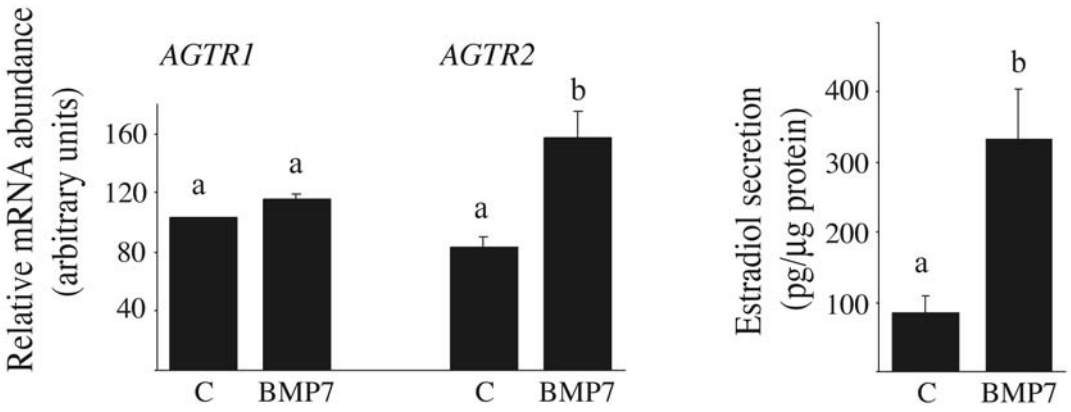

Figure 6. Effect of BMP7 on *AGTR1* and *AGTR2* mRNA expression in, and estradiol secretion from bovine granulosa cells *in vitro.* Cells were cultured in serum-free medium for 6 days in the presence of IGF1 (10ng/ml) alone (C) or with BMP7 (50ng/ml). Gene expression was measured by semiquantitative RT-PCR. Bars with different letters are significantly different ($P<0.05$). Data are means ± SEM of three independent culture replicates.

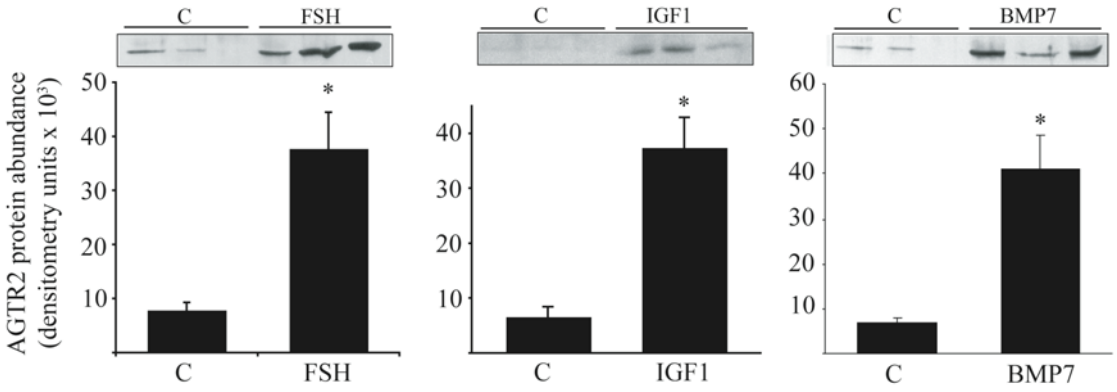

Figure 7. The effect of FSH, IGF1 and BMP7 on AGTR2 protein content in bovine granulosa cells *in vitro*. Cells were cultured in serum-free medium for 6 days in the presence of the stated doses of hormone, and total cell protein was harvested for Western analysis of AGTR2 protein. Bars with asterisks are significantly different from controls ($P<0.05$). The representative Western blot shows samples from one replicate in the same order as the graph. Data are means ± SEM of three independent culture replicates.

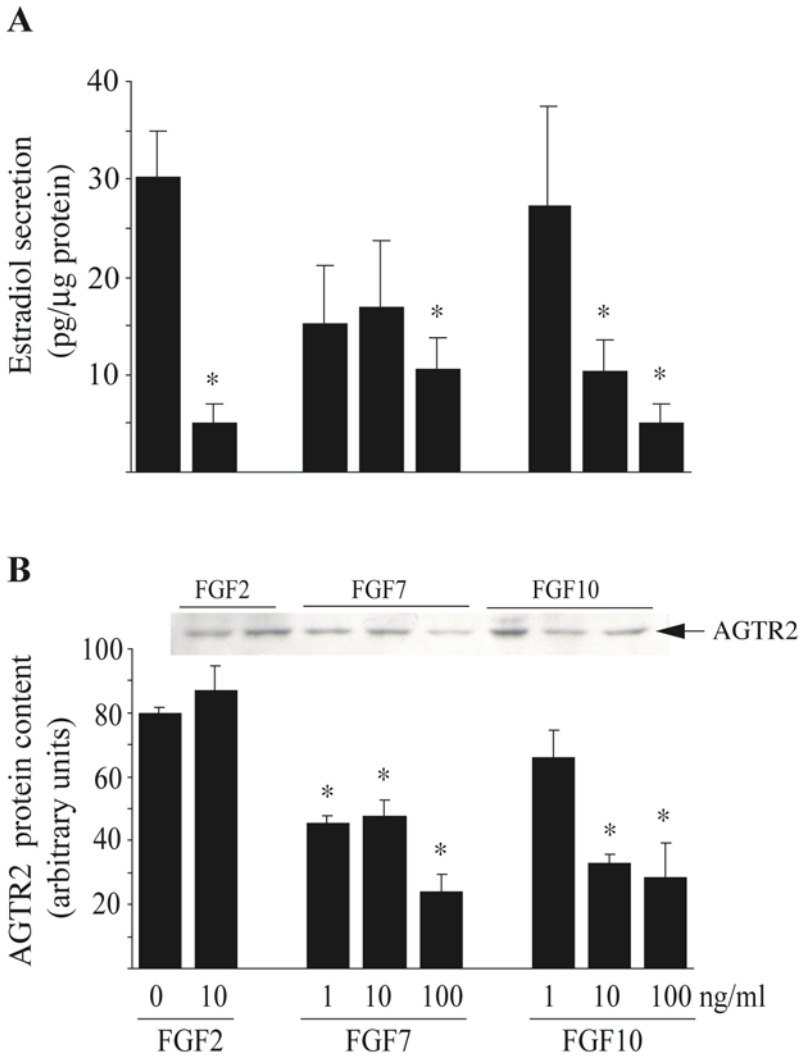

Figure 8. The effect of FGF-2, FGF-7 and FGF-10 on estradiol secretion (**A**) and AGTR2 protein content (**B**) in bovine granulosa cells *in vitro*. Cells were cultured in serum-free medium for 6 days in the presence of the stated doses of hormone, and total cell protein was harvested for Western analysis of AGTR2 protein. Bars with asterisks are significantly different from controls ($P<0.05$). The representative Western blot in panel **B** shows samples from one replicate in the same order as the graph. Data are means ± SEM of three independent culture replicates.

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4. CAPÍTULO 2

ANGIOTENSIN II REGULATES PROTEASE-NEXIN 1 EXPRESSION IN BOVINE GRANULOSA CELLS IN VITRO.

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ANGIOTENSIN II REGULATES PROTEASE-NEXIN 1 EXPRESSION IN BOVINE GRANULOSA CELLS IN VITRO.

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Key words : angiotensin receptor, granulosa cell, follicle

Running head: Angiotensin receptors in bovine follicles

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Abstract

Angiotensin II (AngII) and its receptors (AT1 and AT2) are known to be essential for renal and vascular function, and more recently it has been suggested that these molecules are involved in ovarian follicular development and ovulation. The objective of this study was to determine the physiological role of AngII in the follicle in an agriculturally important species, the cow. Bovine ovaries were obtained from an abattoir and follicles greater than 5 mm diameter dissected. To determine the physiological consequences of AT activation in granulosa cells, cells from small (2-5mm) bovine follicles were cultured in serum-free medium with FSH ± AngII. Semiquantitative RT-PCR was used to measure PN-1 expression and gelatin zymomgraphic was used to measure MMPs active. Cells were maintained in serum-free culture and graded doses of AngII were added. AngII did not alter estradiol or progesterone secretion, nor cell proliferation or MMPs active, but significantly inhibited protease nexin-1 (PN-1) mRNA levels and protein secretion ($P<0.05$). PN-1 is an inhibitor of proteases involved in extracellular matrix remodeling and follicle rupture. We conclude that AngII regulates granulosa PN-1 secretion but do not change MMPs active or estradiol secretion in granulose cells.

Key words: angiotensin II, granulosa cell, follicle, SERPINE2, extracellular matrix

Introduction

The renin-angiotensin system (RAS) consists of the enzyme renin that converts angiotensinogen to angiotensin I (AngI). Angiotensin converting enzyme then cleaves the decapeptide AngI to the octapeptide AngII, which is considered to be the major bioactive peptide of the RAS (reviewed in [1]). An active RAS has been described in the ovary [1], and numerous RAS members are present in follicles, including prorenin [2] and angiotensin receptors. The effects of AngII on blood pressure regulation are mediated through the type 1 receptor (AGTR1, also known as AT1) whereas other

actions are generally mediated through the type 2 receptor (AGTR2, also known as AT2) [1]. In rodents, the predominant form of receptor in the ovary is AGTR2 [3, 4].

To date, most data point to a role for AngII in the induction of ovulation. AngII infusion induced ovulation in perfused rabbit ovaries and AngII antagonists inhibited ovulation in rabbits and rats [5-8]. AngII stimulated prostaglandin (PG) secretion from rabbit ovaries [5, 7] and bovine preovulatory follicles [9]; this may be the mechanism through which AngII induces ovulation, as mice deficient in PG synthase 2 fail to ovulate [10].

AngII may regulate steroidogenesis in the ovary. AngII has been shown to inhibit progesterone secretion from bovine luteal cells [11] and luteinized porcine granulosa cells [12] although AngII either stimulated or inhibited progesterone secretion from human granulosa-lutein cells depending on time of culture [13, 14]. In rat and bovine preovulatory follicles, AngII stimulated progesterone and estradiol secretion [9, 15]. However, other studies have suggested that AngII does not affect progesterone secretion from isolated rat granulosa cells [16]. There may be a role for the RAS in follicle atresia, as AngII inhibited estradiol secretion from cultured rat and rabbit granulosa cells [17, 18], and inducing atresia in rat granulosa cells by withdrawing FSH support increased AngII binding to AGTR2 [19]. This is supported by the expression of *AGTR2* in granulosa cells of atretic but not healthy follicles in rats [3, 4, 20].

The role of AngII in non-ovulatory bovine follicles is not known. Atretic follicles contain more prorenin that healthy follicles [2], suggesting a role for the ovarian RAS in atresia. However, the pattern of expression of AGTR2 differs between rats and cattle. Whereas AGTR2 is expressed in atretic granulosa cells in rats and inhibited by FSH, in cattle AGTR2 expression is higher in healthy follicles and is upregulated by FSH. Part of the difference between studies may be the animal models

used. In rat studies, differentiated granulosa cells are collected from PMSG-primed rats, and in our previous study we used undifferentiated granulosa cells from follicles before the time of expression of LH receptors. The objectives of the present study were to determine the effects of AngII on undifferentiated, nonluteinizing bovine granulosa cells in vitro.

Materials and methods

Granulosa cell culture

The granulosa cell culture system was based on that described by [21], with slight modifications [22]. All materials were obtained from Invitrogen Life Technologies (Burlington, ON, Canada) except where otherwise stated. Briefly, bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir, and were transported to the laboratory in PBS at 35°C containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (1 μg/ml). Cells were collected from small (2-5 mm diameter), and were washed twice by centrifugation at 219 × g for 20 min each, and suspended in α-MEM containing Hepes (20 mM), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma-Aldrich Canada, Oakville, ON, Canada), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1.1 mM), androstenedione (10^{-7} M at start of culture, and 10^{-6} M at each medium change) and insulin (10 ng/ml). Cell viability was estimated with 0.4% Trypan Blue Stain. Cells were seeded into 24-well tissue culture plates (Sarstedt, Montreal, QC) at a density of 1 x 10^6 viable cells per well in 1 ml medium. Cultures were maintained at 37°C in 5% CO_2 in air for 6 days, with 700 μl medium being replaced every 2 days.

To assess the physiological role of AngII, cells were cultured with FSH (1ng/ml) and graded doses of AngII (human AngII; Sigma-Aldrich Canada, Oakville, ON, Canada) added for 6 days of culture. Medium was harvested for steroid assay and Western blotting, and cells were harvested for RNA and DNA extraction. In separate cultures, the effect of AngII on cell proliferation was assessed using the MTT assay (R&D Systems).

Zymography

Gelatin zymography was used to measure matrix metalloproteinase (MMP) enzyme activity in culture medium. Briefly, samples were subjected to electrophoresis at 120 V for 90 min in 10% non-denaturing polyacrylamide gels containing 1mg/ml pig skin gelatin casein (Sigma-Aldrich). After electrophoresis, gels were washed once in 2.5% Triton X-100 for 45 min to remove SDS, and placed in incubation buffer (50 mM Tris, 0.1 M NaCl, 5μM $ZnCl_2$, pH 7.6) at 37°C for 24 h with gentle shaking. The gels were then stained using 0.25% Coomassie Blue in 10% acetic acid, 40% methanol for 1 h, destained in 10% acetic acid, 40% methanol, and then fixed in 10% glycerol. Bands of activity were visualized as clear zones where gelatin degradation occurred, against a dark (blue) background. The volume of medium analyzed was corrected for cell number (total DNA).

Western Blot

Serine protease inhibitor E2 (SERPINE2, also known as protease nexin-1) secretion from cultured granulosa cells was measured by Western blot in spent cultured medium as described [23]. Blots were exposed to X-ray film for image analysis.

Nucleic acid extraction & semi-quantitative RT-PCR

Total RNA and DNA were extracted using Trizol according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm. Total RNA (1 μg) was first treated with 1 U DNase (Promega, Madison, WI) at 37°C for 30 min to digest any contaminating DNA, followed by adding 1 μl of EDTA stop buffer at 65°C for 10 min. The RNA was reverse transcribed in the presence of 1 mM oligo(dT) primer and 4 U Omniscript RTase (Omniscript RT Kit, Qiagen, Mississauga, ON), 0.25 mM dideoxy-nucleotide triphosphate (dNTP) mix, and 19.33 U RNase Inhibitor (Amersham Biosciences, Baie D'Urfé, QC) in a volume of 20 μl at 42°C for 2 h. The reaction was terminated by incubation at 93°C for 5 min.

Bovine-specific primers were used for amplifying *SERPINE2* as described [24], and variability in mRNA amounts was assessed by amplifying histone H2aa1 (*H2AFZ*; [25]). An aliquot (0.4 μl) of the cDNA template was amplified by PCR using 0.2 μl (2.5 U) Taq Polymerase (Amersham Pharmacia Biotech Inc., Oakville, ON, Canada) in a 20-μl PCR buffer (Amersham Pharmacia Biotech Inc.) containing 0.1 mM dNTP mix, and 0.2 μM specific primers. After an initial denaturation step for 3 min at 94°C, target cDNA was amplified with a denaturation step at 94°C for 30 sec (*SERPINE2*) or 45 sec (*H2AFZ*), annealing for 45 sec at 62°C (*SERPINE2*) or 58°C (*H2AFZ*), and elongation at 72°C for 1 min. All reactions were terminated with a final elongation at 72°C for 5

min. Semiquantitative RT-PCR was validated for each gene product. Reactions were performed for 24 cycles for *SERPINE2* and 30 cycles for *H2AFZ.*

The PCR products were separated on 2 % agarose gels with 0.001% ethidium bromide, and visualized under UV light. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was expressed relative to *H2AFZ* mRNA abundance.

Steroid assay

Estradiol was measured in conditioned medium in duplicate as described [26], without solvent extraction. Intra- and inter-assay coefficients of variation were 8.5% and 6.3%, respectively. Progesterone was measured in duplicate as described [27] with mean intra- and inter-assay coefficients of variation were 7.2% and 18%, respectively. The sensitivity of these assays were 10 pg and 4 pg per tube for estradiol and progesterone, equivalent to 0.3 and 20 ng/μg protein, respectively. Steroid concentrations in culture medium were corrected for cell number by expressing per unit mass of total DNA.

Statistical Analysis

Data that did not follow a normal distribution (Shapiro-Wilk test) were transformed to logarithms. Homogeneity of variance was tested with O'Brien and Brown-Forsythe tests. Analysis of analysis was performed with JMP software (SAS Institute) with treatment or follicle group as main effect and culture replicate (where appropriate) as a random variable in the F-test. For gene expression data, housekeeping gene abundance was included as a covariate in the main effects model. Differences between means were tested with the Tukey-Kramer HSD test. Data are presented as means ± SEM.

Results

AngII had no effect on estradiol or progesterone secretion, or on cell proliferation under the present culture conditions (Fig 9a). Granulosa cells secreted tPA, which did not change with dose of AngII, but uPA levels were too low to be accurately quantified. Granulosa cells secreted MMP9 and 2, judged by the apparent M_r of 83,000 and 62,000 respectively, and the activity of these two enzymes was approximately equivalent. No other MMP activity was detected. AngII has no significant effect on MMP activity (Fig 9b). AngII inhibited SERPINE2 secretion at all doses tested (Fig 9c). To determine if this was a result of altered expression of the *SERPINE2* gene, cells were incubated with 10 μM AngII and gene expression determined by RT-PCR; AngII inhibited *SERPINE2* mRNA abundance (Fig 9c).

Discussion.

In rats, AngII is suspected to play a role in follicular atresia based on reports that AGTR2 is expressed only in the granulosa cells of atretic follicles and that AngII inhibits granulosa cell estradiol secretion [18, 20]. In cattle however, AGTR2 is expressed in granulosa cells from healthy follicles [28] and AngII did not alter estradiol or progesterone secretion, or granulosa cell proliferation (present study), suggesting that AngII does not induce atresia in granulosa cells of ruminants.

The effects of AngII on steroidogenesis in follicles are varied. AngII increased progesterone and estradiol secretion in whole preovulatory rat and cow follicles [9, 15]. AngII inhibited progesterone secretion from luteinized porcine granulosa cells [12] and increased progesterone from human granulosa-lutein cells [14]. The response to AngII likely depends on the degree of luteinization/differentiation of the cells, as AngII inhibited progesterone secretion from human granulosa-lutein cells in short-term culture

(2 d) but increased secretion from cells in longer term culture (4 d) [13]. AngII stimulated progesterone secretion from granulosa cells of DES-treated rats [29] but had no effect on cells from PMSG-primed rats [16, 30]. In the present study, we used a model of non-luteinizing, undifferentiated granulosa cells that represent the granulosa layer of growing follicles prior to the acquisition of LH receptors [31, 32]. The present data show clearly that AngII does not alter steroidogenesis or cell proliferation of isolated granulosa cells at this stage of development in cattle.

AngII has been reported to alter expression and secretion of members of the plasminogen activator (PA) and MMP systems in non-ovarian cell types. AngII increased expression of several MMPs and PA inhibitor-1 (PAI-1) in vascular tissues [33-35][36-38]. Granulosa cells from nonovulatory follicles do not express PAI-1, but they do express another PA inhibitor, SERPINE2 [39, 40], and they secrete MMP2 and MMP9 [41]. In the present study, AngII did not affect secreted MMP activity, but inhibited SERPINE2 secretion and mRNA abundance. AngII has also been shown to inhibit SERPINE2 secretion and expression in rat Schwann cells [42].

The relevance of the AngII suppression of SERPINE2 in granulosa cells is unknown. SERPINE2 secretion was shown to be regulated by various growth factors in bovine granulosa cells, and up- or down-regulation was consistently accompanied by concomitant changes in estradiol secretion [43]. The present study shows distinct regulation of SERPINE2 that was not accompanied by changes in estradiol secretion, and demonstrates a very specific role for AngII. This decrease in SERPINE2 secretion was not accompanied by changes in protease activity, but considerable MMP and PA activity is located at the cell surface [44], therefore the measurement of protease activity in culture medium may not reflect potential regulation of enzyme activity within the extracellular matrix.

In conclusion, we have identified a novel role for AngII in granulosa cells. In contrast to rats, in which AngII inhibits estradiol secretion and induces apoptosis in granulosa cells, in cattle AngII had no effect on steroid secretion or cell proliferation. AngII altered only SERPINE2 secretion and expression. The physiological relevance of this remains to be determined.

Acknowledgements

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Figure

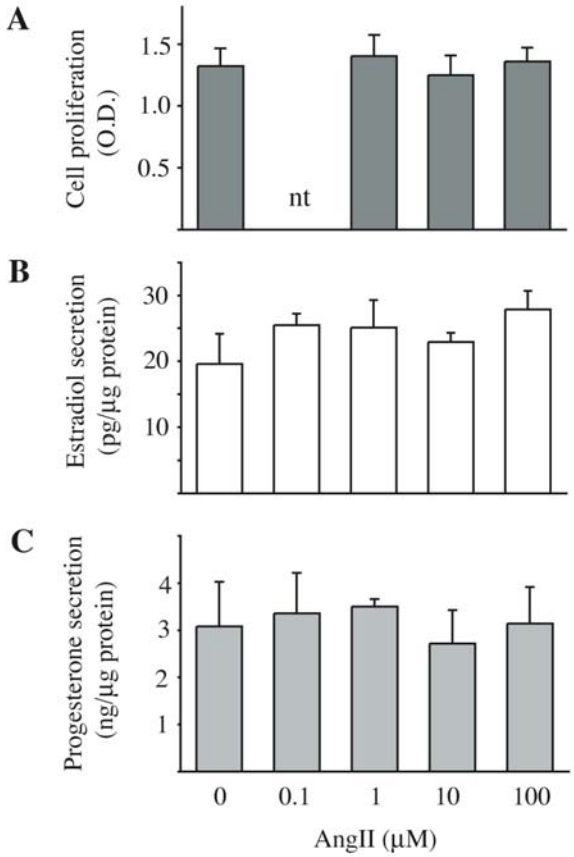

Figure 9a. Effects of AngII on bovine granulosa cell proliferation (**A**), and (**B**) estradiol and (**C**) progesterone secretion. Cells were cultured in serum-free medium for 6 days with FSH (1ng/ml), and the given doses of AngII included from day 4 of culture. Cell proliferation was measured with the MTT assay and steroids were measured by RIA. Data are means ± SEM of three independent culture replicates; nt, not tested.

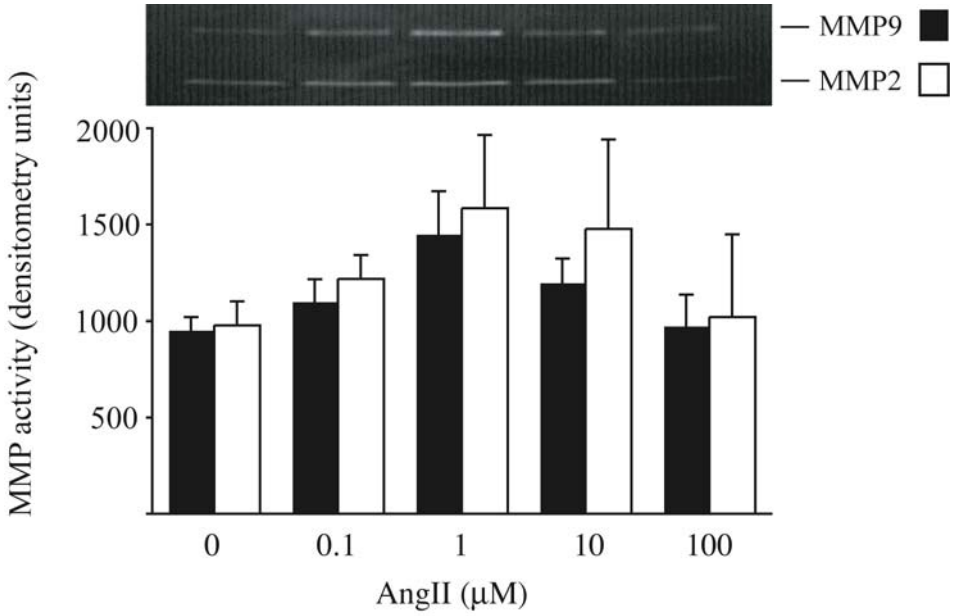

Figure 9b. Effects of AngII on protease activity secreted from bovine granulosa cells. Cells were cultured in serum-free medium for 6 days with FSH (1ng/ml), and the given doses of AngII included from day 4 of culture. Protease activity was measured by gelatin zymography. Proteolytic bands of M_r 83k and 62k are identified as MMP9 and MMP2, respectively (representative gel shown above graph). Data are means ± SEM of three independent culture replicates.

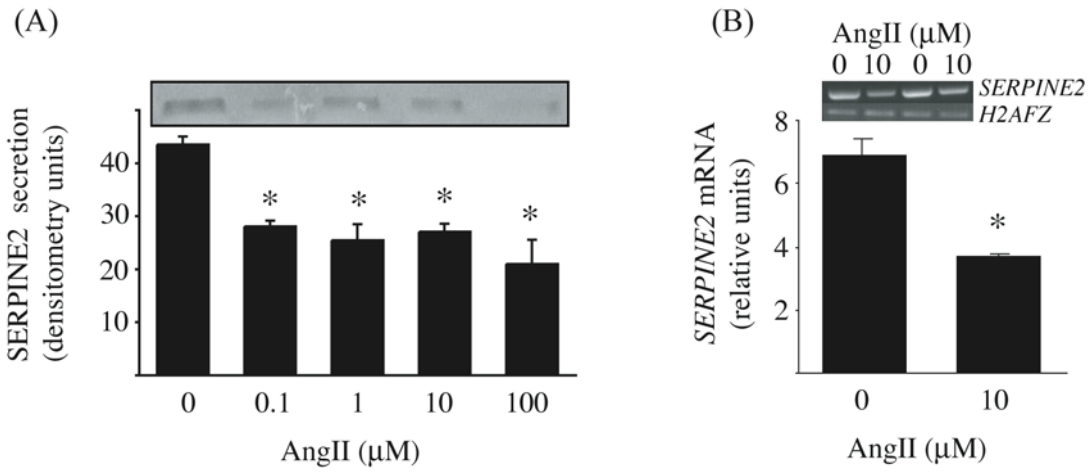

Fig 9c. AngII inhibits (**A**) SERPINE2 secretion and (**B**) gene expression in bovine granulosa cells. Cells were cultured in serum-free medium for 6 days with FSH (1ng/ml), and the AngII (10 μM) added from day 4 of culture. Data are means ± SEM of three independent culture replicates. Asterisks denote means significantly different from control cultures without AngII. A representative Western is shown for one replicate, with samples in the same order as the graph in (**A**). A representative agarose gel is shown to illustrate mRNA abundance in 2 replicates (**B**).

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5. CAPÍTULO 3

THE EXPRESSION OF GENES INVOLVED IN OVULATION ARE REGULATED BY ANGIOTENSIN II IN GRANULOSA CELLS IN VITRO.

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THE EXPRESSION OF GENES INVOLVED IN OVULATION ARE REGULATED BY ANGIOTENSIN II IN GRANULOSA CELLS IN VITRO.

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Key words: ovulation, cox2, serpine2, granulosa cell, follicle

Running head: Angiotensin and ovulation in bovine follicles

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Abstract

Angiotensin II (AngII) induces follicular prostaglandin release in a number of species, and induces ovulation in rabbits. The objective of the present study was to test the hypotheses that AngII, alone or in combination with LH, induces the expression of the enzyme responsible for prostaglandin synthesis, Ptgs2, and the prostaglandin-dependent proteolytic cascade necessary for follicle rupture. Bovine granulosa cells from large (>10 mm) follicles were cultured for 6h, 12h and 24h with LH (100ng/ml) or AngII. These cells expressed Ptgs2 under basal culture conditions, which was not upregulated by either LH or AngII alone. However, LH and AngII in combination significantly enhanced Ptgs2 ($P<0.05$) mRNA and protein accumulation. Expression of the proteolytic enzymes uPA and tPA, and their inhibitor, Serpine2 was also measured by RT-PCR and, as for Ptgs2, the combination of LH and AngII but neither factor alone upregulated expression of all three genes ($P<0.05$). These data suggest that AngII is a physiological co-factor necessary for the expression of genes in granulosa cells that are critical for ovulation.

Introduction

The preovulatory surge of LH initiates complex changes in gene expression in preovulatory follicles, which culminate in ovulation and formation of the corpus luteum. Granulosa cells of preovulatory follicles express LH receptors, and respond in dramatic ways to the LH surge. A considerable body of work in rodents has suggested a probable sequence of events triggered by the LH surge in granulosa cells. One of the early genes upregulated by LH is the progesterone receptor (PR), and expression of this gene is essential for ovulation as demonstrated by studies with PR knock-out mice

(PRKO) [1]. LH also rapidly induced the nuclear factor RIP140, which in turn induces the expression of the proteases cathepsin L and a disintegrin and metalloproteinase with thrombospondin repeats (ADAMTS1), epiregulin, amphiregulin, betacellulin, hyaluronan synthase2 and prostaglandin-endoperoxide synthase2 (Ptgs2, also known as Cox2) [2-4]. Ptgs2 catalyses the synthesis of prostaglandins [5], which are essential for cumulus-oocyte-expansion and ovulation as Ptgs2 knock-out mice fail to ovulate [6].

Down-stream from the above genes is the regulation of proteins involved in the physical rupture of the follicle wall. These include members of the matrix metalloproteinase (MMP) family and their inhibitors (TIMP) [7], and the plasminogen activators (PA) and their inhibitors (PAI). In cattle, hCG transiently upregulated tissue-type PA (tPA), urokinase (uPA) and the inhibitors PAI-1 and protease nexin-1 (Serpine2, also known as PN1) until the time of ovulation when all except uPA decreased [8-10]. Periovulatory changes in several MMPs and TIMPs have also been described in cattle [11, 12]. Owing to the large numbers of proteases and inhibitors and considerable potential for redundancy, the exact role of each protein in ovulation remains unclear. However, injection of the prostaglandin inhibitor indomethacin, which blocks ovulation in cattle [13], into bovine preovulatory follicles resulted in few changes in follicular MMP or TIMP expression but significantly inhibited follicular tPA, uPA and PAI-1 expression [14], suggesting that the PA system is part of the LH-induced mechanism of follicle rupture.

Ovulation can be induced or inhibited by a number of factors other than LH. One example is angiotensin II (AngII), which induced ovulation in perifused rabbit ovaries [15, 16]. Conversely, AngII antagonists inhibit ovulation in rodents, rabbits and cattle [17-19]. The mechanism of action of AngII on ovulation is not clear, although is mediated through the type 2 receptor (AGTR2, also known as AT2) [15, 17]. AngII

stimulated prostaglandin secretion from rabbit ovaries [15, 17] and bovine preovulatory follicles [20]. The objective of the present study was to determine whether AngII stimulates the expression of Ptgs2 in cultured bovine granulosa cells independently or in concert with LH, and to test the hypothesis that AngII stimulates genes involved in follicle rupture.

Materials and methods

Cell culture

All materials were obtained from Invitrogen Life Technologies (Burlington, ON, Canada) except where otherwise stated. Briefly, bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir, and were transported to the laboratory in PBS at 35°C containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (1 μg/ml). Cells were collected from large follicles (>8mm) by aspiration, and were washed twice by centrifugation at 219 × g for 20 min each, and suspended in DMEM-F12, sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma-Aldrich Canada, Oakville, ON, Canada), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1.1 mM), androstenedione (10^{-7} M at start of culture, and 10^{-6} M at each medium change), FSH 1ng/ml, insulin (10 ng/ml) and 2% FCS. Cell viability was estimated with 0.4% Trypan Blue Stain. Cells were seeded into 24-well tissue culture plates (Sarstedt, Montreal, QC) at a density of 1 x 10^6 viable cells per well in 1 ml medium. Cultures were maintained at 37°C in 5% CO_2. After 24h, medium was changed to DMEM-F12 without supplementation for 10 h before adding insulin (10ng/ml) and hormone treatments. Treatments were LH (100ng/ml; NIH) or LH + AngII (100 μM) for the time periods described in Results.

Immunofluorescence

Protein localization in bovine granulosa cells in vitro was performed by immunofluorescence. After cell culture the cells were fixed 4% paraformaldehyde for 20 min, washed 5 times for 5 min in PBS and were blocked in 5% BSA for 1 h and then 1:200 Ptgs2 antibody (cat. PG26, Oxford Biomedical Research, Oxford, MI) was applied for 18 h at 4 C. After the primary antibody, the cells were washed in PBS and then incubated with Cy3-conjugated second antibody (Jackson ImmunoResearch,West Grove, PA) and counterstained with DAPI. Negative controls were performed in the absence of primary antibody. Digital images were captured and mean fluorescence intensity in each field was quantified with ImageJ (NIH). The number of cells in the field was quantified by counting DAPI-stained nuclei, and the staining was expressed as units/cell.

Nucleic acid extraction & semi-quantitative RT-PCR

Total RNA and DNA were extracted using Trizol according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm. Total RNA (1 μg) was first treated with 1 U DNase (Promega, Madison, WI) at 37℃ for 30 min to digest any contaminating DNA, followed by adding 1 μl of EDTA stop buffer at 65°C for 10 min. The RNA was reverse transcribed in the presence of 1 mM oligo(dT) primer and 4 U Omniscript RTase (Omniscript RT Kit, Qiagen, Mississauga, ON), 0.25 mM dideoxy-nucleotide triphosphate (dNTP) mix, and 19.33 U RNase Inhibitor (Amersham Biosciences, Baie D'Urfé, QC) in a volume of 20 μl at 42°C for 2 h. The reaction was terminated by incubation at 93℃ for 5 min.

Bovine-specific primers were used for amplifying uPA , tPA and Serpine2 exactly as described [21]. Primer for bovine Ptgs2 were as published [22]. Variability in mRNA amounts was assessed by amplifying histone H2AFZ (H2AFZ; sense: 5'-AGCGTATTACCCCTGGTCAC -3', antisense: 5'-CCAGGCATCCTTTAGACAGT -3'). An aliquot (1 μl) of the cDNA template was amplified by PCR using 0.25 μl (2.5 U) Taq Polymerase (Amersham Pharmacia Biotech Inc., Oakville, ON, Canada) in a 25-μl PCR buffer (Amersham Pharmacia Biotech Inc.) containing 0.1 mM dNTP mix, and 0.2 μM specific primers. After an initial denaturation step for 3 min at 94°C, target cDNA was amplified with a denaturation step at 94°C for 60 sec (or 45 for *H2AFZ*), annealing for 45 sec at 55°C (tPA), 58°C (*H2AFZ*), 60°C (Ptgs2), 62°C (Serpine2) or 65°C (uPA for 30 sec, and elongation at 72°C for 1 min. All reactions were terminated with a final elongation at 72°C for 5 min. Semiquantitative RT-PCR was validated for each gene product. Reactions were performed for 26 cycles for tPA, 24 cycles for Serpine2 and 30 cycles for uPA, H2AFZ and Ptgs2.

The PCR products were separated on 2 % agarose gels with 0.001% ethidium bromide, and visualized under UV light. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was expressed relative to H2aFZ mRNA abundance.

Steroid assay

Estradiol was measured in conditioned medium in duplicate as described [23], without solvent extraction. Intra- and inter-assay coefficients of variation were 8.5% and 6.3%, respectively. Progesterone was measured in duplicate as described [24] with mean intra- and inter-assay coefficients of variation were 7.2% and 18%, respectively.

The sensitivity of these assays were 10 pg and 4 pg per tube for estradiol and progesterone, equivalent to 0.3 and 20 ng/μg protein, respectively. Steroid concentrations in culture medium were corrected for cell number by expressing per unit mass of total protein.

Statistical Analysis

Data that did not follow a normal distribution (Shapiro-Wilk test) were transformed to logarithms. Homogeneity of variance was tested with O'Brien and Brown-Forsythe tests. Analysis of data was performed with JMP software (SAS Institute) with treatment or follicle group as main effect and culture replicate (where appropriate) as a random variable in the F-test. For gene expression data, housekeeping gene abundance was included as a covariate in the main effects model. Differences between means were tested with the Tukey-Kramer HSD test. Data are presented as means ± SEM.

Results

Steroid secretion from granulosa cells at 6, 12 and 24 h after the addition of LH or LH plus AngII was assessed (Fig 1). Estradiol secretion was no higher at 12 or 24 h than it was at 6 h, indicating no secretion after 6 h. In contrast, progesterone secretion doubled from 6 to 12 h of culture, and then again at 24 h, demonstrating continued secretion of progesterone in culture. AngII did not alter estradiol secretion, but significantly enhanced progesterone secretion at 6 h post-treatment.

As AngII has been reported to increase prostaglandin secretion from bovine follicles in vivo, we next assessed the effects of AngII on Ptgs2 mRNA and protein abundance in granulosa cells in vitro. The addition of LH or AngII alone did not

significantly increase Ptgs2 expression over time, but the addition of AngII to LH-stimulated cells significantly increased Ptgs2 mRNA at 6 and 24 h post-treatment, although not at 12 h (Fig 2A). To assess which AngII receptor mediated the effect of AngII, selective blockers of AGTR1 (Losartan) or of AGTR2 (PD123,319) were added one hour before treatments. PD123,319 effectively inhibited the stimulatory effect of AngII whereas Losartan did not (Fig 2B).

In concordance with the gene expression data, LH or AngII alone did not stimulate Ptgs2 protein accumulation, whereas these two factors combined significantly enhanced Ptgs2 protein accumulation at 6 h post-treatment (Fig 3).

Finally, we assessed the effects of AngII on the PA system. In comparison with LH alone, AngII significantly increased uPA, tPA and Serpine2 expression after 6 h in LH-stimulated cells (Fig 4).

Discussion

The present study clearly shows that AngII stimulates Ptgs2 gene expression and protein accumulation, and expression of the proteases tPA, uPA and the protease inhibitor PN1. AngII has been shown to induce ovulation in rabbit ovaries, and antagonists of angiotensin receptors inhibit ovulation in rabbits, rats and cattle [15-19]. Direct evidence for the induction of ovulation by AngII comes from studies with perfused rabbit ovaries [15, 16]. The rabbit is an induced-ovulator; therefore, the mechanisms involved in ovulation may differ from those in other mammals. Infusion of AngII into preovulatory rabbit and bovine follicles stimulated the release of prostanglandin, suggesting a potential mechanism of AngII action on ovulation [20]. Recently, our group demonstrated that ovulation is blocked when an specific inhibitors

of AngII receptors (saralasin or PD123 319) is injected into ovulatory follicles in cattle [24].

In the present study, we determined whether AngII is capable of inducing ovulation alone or whether an interaction with LH is required. To do so, we used a cell model in which cells from large follicles were cultured for 24 h with serum followed by a serum-free period prior to testing the effects of LH and AngII. Under these conditions, the granulosa cells secreted estradiol and progesterone, expressed Ptgs2, and did not appear morphologically luteinized. Using a similar culture system with pig granulosa cells, Li et al [25] showed that AngII inhibited LH-stimulated progesterone secretion. In the present study, AngII increased progesterone secretion from bovine granulosa cells in short-term (6 h) culture but not in longer-term (12 - 24 h) culture. A similar pattern of stimulation of progesterone was observed in perfused rat ovaries [26].

LH alone had no effect on Ptgs2 protein accumulation in vitro in the present study (Fig 3), which is not accord with the established induction of Ptgs2 by LH in vivo. However, induction of Ptsg2 expression may be an effect of culture, as previously noted in mice [27]. In this previous study, culture of granulosa cells from eCG-primed mice for 4 h induced Ptgs2 expression, which was significantly enhanced by LH. Comparison of Ptgs2 expression in granulosa cells of mice 4 h after hCG injection in vivo with that 4 h after addition of LH in vitro demonstrated that Ptgs2 expression in vivo was significantly higher than in vitro [27]. The authors concluded that a factor(s) in addition to LH is required for optimal Ptgs2 expression. In agreement with this hypothesis, the present data from bovine granulosa cells show that LH alone was not capable of increasing Ptgs2 expression above levels induced by culture, as was also the case for AngII alone, but that the combination of LH and AngII resulted in significantly

increased Ptgs2 expression. Thus, these data suggest that AngII may be one of the previously unknown factors necessary for the chain of events leading to ovulation.

To explore further this hypothesis, we examined the effect of AngII on members of the PA system, prostaglandin-dependent proteases that are likely important for the tissue remodeling involved in follicle rupture. The addition of AngII to LH-treated cells significantly increased expression of tPA, uPA and the inhibitor Serpine2 within 6 h. This is in agreement with the increase in the expression of these genes observed in bovine follicles in vivo following induction of ovulation with hCG or GnRH [8, 10]. As AngII antagonists inhibit prostaglandin release and ovulation in cattle [19, 20] as well as rodents, the likely mechanism is inhibition of prostaglandin-dependent PA activity and the resulting proteolytic degradation of the follicle wall.

In conclusion, the present data strongly suggest that AngII is a mediator of the LH to establish induction of Ptgs2 and to regulate the remodeling of extracellular matrix in bovine granulosa cells. Moreover, there is evidence that AngII is an obligatory co-factor for the LH-induced cascade of events leading to rupture of the follicle wall and ovulation.

Figures

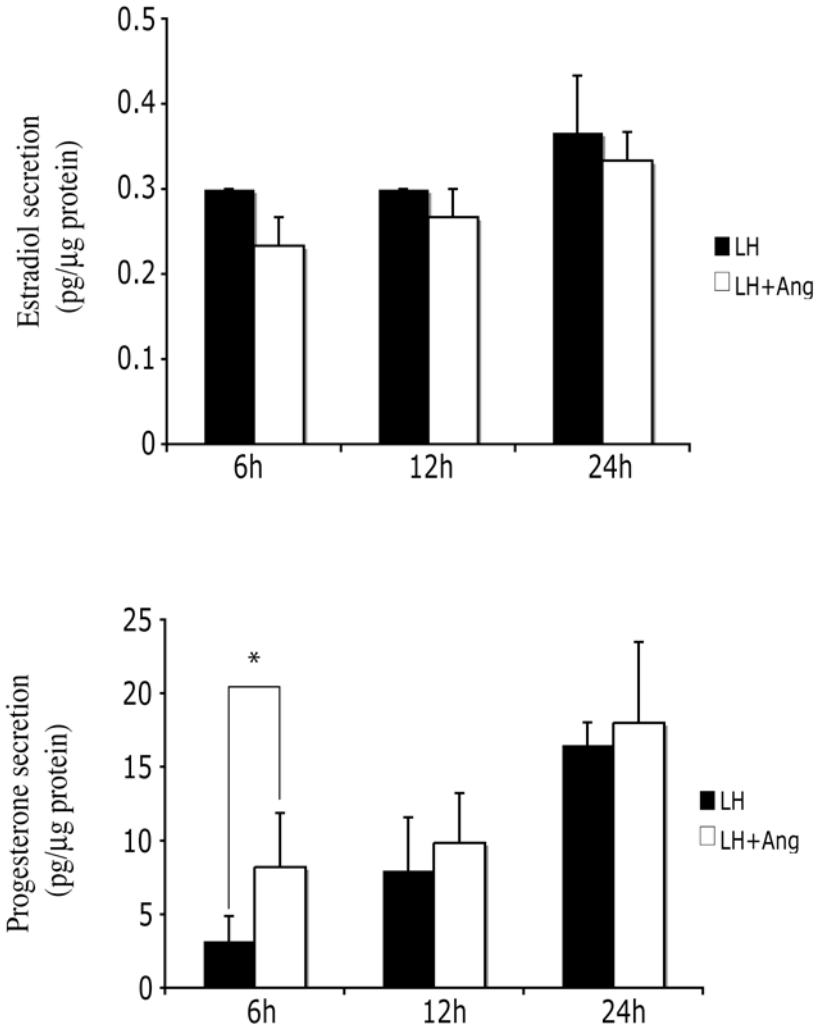

Fig 1. Effect of time in culture on estradiol and progesterone secretion in luteinizing bovine granulosa cells incubated with LH or LH+AngII. Cells from large follicles (>10mm diameter) were cultured with serum for 24 h, and then in serum-free medium for a further 12 h. LH (100ng/ml) and AngII (dose) was then added for the times shown. Means ± SEM are shown for 3 replicate cultures. Asterisk denotes a significant effect of AngII ($P<0.05$).

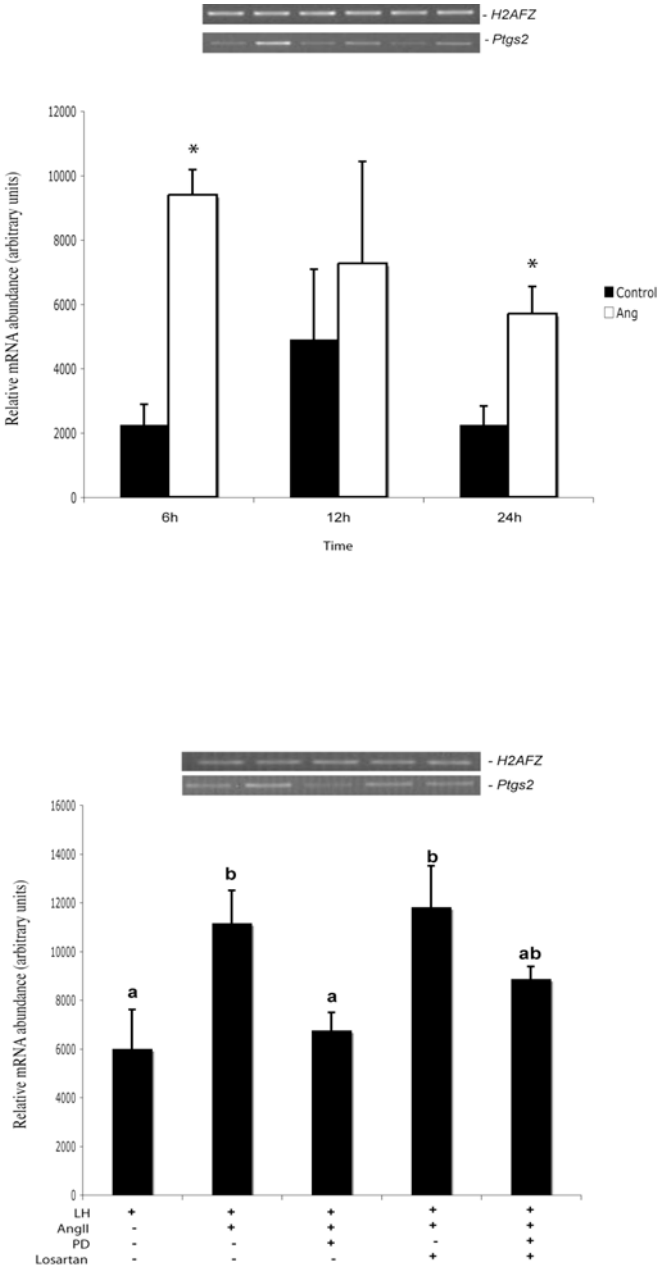

Fig 2. Effect of AngII on Ptgs2 mRNA expression in luteinizing granulosa cells in vitro. Cells were cultured as described in Fig 1, and Ptgs2 mRNA measured by RT-PCR. (A) AngII increased Ptgs2 expression in cells cultured with LH. Asterisks denote differences between LH alone and LH+AngII treatments. (B) Pretreatment with selective AGTR1 (Losartan) and AGTR2 (PD) blockers reveal which AngII receptor mediates the effect of AngII on Ptgs2 expression. Letters above bars denote differences between means ($P<0.05$). Means ± SEM are shown for 3 replicate cultures. A representative gel of PCR products is shown for each gene.

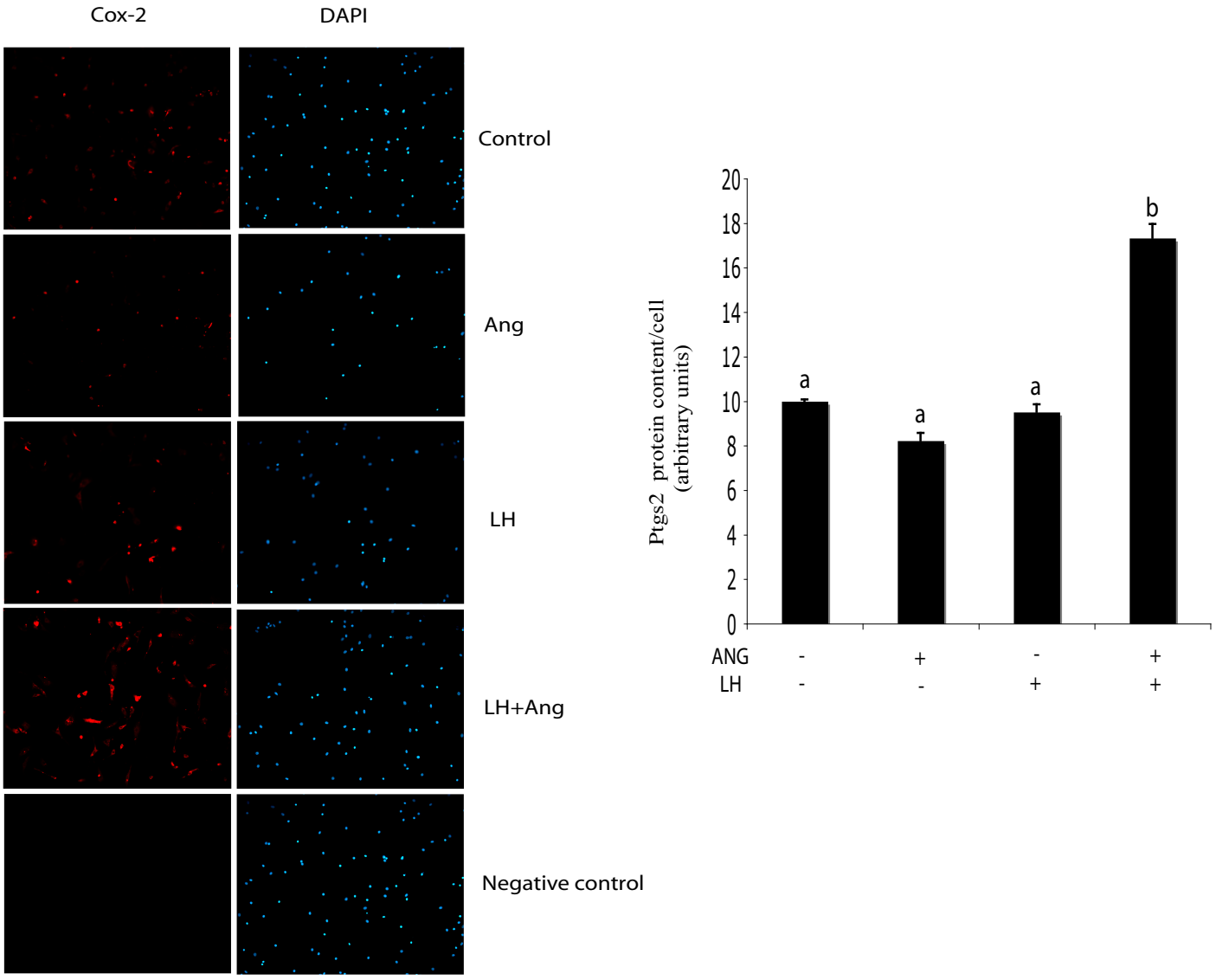

Fig 3. Effect of LH and AngII on Ptgs2 protein as assessed by immunofluorescence in granulosa cells in vitro. Cells were cultured as described in Fig 1, and fixed after 6 h treatment with LH (100 ng/ml), AngII (dose) or both. (A) Representative photomicrographs showing Ptgs2 immunofluorescence determined by Cy3 (red) and cell density determined by DAPI staining (blue). (B) Quantification of Ptgs2 protein corrected for cell number. Means SEM are shown for 3 replicate cultures. Letters above bars denote differences between means ($P<0.05$).

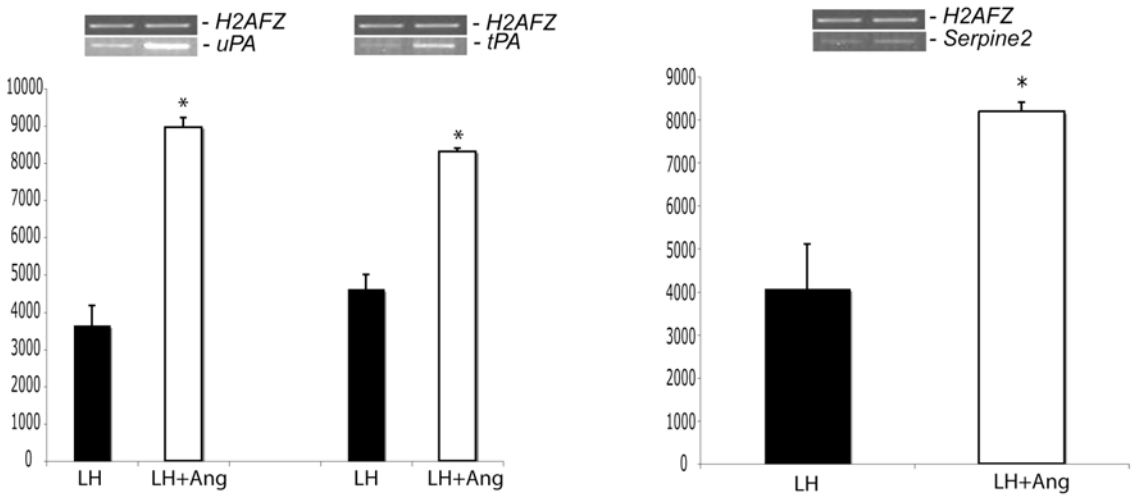

Fig 4. Effect of AngII on expression of members of the PA system in luteinizing granulosa cells in vitro. Cells were cultured as described in Fig 1, and mRNA encoding uPA, tPA and Serpine2 measured by RT-PCR. Asterisks denote differences between LH alone and LH+AngII treatments ($P<0.05$). Means ± SEM are shown for 3 replicate cultures. A representative gel of PCR products is shown for each gene.

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6. DISCUSSÃO

No presente estudo foi demonstrado que o AGTR2 está presente nas células da granulosa de bovinos, que o mRNA para esse gene está aumentado em folículos dominantes e que a expressão na células da granulosa é estimulada por gonadotrofinas. Esses dados evidenciam que a sinalização via AGTR2 está envolvida no crescimento e não na atresia folicular em bovinos. Além disso, foi demonstrado que mRNA AGTR1 e AGTR2 estão presentes em células da teca e granulosa de bovinos.

Em estudos anteriores havia sido demonstrado que a AngII está envolvida com a atresia folicular através da redução na secreção de E2 e da expressão do AGTR2 em células da granulosa de folículos atrésicos (KOTANI et al., 1999; DE GOOYER et al., 2004). No entanto, a expressão do AGTR2 tinha sido detectada somente nas células da teca e não em células da granulosa em folículos bovinos (SCHAUSER et al., 2001a), sugerindo uma via alternativa de ação da AngII em bovinos. As técnicas utilizadas anteriormente não foram sensíveis para detectar o AGTR2 em células da granulosa, vasos e tecidos ricos em receptores de AngII (BRUNSWIG-SPICKENHEIER & MUKHOPADHYAY, 1992a; TOUYZ & SCHIFFRIN, 2000; SCHAUSER et al., 2001b). Neste experimento, foi utilizada a técnica de RT-PCR para investigar a presença de AGTR2, e imunohistoquimica para detectar a proteína tanto nas células da teca quanto nas células da granulosa. A expressão de AGTR1 nas células da teca e nas células da granulosa não diferiu em folículos dominantes e atrésicos, concordando com dados achados em células da teca em bovinos (ACOSTA et al., 1999). Em contraste com estudos em ratos que apresentaram um aumento significativo de AGTR2 nas células da granulosa de folículos atrésicos (DAUD et al., 1988; PUCELL et al., 1991; DE GOOYER et al., 2004), a expressão de AGTR2 foi menor em células da granulosa de folículos atrésicos do que em folículos dominantes.. Esta diferença demonstra uma modificação no sistema fisiológico durante a evolução das espécies.

Células da granulosa quando cultivadas com FSH e fatores de crescimento como BMP-7 e IGF-1 apresentaram maior expressão de mRNA e proteína para AGTR2. Essa relação entre mRNA e proteína sugeri que os níveis de expressão dos receptores sejam regulados nos mesmos níveis de transcrição e tradução. No entanto, a expressão total de receptores pode não corresponder a quantidade de proteína funcional na superfície celular, provavelmente por sofrer uma regulação endócrina ou parácrina que afete níveis de mRNA de AGTR2 e tenha resposta na sinalização via AGTR2. Para avaliar o papel

de fatores de crescimento sobre a expressão de AGTR2, foi testada a ação de 3 tipos de FGFs (FGF-2 (VERNON & SPICER, 1994), FGF-7 (PARROTT & SKINNER, 1998), FGF-10 (BURATINI et al., 2007), que inibiram E2 em células da granulosa cultivadas *in vitro*. Embora todos os FGFs tenham reduzido a secreção de E2, somente o FGF-7 e -10 inibiram os níveis de expressão de proteína AGTR2. Os FGFs possuem efeitos diversos nos diferentes tecidos e atuam através de diferentes receptores. O FGF-2 ativa o receptor FGFR2c, FGFR3c e FGFR4, enquanto, o FGF-7 e -10 ativam predominantemente o receptor FGFR2b (ORNITZ et al., 1996; IGARASHI et al., 1998). Porém, se desconhece os receptores que são utilizados pelos FGFs para a regulação da expressão de AGTR2.

Após estabelecer que os receptores de AngII estejam presentes nas células da granulosa e que são regulados por hormônios e fatores de crescimento envolvidos no desenvolvimento folicular, uma segunda etapa foi iniciada para estabelecer a ação da AngII através da ativação de seus receptores. Células da granulosa cultivadas com FSH na presença ou ausência de AngII não apresentaram diferença na secreção de E2 e P4, bem como na proliferação celular, sugerindo que a AngII não induz a atresia folicular em ruminantes. Para este estudo, foi desenvolvido um modelo de cultivo de células da granulosa não luteinizada e indiferenciada, representando o folículo em crescimento, antes de aquisição de receptores funcionais de LH.

A AngII está associada a alteração da expressão de membros ativos do RME, tais com ativador plasminogênio e metaloproteínases de matriz, aumentando a expressão desses genes em tecidos vasculares (NAKAMURA et al., 2000; ARENAS et al., 2004; BROWATZKI et al., 2005). As células da granulosa de folículos em desenvolvimento não expressam o inibidor do ativador do plasminogênio, mas expressam e secretam MMP2, MMP9 e um inibidor específico das células da granulosa, conhecido como *SERPINE2* (DOW et al., 2002a; SMITH et al., 2005; CAO et al., 2006a). Nós demonstramos que a AngII não altera a atividade de MMPs, mas inibe a expressão de mRNA e secreção de *SERPINE2*, como anteriormente descrito em células de Schawann de ratos (BLEUEL et al., 1995).

A regulação de *SERPINE2* não foi acompanhada por mudança na secreção de E2, demonstrando uma ação específica da AngII no controle do RME em células da granulosa de bovinos.

Em uma terceira etapa, após ter sido mostrado que a ativação de receptores de AngII ocorre através deste peptídeo em células da granulosa provenientes de folículos

pequenos (2-5mm), buscou-se estabelecer os possíveis efeito da AngII em um sistema de cultivo de células da granulosa de folículos grandes (>8mm), mimetizando a ovulação. Esse etapa mostrou que a AngII estimula a expressão de mRNA e proteína para Ptgs2, bem com a expressão para APt, APu e *SERPINE2*.

Existem evidências de que a AngII está envolvida na ovulação em coelhos e vacas (MUKHOPADHYAY et al., 1991; SCHAUSER et al., 2001a; FERREIRA et al., 2007). No presente experimento, nós utilizamos um sistema de cultivo de 24 h sem a presença de soro para demonstrar a ação da AngII associada ou não ao LH, resultando na secreção de E2, P4 e expressão de Ptgs2. Em um sistema de cultivo similar, a AngII inibiu a secreção de P4 estimulada por LH (BÉLANGER et al., 1990). Em contraste, foi demonstrado que AngII aumenta a secreção de P4 e células da granulosa as 6 h porém não as 12 e 24 h após o tratamento. Resultados similares foram observados em ovários de ratos perfundidos com AngII.

A expressão de Ptgs2 não foi alterada pelos tratamentos com LH, não concordando com o fato dessa gonadotrofina aumentar a proteína Ptgs2 *in vivo* (BRIDGES et al., 2006). Em estudos prévios com células da granulosa de camundongos sensibilizados com PMSG *in vitro*, foi encontrado um aumento na expressão de Ptgs2 induzido por LH. No entanto, a indução de Ptgs2 pode ser um efeito do cultivo pela oresença do oócito (JOYCE et al., 2001). A hipótese é que a AngII seja um fator necessário, presente no folículo *in vivo*, que associada ao LH aumenta a expressão de Ptgs2. Essa hipótese é baseada nos resultados que mostram a necessidade dos dois hormônios, LH e AngII, para aumentar a Ptgs2, não havendo resposta positiva quando utilizados isoladamente em cultivo de células da granulosa. Isso sugeri que a AngII possa ser um co-fator necessário na cascata de eventos que desencadeiam a ovulação.

Associado a esses achados, foi verificado o efeito da AngII em genes relacionados ao sistema de RME durante a ovulação. A combinação de LH e AngII aumenta a expressão de ativador do plasminogênio e SERPINE2 6 h após o tratamento. Isto concorda com resultados *in vivo* onde a indução da ovulação com hCG ou GnRH também induziu a expressão desses genes (PUCELL et al., 1991). Assim como antagonistas da AngII inibiram a secreção de prostaglandina e ovulação em bovinos (GUTIERREZ et al., 1997; FERREIRA et al., 2007). O provável mecanismo de ação da AngII deve ser a secreção de prostaglandina e o aumento da atividade do ativador do plasminogênio, resultando na degradação da parede folicular e ovulação.

7. CONCLUSÃO

O receptor AGTR2 está presente nas células da granulosa de bovinos e o mRNA para o receptor AGTR2 está aumentado em folículos dominantes. Além disso, a expressão do mRNA e a tradução da proteína para o AGTR2 são reguladas por FSH, IGF1, BMP7, FGF-7 e FGF-10 em células da granulosa de bovinos cultivadas *in vitro*. AngII também, regula a proteína PN-1, Ptgs2 e ativadores do plasminogênio em células da granulosa, agindo como um co-fator fisiológico necessário para a ovulação.

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