UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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EFEITOS DO ESTRESSE AGUDO E A PARTICIPAÇÃO DO SISTEMA ANGIOTENSINÉRGICO SOBRE A FUNÇÃO REPRODUTIVA EM RATAS: COMPORTAMENTO SEXUAL, OVULAÇÃO E LACTAÇÃO

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“Toda a nossa ciência, comparada com a realidade, é primitiva e infantil - e, no entanto, é a coisa mais preciosa que temos.”

Albert Einstein

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RESUMO

A função reprodutiva constitui-se em um dos diversos estados no qual o estresse pode atuar exercendo efeitos deletérios que colocam em risco a integridade individual e a manutenção da espécie. Dentre os diversos peptídeos que atuam controlando os processos reprodutivos, a angiotensina II (Ang II) possui um papel destacado pela sua atuação no controle de hormônios hipofisários, além de uma importante participação na regulação das respostas ao estresse. Considerando a importância dos mecanismos que controlam as funções reprodutivas e as evidências da influência do estresse sobre esses processos, esta tese teve como objetivo estudar os possíveis efeitos do estresse agudo sobre diferentes aspectos da função reprodutiva em fêmeas e a participação do sistema angiotensinérgico nesses mecanismos. Para isso, foi avaliada a resposta ao estresse de diversos hormônios com funções reprodutivas como a prolactina, o hormônio luteinizante e a progesterona em diferentes fases do processo reprodutivo. A participação do sistema angiotensinérgico foi avaliada através da utilização de antagonistas da Ang II e da quantificação da densidade de receptores de Ang II em núcleos do sistema nervoso central como o núcleo arqueado, o locus coeruleus, o núcleo pré-óptico mediano e o órgão subfornicial em modelos experimentais com diferentes concentrações de estradiol e progesterona. O presente estudo demonstrou que o estresse agudo provoca uma redução na concentração plasmática de prolactina em ratas ovariectomizadas tratadas com estradiol e progesterona e lactantes, sendo esta resposta mediada pelos receptores AT_1 de Ang II no núcleo arqueado. Os esteróides gonadais aumentam a densidade destes receptores de Ang II no núcleo arqueado e a progesterona parece ser a principal moduladora desta regulação. Além disso, esse aumento também ocorre no locus coeruleus, núcleo pré-óptico mediano e órgão subfornicial, já que o tratamento de ratas ovariectomizadas com estradiol e progesterona provoca um aumento na densidade de receptores de Ang II nestes núcleos. Já o estresse agudo na manhã do proestro provocou uma redução nas concentrações plasmáticas de hormônio luteinizante, progesterona e prolactina na tarde do proestro, juntamente com uma redução na ovulação, sendo estes efeitos mediados pelos receptores AT_1 de Ang II. A aplicação de um estresse agudo por contenção durante 1 hora na tarde do proestro provocou uma redução no comportamento sexual, porém esses efeitos não são mediados pelo sistema angiotensinérgico. Em conjunto, esses resultados permitem concluir que o estresse agudo provoca alterações em diferentes aspectos do processo reprodutivo em fêmeas, incluindo efeitos deletérios sobre a lactação, o comportamento sexual, a geração dos picos hormonais pré-ovulatórios e a ovulação. O sistema angiotensinérgico tem uma participação efetiva em diversos mecanismos envolvidos na resposta ao estresse e parece ser um importante regulador dessas respostas.

ABSTRACT

Reproductive functions can be affected by stressful experiences resulting in deleterious effects that can compromise the individual and the species. Ang II is a peptide involved in the control of reproductive functions through regulation of pituitary hormone secretion and that also participates in the stress response mechanisms. Considering the importance of the mechanisms that control reproductive functions and the evidence that reproductive physiology is affected by stress, the present work aimed to test the effects of acute stress on different aspects of female reproductive physiology and the participation of the angiotensinergic system in these mechanisms. For that, we evaluated reproductive hormones, prolactin, luteinizing hormone and progesterone, response to stress in different reproductive stages. The participation of the angiotensinergic system was evaluated through Ang II receptor antagonist administration and by quantifying Ang II receptor density in central nervous system nucleus such as arcuate nucleus, locus coeruleus, median preoptic nucleus and subfornical organ in experimental models with different estradiol and progesterone concentrations. The present results show that acute stress induced a reduction in prolactin plasma levels in estradiol and progesterone treated ovariectomized rats and lactating rats. This reduction is mediated by Ang II AT_1 receptors in the arcuate nucleus. Gonadal steroid replacement increased the Ang II receptor density in the arcuate nucleus and progesterone seems to have a pivotal role in this regulation. Indeed, this increase was also seen in locus coeruleus, median preoptic nucleu and subfornical organ, since treatment of ovariectomized rats with estradiol and progesterone induced an increase in the Ang II receptor density in these nucleus. Acute stress in the morning of proestrus induced a reduction in luteinizing hormone, progesterone and prolactin plasma levels in the afternoon of proestrus, as well as a reduction in the ovulation, through Ang II AT_1 receptors. Restraint stress for 1 hour in the afternoon of proestrus induced a reduction in the sexual behavior, but this effect was not mediated by angiotensinergic system. In summary, the results indicate that acute stress alters different aspects of the female reproductive physiology, including deleterious effects on lactation, sexual behavior, preovulatory hormonal surges and ovulation. Angiotensinergic system participates in different mechanisms involved in the stress response and seems to be an important regulator of these functions.

APRESENTAÇÃO

Esta tese está organizada da seguinte maneira: Introdução; Objetivos; Abordagem Metodológica; Capítulos I ao IV, contendo os artigos científicos publicados e/ou submetidos; Conclusões; Perspectivas e Referências Bibliográficas.

A **Introdução** apresenta o embasamento teórico que nos levou à formulação das hipóteses e propostas de trabalho. A seção **Abordagem Metodológica** mostra uma breve explanação das abordagens experimentais utilizada nos trabalhos. Os materiais e métodos, assim como as referências bibliográficas específicas, encontram-se no corpo de cada trabalho, os quais são apresentados nos **Capítulos** de artigos publicados.

A seção **Conclusões** aborda as conclusões gerais da tese e a seção **Perspectivas** discute as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos durante a realização desta tese.

A seção **Referências Bibliográficas** lista as referências citadas na Introdução da tese.

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ABREVIATURAS

ACTH	Hormônio corticotrófico
Ang II	Angiotensina II
APO	Área pré-óptica
ARC	Núcleo arqueado
CRH	Hormônio liberador de corticotrofina
DA	Dopamina
E_2	Estradiol
EPM	Erro padrão da média
FSH	Hormônio folículo-estimulante
HPA	Eixo hipotálamo-hipófise-adrenal
HPG	Eixo hipotálamo-hipófise-gônadas
ICV	Intracerebroventricular
LC	Locus coeruleus
LH	Hormônio luteinizante
LHRH	Hormônio liberador de hormônio luteinizante
MnPO	Núcleo pré-óptico mediano
NA	Noradrenalina
OVX	Ovariectomizadas
P	Progesterona
PRL	Prolactina
PVN	Núcleo paraventricular
RNAm	RNA mensageiro
SFO	Órgão subfornicial
SNC	Sistema nervoso central

INTRODUÇÃO

A reprodução é um conjunto complexo de processos que ocorrem durante a vida dos animais, iniciando-se com a instalação da puberdade e, no caso das fêmeas, seguindo com o desenvolvimento de variações cíclicas e alterações hormonais coordenadas que resultam no desencadeamento do comportamento sexual e culminam no processo de ovulação, possibilitando a concepção. A continuidade normal desse processo desencadeia passos importantes como a prenhêz, o parto, o comportamento maternal e a lactação, fazendo com que a função reprodutiva obtenha êxito.

A relação entre estresse e função reprodutiva tem sido descrita há décadas através de observações que demonstram uma disfunção do sistema reprodutivo em conseqüência da exposição de um organismo a estímulos estressores. Considerando a importância dos processos reprodutivos para a manutenção das espécies, é de grande relevância que possamos entender as alterações e os mecanismos que relacionam o estresse com a função reprodutiva, sendo este o tema central desta tese.

1. Estresse

Ao longo do tempo, diversos autores têm se dedicado ao estudo do estresse e suas respostas. No entanto, muitas correntes a respeito de qual o significado de estresse e como defini-lo têm sido utilizadas, sem que haja um consenso sobre o tema (Pacák & Palkovits, 2001). A resposta ao estresse, que permite a um organismo enfrentar situações ameaçadoras, consiste de uma rede complexa de sistemas biológicos, que incluem componentes neurovegetativos, endócrinos e comportamentais (Chrousos & Gold, 1992; Charmandari et al., 2005). Chrousos & Gold (1992) definiram estresse como um estado de desarmonia na qual a homeostasia é colocada sob ameaça. Nestas situações, ocorre uma série de respostas adaptativas, físicas e mentais, que se

contrapõem aos efeitos dos estímulos estressantes na tentativa de restabelecer a homeostase (Chrousos & Gold, 1992; López et al., 1999; Charmandari et al., 2005). A função reprodutiva é apenas um dos diversos estados no qual o estresse, seja ele agudo ou crônico, pode atuar exercendo efeitos deletérios que colocam em risco a integridade individual e a manutenção da espécie (Calogero et al., 1998; Dobson & Smith, 2000). As respostas ao estresse classicamente conhecidas são a ativação do eixo hipotálamo-hipófise-adrenal (HPA), que provoca a liberação do hormônio liberador de corticotrofina (CRH) pelo núcleo paraventricular (PVN), de hormônio corticotrófico (ACTH) pela adenohipófise e de corticosterona pela supra-renal, além da liberação de noradrenalina nos terminais simpáticos através da ativação do sistema autonômico simpático e de adrenalina na corrente sanguínea pela medula da glândula supra-renal (Chrousos et al., 1998). Este conjunto de respostas, incluindo a ação das catecolaminas e dos glicocorticóides, induzem alterações em mecanismos fisiológicos como a função cardiovascular e a produção e distribuição de substratos energéticos, dando o suporte necessário para o organismo restabelecer o equilíbrio (Kopin, 1995).

2. Lactação

O período da lactação é parte importante do processo reprodutivo e consiste em um período fisiológico único em que ocorre uma série de alterações neuroendócrinas que incluem a fisiologia do eixo HPA (Toufexis et al., 1996; Douglas, 2005). Dentre essas alterações, encontra-se o fato do eixo HPA ser hiporresponsivo a estímulos estressores devido a uma menor liberação de noradrenalina e uma menor expressão dos receptores adrenérgicos no PVN, o que tem sido entendido como um mecanismo de prevenção a uma exposição excessiva a estímulos estressores neste período (Douglas, 2005). Além disso, uma das principais características do período de lactação é a

presença de altas concentrações plasmáticas de prolactina (PRL), estimulada, em grande parte, pela sucção exercida pelos filhotes nos mamilos da fêmea (Grosvenor et al., 1979; Andrews, 2005). De uma maneira geral, durante a primeira metade da lactação, são secretadas elevadas concentrações de progesterona (P) e PRL, e reduzidas concentrações de estradiol (E_2), hormônio luteinizante (LH) e hormônio folículo-estimulante (FSH) (Smith & Neill, 1977; Taya & Sasamoto, 1981; Taya & Greenwald, 1982; Lee et al., 1989). Assim, havendo um adequado número de filhotes durante o período de lactação, o corpo lúteo secreta P continuamente e a ovulação cíclica é interrompida. No entanto, a remoção da ninhada causa o reaparecimento do ciclo estral e da ovulação em qualquer dia do período lactacional (Taya & Sasamoto, 1986). De qualquer forma, um dos grandes objetivos desse período é a nutrição adequada dos filhotes, através da produção de leite, com papel destacado para a função estimulatória da PRL sobre a síntese de leite (Freeman et al., 2000; Lau 2001). A função de lactogênese claramente requer a secreção de PRL, já que a retirada da hipófise durante a prenhês previne o período lactacional subseqüente. Além disso, ratos sem o gene necessário para a síntese de PRL ou do seu receptor são incapazes de produzir leite (Freeman et al., 2000).

3. Estresse e Prolactina

Em situações de estresse, além das respostas clássicas do eixo HPA, uma variedade de hormônios é liberada e a PRL constitui-se em um dos hormônios que respondem prontamente a estímulos estressantes (Nicoll, 1960; Neill, 1970; Torner & Neumann, 2002). Morishige & Rotchild (1974) demonstraram que a resposta da PRL ao estresse depende das concentrações de PRL pré-estresse no sangue. Se as concentrações pré-estresse são reduzidas o estresse promove uma elevação na PRL plasmática (Neill,

1970; Wakabaiashi et al., 1971; Euker et al., 1975; Leong et al., 1983; Demarest et al., 1985; Bánky et al., 1994; Caldeira & Franci, 2000; Dave et al., 2000). Entretanto, se as concentrações de PRL são elevadas antes da aplicação do estresse, o estresse promove uma diminuição na concentração plasmática de PRL (Grosvenor et al., 1965; Morishige & Rotchild, 1974; Caligaris & Taleisnik, 1983; Poletini, 1998). Além disso, o sistema angiotensinérgico parece ter um papel importante na resposta da PRL ao estresse, já que a secreção de PRL em animais submetidos a um estresse hemorrágico envolve a participação da angiotensina II (Ang II) (Machado et al., 2002). No entanto, a função e a importância fisiológica da PRL em resposta ao estresse ainda é fonte de controvérsias, sendo sugeridas funções de modulação do sistema imunológico, proteção contra o desenvolvimento de úlceras gástricas durante a lactação e manutenção do equilíbrio homeostático afetado por outros hormônios do estresse, sem que haja, no entanto, dados experimentais conclusivos que suportem essas hipóteses (Freeman et al., 2000).

4. Sistema Angiotensinérgico

A relação entre Ang II e a função reprodutiva foi sugerida pela primeira vez quando foram identificados os efeitos desse peptídeo sobre a liberação de hormônios da hipófise anterior em diversos modelos experimentais numa série de trabalhos publicados por Steele & colaboradores (1981, 1982, 1983, 1985 e 1987). Desde então, a Ang II tem sido associada a mecanismos de controle de várias fases do processo reprodutivo. Além disso, a Ang II é um octapeptídeo envolvido em funções de regulação da pressão arterial, equilíbrio hidroeletrolítico, entre outros. Nos últimos 20 anos, além do mecanismo clássico do sistema renina-angiotensina periférico, que já se encontrava bem descrito, muitos laboratórios identificaram sistemas locais presentes em diversos tecidos como a parede arterial, os rins, a supra-renal, a hipófise e as gônadas

(Saavedra, 1992). No entanto, uma das descobertas mais importantes foi a descrição de um sistema renina-angiotensina no sistema nervoso central, atuando inicialmente sobre o aumento da pressão arterial (Bickerton & Buckley, 1961; Saavedra et al., 2004). Atualmente, sabe-se que a Ang II é produzida em vários tecidos, incluindo o cérebro e a hipófise, onde esse peptídeo atua no controle dos hormônios reprodutivos (Saavedra, 1992).

A Ang II exerce suas ações através de receptores que foram classificados farmacologicamente em dois subtipos, receptores AT_1 e AT_2 (Timmermans et al., 1993). Os receptores do tipo AT_1 são subdivididos em duas isoformas distintas, AT_{1A} e AT_{1B}, sendo o AT_{1A} predominante em áreas cerebrais envolvidas no controle da função hipofisária, enquanto o AT_{1B} é expresso na hipófise anterior (Jöhren et al., 1996). Os receptores AT_1 são sensíveis ao bloqueio pelo antagonista losartan (DUP-753) e os receptores AT_2 são sensíveis ao bloqueio por CGP 42112 e PD 123319 (Timmermans et al., 1993). Os receptores de Ang II estão amplamente distribuídos pelo sistema nervoso central (SNC), incluindo núcleos caracteristicamente angiotensinérgicos como o órgão subfornicial (SFO), além de núcleos envolvidos na resposta ao estresse e em funções reprodutivas como o locus coeruleus (LC), o PVN, o núcleo pré-óptico mediano (MnPO) e o núcleo arqueado (ARC) (Lenkei et al., 1997). Além disso, evidências demonstram que os receptores de Ang II no SNC e na hipófise são modulados pelos esteróides gonadais. A densidade de receptores de Ang II no ARC dorsomedial é maior no estro em comparação com as outras fases do ciclo estral (Seltzer et al., 1993) e o tratamento de ratas ovariectomizadas (OVX) com E_2 e P aumenta a densidade de receptores e a expressão do seu RNA mensageiro (RNAm) no ARC, enquanto que na hipófise ocorre uma diminuição desses receptores (Jöhren et al., 1997). No entanto, o tratamento de ratas OVX apenas com E_2 induz uma redução na densidade de receptores

de Ang II na hipófise (Chen & Printz, 1983; Seltzer et al., 1992) e no SFO (Kisley et al. 1999).

5. Sistema Angiotensinérico e o Estresse

As ações do estresse e dos glicocorticóides sobre neuropeptídeos têm sido muito estudadas. Existem evidências que implicam um papel da Ang II na regulação das respostas ao estresse (Fitzsimons, 1980; Saavedra et al., 1986; Saavedra et al., 2004). Estudos demonstraram que o estresse aumenta os sítios de ligação para Ang II no PVN e no SFO, e isso pode ocorrer devido a um aumento nas concentrações circulantes de Ang II e de glicocorticóides (Castren & Saavedra, 1988). Evidências sugerem que os glicocorticóides regulam a expressão desses receptores no PVN (Aguilera et al., 1995; Leong et al., 2001). A adrenalectomia, em ratos, reduz a expressão do RNAm para o receptor AT_1 nesse núcleo e a administração de glicocorticóides reverte esse efeito (Aguilera et al., 1995). A ativação de receptores AT_1 no PVN, eminência mediana, hipófise e supra-renal resulta na síntese e secreção aumentada de CRH, secreção de ACTH e de corticosterona, respectivamente, ressaltando a participação da Ang II central na resposta ao estresse. Estímulos psicológicos e sensoriais através de vias envolvendo o córtex cerebral, complexo amigdalóide e hipocampo também atuam para que ocorra a ativação do eixo HPA em diferentes níveis (Saavedra et al., 2004). O bloqueio dos receptores centrais (AT_1) de Ang II, através de uma injeção intracerebroventricular (ICV), diminui as concentrações plasmáticas de noradrenalina, adrenalina e dopamina (DA) após estresse por imobilização, evidenciando o seu papel na regulação da ativação simpática em resposta a estímulos estressores (Jezova et al. 1998). Além disso, o estresse por imobilização aumenta a atividade da renina plasmática (Jindra & Kvetnansky, 1982), indicando uma ativação do sistema renina-angiotensina periférico e

a própria Ang II aumenta significativamente no plasma e no SNC após estresse agudo ou crônico (Yang et al., 1996).

6. O Sistema Angiotensinérgico e a Secreção de Prolactina

A PRL é um hormônio polipeptídico sintetizado e secretado por células especializadas da hipófise anterior denominadas lactotrofos. Dentre as múltiplas funções desse hormônio, dois grandes grupos podem ser didaticamente distinguidos: funções reprodutivas (lactação, função luteal e comportamento reprodutivo) e homeostáticas (resposta imune, osmorregulação e angiogênese) (Freeman et al., 2000). A Ang II contribui para a regulação fisiológica da secreção de PRL, tanto através da Ang II central (Myers & Steele, 1989, 1991), quanto da Ang II sintetizada perifericamente (Aguilera et al., 1982; Anderson & Cronin, 1990). Em ratas OVX tratadas com E_2, o pico induzido de PRL é significativamente atenuado se os animais são tratados com oligonucleotídeos antisense para o RNAm da Ang II, demonstrando a participação do sistema angiotensinérgico na secreção da PRL (Yuan & Pan, 2002). No entanto, este mecanismo de controle da secreção de PRL parece ser antagônico, já que a Ang II na hipófise anterior estimula a sua secreção (Steele et al., 1981; Aguilera et al., 1982; Steele & Myers, 1990), enquanto que a Ang II central possui efeito inibitório sobre a secreção de PRL (Steele et al., 1981; 1982; Myers & Steele, 1989, 1991). Essa inibição pela Ang II central parece ser através da liberação de DA e ativação de receptores em neurônios dopaminérgicos no ARC (Jöhren et al., 1997; Mounzih et al., 1994). Esse efeito é produzido através de receptores do tipo AT_{1A} de Ang II e é regulado pelas concentrações de esteróides gonadais, já que ratas OVX tratadas com E_2 e P apresentam um aumento nas concentrações desses receptores no ARC quando comparadas a ratas OVX tratadas com placebo (Jöhren et al., 1997). Este aumento na densidade de

receptores de Ang II pode, desta forma, promover um aumento da atividade do sistema angiotensinérgico no ARC, aumentando a atividade dopaminérgica e, consequentemente, diminuindo a secreção de PRL (Seltzer et al., 1993).

7. O Ciclo Estral da Rata

A rata, assim como a mulher, apresenta ovulação cíclica espontânea independente de alterações sazonais ou de atividade sexual. No entanto, o ciclo das ratas é composto por quatro fases definidas com duração média total de quatro dias (Matthews & Kenyon, 1984), o que, aliado à semelhança com o perfil hormonal apresentado pela mulher e ao fato de serem animais de pequeno porte e fácil manutenção, tornam a rata um excelente modelo experimental para estudos de reprodução. Dessa forma, as evidências sobre o controle do ciclo ovariano de mamíferos que possuem ovulação espontânea são baseadas, em grande parte, em estudos sobre o controle do ciclo estral da rata.

O ciclo estral é composto por quatro fases distintas: proestro, estro, diestro 1 (ou metaestro) e diestro 2 (ou diestro). A fase de proestro dura cerca de 12 a 14 horas e estende-se do início da manhã até o início da noite, sendo no final desta tarde que ocorrem os picos hormonais pré-ovulatórios. O estro tem duração aproximada de 25 a 27 horas e, apesar de constituir-se em um longo período, apenas no início da noite até a madrugada (quando irá ocorrer a ovulação) a rata é receptiva ao macho, período este comumente denominado como estro comportamental. Após o estro, teremos um período denominado de diestro 1, com duração de 6 a 8 horas, seguido pelo diestro 2, que dura de 55 a 57 horas, reiniciando, após, um novo ciclo (Freeman, 1994).

As fases do ciclo estral podem ser facilmente identificadas através da análise diária das mudanças na mucosa vaginal. Considerando que as células do epitélio vaginal são altamente responsivas às concentrações de E_2 e P, a presença de células nucleadas, leucócitos ou células corneificadas (Matthews & Kenyon, 1984) possibilita a identificação de cada uma das fases (Figura 1).

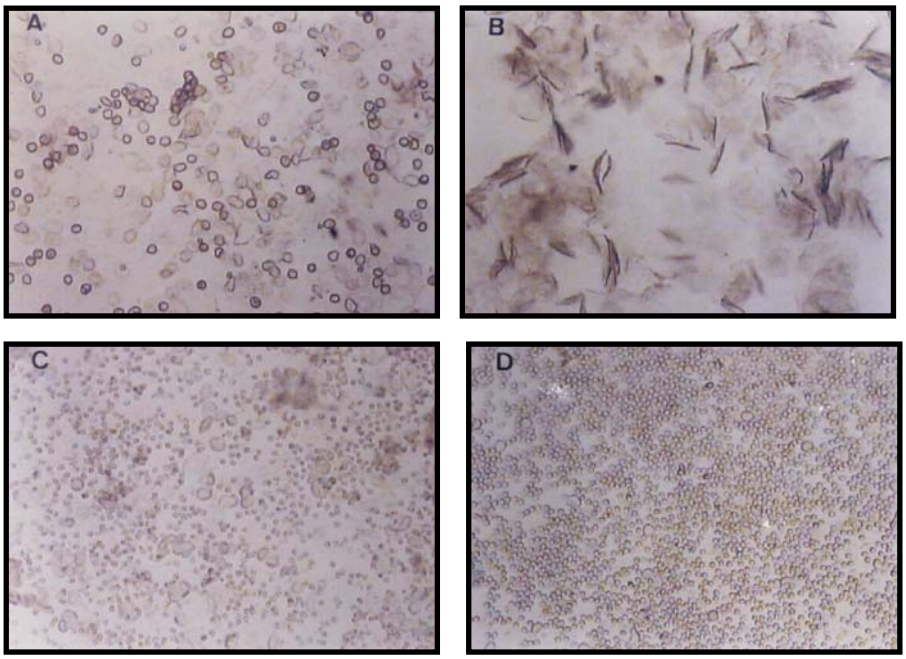

Figura 1. Fotomicrografia de esfregaço vaginal de ratas com ciclos regulares de 4 dias. Todos os esfregaços foram colhidos entre 8:00 e 10:00 horas da manhã. **A**: Proestro; **B**: Estro; **C**: Diestro 1; **D**: Diestro 2.

8. O Perfil Hormonal do Ciclo Estral

As gonadotrofinas, LH e FSH, são glicoproteínas produzidas e secretadas pela adenohipófise, em um conjunto de células especializadas denominadas gonadotrofos, e que atuam na regulação da função ovariana (Clayton & Catt, 1981). No ciclo estral, as concentrações de LH e FSH permanecem baixas durante quase todo o ciclo. A secreção de LH aumenta antes da ovulação, o que chamamos de "pico pré-ovulatório". Esse

aumento, induzido pelo efeito de retroalimentação positiva do E_2 no eixo hipotálamo-hipófise-gônadas (HPG), ocorre na tarde do proestro e pode ser mimetizado em ratas OVX através da reposição com E_2 (Freeman et al., 1976). Assim, apenas no início da tarde do proestro, em torno de 15 horas, haverá um aumento nas concentrações de LH e FSH que culminará com um pico ao redor das 17 horas, sendo este o responsável pela indução da ovulação algumas horas depois. Já as concentrações de E_2 são baixas entre o estro e a manhã do diestro 1 e começam a aumentar a partir da tarde do diestro 1, atingindo seus maiores valores perto do meio dia do proestro e retornando a valores basais na madrugada do estro. A P apresenta um pico na tarde do proestro e início do estro, muito próximo ao pico pré-ovulatório do LH e de origem folicular. Além disso, um segundo pico, de origem luteal, se estende do diestro 1 até a madrugada do diestro 2. Por fim, a PRL também apresentará um pico pré-ovulatório, na tarde do proestro, juntamente com o pico de gonadotrofinas (Smith et al., 1975) (Figura 2).

9. Sistema Angiotensinérgico e a Secreção de LH

O LH sofre influência do sistema angiotensinérgico. Phillips et al. (1992) demonstraram que há um aumento no conteúdo de Ang II no hipotálamo, no líquor e no fluido intersticial do PVN, todos às 13:30 h, precedendo o pico de gonadotrofinas induzido pela injeção de E_2 e P. Da mesma forma, Ghazi et al. (1994), utilizando ratas ciclando na fase de proestro demonstraram um aumento de Ang II no líquor e fluido intersticial na área pré-óptica (APO) entre 12:00 e 13:30 h, precedendo o pico pré-ovulatório que ocorre nessa tarde. A injeção ICV de saralasina (antagonista inespecífico dos receptores de Ang II) ou enalapril (inibidor da enzima conversora) na tarde do proestro inibe o pico do LH e bloqueia a ovulação em ratas ciclando, demonstrando a participação do sistema angiotensinérgico central na produção do pico de LH na tarde

do proestro e no mecanismo de ovulação (Steele et al., 1983). A injeção ICV de Ang II em ratas afeta a liberação de LH de uma maneira dependente dos hormônios ovarianos E_2 e P. Em ratas OVX, a Ang II inibe a liberação pulsátil de LH de uma forma dose-dependente, mas em ratas OVX tratadas com E_2 e P a Ang II estimula a liberação de LH. Essa ação estimulatória sobre a secreção de LH ocorre no hipotálamo anterior, região preóptica, atuando via noradrenalina (NA) nos neurônios noradrenérgicos que terminam nesta área (Steele et al., 1987).

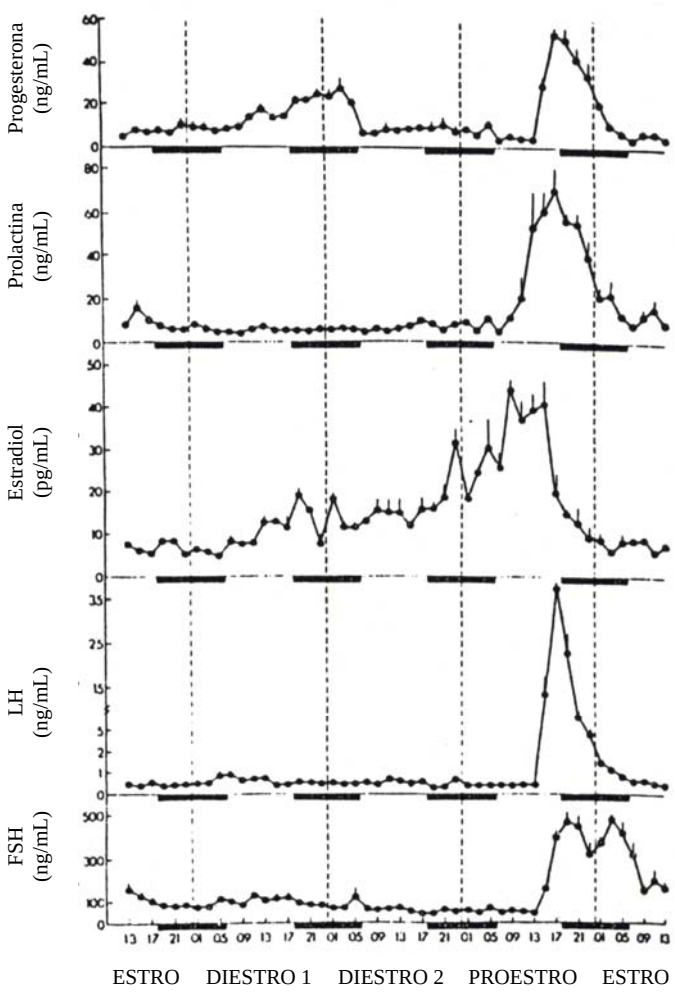

Figura 2. Concentrações plasmáticas (média±EPM) de progesterona, prolactina, estradiol, LH e FSH, obtidas em intervalos de 2 horas, durante os quatro dias do ciclo estral de ratas. O traço mais largo no eixo horizontal representa o período escuro do ciclo diário claro-escuro, e os números abaixo das barras o horário do dia referente a um ciclo de 24 horas (Smith et al., 1975).

10. Estresse e Gonadotrofinas

Estresse de origem física ou emocional pode provocar interferências em funções reprodutivas (Chatterton, 1990). A ativação do eixo HPA em resposta a estímulos estressores provoca uma ruptura na fisiologia normal do eixo HPG, levando a efeitos supressivos sobre a função reprodutiva (Wingfield & Sapolsky 2003, Rivier & Rivest 1991). O efeito supressivo do estresse crônico sobre o eixo HPG ocorre, provavelmente, pela manutenção de concentrações elevadas de CRH e, consequentemente, de ACTH e glicocorticóides, este produzindo efeito inibitório sobre a secreção de LH (Calogero et al., 1998).

De uma maneira geral, a secreção de gonadotrofinas pode ser alterada por estímulos estressores. A resposta do LH ao estresse agudo tem sido estudada preferencialmente em machos e/ou fêmeas OVX (Ajika et al., 1972; Krulich et al., 1974; Euker et al., 1975; Siegel et al., 1981; Mcgivern & Redei, 1994; Briski, 1996). Estímulos estressores podem provocar respostas variáveis sobre a secreção de LH (aumento, diminuição ou ausência de resposta) de acordo com o modelo de estresse utilizado (Briski, 1996). Neil (1970) utilizando anestesia por éter seguido de laparotomia mostrou que fêmeas, nas diversas fases do ciclo, não apresentavam alterações na concentração de LH, enquanto que Turpen et al. (1976) mostraram que estresse por éter (3 min) na tarde do proestro provocava um aumento significativo na concentração de LH. No entanto, estresse por contenção (5 a 7h) na tarde do proestro bloqueia o pico de LH e a ovulação (Roozendaal et al., 1995). Já mulheres na menopausa, tratadas com E_2, o estresse através da injeção de endotoxinas provoca um aumento na concentração de LH que é dependente de E_2 (Puder et al., 2000).

Da mesma forma, a resposta do FSH ao estresse também apresenta ampla variação na literatura, podendo haver aumento, diminuição ou ausência de resposta. Em

fêmeas OVX, estresse por anestesia com éter e laparotomia aumenta a secreção de FSH (Ajika et al.,1972), assim como estresse por éter (2 min) aplicado em ratos machos (Krulich et al., 1974). Já estresse através de som, luz ou calor não provoca alterações na secreção de FSH (Siegel et al., 1981), da mesma forma que estresse por éter (3 min) não altera FSH em machos e fêmeas tanto na manhã quanto na tarde do proestro (Turpen et al., 1976). No entanto, a contenção (5 a 7h) suprime o aumento do FSH na tarde do proestro (Roozendaal et al., 1995).

11. Comportamento Sexual

Durante o ciclo estral normal na rata, as alterações hormonais induzem o aparecimento de uma série de comportamentos que indicam a receptividade da fêmea, os quais incluem investigação dos genitais do macho, pequenas corridas e saltos, vocalizações e contatos físicos efêmeros, além da expressão do comportamento de lordose (Ellingsen & Agmo, 2004; Pfaff et al., 1994). Este último, o reflexo de lordose, é uma postura estereotipada adotada pela fêmea receptiva em resposta à estimulação táctil do macho através do seu comportamento de monta (Truitt et al., 2003). A lordose, que é um importante parâmetro para avaliação da receptividade, é resultado da ativação de um circuito neural e dependente dos esteróides gonadais (Flanagan-cato et al., 2001). Durante o ciclo estral, este comportamento é dependente de uma elevação na concentração plasmática de E_2 seguida por um aumento nas concentrações de P. Dessa forma, a retirada dos ovários abole o comportamento sexual, sendo a sua reinstalação possível apenas após a reposição com E_2 e P. No entanto, o tratamento isolado com E_2 induz somente alguns aspectos do comportamento sexual, enquanto o tratamento com E_2 seguido de P irá induzir o conjunto de comportamentos proceptivos na sua totalidade (Auger, 2001; Mani, 2001). Além dos esteróides gonadais, a PRL, que é um importante

hormônio do estresse, também exerce influências sobre o comportamento sexual, apesar da sua atuação na regulação desses mecanismos ainda ser controversa (Freeman et al., 2000). Evidências sugerem que o bloqueio do pico pré-ovulatório de PRL na tarde do proestro diminui a receptividade sexual, apontando uma função estimulatória importante para a indução da receptividade normal em ratas ciclando (Witcher & Freeman, 1985).

Além disso, da mesma forma que os hormônios reprodutivos, o comportamento sexual também é alterado pelo estresse (Plas-Roser & Aron 1981). A ativação do eixo HPA e a conseqüente liberação de CRH diminuem o comportamento sexual (Rivier & Vale, 1984). Estresse por contenção diminui o comportamento de lordose em ratas OVX tratadas com E_2 e parece que tanto E_2 como a P, através de um efeito dose-dependente, reduzem a diminuição do comportamento sexual induzida pelo estresse (Truitt et al., 2003; White & Uphouse, 2004).

12. Ovulação

O ciclo reprodutivo da rata tem como marco principal o processo de ovulação. Os processos de crescimento e maturação folicular, assim como o funcionamento do corpo lúteo, são coordenados diretamente pelas gonadotrofinas (Freeman, 1994). A ovulação, que é o ápice do processo de maturação folicular, depende fundamentalmente do pico pré-ovulatório de gonadotrofinas, seguido pela formação do corpo lúteo (com função secretora de E_2 e P) que será mantido pela ação do LH e regredirá em caso de não fecundação (Freeman, 1994; Levine, 1997). Ratas com ciclo estral regular apresentam, geralmente, uma ovulação na manhã do estro com um número de oócitos entre 10 e 14 a cada ciclo (Gomes et al., 1999) (Figura 3).

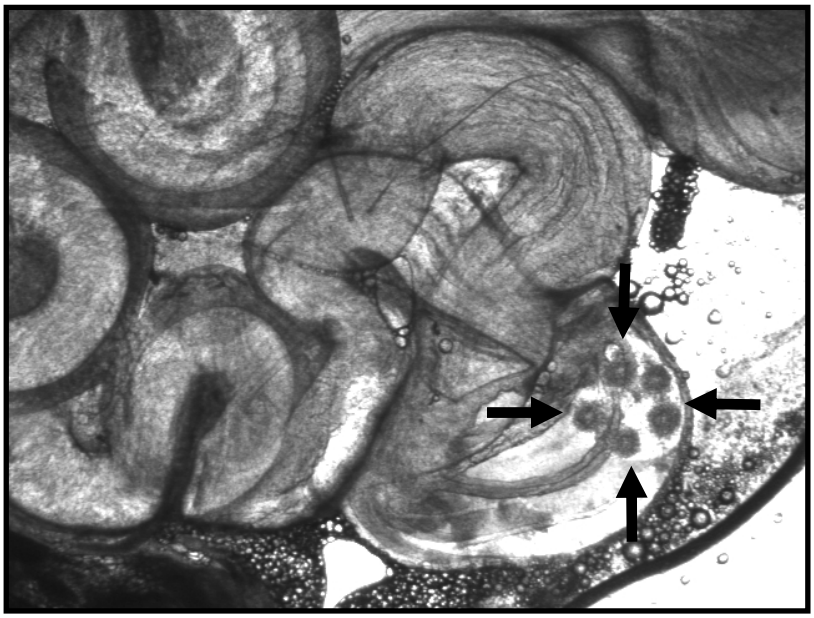

Figura 3. Fotomicrografia do oviduto direito de uma rata com ciclo estral regular de 4 dias na manhã do estro. O ovário foi retirado, colocado entre duas lâminas de vidro e comprimido para análise em microscópio óptico. As setas indicam a localização dos oócitos.

O aumento da concentração de LH na tarde do proestro é o evento chave para o desencadeamento do processo de ovulação. Este pico pré-ovulatório de LH depende da liberação pulsátil do hormônio liberador de LH (LHRH) e estes processos, por sua vez, são produzidos pelos efeitos das ações de retroalimentação positiva do E_2 e pelos sinais neurais específicos que iniciam os picos (Levine, 1997). Além do aumento na secreção de LHRH, outros eventos são importantes para a geração do pico pré-ovulatório e a conseqüente ovulação. Evidências sugerem que um aumento de NA em regiões hipotalâmicas que contêm neurônios LHRH são fundamentais para a geração do pico de LH (Rance & Barraclough, 1981). Esta NA parece ser originada de um importante núcleo noradrenérgico denominado LC, já que a estimulação elétrica do LC potencia a liberação de LH induzida pela estimulação da APO (Gitler & Barraclough, 1987). Além

disso, lesão eletrolítica do LC diminui o conteúdo de NA no hipotálamo diminuindo a liberação pulsátil e o pico pré-ovulatório de LH (Anselmo-Franci et al., 1997; 1999). Da mesma forma, conforme exposto no item 9 (Sistema Angiotensinérgico e a Secreção de LH), o sistema angiotensinérgico participa da geração do pico pré-ovulatório de LH, já que há um aumento de Ang II no líquor e na APO precedendo o pico de LH que ocorre na tarde do proestro (Ghazi et al., 1994) e que o bloqueio desse aumento de Ang II no meio dia do proestro inibe o pico do LH e bloqueia a ovulação em ratas ciclando (Steele et al., 1983).

Considerando a importância do sistema reprodutivo para manutenção da espécie, a complexidade dos mecanismos fisiológicos que o controlam e os efeitos deletérios que podem advir do estresse inerente à vida de todas as espécies, assim como as diversas evidências que demonstram a participação da Ang II tanto em processos fisiológicos da função reprodutiva como em resposta a estímulos estressores, formulou-se a hipótese de que o estresse agudo poderia causar alterações em diferentes aspectos da função reprodutiva em fêmeas e de que o sistema angiotensinérgico poderia participar da regulação desses mecanismos.

OBJETIVOS

OBJETIVO GERAL

Estudar os efeitos do estresse agudo sobre diferentes aspectos da função reprodutiva em fêmeas e a participação do sistema angiotensinérgico nesses mecanismos.

OBJETIVOS ESPECÍFICOS

1. Avaliar a participação da Ang II central na resposta da prolactina ao estresse em dois modelos experimentais que apresentam prolactina basal elevada ($OVXE_2P$ e 7º dia de lactação);

2. Avaliar a densidade de receptores de Ang II no núcleo arqueado em modelos experimentais com diferentes concentrações de estradiol e progesterona (OVX, $OVXE_2P$, dia 7 de lactação e dia 20 de lactação);

3. Avaliar a modulação dos receptores de Ang II pelos esteróides gonadais, estradiol e progesterona, em áreas do sistema nervoso central envolvidas no controle de funções reprodutivas e homeostáticas (locus coeruleus, núcleo pré-óptico mediano e órgão subfornicial);

4. Avaliar os efeitos do estresse agudo no dia do proestro sobre o comportamento sexual, a ovulação e o perfil hormonal (hormônio luteinizante, progesterona, prolactina e corticosterona) em ratas com ciclo estral regular. Além disso, avaliar a participação do sistema angiotensinérgico nas alterações provocadas pelo estresse.

ABORDAGEM METODOLÓGICA

Nesta seção, será feita uma breve descrição da abordagem metodológica utilizada em cada um dos trabalhos que compõe esta tese. A descrição completa dos materiais e métodos utilizados encontra-se no corpo de cada trabalho nos capítulos de Artigos Publicados.

Todos os animais utilizados nos experimentos que compõe esta tese são ratos Wistar machos e fêmeas provenientes do biotério do Instituto de Ciências Básicas da Saúde da UFRGS. Os animais foram mantidos num ambiente de temperatura (24±2 ºC) e luz (6:00 às 18:00h) controladas, com água e ração *ad libitum*. Todos os experimentos seguiram o Guia para Cuidado e Uso de Animais de Laboratório do Instituto Nacional de Saúde dos EUA – NIH (1986).

1. Artigo I

Este trabalho teve como objetivo avaliar a participação da Ang II central na resposta da PRL ao estresse em modelos experimentais que apresentam PRL basal elevada. Para isto, foram utilizadas ratas OVX tratadas com E_2 e P ($OVXE_2P$) e ratas no dia 7 de lactação, visto que ambos são modelos com concentrações plasmáticas de PRL elevadas. Os animais foram implantados com cânulas bilaterais no ARC através de cirurgia estereotáxica e submetidos ao estresse por éter durante 1 minuto. O sangue foi coletado através de decapitação e a concentração de PRL plasmática dosada através de radioimunoensaio.

2. Artigo II

Este trabalho teve como objetivo avaliar a densidade de receptores de Ang II no ARC em modelos experimentais com diferentes concentrações de E_2 e P. Para isto,

foram utilizadas ratas OVX tratadas com veículo, OVX tratadas com E_2 e P, ratas no dia 7 de lactação e no dia 20 de lactação. Os animais foram decapitados e tiveram o sangue coletado e o cérebro removido e congelado. Os cérebros foram cortados em criostato e a densidade de receptores de Ang II no ARC foi determinada através da técnica de autoradiografia. As concentrações plasmáticas de E_2 e P foram dosadas através de radioimunoensaio.

3. Artigo III

Este trabalho teve como objetivo avaliar a modulação dos receptores de Ang II pelos esteróides gonadais, E_2 e P, em áreas do SNC envolvidas no controle de funções reprodutivas e homeostáticas. Foram utilizadas ratas OVX tratadas com veículo e OVX tratadas com E_2 e P. Os animais foram decapitados e tiveram seus cérebros removidos e congelados. Os cérebros foram cortados em criostato e a densidade de receptores de Ang II no LC, MnPO e SFO foram determinadas através da técnica de autoradiografia.

4. Artigo IV

Este trabalho teve como objetivo avaliar os efeitos do estresse agudo no dia do proestro sobre o comportamento sexual, a ovulação e o perfil hormonal em ratas com ciclo estral regular, e avaliar a participação do sistema angiotensinérgico nas alterações provocadas pelo estresse. Para isto, foram utilizadas fêmeas adultas com ciclo estral regular e testados os efeitos de diferentes paradigmas de estresse na manhã ou na tarde do proestro (contenção por 10 minutos, contenção por 1 hora e éter por 1 minuto) sobre o comportamento sexual e a ovulação. Com base nos resultados, foi testada a participação do sistema angiotensinérgico na redução da ovulação induzida pelo estresse aplicado na manhã do proestro e no perfil hormonal do final da manhã e tarde do

proestro, através da dosagem das concentrações plasmáticas de LH, P, PRL e corticosterona por radioimunoensaio. Por fim, também foi testada a participação do sistema angiotensinérgico nos efeitos do estresse aplicado na tarde do proestro sobre o comportamento sexual. A ovulação foi avaliada através da contagem do número de oócitos em microscópio óptico e o comportamento sexual através do quociente de lordose (número de lordoses dividido pelo número de montas) após análise dos registros efetuados em fita cassete por 15 minutos.

CAPÍTULO I

Angiotensin II receptors in the arcuate nucleus mediate stress-induced reduction of prolactin secretion in steroid-primed ovariectomized and lactating rats.

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Research report

Angiotensin II receptors in the arcuate nucleus mediate stress-induced reduction of prolactin secretion in steroid-primed ovariectomized and lactating rats

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Abstract

Angiotensin II (Ang II) is a peptide that exerts an inhibitory effect upon pituitary prolactin (PRL) release through the hypothalamic arcuate nucleus (ARC). Since both PRL and Ang II are known to be affected by stress, the experiments reported here were conducted to investigate the possible participation of Ang II in the stress-induced response of PRL in situations in which pre-stress PRL levels are high, as during the PRL surge induced by estradiol (E_2) and progesterone (P) in ovariectomized rats ($OVXE_2P$) and lactating females on day 7 post-partum. Adult female rats were stereotactically implanted with bilateral guide-cannulae in the ARC; 3 days later, they were microinjected with saline or losartan and, after a 15-min interval, they were submitted to stress by ether inhalation during 1 min. Five minutes after stress, trunk blood samples were collected. Plasma PRL was measured by radioimmunoassay (RIA). In $OVXE_2P$ and lactating rats, a significant reduction in PRL levels was detected after stress compared to non-stressed animals. The microinjection of losartan in the ARC before stress blocked the reduction of PRL in both $OVXE_2P$ and lactating females. In conclusion, the stress-induced reduction of plasma PRL in $OVXE_2P$ and lactating rats is mediated by Ang II through AT_1 receptors in the ARC.

Theme: Endocrine and autonomic regulation
Topic: Neuroendocrine regulation: other

Keywords: Prolactin; Stress; Angiotensin II; Arcuate nucleus; Lactation; Rat

1. Introduction

Prolactin (PRL) secretion in response to stress depends on its level before stimulation [16,31]. When the pre-stress level of PRL is low, stress increases plasma PRL [4,11,35,50]. In contrast, when the pre-stress basal level is high, during lactation, for instance, stress induces a marked reduction in plasma PRL [5,17,31]. Thus, PRL secretion shows a state-dependent stress response.

In women, stress influences lactation [24]. Stress during labor and delivery delays the onset of lactation [9]. In mothers of preterm infants, a stressful condition shows a deficiency in lactation due to PRL reduction [8]. In addition, psychosocial stress in postpartum women reduces PRL secretion, which leads to alterations in normal lactation mechanisms [18].

Lactation is characterized by dramatic changes in the hypothalamic–pituitary–gonadal axis [32]. In rats, suckling stimulation increases plasma PRL, especially in the first half of lactation with the highest concentration on day 7 [49], this effect being, at least in part, due to the suppression of tuberoinfundibular dopamine (TIDA) neural activity in the hypothalamic arcuate nucleus (ARC) [10,11,19].

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Progesterone (P) injection induces a very significant PRL surge in estradiol (E_2)-primed ovariectomized (OVX) rats. This increase of PRL secretion is caused by the action of ovarian steroids directly stimulating PRL release from lactotrophs and also inhibiting the activity of hypothalamic TIDA neurons [12]. On the other hand, during lactation, when plasma levels of P are high, whereas E_2 levels are low [41,48,49], suckling appears to be the major stimulus that activates mechanisms inhibiting TIDA neuronal activity and thereby removing the inhibitory influence on the secretion of PRL [10].

Angiotensin II (Ang II) is also a major stress hormone that increases in the plasma as well as in the central nervous system in response to stress stimulation [51]. Stress increases the density of Ang II binding sites in the hypothalamic paraventricular nucleus and the subfornical organ of rats [7], and also increases renin activity [20].

Ang II seems to have a dual role in PRL secretion [33]. Indeed, in the anterior pituitary, Ang II stimulates PRL release directly in the lactotroph [1,45], while in the ARC, Ang II exerts an inhibitory effect [33,45,46]. The Ang II inhibitory effect on PRL release is mediated by an increased dopamine release into the pituitary portal blood system through its excitatory action on the TIDA neurons [46]. A previous study [21] showed that Ang II type 1 receptors (AT_1) mRNA are co-localized with tyrosine hydroxylase positive neurons in the ARC. AT_1 receptors in both the pituitary and ARC are regulated by E_2 and P, so that in the absence of gonadal steroids, as in OVX rats, there is a greater expression of the receptors in the pituitary than in the ARC that shows a low expression. Conversely, in OVX rats treated with E_2 and P ($OVXE_2P$), an increased expression of AT_1 receptors is induced in the ARC, but not in the pituitary, confirming the hypothesis of a dual role of Ang II in the regulation of PRL release [21]. Indeed, during lactation the ARC expresses Ang II receptors, as well as the angiotensinogen mRNA, although at a lower level than a female in diestrus [42,43].

The present study aimed to test the hypothesis that Ang II modulates the reduction of PRL plasma levels in response to stress in two situations in which PRL secretion has already been activated. For that, we infused the AT_1 receptor antagonist, losartan, in the ARC nucleus of $OVXE_2P$ and lactating rats, and measured plasma PRL after ether stress. Considering that Ang II is activated in response to stress [51] and it exerts a central inhibitory effect on PRL secretion [33,45,46] through the ARC nucleus, we hypothesized that Ang II could explain the stress-induced reduction of PRL in situations when basal pre-stress PRL levels are high.

2. Materials and methods

2.1. Animals

Adult female (195–280 g) and pregnant Wistar rats (220–300 g) were obtained from the colony of the Federal University of Rio Grande do Sul. Animals were housed individually in a temperature-controlled room (22 ± 1 °C) with a 12:12-h light–dark cycle (lights on at 06:00 h) and free access to food (Rodent chow—Nutrilab, Colombo, PR, Brazil) and water. All animal procedures were carried out in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals [34] and the Research Committee of the University approved them.

2.2. Anesthetic

Animals were anesthetized with ketamine (Francotar, Parke-Davis, São Paulo, Brazil—100 mg/kg, i.m.) and xylasine (Rompun, Bayer, São Paulo, Brazil—50 mg/kg, i.m.) before ovariectomy and stereotaxic surgery procedures.

2.3. Experimental design

2.3.1. Experiment 1: effect of stereotaxic surgery on PRL secretion of $OVXE_2P$ and lactating rats

To avoid the possibility that stereotaxic surgery procedure could influence the PRL secretion, an experiment was performed comparing $OVXE_2P$ and lactating females microinjected with saline through bilateral guide-cannulae implanted in the ARC with $OVXE_2P$ and lactating females that were not submitted to the stereotaxic surgery.

2.3.1.1. $OVXE_2P$ rats. After anesthesia, adult female rats were ovariectomized. Two weeks after ovariectomy, the animals were treated subcutaneously with estradiol benzoate (5 μg/rat in 0.2 ml corn oil; Benzoginoestril, Sarsa, Rio de Janeiro, Brazil) for 3 consecutive days at 09:00 h and with progesterone (25 mg/rat in 0.2 ml corn oil; Sigma) on the 4th day at 10:00 h. On day 4 of the gonadal steroid replacement protocol at 16:00 h, trunk blood samples were collected to measure PRL by radioimmunoassay (RIA). Two groups were studied: (1) no stereotaxic surgery ($n = 6$)—animals were decapitated without any other intervention and (2) ARC saline microinjection ($n = 6$)—animals were stereotactically implanted on day 1 of the gonadal steroid replacement protocol with bilateral guide-cannulae in the ARC, and 3 days later microinjected with saline (0.2 μl) 20 min before decapitation.

2.3.1.2. Lactating rats. Female rats had the day of parturition designated as day 0 of lactation. On the 1st day of lactation, the number of pups was adjusted to 8. Only females with at least eight pups were used in the experiment. On day 7 post-partum at 09:00 h, animals were decapitated and trunk blood was collected to measure PRL. Two groups were studied in this experiment: (1) no stereotaxic surgery ($n = 8$)—animals were decapitated without any other intervention and (2) ARC saline microinjection ($n = 10$)—animals were stereotactically implanted on day 4 post-partum with bilateral guide-cannulae in the

ARC, and 3 days later microinjected with saline (0.2 μl) 20 min before decapitation.

2.3.2. Experiment 2: effect of losartan microinjection in the ARC on stress-induced PRL secretion in $OVXE_2P$ rats

Adult female rats were ovariectomized and had the gonadal steroids replaced ($OVXE_2P$), as described in Experiment 1. On day 4 of the gonadal steroid replacement protocol at 16:00 h, animals were killed by decapitation and trunk blood samples were collected. Rats were divided into four groups as follows: (1) no-implantation, no-stress ($n=22$); (2) no-implantation + stress ($n=13$)—animals were submitted to 1 min ether stress 5 min before decapitation; (3) saline + stress ($n=16$) and (4) losartan + stress ($n=15$)—animals were stereotactically implanted (day 1 of the replacement protocol) with bilateral guide-cannulae in the ARC and microinjected with saline (NaCl 0.15 M; 0.2 μl) or losartan (10 nM; 0.2 μl), respectively, 15 min before ether stress which was applied 5 min before decapitation. Plasma PRL was measured in all groups.

2.3.3. Experiment 3: effect of losartan microinjection in the ARC on the stress-induced PRL secretion in lactating rats

Lactating female rats were used in this experiment (parturition day and pup adjustment as described in Experiment 1). On day 7 post-partum at 09:00 h, animals were killed by decapitation and trunk blood samples were collected. Animals were divided into four groups as follows: (1) no-implantation, no-stress ($n=12$); (2) no-implantation + stress ($n=12$)—animals were submitted to 1 min ether stress 5 min before decapitation; (3) saline + stress ($n=17$); and (4) losartan + stress ($n=13$)—animals were stereotactically implanted (day 4 of lactation) with bilateral guide-cannulae in the ARC and microinjected with saline (NaCl 0.15 M; 0.2 μl) or losartan (10 nM; 0.2 μl), respectively, 15 min before ether stress which was applied 5 min before decapitation. Plasma PRL was measured in all groups. After stereotaxic surgery, ARC microinjection and stress stimulus, the animals were returned to their cages and remained with the litter until the time of decapitation. Considering that a non-specific effect of implantation surgery would occur and compromise the model for a PRL study, we have observed typical maternal behaviors after the implantation procedure (day 4). Twice a day we monitored some pronurturant behaviors (nest repair, carrying pups to the nest and licking) and animals that did not reestablish the expected maternal behaviors until day 6 of lactation, evidenced by deficient nest repair and retrieval of the pups to the nest, were excluded from the study.

2.4. Stereotaxic surgery and histology

After anesthesia, bilateral guide-cannulae (15 × 0.6 mm) were inserted 2 mm above the ARC using a stereotaxic instrument (David Kopf) with the head at the zero point of the incisor bar. The dorsal surface of the skull was exposed and holes (2 mm) were drilled bilaterally 3.0 mm lateral to the sagittal line and 3.3 mm posterior to the bregma. The cannulae were angled 17°, lowered to a depth of 8.0 mm below the dura mater and held in place by two stainless steel screws and dental cement. The coordinates used were determined in accordance with the Paxinos and Watson [36] atlas. After surgery, animals were returned to their respective cages. At the end of the experiment, after decapitation, the brains were removed, fixed in 4% formaldehyde and sectioned using a vibratome (100-μm frontal sections). Sections were stained with cresyl violet and the position of each cannula was determined. Only animals with both cannulae positioned in the nucleus were used.

2.5. Drug administration

Losartan (10 nM—DUP 753, Du Pont), an antagonist for Ang II AT_1 receptor, was diluted in saline. Microinjection was manually performed in each side at a volume of 0.2 μl using a 10-μl Hamilton syringe connected by polyethylene tubing (PE 10) to an injecting needle that remained in the nucleus for 1 min after injection. The injecting needle (17 × 0.3 mm) was 2.0 mm longer than the guide-cannula, which was 2.0 mm above the nucleus, so that only the injecting needle penetrated the nucleus. The tubing and needle had been previously filled with the solution that was slowly infused.

2.6. Stress and blood collection

Ether stress consisted of placing the animals in a jar saturated with ether vapor for 1 min. In all experiments, trunk blood samples were collected in tubes filled with heparin 5.000 I.U. (heparin/ml) after decapitation and were centrifuged. Individual plasma samples were stored frozen at − 20 °C until the day of the assay.

2.7. Radioimmunoassay

Plasma PRL was determined by double antibody RIA using specific kits provided by The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Baltimore, MD, USA). PRL antisera was anti-rat PRL-S9 and the reference preparations was PRL-RP3. The lower limit of detection was 0.09 ng/ml and the intra- and inter-assay coefficients of variation were 4% and 11.7%, respectively. All samples were measured in duplicate and, when needed, at different dilutions.

2.8. Statistics

All data were expressed as mean ± S.E.M. and analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc analysis using a Newman–Keuls test for multiple comparisons. When appropriate, the difference between two

Table 1
Mean (± S.E.M.) plasma PRL concentrations in no stereotaxic surgery and ARC saline microinjected animals in both $OVXE_2P$ and lactating female rats

	Group	PRL (ng/ml)
$OVXE_2P$	No stereotaxic surgery ($n=6$)	772.5 ± 120.8
	ARC saline microinjection ($n=6$)	833.2 ± 135.7
Lactating	No stereotaxic surgery ($n=8$)	431.0 ± 94.69
	ARC saline microinjection ($n=10$)	389.4 ± 21.25

groups was tested by the Student's *t*-test. In all cases, differences were considered significant when $p < 0.05$.

3. Results

Effects of stereotaxic surgery and ARC saline microinjection effect on PRL plasma levels in $OVXE_2P$ and lactating animals are presented in Table 1 (Experiment 1). No significant change was detected in $OVXE_2P$ [$t(10) = 0.334$] and lactating [$t(16) = 0.476$] animals that received a saline microinjection in the ARC compared to non-stereotaxic surgery control animals. Thus, stereotaxic procedure and ARC microinjection of saline did not change plasma PRL levels in both models used in these experiments.

Figs. 1 and 2 show, respectively, the plasma PRL in four groups of $OVXE_2P$ (Experiment 2) and lactating (Experiment 3) rats: no-implantation and no-stress; no-implantation + stress; previously microinjected in the ARC with saline and stressed (saline + stress) and previously microinjected with losartan in the ARC and stressed (losartan + stress). One-way ANOVA revealed a significant effect for groups (no-implantation and no-stress; no-implantation + stress; saline + stress; and losartan + stress) in both $OVXE_2P$ rats [$F(3,62) = 7.004$, $p < 0.05$] and lactating rats [$F(3,50) = 6.816$, $p < 0.05$] were detected in plasma PRL. The Newman–Keuls post hoc analysis revealed that ether stress induced a significant reduction in plasma PRL levels compared to the no-stress group. Microinjection with saline in the ARC 15 min before stress did not alter the stress-induced reduction of PRL since no difference was detected when the saline + stress group was compared to the stress group. Losartan microinjection in the ARC 15 min before stress blocked the stress-induced reduction of PRL since no significant differences were detected compared to the no-implantation and no-stress group. Moreover, the plasma PRL in the losartan + stress group was significantly higher than in the no-implantation + stress and saline + stress groups.

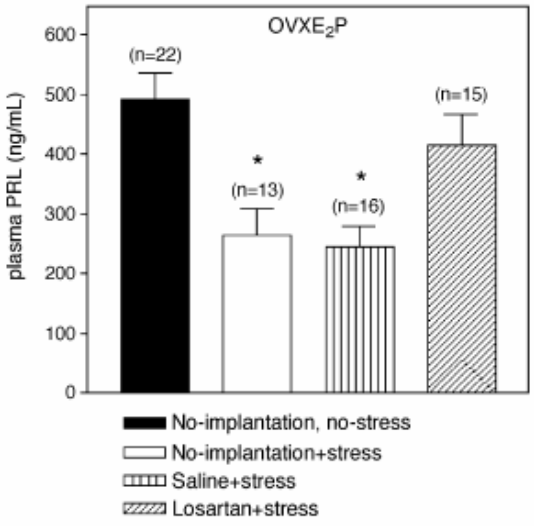

Fig. 1. Effects of microinjections of saline (NaCl 0.15 M; 0.2 μl) and losartan (10 nM; 0.2 μl) in the ARC on the stress-induced (1 min ether) response of plasma PRL (mean ± S.E.M.) in ovariectomized rats treated with estradiol and progesterone (OVXE2P). Animals were decapitated 5 min after stress. *$p < 0.05$ compared to no-implantation, no-stress and losartan + stress groups.

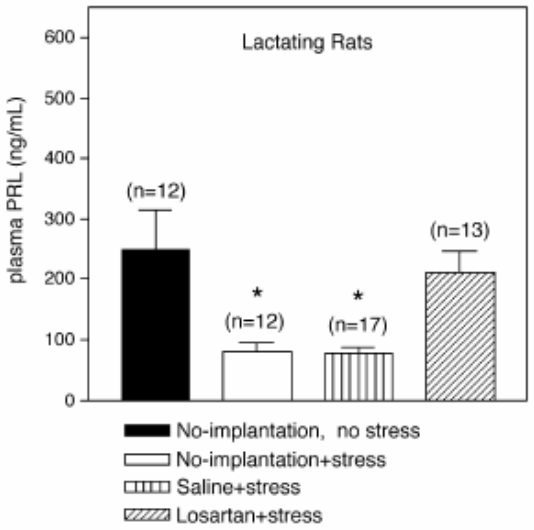

Fig. 2. Effects of ARC microinjections of saline (NaCl 0.15 M; 0.2 μl) and losartan (10 nM; 0.2 μl) on the stress-induced (1 min ether) response of plasma PRL levels (mean ± S.E.M.) in lactating (7th day post-partum) rats. Animals were decapitated 5 min after stress. *$p < 0.05$ compared to no-implantation, no-stress and losartan + stress groups.

4. Discussion

The present study provides evidence that Ang II in the ARC plays a critical role in the stress-induced decrease of plasma PRL, since it demonstrated that the stress-induced reduction of plasma PRL was blocked by the microinjection of an AT_1 receptor antagonist, losartan, in the ARC of $OVXE_2P$ and lactating rats.

Our findings that stress reduces PRL levels in $OVXE_2P$ and lactating rats are consistent with various studies demonstrating that stress induces a marked reduction in plasma PRL when pre-stress PRL levels are high [5,17,31].

Previous studies showed that losartan, a selective antagonist of the AT_1 Ang II receptor [37], completely blocked the effects of Ang II when microinjected in nanomolar concentrations into brain areas involved with blood pressure responses, as well as stress-induced blood pressure responses [2,15,23]. It also blocks the stimulatory and inhibitory action of Ang II in the medial preoptic area on LH and PRL secretion, respectively [13]. Kirby et al. [22] showed that losartan microinjected in the forebrain blocked the AT_1 receptors for at least 1 h, while Kubo et al. [23] demonstrated that the effects of losartan were clearly seen 5 min after its microinjection. These findings indicate that both the dose (10 nM) and the interval (15 min) between the injection and the stress stimulation used in our experiments were probably effective in blocking the angiotensinergic receptors in the ARC.

Our results show that the stress-induced reduction of plasma PRL in the $OVXE_2P$ and lactating females involves the effect of Ang II on its AT_1 receptors localized in ARC nucleus neurons. Autoradiographic studies have shown that ARC expresses only the AT_1 receptor subtype and there is no AT_2 subtype in this nucleus in males and females [21,25,40]. Expression of AT_1 receptor [21,40] and its mRNA in the ARC [21] is up-regulated by E_2 and P treatment of OVX animals. Moreover, it is suggested that the increased expression of AT_1 receptors in the ARC would occur in the dopaminergic cells, since in situ hybridization studies showed the co-localization of AT_1 receptor mRNA and tyrosine hydroxylase immunoreactivity in those neurons [21]. Thus, we may suggest that the infusion of losartan in the ARC prevented the expected decrease in plasma PRL after stress by reducing dopamine release into the hypothalamic–hypophysial portal system and consequently blocking the stress-induced decrease of PRL.

Previous studies have shown that intracerebroventricular injection of Ang II reduces plasma PRL levels in ovarian steroid-primed female rats [44,46]. Furthermore, the substantial increase in the Ang II content in the hypothalamus, as well as in plasma circulating levels, after different stress paradigms [51], contribute to the hypothesis of an angiotensinergic effect in the stress-induced response of PRL in the $OVXE_2P$ and lactating females. The ARC together with the paraventricular nucleus participates in pathways that regulate the hypothalamic–pituitary–adrenal axis [6,26] and is involved in the PRL response to acute stress stimulation [38]. In diestrus cycling female, stress reduces TIDA neuronal activity and thus increases PRL levels [11,30]. However, in the $OVXE_2P$ and lactating females, it is unlikely that this mechanism explains the PRL responses shown in the present study. In our models, dopaminergic activity in both $OVXE_2P$ and lactating rats is, at least, partially inhibited, which contributes to the high circulating plasma PRL levels found during lactation and after E_2 and P treatment in OVX animals [10,12].

Lactating rats do express Ang II receptors and angiotensinogen mRNA in the midcaudal ARC, however, the expression is lower than in a diestrus cycling rat [42,43], which could indicate a less important role of Ang II in PRL secretion in lactating females than cycling ones. However, the ARC of lactating females has one of the highest density concentrations of angiotensinogen mRNA compared to other brain areas, in the same way as in the diestrus phase of cycling females [43]. Nevertheless, in the present study, losartan in the ARC was able to block PRL response to stress in the lactating rats.

On the other hand, stress stimulation increases Ang II in the central nervous system [51]. However, the exact mechanisms of Ang II production in the brain are still poorly understood [25,29]. Within the central nervous system, angiotensinogen synthesis occurs in glial cells, especially in the hypothalamus [3,47]. Renin mRNA is also present in the brain, although its concentration seems to be low [14] and its spatial relationship to centrally synthesized angiotensinogen is unclear [29]. The angiotensin converting enzyme is located extensively in the central nervous system, with the highest concentration found in the circumventricular organs [39], where angiotensin I from peripheral circulation may be locally converted to Ang II [29].

In the rat brain, the immunohistochemical identification of Ang II reveals an extensive system of Ang II-immunoreactive cells and fibers in specific regions, for instance, the median eminence and the circumventricular organs. In contrast, the ARC has only a few immunoreactive cells [28]. Considering that the ARC is not part of the subfornical organ pathways [27,29], the peripheral effect of Ang II through these neurons is unlikely to explain our results. Thus, so far it has not been possible to define the precise origin of the Ang II that would modulate PRL secretion, although a local production of Ang II could occur, considering the expression of angiotensinogen in the ARC [43].

In the present study, losartan microinjection into the ARC did not induce a higher PRL level than in the control non-stressed animals, which suggests that the action of Ang II on the PRL secretion is not tonic in these models. This result is in agreement with the previous findings [46] in which pretreatment of OVX-estradiol-primed rats with saralasin, an unspecific Ang II antagonist, did not modify plasma PRL basal levels, although it suppressed the inhibitory effect of exogenous Ang II in the PRL secretion. Thus, Ang II seems to act on PRL secretion in response to stress, but would not have a constant inhibitory action on PRL secretion in $OVXE_2P$ and lactating rats.

In conclusion, our results show that stress-induced PRL reduction in both high PRL levels, $OVXE_2P$ and lactating rats, is mediated by Ang II AT_1 receptors in the ARC. Our findings that the decrease of PRL secretion in the rat caused by stress can be avoided by blocking AT_1 receptors could

contribute to further studies on lactation deficiency due to the influence of stress in women.

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CAPÍTULO II

Estradiol and progesterone modulation of angiotensin II receptors in the arcuate nucleus of ovariectomized and lactating rats.

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Estradiol and progesterone modulation of angiotensin II receptors in the arcuate nucleus of ovariectomized and lactating rats.

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Abstract

The expression of angiotensin II (Ang II) receptors in the brain is modulated by estradiol and progesterone. Considering that Ang II plays a critical role in controlling prolactin secretion and that neurons in the arcuate nucleus (ARC) are the main regulator of this function, the present study aimed to evaluate ARC Ang II receptor binding in 2 experimental models with different estradiol and progesterone plasma levels. Animals were divided into 4 groups: ovariectomy (OVX) plus oil vehicle, OVX plus estradiol and progesterone replacement, lactating rats on day 7 post-partum and lactating rats on day 20. Animals were killed by decapitation and the brains were removed. Ang II receptors were quantified by autoradiography in ARC. Trunk blood samples were collected and plasma estradiol and progesterone were measured by radioimmunoassay. Treatment of OVX rats with estradiol and progesterone increased Ang II receptor binding when compared to OVX vehicle-treated animals. Plasma estradiol (r=+0.77) and progesterone (r=+0.87) were highly correlated with Ang II receptors in ovariectomized animals. Lactating rats (day 20) showed a significant decrease in Ang II receptor binding and plasma progesterone when compared to lactating rats (day 7), however no difference was seen in plasma estradiol. Plasma levels of progesterone (r=+0.81), but not estradiol (r=+0.32), were highly correlated with Ang II receptors in lactating rats. In conclusion, present results show that ARC Ang II receptors decreases on day 20 of lactation compared to day 7 and are highly correlated with plasma progesterone, indicating a pivotal role for progesterone in this regulation.

Section: Neurophysiology, Neuropharmacology and other forms of Intercellular Communication

Key Words: Angiotensin II receptors; Arcuate nucleus; Estradiol; Progesterone; Lactation; Ovariectomy.

1. Introduction

Angiotensin II (Ang II) is an octapeptide involved in functions such as blood pressure regulation, hydric balance and reproductive control through modulation of pituitary hormones (Saavedra, 1992). It exerts its functions through two different subtypes of receptors pharmacologically classified as AT_1 and AT_2 (Timmermans et al., 1993). Ang II AT_1 receptors have been subdivided into two different isoforms, AT_{1A}, located in brain areas involved with the pituitary function, and AT_{1B}, present in the anterior pituitary (Jöhren et al., 1996). Ang II receptors are widely distributed in the central nervous system including important nuclei involved in reproductive functions, such as the arcuate nucleus (Lenkei et al., 1997), which is one of the major regulator of prolactin secretion (Freeman et al., 2000) .

We have previously demonstrated (Donadio et al., 2004) that Ang II receptors in the arcuate nucleus mediate stress-induced reduction of prolactin secretion in steroid-primed ovariectomized (OVX) and lactating rats. In lactating rats, suckling stimulation increases plasma prolactin through the suppression of tuberoinfundibular dopamine neural activity in the hypothalamic arcuate nucleus (Demarest et al., 1983, 1985; Hoffman et al., 1994). However, the increase of prolactin secretion in steroid-primed OVX rats is caused by the action of ovarian steroids directly stimulating prolactin release from lactotrophs and also inhibiting the activity of hypothalamic tuberoinfundibular dopamine neurons (Demaria et al., 2000). On the other hand, it should be noted that in spite of the similarity of high prolactin plasma levels in these two experimental models, there are profound differences regarding estradiol and progesterone plasma concentrations. Injection of estradiol and progesterone in OVX animals, as expected, results in high steroid plasma levels. In contrast, lactating rats show very low estradiol plasma levels and high progesterone plasma concentrations

(Smith & Neill, 1977; Taya & Sasamoto, 1981; Taya & Greenwald, 1982). Indeed, reproductive hormone plasma concentrations in lactating animals change throughout the lactation period, with prolactin and progesterone showing high levels on day 7 and then decreasing gradually until day 20 of lactation, in contrast with the low estradiol plasma levels during the whole lactation period (Taya & Sasamoto, 1981; Taya & Greenwald, 1982).

There is consistent evidence that Ang II receptor expression in specific brain areas and in the anterior pituitary is under the control of reproductive hormones. Seltzer et al. (1993) showed an increased expression of dorsomedial arcuate nucleus AT_1 receptors in cycling female rats on the day of estrus, which suggests the modulation of brain AT_1 receptors by ovarian hormones. Treatment of estrogen-primed OVX rats with progesterone upregulates Ang II receptors in selective brain areas involved in the inhibition of pituitary prolactin release, whereas estrogen treatment downregulates the expression of Ang II receptors in the anterior pituitary (Chen & Printz, 1983; Seltzer et al., 1992). Indeed, Ang II AT_1 receptors and the expression of its mRNA are induced in the arcuate nucleus of OVX rats treated with estradiol and progesterone. Conversely, in the absence of gonadal steroids, as in OVX rats, there is a greater expression of the receptors in the pituitary than in the arcuate nucleus that presents a low expression (Jöhren et al., 1997). We have demonstrated that estradiol and progesterone upregulate Ang II receptors in other brain areas that are involved in homeostatic and reproductive functions, such as the locus coeruleus, median preoptic nucleus and subfornical organ of ovariectomized rats (Donadio et al., 2005). On the other hand, lactating rats also express Ang II receptors, as well as the angiotensinogen mRNA, in several brain regions including the arcuate nucleus, although at a lower level than a female in diestrus (Speth et al., 1999, 2001).

Considering the importance of arcuate nucleus Ang II receptors in the control of prolactin secretion, especially in the lactation period, the evidence that specific brain Ang II receptors are modulated by gonadal steroids and the differences in estradiol and progesterone plasma levels between OVX and lactating rats and also throughout the lactation period, the purpose of the present study was to evaluate arcuate nucleus Ang II receptor binding in OVX rats treated with estradiol and progesterone or oil vehicle and in lactating rats on day 7 and 20 post-partum. For that, we measured arcuate nucleus Ang II receptor density by autoradiographic binding and correlated with the ovarian steroid plasma levels.

2. Results

Autoradiographic analysis of tissue sections in OVX vehicle-treated, OVX treated with estradiol and progesterone ($OVXE_2P$) and lactating animals in both days 7 and 20 post-partum revealed the presence of specific Ang II receptors localized in the arcuate nucleus. Figure 1 illustrates the film autoradiographs of coronal brain sections showing total [^{125}I]Sar^1-Ang II receptor binding in the arcuate nucleus in OVX, $OVXE_2P$ and lactating rats. Figure 2 shows estradiol plasma levels, progesterone plasma levels and the [^{125}I]Sar^1-Ang II receptor binding expressed in femtomoles per milligram of protein in the arcuate nucleus of OVX rats treated with oil vehicle and $OVXE_2P$ animals. As expected, the $OVXE_2P$ group showed an increased estradiol [$t(10)=5.001$, $P=0.0005$] (figure 2A) and progesterone [$t(10)=10.56$, $P<0.0001$] (figure 2B) plasma concentrations when compared to OVX animals. Treatment of OVX rats with estradiol and progesterone produced an increased Ang II receptor binding in the arcuate nucleus when $OVXE_2P$ was compared to OVX group [$t(10)=11.85$, $P<0.0001$] (figure 2C).

Figure 3 shows estradiol plasma levels, progesterone plasma levels and the [^{125}I]Sar^{1}-Ang II receptor binding in the arcuate nucleus of lactating rats on day 7 and lactating rats on day 20 of lactation. No significant differences in estradiol plasma concentration were found when lactation day 7 animals were compared to lactation day 20 [t(10)=0.133] (figure 3A). Lactating rats on day 20 showed decreased progesterone plasma levels when compared to lactating rats on day 7 [t(10)=4.396, *P*=0.001] (figure 3B). Indeed, Ang II receptor binding in the arcuate nucleus was significantly decreased in lactating rats on day 20 when compared to lactating rats on day 7 [t(10)=2.930, *P*=0.01] (figure 3C).

Plasma concentrations of estradiol (r=+0.77, *P*=0.001) and progesterone (r=+0.87, *P*=0.0001) were highly correlated with the Ang II receptor binding in the arcuate nucleus of ovariectomized rats (figure 4A and B, respectively). However, in lactating animals progesterone (r=+0.81, *P*=0.0007), but not estradiol (r=+0.15), plasma levels were observed to be highly correlated with Ang II receptor binding in the arcuate nucleus (figure 5B and A, respectively).

3. Discussion

The results obtained in the present study confirm previous observations of the presence of Ang II receptors in the arcuate nucleus (Lenkei et al., 1997; Jöhren et al., 1997; Mackinley et al., 2003). We also demonstrate that Ang II receptor binding in the arcuate nucleus decreases on day 20 of lactation compared to day 7 and that this regulation correlates predominantly with progesterone plasma concentration rather than estradiol plasma levels. Thus, it seems that progesterone plays a pivotal role in the upregulation of Ang II receptors since previous work using experimental models where estradiol was injected alone demonstrated an Ang II receptor down-regulation in the

central nervous system (Kisley et al., 1999), in contrast with studies that used an estradiol plus progesterone paradigm and demonstrated an Ang II receptor upregulation (Jöhren et al., 1997; Donadio et al., 2005). Considering the well-known effect of estradiol in promoting the expression of progesterone receptors (Scott et al., 2000; Turgeon & Waring, 2000), the Ang II receptor upregulation in these models would be a result of the crucial role of estradiol inducing the progesterone receptor and the progesterone action to modulate the expression of Ang II receptors in the brain. Indeed, as previously described (Jöhren et al., 1997), we have demonstrated here that treatment of OVX rats with estradiol and progesterone upregulates Ang II receptors in the arcuate nucleus, as well as it was demonstrated in the locus coeruleus, median preoptic nucleus and subfornical organ (Donadio et al., 2005).

Another important aspect to be considered is that lactating rats show different patterns of ovarian steroid hormone throughout the lactation period (Taya & Sasamoto, 1981; Taya & Greenwald, 1982). Our results show that on both days of lactation the animals studied presented low estradiol plasma levels and no differences were seen between day 7 and 20 of lactation. However, we found a decreased progesterone plasma concentration on day 20 of lactation when compared to day 7 of lactation, as well as lower Ang II receptor binding in lactating rats on day 20. Thus, in lactating rats, progesterone, but not estradiol, plasma levels were highly correlated with Ang II receptor binding, contributing to the hypothesis of a pivotal role of progesterone in the modulation of Ang II receptor binding in the central nervous system. The present results suggest that progesterone may act on arcuate progesterone receptors to induce the expression of AT_1 receptors. The progesterone receptor is a transcription factor and a member of a large family of structurally related gene products known as the nuclear receptor superfamily (Conneely & Lydon, 2000; Schumacher et al., 1999). Generally it

acts by increasing the transcription of specific genes after binding to selective intracellular receptors, although direct action on the cellular membrane is also possible (Schumacher et al., 1999). However, so far it has not been possible to define the precise mechanism by which progesterone may act to induce the Ang II receptor in the arcuate nucleus, although genomic actions appear plausible.

Lactation is characterized by dramatic changes in the hypothalamic-pituitary-gonadal axis (Murata et al., 1991) and also by changes in the patterns of all reproductive hormones (Smith & Neill, 1977; Taya & Sasamoto, 1981; Taya & Greenwald, 1982). Previous studies demonstrated that lactating rats express Ang II receptors and angiotensinogen mRNA in the arcuate nucleus, however, with a lower expression when compared to a diestrus cycling rat (Speth et al., 1999, 2001). Our results show that lactating rats do present Ang II receptors in the arcuate nucleus and that this expression decreases during the lactation period, since we have demonstrated lower Ang II receptor binding on day 20 of lactation when compared to day 7. It is already known that Ang II acts to control prolactin release (Steele et al., 1981; Steele, 1992; Freeman et al., 2000) and considering the importance of prolactin secretion, especially during the lactation, Ang II receptor changes in this period would be involved in the regulation of prolactin secretion.

The tuberoinfundibular dopamine neurons in the arcuate nucleus are considered to be the major physiological regulator of prolactin secretion (Freeman et al., 2000). Ang II AT_1 receptors and mRNA are co-localized with tyrosine hydroxylase-positive neurons in the arcuate nucleus (Jöhren et al., 1997) and there is evidence that the Ang II inhibitory effect on prolactin release is mediated by an increased dopamine release into the pituitary portal blood system through its excitatory action on tuberoinfundibular dopamine neurons (Steele et al., 1982). Our findings that arcuate

nucleus Ang II receptors are regulated by estradiol and progesterone in both ovariectomized and lactating animals contributes to explain the different prolactin responses in specific situations such as the response to stress stimulation, since we have previously demonstrated that Ang II participates in the regulation of prolactin during stress (Donadio et al., 2004) and that prolactin response to stress depends on its level before stimulation (Freeman et al., 2000; Morishige & Rothchild, 1974).

In conclusion, present results show that Ang II receptors in the arcuate nucleus decreases on day 20 of lactation compared to day 7 and are highly correlated with progesterone plasma levels, confirming that these receptors are regulated by ovarian steroid hormones, estradiol and progesterone, in ovariectomized and lactating rats. The study provides a contribution to the hypothesis of a critical role of progesterone in the regulation of central Ang II receptors in animals with different hormone backgrounds.

4. Experimental Procedure

4.1. Animals

Adult female (180-280 g) and pregnant Wistar rats (220-300 g) were obtained from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil). Animals were housed individually in a temperature-controlled room (22 ± 1°C) with a 12:12 h light-dark cycle (lights on at 06:00 h) and free access to food (Rodent chow – Nutrilab, Colombo, PR, Brazil) and water. All animal procedures were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the Research Committee of the University approved them.

4.2. Anesthetic

The animals were anesthetized with ketamine (Francotar, Parke-Davis, São Paulo, Brazil – 100 mg/Kg, i.m.) and xylasine (Rompun, Bayer, São Paulo, Brazil – 50 mg/Kg, i.m.) before ovariectomy.

4.3. Experimental Design

Adult female rats were ovariectomized after anesthesia. Two weeks after ovariectomy, the animals were divided into two groups (OVX plus estradiol / progesterone replacement and OVX plus oil vehicle). Hormones and vehicle were injected subcutaneously. Estradiol benzoate (5 μg/rat in 0.2 mL corn oil; Benzoginoestril, Sarsa, Rio de Janeiro, Brazil) was injected for 3 consecutive days at 09:00 h and progesterone (25 mg/rat in 0.2 mL corn oil; Sigma) was injected on the 4th day at 10:00 h. On day 4 of the gonadal steroid replacement protocol at 16:00 h, animals in both groups were killed by decapitation. Pregnant rats had the day of parturition monitored and designated as day 0 of lactation. On the 1st day of lactation, the number of pups was adjusted to 8. Only females with at least 8 pups were used in the experiment. On day 7 or 20 post-partum at 09:00 h, animals were killed by decapitation. In all experimental groups the brains were immediately removed after decapitation, frozen by immersion in isopentane at -30°C and stored at -80°C. Consecutive 20-μm-thick coronal brain sections were cut at -20°C in a cryostat and brain regions selected according to the Paxinos and Watson (1997) rat brain atlas. Sections were thaw-mounted on gelatin-coated glass slides, dried overnight in a desiccator at 4°C and stored at -20°C until processed for receptor autoradiography. Trunk blood samples were also collected after animals were decapitated to measure estradiol and progesterone by radioimmunoassay (RIA).

4.4 Quantitative receptor autoradiography

Sar^1-Ang II (Peninsula Laboratories, Belmont, CA) was iodinated by New England Nuclear (Boston, MA) to a specific activity of 2000 Ci/mmol. Brain sections containing the arcuate nucleus were preincubated for 15 min at 22°C in 10 mM sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na_2EDTA, 0.005%

bacitracin, and 0.2% proteinase-free BSA, and then incubated for 120 min in fresh buffer containing 5×10^{-10} M [^{125}I]Sar^{1}-Ang II (total binding). Nonspecific binding was determined in consecutive sections by incubation in the presence of 5×10^{-6} M unlabeled Ang II. After incubation, sections were washed four times in ice-cold 50 mM Tris-HCl, pH 7.4, followed by a dip in ice-cold distilled water and dried under air. The dry, labeled sections were exposed for several days to Hyperfilm-^{3}H (Amersham, Arlington Heights, IL) in X-ray cassettes. Films were developed with D19 Kodak developer (Eastman, Kodak, Rochester, NY) for 4 min at 4ºC and fixed in a Kodak fixer (Eastman, Kodak) for 4 min at 22ºC. [^{125}I]Sar^{1}-Ang II binding was quantified by measuring optical densities in the autoradiograms by computerized microdensitometry using the public domain National Institutes of Health Image program (developed at National Institutes of Health and available on the Internet at *http://rsb.info.nih.gov/nih-image/*). Optical densities were transformed to corresponding values of femtomoles per milligram of protein (Nazarali et al., 1989).

4.5. Radioimmunoassay

Plasma estradiol and progesterone were determined by double antibody RIA using the Estradiol and Progesterone Maia KITs (Biochem Immunosystems, Serotec, Italy). The intra-assay coefficients of variation were 2.5% for estradiol and 2.5% for progesterone.

4.6. Statistics

All data were expressed as mean ± S.E.M. and the difference between two groups was tested by the Student *t* test. A Pearson's correlation analysis was performed between the plasma hormone concentrations (E_2 e P) and Ang II receptor binding in the ARC. In all cases, differences were considered significant when $P<0.05$.

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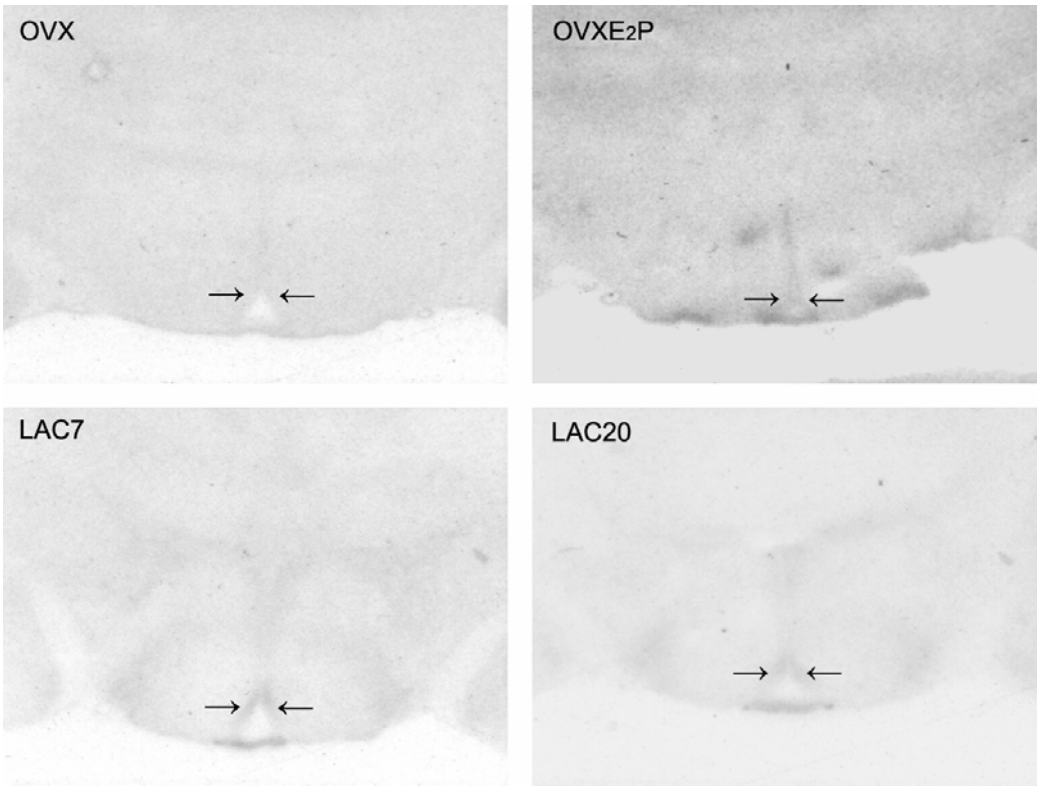


Figure 1

Figure 1. Film autoradiographs of coronal brain sections showing total Ang II receptor binding in OVX (left top), $OVXE_2P$ (right top), lactating day 7 (left bottom) and lactating day 20 (right bottom) rats. Arrows indicate specific Ang II receptor binding in arcuate nucleus in all experimental groups. Brain sections were incubated with 5×10^{-10} M [^{125}I]Sar^1-Ang II (total binding).

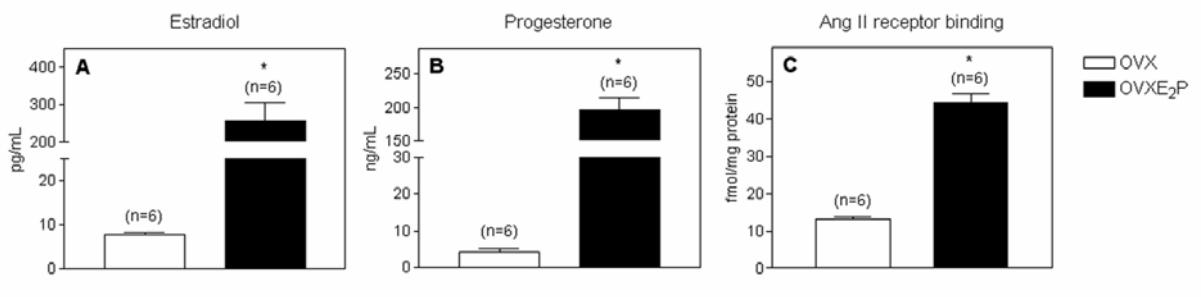
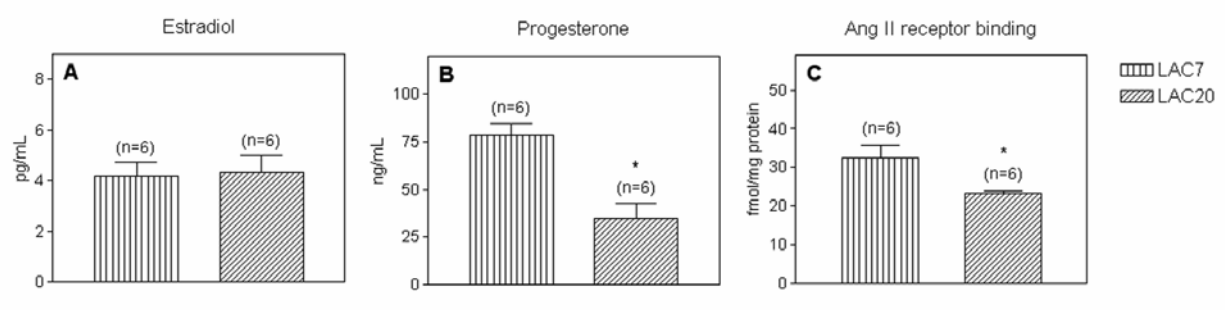

Estradiol
Progesterone
Ang II receptor binding
A
B
C
pg/mL
ng/mL
fmol/mg protein
(n=6)
*
OVX
OVXE2P

Figure 2

Figure 2. Estradiol (A) plasma concentrations (pg/mL), progesterone (B) plasma concentrations (ng/mL) and the [^{125}I]Sar^{1}-Ang II receptor binding (quantitative autoradiography expressed in femtomoles per milligram of protein) in the arcuate nucleus (C) of OVX (vehicle treated) and OVXE_2P rats. All data were expressed as mean ± S.E.M. and the difference between groups was tested by the Student *t* test. The number of animals (n) is given in parentheses. * $P<0.05$ when compared to the OVX group.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A
pg/mL
8
6
4
2
0
(n=6)
(n=6)
Progesterone
B
ng/mL
100
75
50
25
0
(n=6)
*
(n=6)
Ang II receptor binding
C
fmol/mg protein
50
40
30
20
10
0
(n=6)
*
(n=6)
LAC7
LAC20

Figure 3

Figure 3. Estradiol (A) plasma concentrations (pg/mL), progesterone (B) plasma concentrations (ng/mL) and the [^{125}I]Sar^{1}-Ang II receptor binding (quantitative autoradiography expressed in femtomoles per milligram of protein) in the arcuate nucleus (C) of lactating rats day 7 and lactating rats day 20 of lactation. All data were expressed as mean ± S.E.M. and the difference between groups was tested by the Student *t* test. The number of animals (n) is given in parentheses. * $P<0.05$ when compared to the LAC7 group.

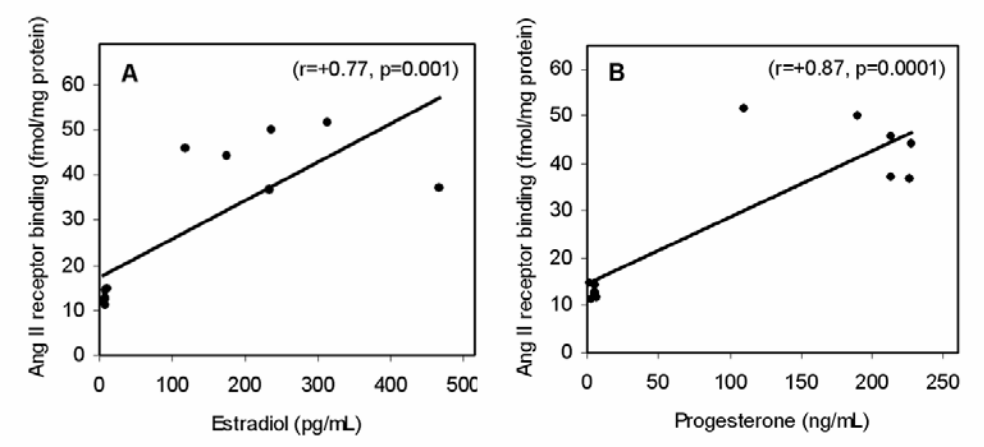
A
(r=+0.77, p=0.001)
Ang II receptor binding (fmol/mg protein)
Estradiol (pg/mL)
B
(r=+0.87, p=0.0001)
Ang II receptor binding (fmol/mg protein)
Progesterone (ng/mL)

Figure 4

Figure 4. Scattergram of the correlation between estradiol (pg/mL) (A) and progesterone (ng/mL) (B) plasma concentrations and the Ang II receptor binding (fmol/mg protein) in the ARC of $OVXE_2P$ rats. A Pearson's correlation analysis was performed in both cases.

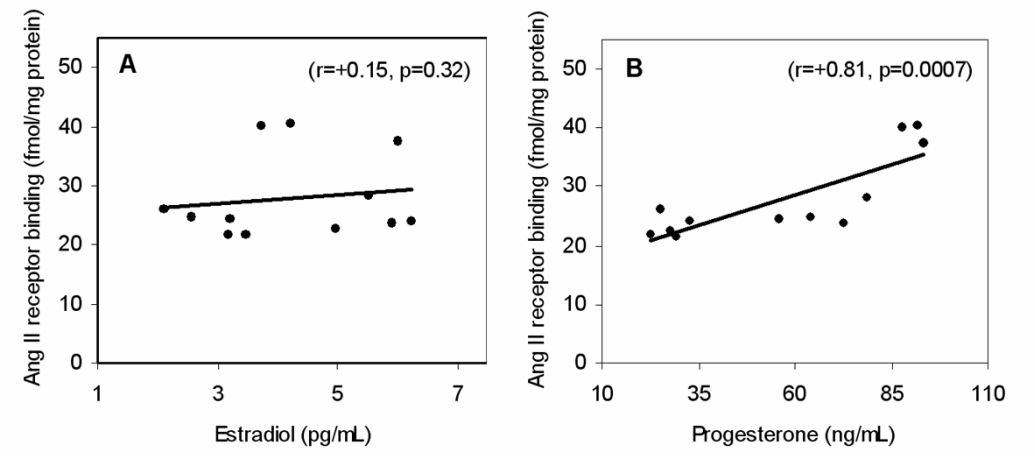
A
(r=+0.15, p=0.32)
Ang II receptor binding (fmol/mg protein)
Estradiol (pg/mL)
B
(r=+0.81, p=0.0007)
Ang II receptor binding (fmol/mg protein)
Progesterone (ng/mL)

Figure 5

Figure 5. Scattergram of the correlation between estradiol (pg/mL) (A) and progesterone (ng/mL) (B) plasma concentrations and the Ang II receptor binding (fmol/mg protein) in the ARC of lactating rats. A Pearson's correlation analysis was performed in both cases.

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Research Report

Angiotensin II receptors are upregulated by estradiol and progesterone in the locus coeruleus, median preoptic nucleus and subfornical organ of ovariectomized rats

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Abstract

Angiotensin II (Ang II) receptors in specific brain areas and in the anterior pituitary are controlled by reproductive hormones. Since Ang II also plays a role in controlling reproductive functions, such as luteinizing hormone and prolactin secretion, the objective of the present study was to evaluate the regulation of Ang II receptors by estradiol (E_2) and progesterone (P) in areas of the brain involved in homeostatic and reproductive functions, such as the locus coeruleus (LC), median preoptic nucleus (MnPO) and subfornical organ (SFO). Adult female rats were ovariectomized under anesthesia and divided into 2 groups after 2 weeks: OVX plus E_2/P replacement ($OVXE_2P$) and OVX plus oil vehicle (OVX). E_2 was injected for 3 consecutive days followed by an injection of P on the 4th day. Animals were killed by decapitation and the brains were removed and frozen. Consecutive coronal brain sections were cut in a cryostat and Ang II receptors were quantified by autoradiography in the MnPO, LC and SFO. Treatment of OVX rats with E_2 and P induced a significant increase in the Ang II receptor binding (fmol/mg protein) in the MnPO (OVX: 4.48 ± 0.58 and $OVXE_2P$: 9.89 ± 1.65), LC (OVX: 2.72 ± 0.37 and $OVXE_2P$: 8.03 ± 0.9) and SFO (OVX: 5.45 ± 0.66 and $OVXE_2P$: 10.73 ± 1.79) compared to OVX animals treated with the vehicle, $P < 0.05$. In conclusion, these results show that Ang II receptors are upregulated by E_2 and P in the LC, MnPO and SFO of ovariectomized rats.

Theme: Neurotransmitters, modulators, transporters and receptors
Topic: Receptor modulation, up- and downregulation

Keywords: Angiotensin II receptor; Locus coeruleus; Median preoptic nucleus; Subfornical organ; Estradiol; Progesterone

1. Introduction

Angiotensin II (Ang II) is an octapeptide involved in functions such as blood pressure regulation, hydric balance and reproductive control through modulation of pituitary hormones [32]. It performs its functions through two different subtypes of receptors pharmacologically classified as AT_1 and AT_2 [41]. AT_1 Ang II receptors have been subdivided into two different isoforms AT_{1A}, located in brain areas involved in the pituitary function, and AT_{1B}, present in the anterior pituitary [17]. Ang II receptors are widely distributed in the central nervous system including well known angiotensynergic sites such as the subfornical

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organ (SFO), as well as the brain nucleus that participates in the control of reproductive function, such as the locus coeruleus (LC), the median preoptic nucleus (MnPO) and the arcuate nucleus (ARC) [21].

There is consistent evidence that Ang II receptor expression in specific brain areas and in the anterior pituitary is controlled by reproductive hormones. Seltzer et al. [35] showed an increased expression of dorsomedial ARC AT_1 receptors in cycling female rats on the day of estrus, which suggests modulation of brain AT_1 receptors by ovarian hormones. Treatment of estrogen-primed ovariectomized (OVX) rats with progesterone (P) upregulates Ang II receptors in selective brain areas involved in the inhibition of pituitary prolactin release, whereas estrogen treatment downregulates the expression of Ang II receptors in the anterior pituitary [6,34]. Indeed, Ang II AT_1 receptors and the expression of their mRNA are induced in the ARC of OVX rats treated with estradiol (E_2) and P. Conversely, in the absence of gonadal steroids, as in OVX rats, there is a greater expression of the receptors in the pituitary than in the ARC that shows a low expression [18].

Besides Ang II receptor modulation by reproductive hormones and its localization in areas involved in the control of reproduction, extensive evidence supports a reproductive role for Ang II, including regulation of luteinizing hormone (LH) secretion [8,40], prolactin (PRL) secretion [25,39], participation in the PRL regulation during the lactation period [7] as well as the rise of Ang II in the central nervous system prior to the LH surge during the afternoon of proestrus [13,30].

Considering Ang II participation in reproductive functions such as the control of LH and PRL release [38], as well as the modulation of specific brain Ang II receptors by gonadal steroids, the purpose of the present study was to evaluate the regulation of Ang II receptors by E_2 and P in other brain areas involved in homeostatic and reproductive functions, such as the LC, MnPO and SFO. The SFO is one of the circumventricular organs localized outside the blood–brain barrier and involved in the central regulation of cardiovascular function and fluid homeostasis [10]. The nucleus presents a high AT_1 receptor density [21] and may be a direct connection between the peripheral and central angiotensynergic systems, since it responds to stimulation by circulating Ang II [15]. Moreover, there is a wide distribution of Ang II immunoreactive fibers in the brain, including efferents from the SFO to the MnPO and thence to the paraventricular nucleus and supraoptic nucleus [22,24]. The LC, a small pontine nucleus, is a major cluster of noradrenergic neurons in the central nervous system [11] and has a broad, diffuse action on several central structures including the brain nucleus involved in the secretion of LH-releasing hormone [4]. Indeed, the LC has been implicated in the control of LH secretion, and is thus a major modulator of the hypothalamic–pituitary–gonadal axis. Electrical stimulation of the LC potentiates the release of LH induced by stimulation of the medial preoptic area (MPOA) [14]. Moreover, an electrolytic lesion of the LC decreases the noradrenaline content in the medial basal hypothalamus and blocks the pulsatile release of LH in OVX rats, preovulatory gonadotropin surges, as well as surges induced by steroids in OVX animals [1,2,12]. Pulsatile gonadotropin secretion is controlled by a large number of neurons localized in the hypothalamus. The preoptic area plays a crucial role in controlling gonadotropin secretion since it contains a high number of LH-releasing hormone positive neurons [23]. Ang II receptors are present in several nuclei of the preoptic area including the MnPO that shows AT_1 binding and a high AT_1 mRNA expression [21] and also shows LH-releasing hormone positive cells [3]. Moreover, the LC is also influenced by the central angiotensynergic system [36] and presents Ang II AT_2 receptor binding as well as the expression of its mRNA [21].

Thus, to evaluate Ang II receptor modulation by reproductive hormones in these specific brain regions, LC, MnPO and SFO, we measured Ang II receptor density by autoradiographic binding in OVX and OVX rats treated with E_2 and P.

2. Material and Methods

2.1. Animals

Adult female (180–280 g) Wistar rats were obtained from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil). Animals were housed individually in a temperature-controlled room (22 ± 1 °C) with a 12:12 h light–dark cycle (lights on at 06:00 h) and free access to food (Rodent chow-Nutrilab, Colombo, PR, Brazil) and water. All animal procedures were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [26] and the Research Committee of the University approved them.

2.2. Anesthetic

Animals were anesthetized with ketamine (Francotar, Parke-Davis, São Paulo, Brazil −100 mg/kg, i.m.) and xylasine (Rompun, Bayer, São Paulo, Brazil −50 mg/kg, i.m.) before ovariectomy.

2.3. Experimental design

Adult female rats were ovariectomized after anesthesia. Two weeks after ovariectomy, the animals were divided into two groups (OVX plus estradiol/progesterone replacement and OVX plus oil vehicle). Hormones and vehicle were injected subcutaneously. Estradiol benzoate (5 μg/rat in 0.2 mL corn oil; Benzoginoestril, Sarsa, Rio de Janeiro, Brazil) was injected for 3 consecutive days at 09:00 h and progesterone (25 mg/rat in 0.2 mL corn oil; Sigma) was injected on the 4th day at 10:00 h. On day 4 of the gonadal

steroid replacement protocol at 16:00 h, animals in both groups were killed by decapitation and the brains were immediately removed, frozen by immersion in isopentane at −30 °C and stored at −80 °C. Consecutive 20-μm-thick coronal brain sections were cut at −20 °C in a cryostat and brain regions were selected according to the Paxinos and Watson rat brain atlas [29]. Sections were thaw-mounted on gelatin-coated glass slides, dried overnight in a desiccator at 4 °C and stored at −20 °C until processed for receptor autoradiography.

2.4. Quantitative receptor autoradiography

Sar^1–Ang II (Peninsula Laboratories, Belmont, CA) was iodinated by New England Nuclear (Boston, MA) to a specific activity of 2000 Ci/mmol. Brain sections containing MnPO, LC and SFO were preincubated for 15 min at 22 °C in 10 mM sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na_2EDTA, 0.005% bacitracin and 0.2% proteinase-free BSA, and then incubated for 120 min in fresh buffer containing 5×10^{-10} M [^{125}I]Sar^1–Ang II (total binding). Nonspecific binding was determined in consecutive sections by incubation in the presence of 5×10^{-6} M unlabeled Ang II. After incubation, sections were washed four times in ice-cold 50 mM Tris–HCl, pH 7.4, followed by a dip in ice-cold distilled water and air-dried. The dry, labeled sections were exposed for several days to Hyperfilm-^{3}H (Amersham, Arlington Heights, IL) in X-ray cassettes. Films were developed with D19 Kodak developer (Eastman, Kodak, Rochester, NY) for 4 min at 4 °C and fixed in a Kodak fixer (Eastman, Kodak) for 4 min at 22 °C. [^{125}I]Sar^1–Ang II binding was quantified by measuring optical densities in the autoradiograms by computerized microdensitometry using the public domain National Institutes of Health Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Optical densities were transformed into corresponding values of femtomoles per milligram of protein [27].

2.5. Statistics

All data were expressed as mean ± SEM and the difference between two groups was tested by the Student *t* test. In all cases, differences were considered significant when $P < 0.05$.

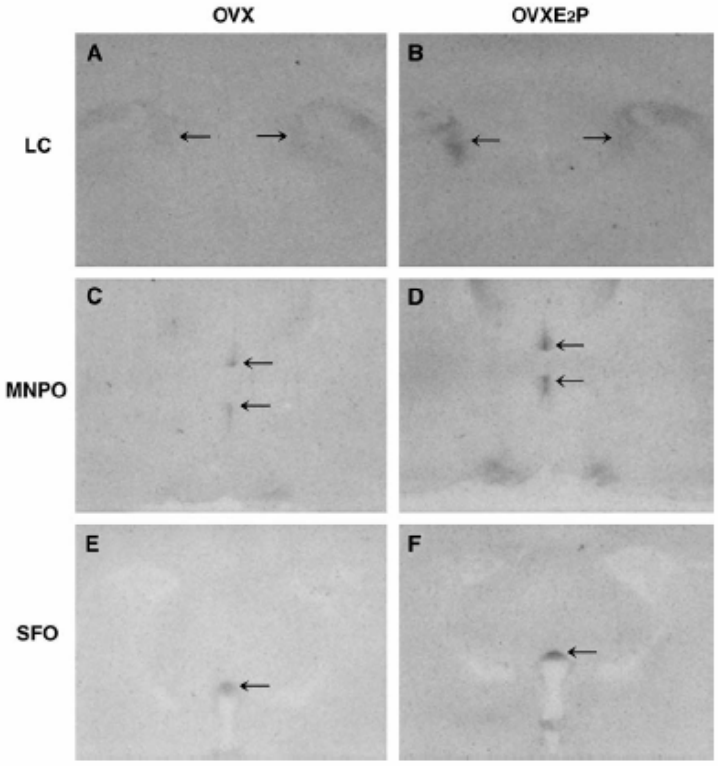

Fig. 1. Film autoradiographs of coronal brain sections showing total Ang II receptor binding in OVX (left) and $OVXE_2P$ (right) rats. Arrows indicate specific Ang II receptor binding in the locus coeruleus of OVX (A) and $OVXE_2P$ (B) animals; in the median preoptic nucleus of OVX (C) and $OVXE_2P$ (D); and in the subfornical organ of OVX (E) and $OVXE_2P$ (F). Brain sections were incubated with 5×10^{-10} M [^{125}I]Sar^1–Ang II (total binding).

3. Results

Autoradiographic analysis of tissue sections in OVX and $OVXE_2P$ animals revealed the presence of specific Ang II receptors localized in the brain areas studied, LC, MnPO and SFO. Fig. 1 illustrates the film autoradiographs of coronal brain sections showing total [^{125}I]Sar^1–Ang II receptor binding in the LC, MnPO and SFO in OVX and $OVXE_2P$ animals. Specific [^{125}I]Sar^1–Ang II receptor binding in each brain nucleus studied is indicated by an arrow and the effect of the gonadal steroid replacement is seen markedly among OVX and $OVXE_2P$ groups. Fig. 2 shows the quantitative effect of three consecutive days of E_2 treatment plus one day of P in OVX rats in the [^{125}I]Sar^1–Ang II receptor binding expressed in femtomoles per milligram of protein in the LC, MnPO and SFO. Ang II receptor binding in vehicle-treated OVX rats was low and estradiol/progesterone treatment of OVX rats produced increased Ang II receptor binding when compared to vehicle-treated OVX rats in the LC (OVX: 2.72 ± 0.37 and $OVXE_2P$: 8.03 ± 0.9), [$t(10) = 5.417$, $P < 0.05$]; MnPO (OVX: 4.48 ± 0.58 and $OVXE_2P$: 9.89 ± 1.65), [$t(10) = 3.078$, $P < 0.05$] and SFO (OVX: 5.45 ± 0.66 and $OVXE_2P$: 10.73 ± 1.79), [$t(10) = 2.753$, $P < 0.05$]. The LC showed the most pronounced effect with a threefold increase in [^{125}I]Sar^1–Ang II receptor binding after E_2 and P replacement in OVX animals.

4. Discussion

The results obtained in the present study confirm previous observations of the presence of Ang II receptors in the brain areas studied, LC, MnPO and SFO [21,24,31]. We have also demonstrated that treatment of OVX rats with E_2 and P upregulates Ang II receptors in the LC, MnPO and SFO. This effect is similar to the one previous described for the ARC, where E_2 and P also induced an upregulation of the Ang II receptors, conversely to the decrease seen in the pituitary after the gonadal steroid treatment [18]. Earlier evidence indicated that alterations in the balance of reproductive hormones resulted in changes in the activity of brain Ang II system [32,38]. The number of Ang II AT_1 receptors changes during the estrous cycle in the dorsomedial ARC, with the highest Ang II receptor binding found in the estrus [35]. On the other hand, Kisley et al. [19] showed that treatment of OVX rats for 2 consecutive days with E_2 resulted in decreased AT_1 receptor binding in the SFO. However, the hormone treatment paradigm utilized in this experiment is different from the regimen we used, since E_2 alone was injected and we used E_2 and P. As such, we found upregulation in all three areas studied, including the SFO, as was previously demonstrated for the ARC [18] where increased Ang II receptor binding and mRNA expression were seen in OVX rats that were also treated with E_2 and P. Although no further conclusions are possible from the present experiments, apparently, in the central nervous system, E_2 decreases Ang II receptor binding [19], while treatment with both E_2 and P, as demonstrated by the present results, would increase the Ang II receptor density. Considering that E_2 promotes the expression of P receptors [33,42] and that a P role for the expression of Ang II receptors in the brain was also suggested [18,35], we believe that E_2 is crucial to induce the P receptor and that P would be

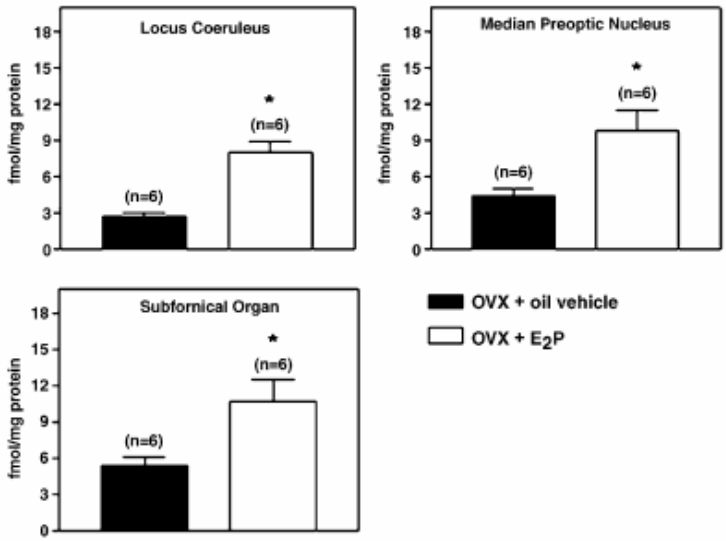

Fig. 2. Effect of estradiol and progesterone treatment of OVX rats on the [^{125}I]Sar^1–Ang II receptor binding in the locus coeruleus, median preoptic nucleus and subfornical organ. Each bar represents the quantitative autoradiography expressed in femtomoles per milligram of protein mean ± SEM. The number of animals (n) is given in parentheses. *$P < 0.05$ when compared to the OVX group in the same brain area.

responsible for inducing the expression of Ang II receptors in the brain. However, further studies should be conducted to answer the question.

It is well-known that the LC, a major cluster of noradrenergic neurons in the central nervous system, is implicated in stress-related responses. LC neurons increase their activity dramatically in response to certain stressful stimuli and are involved in both conduction of stress signals to forebrain areas and in the organization of stress responses [28]. On the other hand, Ang II is a major stress hormone that increases in the plasma as well as in the central nervous system in response to stress stimulation [43]. Stress increases the density of Ang II binding sites in the hypothalamic paraventricular nucleus and the SFO of rats [5] and also increases renin activity [16]. Indeed, in lactating and steroid-primed OVX rats, the Ang II receptors in the ARC mediate the stress-induced reduction of prolactin secretion [7]. Considering that a number of behaviors have been shown to be influenced by the stage of the estrous cycle and also by stressful stimuli, our present results that demonstrate the Ang II receptor modulation by ovarian hormones in the LC could contribute to the hypothesis that these receptors participate in the modulation of stress-induced responses in female rats with different sexual hormone backgrounds.

It is already known that Ang II controls PRL release and that ovarian steroids modulate the expression of its receptor in the brain areas involved in this function [18]. Our results indicating that Ang II receptors are induced in the LC by E_2 and P may suggest that Ang II could participate in the regulation of LH secretion, since the LC is considered a major modulator of the hypothalamic–pituitary–gonadal axis and has been implicated in the control of LH secretion [1,2,12,14]. Indeed, intracerebroventricular Ang II injection can influence the LH secretion in a function that depends on the gonadal steroids [37,40]. Moreover, our results also show that E_2 and P induced Ang II receptors in the MnPO, which is a nucleus involved in hydric balance control that also shows LH-releasing hormone positive cells [3].

Reproduction in the female imposes heavy demands on the body fluids to meet the needs of the developing fetus in utero and to support lactation after parturition [10]. Ovarian steroids are involved in the regulation of Ang II-induced water intake in the rat [20] and variations in drinking behavior were also demonstrated during the estrous cycle [9]. Indeed, SFO and MnPO are considered the most important sites for the Ang II action that controls water intake and sodium appetite [10] and our results indicate that E_2 and P can act to induce Ang II receptors in both brain regions, which contributes to the hypothesis of an Ang II modulation of drinking behavior during different reproductive stages.

In conclusion, these results show that treatment of OVX rats with E_2 and P upregulates Ang II receptors in brain areas other than the ARC, such as the LC, MnPO and SFO. The study provides a contribution to understanding the different Ang II actions in the female estrous cycle, as well as during different reproductive stages.

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CAPÍTULO IV

Effect of acute stress in the day of proestrus on sexual behavior and ovulation in female rats: participation of angiotensinergic system.

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Effects of acute stress on the day of proestrus on sexual behavior and ovulation in female rats: participation of the angiotensinergic system.

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Short title: Effects of acute stress on the day of proestrus

Key Words: stress, ovulation, sexual behavior, female rats, estrous cycle, angiotensin II, LH, progesterone, prolactin.

Abstract

Physical or emotional stress can affect female reproductive physiology and behavior. Angiotensin II (Ang II) is a hormone that participates in the stress response and also in the control of reproductive hormones. The present study aimed at evaluating the effects of acute stress in the morning and afternoon of proestrus on the sexual behavior and ovulation in female rats and the participation of Ang II in the stress-induced effects. Adult female Wistar rats with regular estrous cycles were used. Different stress protocols were tested in the morning and in the afternoon of proestrus: restraint stress 10 min; restraint stress 1 h and ether stress, respectively. Different animals were used in each stress group tested. The participation of Ang II in the effects of stress in the morning and in the afternoon of proestrus was evaluated by injecting Ang II receptor antagonists (losartan and PD 123319) 15 min before stress stimulation. The lordosis quotient (lordosis/mounts) was recorded and the number of oocytes was counted in the morning of estrous. Blood samples were collected to test the effects of stress in the morning of proestrus on plasma hormones: luteinizing hormone (LH), progesterone (P), prolactin (PRL) and corticosterone. All types of stress in the morning of proestrus induced a reduction in the number of oocytes, but no difference was found in the lordosis quotient. Restraint stress for 1 h in the afternoon of proestrus induced a significant reduction in the lordosis quotient, but no effect on the number of oocytes was found. Peripheral and central losartan, but not PD 123319, injections partially reverted the effects of stress in the morning of proestrus on ovulation. Ang II antagonists had no effect on stress-induced reduction in the lordosis behavior. Acute stress in the morning of proestrus also reduced LH, P and PRL surges later on the same day. The reduction in LH and P was mediated by Ang II AT_1 receptors. In conclusion, acute stress on the day of proestrus can affect female reproductive physiology.

Moreover, the angiotensinergic system, through AT_1 receptors, participates in the effects of acute stress in the morning of proestrus.

Introduction

The reproductive cycle in the female is a recurring set of events that culminates in the ability to ovulate eggs, mate and achieve fertilization (Schwartz 2000). The preovulatory gonadotropin surge in the proestrus is a key event for ovulation. This process depends on both the positive-feedback actions of preovulatory estradiol (E_2) secretion and specific neural signals to initiate the surge (Levine 1997). Indeed, the preovulatory luteinizing hormone (LH) surge in the proestrus is preceded by important events such as an increased LH-releasing hormone (LHRH) secretion (Levine 1997), an increased noradrenaline turnover rate in hypothalamic areas containing LHRH-releasing cells (Rance & Barraclough 1981), as well as an angiotensin II (Ang II) rise in the central nervous system prior to the LH surge in the afternoon of proestrus (Ghazi *et al.* 1994). Besides controlling ovulation, the ovarian steroid hormones E_2 and progesterone (P) have profound modulatory influences on neural circuits that regulate sexual behavior (Mani 2001, Flanagan-Cato *et al.* 2001). During the estrous cycle, sexual behavior depends on an increase in serum E_2 followed by an increase in P concentration. Indeed, administration of E_2 followed by P to ovariectomized rats induces the full complement of proceptive behaviors, while treatment with E_2 alone can induce only some aspects of sexual behavior (Mani 2001, Auger 2001).

It is well known that reproductive functions such as LH secretion and sexual behavior can be affected by stressful experiences (Wingfield & Sapolsky 2003, Plas-Roser & Aron 1981). Stress activates the hypothalamic-pituitary-adrenocortical (HPA) axis and disrupts the hypothalamic-pituitary-gonadal (HPG) axis, leading to suppressive effects on female reproductive physiology and behavior (Wingfield & Sapolsky 2003, Rivier & Rivest 1991). The suppressive effect of stress on the HPG axis, especially in chronic stress models, is believed to be due primarily to the influence of elevated levels

of corticotropin-releasing hormone (CRH) and glucocorticoids (Calogero *et al.* 1998). Women submitted to intense exercise, which is considered physiological stress, present many reproductive abnormalities including delayed menarche, amenorrhea and infertility (Warren & Perlroth 2001). Indeed, stress-induced activation of the HPA axis is associated with the stimulation of LH release in estrogen-treated women (Puder *et al.* 2000). Although it is well established that chronic stress affects reproductive functions such as the LH secretion, the effects of acute stress remain controversial. Acute stress can elicit variable patterns of LH release, for instance increased, unaltered or decreased circulating LH levels, depending on the paradigm and the magnitude of the stress (Briski 1996).

One of the major stress hormone, Ang II increases in the plasma as well as in the central nervous system in response to stress stimulation (Yang *et al.* 1996). Stress increases the density of Ang II binding sites in the hypothalamic paraventricular nucleus and the subfornical organ of rats (Castren & Saavedra 1988), and also increases renin activity (Jindra & Kvetnansky 1982). Moreover, it is well established that Ang II is involved in the control of reproductive functions, including the regulation of LH secretion. Intracerebroventricular (ICV) infusion of Ang II can either suppress or facilitate the release of LH, with the direction of the LH response determined by the gonadal steroid background of the animal (Steele *et al.* 1985). Indeed, Ang II seems to have a crucial role in the mechanisms involved in ovulation, since there is a physiological rise of Ang II in the central nervous system prior to the LH surge during the afternoon (between 1200 and 1330 h) of the proestrus (Ghazi *et al.* 1994). Thus, Ang II also seems to have a stimulatory effect on LH secretion on the day of proestrus, since treatment with the Ang II receptor antagonist saralasin has been shown to reduce LH release and inhibit ovulation (Steele *et al.* 1983).

Considering the controversial results of acute stress on LH secretion and the evidence that activation of HPA axis can disrupts HPG axis, leading to suppressive effects on female reproductive physiology, the present study aimed at testing the effects of different acute stress paradigms on the day of proestrus on ovulation, sexual behavior and reproductive hormones release, such as LH, P and PRL. Moreover, we studied the participation of the angiotensinergic system on the stress-induced effects, since Ang II is a major stress hormone and also influences LH secretion and ovulation.

Materials and Methods

Animals

Adult female (180-280 g) Wistar rats were obtained from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil). Animals were housed individually in a temperature-controlled room (22 ± 1°C) with a 12:12 h light-dark cycle (lights on at 0600 h) and free access to food (Rodent chow – Nutrilab, Colombo, PR, Brazil) and water. All animal procedures were carried out in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and the Research Committee of the University approved them.

Experiment 1: Effects of acute stress in the morning and in the afternoon of proestrus on sexual behavior and number of oocytes.

Adult female rats with 3 regular estrous cycles were submitted to acute stress in the morning (1000 h) or in the afternoon (1600 h) of proestrus. The animals were submitted to one of the 3 different stress paradigms tested: restraint stress 10 min, restraint stress 1 h and ether stress 1 min. Different animals were used in each stress paradigm group tested. In the evening of proestrus (from 2000 to 2100 h) sexual

behavior was recorded for 15 min and the total number of lordosis and the total number of mounts and intromissions were analyzed in all experimental groups. In the morning of estrus (9000 h) animals were decapitated, the ovaries removed and the oviduct dissected and squashed between two microscope slides. The number of oocytes of both oviduct ampullae was counted under the microscope (Zeiss, Goettingen, Germany) with a 2·5 × lens.

Experiment 2: Participation of the angiotensinergic system in the effects of acute stress in the morning of proestrus on the number of oocytes.

Adult female rats with 3 regular estrous cycles were used in all cases. In order to test the participation of the angiotensinergic system animals were submitted to peripheral or central Ang II antagonist injections. Intraperitoneal injections were performed 15 min before stress according to the following experimental groups: control (no stress and no injection); saline (0.9%) injection, no stress; saline + stress; PD 123319 (3 mg/Kg) + stress and Losartan (10 mg/Kg) + stress. In order to perform central injections animals had a guide cannula implanted into the right lateral ventricle by stereotaxic surgery. In the third regular estrous cycle after the surgery, animals were submitted to an intracerebroventricular (ICV) injection 15 min before stress according to the following experimental groups: control (no surgery and no stress); surgery, no stress; surgery + stress; saline ICV (0.9%) + stress and Losartan ICV (5 μg/2 μl) + stress. In all cases the stress paradigm used in this experiment was restraint 1 h in the morning (1000 h) of proestrus. In the morning of estrus (9000 h) animals were decapitated, the ovaries removed and the number of oocytes was counted as described in experiment 1.

Experiment 3: Effect of acute stress in the morning of proestrus on LH, P, PRL and corticosterone plasma concentrations.

In the afternoon of diestrus, between 1600 and 1800 h, the animals with three regular estrous cycles were submitted to jugular vein cannulation. At time of the experiment, a length of polyethylene tubing (PE-50) was connected to the jugular catheter, filled with heparinized saline (200 I.U. heparin/mL) and the rats allowed to remain undisturbed in their cages for an additional 30 min until the beginning of the experiment. Blood samples (0.4 mL) were collected every hour (1000–1800 h) from the morning to the afternoon of the proestrus in plastic heparinized syringes. Animals were divided into three experimental groups: control, no stress; saline + stress – animals were infused through the jugular vein with saline 0.9% 15 min before stress; Losartan + stress - animals were infused through the jugular vein with losartan (10 mg/kg) 15 min before stress. Immediately after the first blood sample was collected (1000 h) animals were submitted to restraint stress for 1 h, except for the control group where animals remained undisturbed in their cages. The second blood sample was collected immediately after the stress period (1100 h). After each blood sample was taken, 0.4 mL 0.9% NaCl was injected to replace the volume removed. The blood samples were centrifuged and plasma was separated and stored frozen at -80°C until assayed for LH, P, PRL and corticosterone.

Experiment 4: Participation of the angiotensinergic system in the effects of acute stress in the afternoon of proestrus on the sexual behavior.

Adult female rats with 3 regular estrous cycles were used in all cases. Intraperitoneal Ang II antagonist injections were performed 15 min before stress according to the following experimental groups: control (no stress and no injection);

saline (0.9%) injection, no stress; saline + stress; Losartan (10 mg/Kg) and PD 123319 (3 mg/Kg) + stress. In all cases the stress paradigm used in this experiment was restraint 1 h in the afternoon (1600 h) of proestrus. In the evening of proestrus (from 2000 to 2100 h) sexual behavior was recorded for 15 min and the total number of lordosis and the total number of mounts and intromissions were analyzed in all experimental groups.

Estrous Cycle

Vaginal smears were taken daily after the 70th day of age and only rats showing at least 3 consecutive regular 4-day estrous cycles were used.

Stereotaxic surgery

The animals were anesthetized with ketamine (Francotar, Parke-Davis, São Paulo, Brazil – 100 mg/Kg, i.m.) and xylasine (Rompun, Bayer, São Paulo, Brazil – 50 mg/Kg, i.m.) and unilateral guide-cannulae (10 x 0.6 mm) were inserted into the right lateral ventricle using a stereotaxic instrument (David Kopf) with the head at the zero point of the incisor bar. The dorsal surface of the skull was exposed and a hole (2 mm) was drilled 1.4 mm lateral to the sagittal line and 0.9 mm posterior to the bregma. The cannula was lowered to a depth of 3.2 mm below the dura mater and held in place by 2 stainless steel screws and dental cement. The coordinates used were determined in accordance with the Paxinos and Watson (1997) atlas. To determine the accuracy of lateral ventricle placement, Ang II, 50 ng in 2μL saline, was injected and the animals offered water to drink. The water intake within 5 min of the injection was presumptive evidence of the correct positioning of the cannula to allow diffusion of the Ang II to dipsogenic brain sites. Testing was performed in the home cage of the animal 72 h after the stereotaxic procedure (Steele 1990, Hogarty *et al.* 1992).

Jugular cannulation and blood sampling

The animals were anesthetized with tribromoethanol (Aldrich; 1 mL of a 2·5% solution/100 g body weight i.p.; Poletini *et al.* 2003) and a silastic cannula was inserted through the external jugular vein into the right atrium according to the technique of Harms and Ojeda (1974). Blood samples (0.4 mL) were collected in heparinized syringes with an equivalent volume of 0·9% NaCl (saline) solution replaced after each bleeding. Plasma was separated by centrifugation and stored at -80°C until the assay.

Sexual behavior

The adult females were tested with a proven breeder male in the observation cage (70 X 70 X 35 cm). During the dark period of the cycle (from 2000 to 2100 h) the rats were videotaped for 15 min and the total number of lordosis exhibited by the female as well as the total number of mounts and intromissions exhibited by the male were recorded. The lordosis quotient, which is an index of female sexual receptiveness, was calculated by dividing the number of lordosis by the number of mounts (Sodersten & Hansen 1977).

Drug administration

Losartan (DUP 753, Du Pont), an antagonist for the Ang II AT_1 receptor, and PD 123319 (Sigma), an antagonist for the Ang II AT_2 receptor, were diluted in saline. Central (ICV) injections were manually performed in a volume of 2 μL using a 10-μL Hamilton syringe connected by polyethylene tubing (PE 10) to an injecting needle that remained in the right ventricle for 1 min after injection. The injecting needle (11 x 0.3 mm) was 1.0 mm longer than the guide-cannula. The tubing and needle had been previously filled with the solution that was slowly infused.

Stress paradigms

Restraint stress consisted of placing the animals in a plastic cylinder (4.7 cm diameter and 17.7 cm large) for 10 min or 1 h according to the experimental group previously described. Ether stress consisted of placing the animals in a jar saturated with ether vapor for 1 min.

Radioimmunoassay

Plasma LH and PRL were determined by double antibody radioimmunoassay using specific kits provided by National Hormone and Peptide Program (National Institutes of Diseases Digestive and Kidney, USA). The reference preparation was LH-RP_3 and PRL RP_3. The lower detection limit was 0.05 ng/mL for LH and 0.2 ng/mL for PRL. The intra-assay coefficient of variation was 4% for LH and 3.5% for PRL. The concentration of plasma progesterone was determined by double antibody radioimmunoassay using specific kits provided by Maia (BioChem ImmunoSystems, Itália S.P.A). The lower limit for detection was 0.3 ng/mL and the intra-assay coefficient of variation was 4%. RIA for corticosterone required plasma extraction using ethanol. The antibody and standard used were provided by Sigma (USA), and the ^{3}H labeled hormone was from Amersham (USA). The lower limit for detection was 2 ng/mL and the intra-assay coefficient of variation was 5%.

Statistics

All data were expressed as mean ± S.E.M. The number of oocytes and the lordosis quotient were analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc analysis using a Newman-Keuls test for multiple comparisons. The

significance of differences of LH, P, PRL and corticosterone between groups and among the blood samples collected was determined by two-way ANOVA with repeated measures followed by the Newman-Keuls test for multiple comparisons. The areas under the curve (AUCs) for plasma LH, P, PRL and corticosterone in all groups were calculated and the significance of differences between the groups was determined by one-way ANOVA followed by the Newman-Keuls test. In all cases, differences were considered significant when $P \leq 0.05$.

Results

Experiment 1: Effects of acute stress in the morning and in the afternoon of proestrus on sexual behavior and number of oocytes.

Figure 1 shows the effects of different acute stress paradigms in the morning (1000 h) and in the afternoon (1600 h) of the proestrus on the number of oocytes and sexual behavior. One-way ANOVA revealed a significant effect for groups in the number of oocytes when stress was performed in the morning of proestrus ($F(3,53)=8.98$, $P<0.0001$). The Newman–Keuls post hoc analysis revealed that all stress paradigms tested in the morning of the proestrus (restraint 10 min, restraint 1 h and ether 1 min) caused a significant reduction in the number of oocytes when compared to the control group. No difference was detected in the lordosis quotient when stress was applied in the morning of the proestrus ($F(3,53)=1.528$). In the afternoon of the proestrus no significant difference was detected in the number of oocytes when animals were submitted to either restraint 10 min, restraint 1 h or ether 1 min ($F(3,58)=2.043$). However, one-way ANOVA revealed a significant effect for groups in the lordosis quotient when stress was performed in the afternoon of proestrus ($F(3,58)=3.243$, $P=0.02$). Restraint for 1 h in the afternoon of the proestrus induced a significant

reduction in the lordosis quotient when compared to the control group and no difference was detected when animals were submitted to restraint 10 min or ether 1 min in the afternoon of the proestrus.

Experiment 2: Participation of the angiotensinergic system in the effects of acute stress in the morning of proestrus on the number of oocytes.

Figure 2 shows the effect of peripheral Ang II antagonist injections on the effects of restraint (1 h) stress in the morning of proestrus on the number of oocytes. One-way ANOVA revealed a significant effect for groups ($F(4,44)=39.85$, $P<0.0001$) and the Newman–Keuls post hoc analysis revealed that restraint stress induced a significant reduction in the number of oocytes when saline+stress was compared to both control and saline, no stress. Injection of the Ang II AT_1 antagonist losartan prior to the stress partially reverted the stress-induced reduction in the number of oocytes. Significant differences were seen when the losartan group was compared to the control group, saline, no stress, saline+stress and PD+stress. Injection of the Ang II AT_2 antagonist PD alone had no significant effects on the stress-induced reduction in the number of oocytes. Figure 3 shows the effect of centrally injected losartan on the effects of restraint (1 h) stress in the morning of the proestrus on the number of oocytes. One-way ANOVA revealed a significant effect for groups ($F(4,42)=29.40$, $P<0.0001$) and the Newman–Keuls post hoc analysis revealed that ICV injection of losartan partially reverted the stress-induced reduction in the number of oocytes. Significant differences were found when the losartan group was compared to the control group, surgery, no stress, surgery+stress and saline+stress group.

Experiment 3: Effects of acute stress in the morning of proestrus on LH, P, PRL and corticosterone plasma concentrations.

Figure 4 shows the effect of peripheral Ang II antagonist losartan injection on the effects of 1 h restraint stress in the morning of proestrus on LH, P, PRL and corticosterone plasma concentrations. Significant main effects for group ($F(2,27)=11.30$, $P=0.0002$), time of day ($F(8,216)=37.34$, $P<0.0001$) and interaction among groups and time of day ($F(16,216)=5.69$, $P<0.0001$) were detected for LH plasma concentrations (Fig. 4A). *Post hoc* analysis revealed an increase in LH plasma concentration in the control and losartan+stress groups at 1600, 1700 and 1800 h when compared to previous time (from 1000 to 1500 h). A significant decrease was seen in saline+stress animals when compared to control and losartan+stress at 1600, 1700 and 1800 in the afternoon of the proestrus. The AUC of LH is shown in Fig 4B and saline+stress group had a significantly smaller AUC ($F(2,27)=10.61$, $P<0.0001$) than control and losartan+stress groups.

Figure 4C shows P plasma concentrations in the proestrus. Significant main effects for group ($F(2,25)=3.81$, $P=0.03$), time of day ($F(8,200)=9.57$, $P<0.0001$) and interaction among groups and time of day ($F(16,200)=3.77$, $P<0.0001$) were detected. *Post hoc* analysis revealed a significant increase in P plasma concentrations at 1100 h in saline+stress and losartan+stress groups when compared to the time 1000 h in the same group. A significant increase in P plasma concentrations was seen in the afternoon of the proestrus in control and losartan+stress animals when times 1700 and 1800 were compared to times from 1300 to 1500 h in the same group. Saline+stress group had a significant decrease in P plasma levels when compared to control animals at time 1800

h. The AUC of P showed a significant decrease ($F(2,25)=6.926$, $P<0.004$) in the saline+stress group when compared to the control and losartan+stress groups (Fig. 4D).

Figure 4E shows PRL plasma concentrations on the day of proestrus. Significant main effects for group ($F(2,28)=7.26$, $P=0.002$), time of day ($F(8,224)=31.82$, $P<0.0001$) and interaction among groups and time of day ($F(16,224)=1.91$, $P=0.02$) were detected. *Post hoc* analysis revealed that PRL plasma concentration in the control group was higher from 1400 until 1800 h compared with the previous times. Saline+stress and losartan+stress groups also showed a significant increase in PRL plasma concentration when time 1500 h was compared to previous times (from 1000 to 1300) in the same group. However, a significant decrease was seen when saline+stress and losartan+stress groups were compared to the control group at time 1600 h. The AUC of P is shown in Fig. 4F. The saline+stress group had a significantly smaller AUC ($F(2,28)=7.386$, $P<0.002$) compared to the control and losartan+stress groups.

Figure 4G shows corticosterone plasma concentrations in the proestrus. Significant main effects were detected for group ($F(2,28)=4.44$, $P=0.02$) and time of day ($F(8,224)=14.95$, $P<0.0001$). Newman-Keuls *post hoc* analysis revealed significant differences between control and saline+stress groups and between losartan+stress and saline+stress groups. Significant differences were also detected when times 1000 to 1200 h were compared with times from 1300 to 1800 h in the proestrus. However, no significant interaction among groups and time of day ($F(16,224)=1.44$,) was detected. The AUC of corticosterone was significantly higher ($F(2,28)=4.354$, $P<0.02$) in saline+stress group when compared to control and losartan+stress groups (Fig. 4H).

Experiment 4: Participation of the angiotensinergic system in the effects of acute stress in the afternoon of proestrus on the sexual behavior.

Figure 5 shows the effect of peripheral Ang II antagonist injections on the effects of restraint (1 h) stress in the afternoon of proestrus on the lordosis quotient. One-way ANOVA revealed a significant effect for groups ($F(3,55)=13.56$, $P<0.0001$) and the Newman–Keuls post hoc analysis revealed that restraint stress induced a significant reduction in the lordosis quotient when saline+stress was compared to both control and saline, no stress. Co-injection of Ang II AT_1 antagonist losartan and Ang II AT_2 antagonist PD 123319 prior to the stress had no significant effects on the stress-induced reduction in the lordosis quotient.

Discussion

The study provides evidence that acute stress on the day of proestrus can affect sexual behavior and ovulation, since it demonstrates a reduced number of oocytes when animals were submitted to acute stress in the morning of proestrus and a decreased lordosis quotient when stress was applied in the afternoon of proestrus.

It is well known that chronic stress has a suppressive effect on the HPG axis (Rivier & Rivest 1991, Calogero *et al.* 1998, Wingfield & Sapolsky 2003). However, effects of acute stress, especially on the day of proestrus, are poorly understood. Roozendaal *et al.* (1995) demonstrated that restraint stress from 5 to 7 h on the day of proestrus resulted in a strong inhibition of the LH surge, as well as a complete absence of ovulation. Our results showed that different paradigms of stress applied in the morning of proestrus can induce a reduction in the number of oocytes, since both 10 min and 1 h restraint stress, as well as 1 min ether stress, significantly reduced ovulation. However, when animals were submitted to stress in the afternoon of the

proestrus (1600 h), at the time of the LH surge, no significant differences were found in the number of oocytes in all types of stress tested.

Ovulation is initiated by a gonadotrophin surge on the afternoon of proestrus with the preovulatory levels of LH playing a crucial role in this mechanism (Greig & Weisz 1973, Ishikawa 1992). Our results showed a decreased LH surge in the afternoon of proestrus in animals submitted to 1 h restraint stress in the morning of proestrus, which explains the decreased number of oocytes found in these animals. Considering that the magnitude of the LH surge is considerably larger than needed to cause ovulation (Greig & Weisz 1973), the reduced LH surge demonstrated when animals were submitted to stress was able to induce ovulation, although with a significant reduction in the number of oocytes.

Acute stress can elicit variable patterns of LH release, such as increased, unaltered or decreased circulating LH levels, depending on the paradigm and the magnitude of the stress (Briski 1996). We have found no difference in the LH plasma concentration after 1 h restraint stress in the morning of proestrus, but significant reduction in the afternoon of proestrus, which indicates the stress-induced influence on mechanisms responsible for the LH surge in the afternoon of proestrus rather than an acute effect on the LH plasma concentration. On the other hand, it has been shown that restraint stress (1 h) could inhibit the pulsatile LH secretion in ovariectomized Wistar rats (Li *et al.* 2004). Considering the importance of pulsatility in the LH secretion, the disruption in the LH pulses could also play a role in the decreased LH secretion and ovulation seen in our results.

Preovulatory LH surge in the proestrus is preceded by major events such as an increased LHRH secretion (Levine 1997), an increased noradrenaline turnover rate in hypothalamic areas containing LHRH-releasing cells (Rance & Barraclough 1981), as

well as an Ang II rise in the central nervous system prior to the LH surge (Ghazi *et al.* 1994, Phillips *et al.* 1992). Our results show that previous injection of losartan, an Ang II AT_1 antagonist, partially restored the stress-induced decrease in the number of oocytes, showing evidence of the participation of the angiotensinergic system. It is well known that Ang II is a major stress hormone that increases in the plasma as well as in the central nervous system in response to stress stimulation (Yang *et al.* 1996) and that renin activity is also increased after stress (Jindra & Kvetnansky 1982). Considering that there is a physiological rise of Ang II in the cerebrospinal fluid and interstitial fluid from the preoptic area that occurs between 1200 and 1330 h in proestrus (Ghazi *et al.* 1994) and that the blockage of central Ang II receptors in the afternoon of proestrus with saralasin, a non-specific Ang II receptor antagonist, attenuates the LH surge and inhibits ovulation (Steele *et al.* 1983), the stress-induced reduction in the number of oocytes and LH secretion could be the result of an Ang II rise induced by stress (from 1000 to 1100 h) before the physiological rise, which could be causing an Ang II receptor desensitization (Gebke *et al.* 1998, Iglesias *et al.* 2001) and preventing the effects of the Ang peak between 1200 and 1330 h in proestrus. Our results show that either central or peripheral injection of losartan was able to prevent the stress-induced reduction on ovulation. However, considering that the Ang II rise necessary for the LH surge and the ovulation occurs in the central nervous system (Ghazi *et al.* 1994) and that losartan is able to cross the blood brain barrier (Li *et al.* 1993, Wang *et al.* 2003), we believe that an angiotensinergic central effect could be more effective to explain our results, however a peripheral effect can not be ruled out.

Another aspect to be considered is that endogenous Ang II in the brain plays an important role in the control of sympathetic-adrenomedullary system activity during stress. Losartan-pretreated (ICV) rats show significant lower plasma norepinephrine,

epinephrine and dopamine levels after immobilization stress (Jezova *et al.* 1998). Norepinephrine is known to have a suppressive effect on LH secretion through stimulation of CRH in the paraventricular nucleus (Tsukumura *et al.* 1994), and an increased dopaminergic tone can also stimulate CRH release increasing the endogenous opioid peptides and then suppressing the LHRH release (Calogero *et al.* 1998). The stimulatory effect of Ang II on the sympathetic-adrenomedullery system during stress could be another possible explanation for the effect of losartan in preventing stress-induced reduction in the number of oocytes and in LH secretion. On the other hand, corticosterone secretion in response to stress seems not to be affected by the blockage of Ang II receptors (Jezova *et al.* 1998), which was confirmed by our results showing an increase in plasma corticosterone levels after stress that is independent of the losartan pretreatment. However, we have shown a significant increase in the corticosterone AUC in the afternoon of the proestrus in saline-treated animals submitted to stress, which can also play a role in the inhibition of the HPG axis (Calogero *et a*l. 1998).

Progesterone is another major hormone for reproduction with contradictory effects regarding the ovulation process, since it has a well-established contraceptive effect and also a stimulatory effect on LH surge in the afternoon of proestrus (Zalányi 2001). We have demonstrated here that 1 h restraint stress increased P secretion independent of Ang II AT_1 receptor blockage. However, in the afternoon of proestrus animals previously exposed to stress showed a significant reduction in plasma P concentration with the participation of Ang II AT_1 receptors in this effect. Although plasma P in the afternoon of proestrus seems unnecessary for the preovulatory LH rise, since estradiol is able to induce an LH surge in ovariectomized and adrenalectomized rats (Micevych *et al.* 2003), P has an important role in the mechanism of follicular rupture (Zalányi 2001) which is necessary for the normal ovulation processes and could

play a role in our results, considering that stress in the morning of proestrus induced a significant reduction in the physiological P rise in the afternoon of proestrus.

Sexual behavior, as well as ovulation, is a crucial part of reproductive physiology that can be accessed by measuring the number of lordosis postures that the female assumes when mounted by a conspecific male. The female sexual behavior depends on ovarian steroid hormones, E_2 and P, since ovariectomized female rats present abolished sexual behavior (Mani 2001, Auger 2001). Restraint stress is also known to induce disruptive effects on lordosis behavior (Truitt *et al.* 2003, White & Uphouse 2004). Our results show that stress in the morning of proestrus had no effect on sexual behavior. However, 1 h restraint stress in the afternoon of proestrus induced a significant reduction in the lordosis quotient that was not seen when 10 min restraint or 1 min ether stress was performed. In spite of the pivotal role of P in the modulation of sexual behavior, our present results, as well as others (Puder *et al.* 2000), show that stress increases P secretion, which makes unlikely that a stress-induced effect on the P surge would explain the reduction in the lordosis quotient. However, PRL is also known to influence reproductive behaviors. Although its role in the control of female sexual behavior is quite controversial (Freeman *et al.* 2000), there is evidence showing that, in the afternoon of proestrus, suppression of the PRL surge dramatically attenuates sexual receptivity (Witcher & Freeman 1985). Stress induces a paradoxical effect on PRL secretion (Morishige & Rothchild 1974, Freeman *et al.* 2000) and when the pre-stress PRL levels are high, during the proestrus afternoon or lactation period, for instance, stress induces a marked reduction in plasma PRL (Morishige & Rothchild 1974, Donadio *et al.* 2004). Considering that the decreased lordosis quotient was seen when stress was applied at the time of the PRL surge, our results could be due to an attenuation in the physiological plasma PRL surge that is necessary for normal sexual

behavior. However, further studies should be conducted to answer the question. Although there is evidence that Ang II participates in the regulation of male sexual behavior (Breigeiron *et al.* 2002), our results show that stress-induced reduction in the lordosis quotient does not involve the peripheral angiotensinergic system, however a central effect cannot be ruled out.

In conclusion, the present results show that acute stress in the morning of proestrus induces a reduction in the number of oocytes and in the profile of several reproductive hormones, LH, P and PRL. These effects are mediated by Ang II AT_1 receptors. In the afternoon of the proestrus, 1 h restraint stress reduced sexual behavior and this effect seems not to be mediated by Ang II receptors. We may conclude that acute stress on the day of proestrus can disrupt normal female reproductive physiology.

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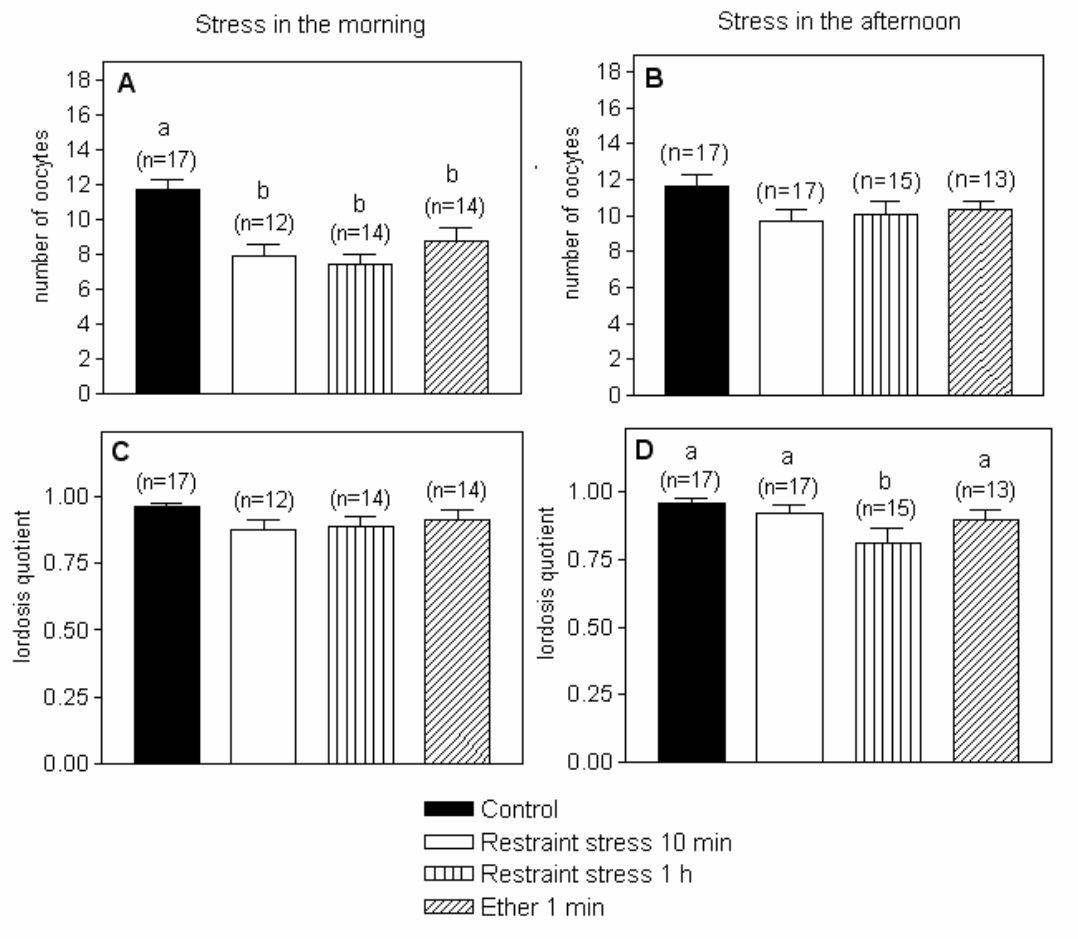
Stress in the morning
Stress in the afternoon
A
B
C
D
number of oocytes
lordosis quotient
a (n=17)
b (n=12)
b (n=14)
b (n=14)
(n=17)
(n=17)
(n=15)
(n=13)
(n=17)
(n=12)
(n=14)
(n=14)
a (n=17)
a (n=17)
b (n=15)
a (n=13)
Control
Restraint stress 10 min
Restraint stress 1 h
Ether 1 min

Figure 1

Figure 1. Effects of acute stress in the morning of proestrus on ovulation (A) and sexual behavior (C); animals were submitted to stress at 1000 h. Effect of acute stress in the afternoon of proestrus on ovulation (B) and sexual behavior (D); animals were submitted to stress at 1600 h. In all cases animals were divided into four groups: control; restraint stress for 10 min; restraint stress for 1 h; ether stress for 1 min. All data (mean ± S.E.M.) were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test (significance accepted at $P \leq 0.05$). Different letters indicate significant differences. The number of animals (n) is given in parentheses.

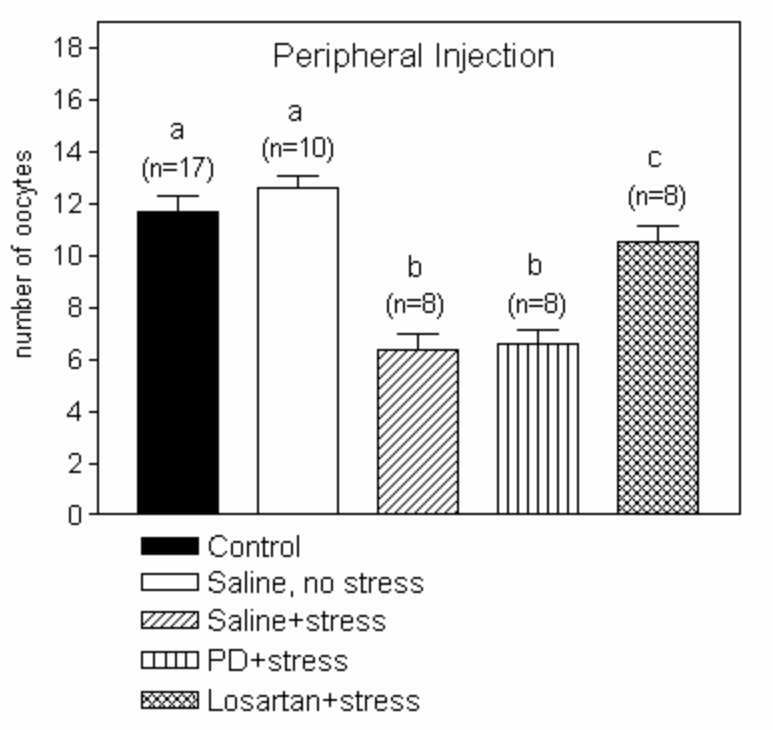
Peripheral Injection
number of oocytes
18
16
14
12
10
8
6
4
2
0
a
(n=17)
a
(n=10)
b
(n=8)
b
(n=8)
c
(n=8)
Control
Saline, no stress
Saline+stress
PD+stress
Losartan+stress

Figure 2

Figure 2. Effects of intraperitoneal injection of Ang II antagonists on the effects of acute stress in the morning of proestrus on ovulation. Animals were submitted to restraint stress for 1 h at 1000 h and pre-injected 15 min before with saline (0.9%), losartan (AT_1 antagonist, 10 mg/kg) or PD123319 (AT_2 antagonist, 3 mg/kg), according to the following groups: control; saline, no stress; saline+stress; PD+stress; losartan+stress. Data (mean ± S.E.M.) were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test (significance accepted at $P \leq 0.05$). Different letters indicate significant differences. The number of animals (n) is given in parentheses.

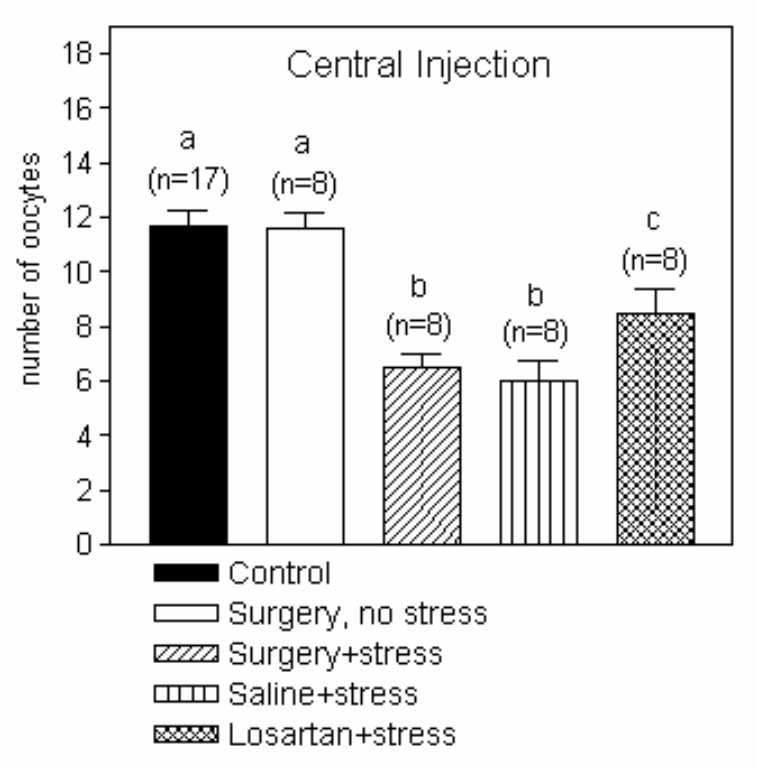
Central Injection
number of oocytes
18
16
14
12
10
8
6
4
2
0
a
(n=17)
a
(n=8)
b
(n=8)
b
(n=8)
c
(n=8)
Control
Surgery, no stress
Surgery+stress
Saline+stress
Losartan+stress

Figure 3

Figure 3. Effects of intracerebroventricular injection of the Ang II antagonist losartan on the effects of acute stress in the morning of proestrus on ovulation. Animals were submitted to restraint stress for 1 h at 1000 h and pre-injected 15 min before with saline (0.9%) or losartan (AT_1 antagonist, 5 μg/2 μl) according to the following groups: control; surgery, no stress; surgery+stress; saline+stress; losartan+stress. Data (mean ± S.E.M.) were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test (significance accepted at $P \leq 0.05$). Different letters indicate significant differences. The number of animals (n) is given in parentheses.

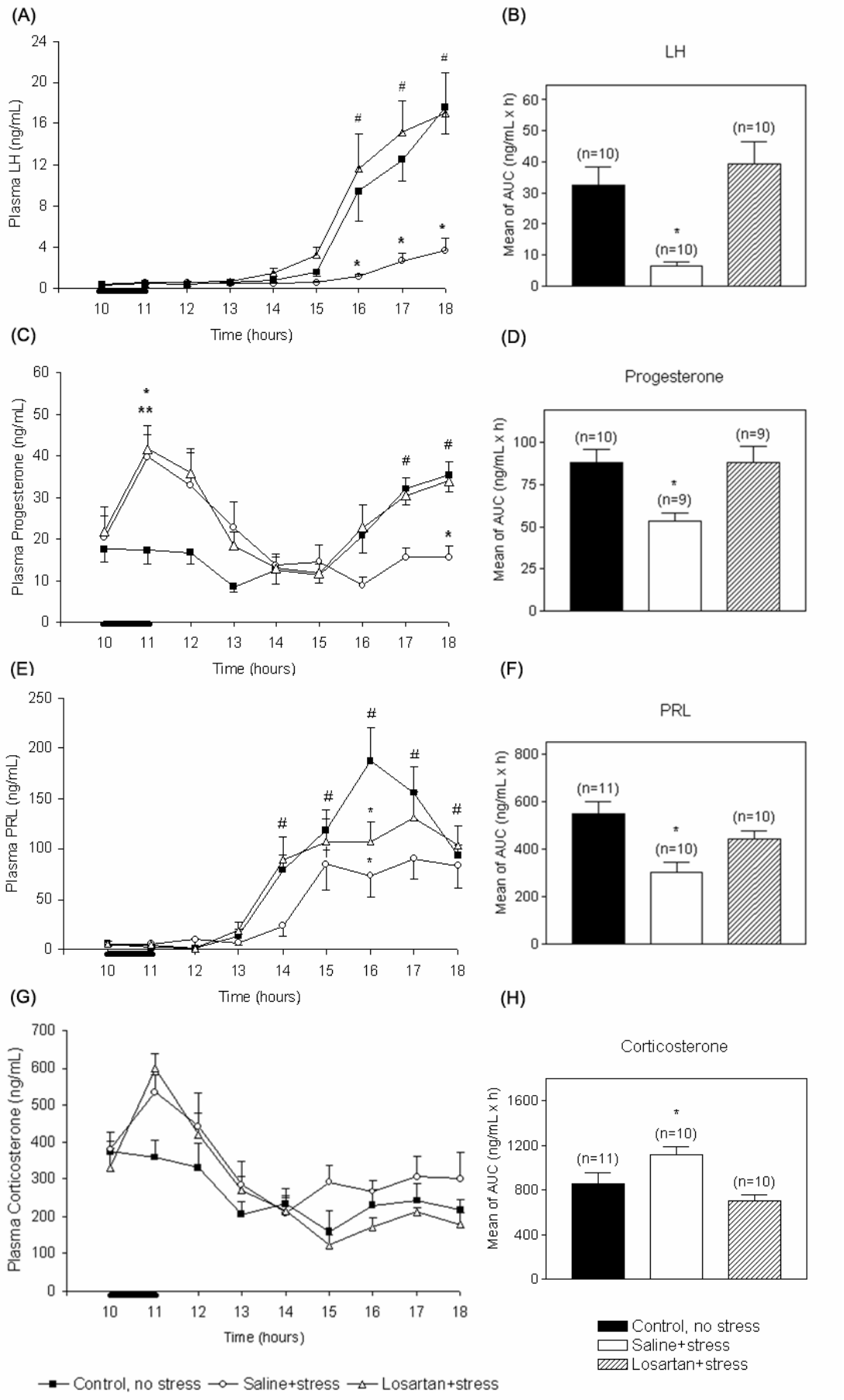
(A)
Plasma LH (ng/mL)
Time (hours)
(B)
LH
Mean of AUC (ng/mL x h)
(n=10)
(n=10)
(n=10)
(C)
Plasma Progesterone (ng/mL)
Time (hours)
(D)
Progesterone
Mean of AUC (ng/mL x h)
(n=10)
(n=9)
(n=9)
(E)
Plasma PRL (ng/mL)
Time (hours)
(F)
PRL
Mean of AUC (ng/mL x h)
(n=11)
(n=10)
(n=10)
(G)
Plasma Corticosterone (ng/mL)
Time (hours)
(H)
Corticosterone
Mean of AUC (ng/mL x h)
(n=11)
(n=10)
(n=10)
Control, no stress
Saline+stress
Losartan+stress

Figure 4

Figure 4. Effects of acute stress (1 h restraint) in the morning of proestrus on LH, P, PRL and corticosterone plasma concentrations and AUC of LH, P, PRL and corticosterone. The blood samples (0.4 mL) were collected hourly from 1000 to 1800 h from a jugular catheter. (A, C, E, G) Plasma hormone concentrations (mean ± S.E.M.); data were analyzed using two-way ANOVA with repeated measures followed by the Newman-Keuls test. (A) Plasma LH concentration. * Indicates a significant difference from control and losartan+stress groups at the same time. # Indicates a significant difference in control and losartan+stress groups from previous times in the same group. (C) Plasma P concentration. * Indicates a significant difference from control group at the same time. # Indicates a significant difference in control and losartan+stress groups compared to times 1400 and 1500 in the same group. ** Indicates a significant difference in saline+stress and losartan+stress groups compared to time 1000 in the same group. (E) Plasma PRL concentration. * Indicates a significant difference from control group at the same time. # Indicates a significant difference in control group from previous times in the same group. Black horizontal bar represents the stress period. (B, D, F, H) Each bar represents the mean ± S.E.M. for AUC hormones. Data were analyzed by one-way ANOVA followed by the Newman-Keuls test. * Indicates significant difference from control and losartan+stress groups. The number of animals (n) is given in parentheses. In all cases significance accepted at $P \leq 0.05$.

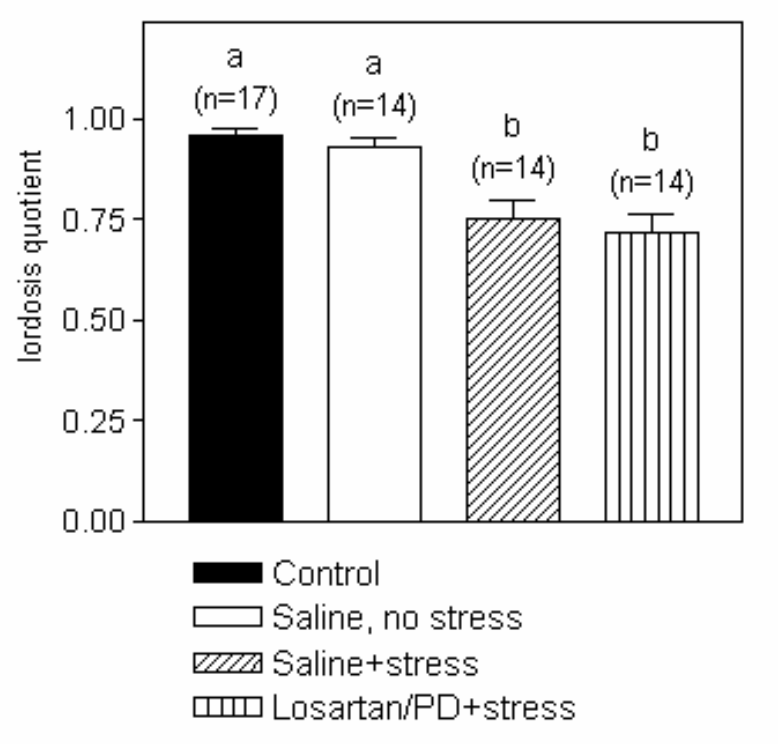
a
(n=17)
a
(n=14)
b
(n=14)
b
(n=14)
lordosis quotient
1.00
0.75
0.50
0.25
0.00
Control
Saline, no stress
Saline+stress
Losartan/PD+stress

Figure 5

Figure 5. Effects of intraperitoneal injection of Ang II antagonists on the effects of acute stress in the afternoon of proestrus on sexual behavior. Animals were submitted to restraint stress for 1 h at 1600 h in the afternoon and pre-injected 15 min before with saline (0.9%), losartan (AT_1 antagonist, 10 mg/kg) + PD123319 (AT_2 antagonist, 3 mg/kg), according to the following groups: control; saline, no stress; saline+stress; losartan/PD+stress. Data (mean ± S.E.M.) were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test (significance accepted at $P \leq 0.05$). Different letters indicate significant differences. The number of animals (n) is given in parentheses.

From: espere@subs-espere.org
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Dear Dr Sanvitto

Thank you for submitting your paper entitled “Effects of acute stress on the day of proestrus on sexual behavior and ovulation in female rats: participation of the angiotensinergic system” to Journal of Endocrinology using the Espere System.

Your paper is now with the editorial office and will shortly be assigned a manuscript number. Please use this number when referring us to your paper.
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CONCLUSÕES

Os resultados obtidos neste trabalho permitem concluir que:

O estresse agudo provoca alterações em diferentes aspectos do processo reprodutivo em fêmeas, incluindo efeitos deletérios sobre a lactação, o comportamento sexual, a geração dos picos hormonais pré-ovulatórios e a própria ovulação. Os resultados demonstram alguns desses efeitos ainda desconhecidos e apontam o sistema angiotensinérgico como um importante regulador dessas respostas.

1. Artigo I:

    1.1. O estresse agudo provoca uma redução na concentração plasmática de prolactina em ratas ovariectomizadas tratadas com estradiol e progesterona e lactantes;

    1.2. A diminuição da prolactina em resposta ao estresse é mediada pelos receptores AT_1 de Ang II no núcleo arqueado.

Estes dados confirmam a prolactina como um hormônio envolvido nas respostas do estresse e apontam a participação do sistema angiotensinérgico na regulação da secreção de prolactina em reposta a estímulos estressores em situações aonde a concentração basal deste hormônio é elevada, destacando-se, neste caso, o período da lactação.

2. Artigo II:

    2.1. Os receptores de Ang II no núcleo arqueado são modulados pelos esteróides gonadais, estradiol e progesterona, em ratas ovariectomizadas e lactantes;

2.2. Os receptores de Ang II no núcleo arqueado variam ao longo do período de lactação apresentando uma menor densidade no final deste período (dia 20);

2.3. Existe uma forte correlação entre a concentração plasmática de progesterona e a densidade de receptores de Ang II no núcleo arqueado, evidenciando que a modulação desses receptores parece ser resultado de um papel preponderante da progesterona.

Esses dados confirmam a regulação dos receptores centrais de Ang II pelos esteróides gonadais e evidenciam a progesterona como principal moduladora desta função. Além disso, a variação na densidade de receptores de Ang II ao longo do período de lactação contribui para a hipótese da participação do sistema angiotensinérgico na regulação da prolactina em diferentes estágios do processo reprodutivo.

3. Artigo III:

3.1. O tratamento de ratas ovariectomizadas com estradiol e progesterona provoca um aumento na densidade de receptores de Ang II no locus coeruleus, núcleo pré-óptico mediano e órgão subfornicial;

3.2. Da mesma forma que havia sido demonstrado para o núcleo arqueado, os resultados mostram que, no sistema nervoso central, os receptores de Ang II sofrem uma “up-regulation” pelo estradiol e progesterona.

A modulação dos receptores de Ang II pelos esteróides gonadais em áreas envolvidas com funções reprodutivas e de resposta ao estresse, coloca o sistema

angiotensinérgico como possível regulador destas funções e contribui para a hipótese da Ang II ter um papel modulador nas diferentes respostas neuroendócrinas e comportamentais ao longo dos diferentes estágios reprodutivos pelos quais passam as fêmeas.

4. Artigo IV:

4.1. O estresse agudo no dia do proestro diminui a função reprodutiva em fêmeas ciclando;

4.2. O estresse agudo na manhã do proestro provoca uma redução nas concentrações plasmáticas de hormônio luteinizante, progesterona e prolactina e uma redução na ovulação, porém não altera o comportamento sexual;

4.3. Os efeitos do estresse agudo na manhã do proestro são mediados pelos receptores AT_1 de Ang II;

4.4. O estresse agudo por contenção (1 hora) na tarde do proestro reduz o comportamento sexual, porém não provoca alterações na ovulação.

4.5. Os efeitos do estresse agudo na tarde do proestro sobre o comportamento sexual não são mediados pelo sistema angiotensinérgico.

Esse conjunto de dados indica efeitos deletérios do estresse agudo no dia do proestro, evidenciando que os mecanismos envolvidos na geração dos picos pré-ovulatórios, do comportamento sexual e da ovulação podem ser influenciados por estímulos estressores. O sistema angiotensinérgico é um componente importante na mediação de alguns desses efeitos induzidos pelo estresse agudo no dia do proestro.

PERSPECTIVAS

Com base nos resultados apresentados nesta tese, são sugeridos alguns experimentos que poderiam dar continuidade ao estudo da relação entre estresse, função reprodutiva e sistema angiotensinérgico.

- considerando que o sistema angiotensinérgico participa da mediação da resposta da prolactina ao estresse durante a lactação e que existem variações importantes da densidade de receptores de Ang II no núcleo arqueado, seria interessante avaliar a resposta da prolactina ao estresse agudo em outras fases do período de lactação;

- levando-se em consideração o potencial aplicado da Ang II como mediador da diminuição da secreção de prolactina em resposta ao estresse na lactação e considerando que o antagonista AT_1 losartan é permeável à barreira hematoencefálica, seria interessante avaliar os efeitos da injeção periférica do antagonista dos receptores AT_1 de Ang II na resposta da prolactina ao estresse em ratas lactantes e $OVXE_2P$.

- considerando a redução no pico pré-ovulatório de hormônio luteinizante e na ovulação induzida pelo estresse e a importância do pico de Ang II que ocorre ao meio dia do proestro para estes processos, seria pertinente avaliar o pico de Ang II que ocorre nesse período em ratas que são submetidas a estresse agudo na manhã do proestro.

- considerando a redução no comportamento sexual induzida pelo estresse na tarde do proestro e o importante papel regulador da prolactina neste mecanismo, seria interessante avaliar o perfil hormonal das ratas ciclando submetidas ao estresse agudo na tarde do proestro, através de coletas seriadas de sangue, com atenção especial à resposta da prolactina.

- levando-se em consideração os resultados que indicam uma modulação "tardia" da secreção de corticosterona em resposta ao estresse pelo sistema

angiotensinérgico, tendo em vista que a concentração de corticosterona é maior na tarde do proestro nas ratas estressadas tratadas com salina em comparação com as ratas tratadas com losartan, seria interessante investigar a participação da Ang II como estimulador da síntese de corticosterona, através de efeitos de transcrição gênica, em resposta a estímulos estressores.

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