Blockade of Angiotensin Receptor Subtypes in Arcuate Uterine Artery of Pregnant and Postpartum Rats

Jean St-Louis, Benoit Sicotte, Sophie Bédard, Michèle Brochu

Abstract—During pregnancy, uterine circulation undergoes hypertrophy and hyperplasia. We investigated the effects of angiotensin (Ang) II receptor subtype (AT₁/AT₂) blockade on increased responses to the peptide during reversible remodeling of the uterine vasculature in pregnant and postpartum rats. Uterine arcuate arteries were set up in wire myographs for microvessel and submitted to a tension equivalent to 50 mm Hg transmural pressure. Cumulative concentration-response curves to Ang II were measured in the absence and presence of losartan on the same vascular segment. A similar protocol was repeated in the presence of PD 123,319, an AT₂ receptor blocker, again in the absence and presence of losartan. Responses to Ang II on the arcuate artery increased markedly during pregnancy and returned to the prepregnant level within 12 days postpartum. Losartan (10⁻⁷ mol/L) produced a parallel right shift of the concentration-response curve to Ang II in all groups of tissues, but potency of the AT₁ receptor blocker was reduced at the end of pregnancy and in the early postpartum period. PD 123,319 (10⁻⁷ mol/L) significantly increased maximum response to Ang II in arterial segments of the nonpregnant, term-pregnant, and 5 days postpartum rats. AT₁ receptor expression was decreased in arcuate arteries of term-pregnant rats. These results show that contractile responses to Ang II on the uterine arcuate artery of the rat are mediated by the AT₁ receptor and that blockade of AT₂ receptors potentiated responses to the peptide. They also indicate that, in uterine vessels, AT₂ receptor stimulation interferes with Ang II responses, but this effect is decreased in uterine arcuate arteries in the peripartum period. (Hypertension. 2001;38:1017-1023.)

Key Words: angiotensin receptors, angiotensin arteries, uterine losartan pregnancy

Pregnancy in humans and rats is different in many respects, such as duration, hormonal environment (dependence on corpus luteum versus endocrine implication of the placenta), structure of the uterus, and number of fetuses. However, to accommodate the progressively increasing needs of the growing fetus(es), the uterine circulation undergoes important structural changes in both species. It is conceivable that the influences exerted on the uterine blood vessels and the mechanisms responsible for these changes in uterine vascular remodeling are similar. The increase in size of the uterine vessels, which is required to account for the huge increase in uterine blood flow, has been characterized in the rat as hypertrophy and hyperplasia, indicating that vascular remodeling may be a physiological (in contrast to pathological in vascular injuries) and reversible process. Indeed, growth of uterine vascular wall is almost completely reversible on a time basis similar to its induction. It is of major importance to understand the physiological mechanisms responsible for the changes in the uterine vasculature during pregnancy and postpartum to better understand the influences that may be targeted in pathological conditions.

Angiotensin (Ang) II is involved in many physiological and pathological processes. The development of nonpeptide inhibitors for the octapeptide has revealed the existence of 2 types of Ang II receptors: AT₁ mediates the known effects of the peptide, whereas AT₂ is still the object of intensive studies to uncover its physiological role. AT₂ receptor subtype is suspected to exert trophic effects on vascular smooth muscle cells. Moreover, AT₂ receptors appear to exert this action either directly or via the release of local active substances such as kinins or NO. We have reported that the uterine arcuate artery of the rat undergoes an increase in diameter that is manifested from the 14th day of pregnancy, reaching a maximum (almost double) at the eve of parturition. This process was half reversed by 5 days postpartum. Parallel to these changes, there was an increase in responses to Ang II and phenylephrine, which were linearly related to increased diameter of the vessels, but this was not observed with the increase responses to KCl. We postulated that remodeling of the uterine vasculature is accompanied by changes in reactivity to Ang II that are linked to receptor subtype regulation. To test this hypothesis, we measured the effects of Ang II receptor blockade (AT₁ and AT₂) on contractile responses to the peptide on isolated uterine arcuate arteries obtained during pregnancy and postpartum in the rat. The data presented herein suggest that both
receptor subtypes are involved in the regulation of the vasoconstriction induced by Ang II.

Methods
Pregnant Sprague-Dawley rats (bred in our facilities after purchase from Charles Rivers Canada, St Constant, Quebec) were used on days 14 and 22 of gestation and on days 5 and 12 postpartum. The protocol was approved by the local animal care committee.

Myotropic Responses of the Uterine Arcuate Artery
Uterine horn and attached vasculature were rapidly placed in cold oxygenated Krebs’ bicarbonate solution. An arcuate artery, at mid-point of the uterine arcade, was cleaned of adhering tissues under a stereomicroscope. A section of ~1.5 mm was isolated, and 2 tungsten wires (20 μm in diameter) were inserted into the lumen to secure the vessel to supports of a myograph system. One support was attached to a strain gauge force transducer (TRN 001, Kent Scientific), and the other was connected to a micrometer device. Force was recorded on a personal computer through data acquisition package (Workbench, Kent Scientific). The arcuate artery segment was bathed in 7 mL circulating Krebs’ bicarbonate solution ([in mmol/L] NaCl 118, KCl 4.65, NaHCO₃ 25, CaCl₂ 2.5, MgSO₄ 1.18, KH₂PO₄ 1.18, dextrose 5.5) bubbled with 95% O₂/5% CO₂; pH was 7.4. A passive length-tension relationship was generated by increasing the distance (by steps of 25 μm) between the supports of the myograph. Results were fitted to a passive exponential model that intersected the straight line of Laplace (for a pressure of 50 mm Hg) at the diameter used as passive tension. After 30 minutes of equilibration at this tension, vessels were challenged with 10⁻⁶ mol/L phenylephrine. In addition to this response, 10⁻⁷ mol/L carbachol was given to document the presence of functional endothelium. The steady state mRNA levels of AT₁ and AT₂ were measured after 10 minutes of preincubation with PD 123,319 (10⁻⁵ mol/L), an antagonist for both receptors.

RNase Protection Assay
Total cellular RNA was extracted by modification of the method of Chomczynski and Sacchi with the use of TRIzol reagent (Gibco BRL). Whole uterine vascular beds from nonpregnant and term-pregnant rats were cleaned of fat and membranes. Final RNA pellets were dissolved in an appropriate volume of 100% formaldehyde and stored at −20°C. The RNA concentration was determined from absorbance measurements at 260 nm.

A 182-base Drai fragment excised from pcDNA1 plasmid (kindly provided by Dr Kathy Griendling, Emory University, Atlanta, Ga) was used for the preparation of a high-activity RNA antisense probe for the AT₁ receptor. A 215-base HindIII-Xbal fragment excised from pcDNA1/Amp plasmid (from Gaétan Guillemette, Université de Sherbrooke, Sherbrooke, Québec, Canada) was cloned into the multiple cloning sites of the Bluescript vector, linearized with PvuII, and used for the preparation of a high-activity RNA antisense probe for the AT₂ receptor. A 334-base fragment of β-actin gene (Ambion RNA, Austin, Tex) was used as an internal control. The antisense probes were prepared by transcription in vitro with the Ambion MaxScript technique (Ambion RNA) in the presence of [α-³²P]UTP (Mandel) and T7 polymerase for AT₁, receptor, and SP6 polymerase for AT₂, and for β-actin. The radioactive probes were separated from unincorporated [α-³²P]UTP by washing with 75% ethanol/25% sodium acetate.

For the hybridization step, 25 μg of cellular RNA was combined with the antisense probes (100 000 cpm for AT₁, and AT₂, 15 000 cpm for β-actin) in a buffer containing 5× PIPES (200 mmol/L PIPES, pH 6.4), 2 mol/L NaCl, 5 mmol/L EDTA, and 80% of deionized formamide. This mix was incubated at 65°C for 10 minutes. After denaturation of RNAs (85°C, 5 minutes), hybridization was performed overnight at 47°C. Nonhybridized RNAs were digested by T1/A RNase (RNase cocktail from Ambion RNA company) in a RNase digestion buffer containing 1 mol/L Tris-HCl (pH 7.5), 0.5 mol/L EDTA, and 0.3 mol/L NaCl for 1 hour at 30°C. After treatment with 20% SDS and 10 mg/mL proteinase K for 15 minutes at 37°C, the RNAs hybridized were purified by phenol/chloroform extraction, precipitated in 100% ethanol, and resuspended in 10 μL of electrophoresis buffer containing 80% deionized formamide, 1 mmol/L EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, and 0.1% SDS. RNAs were denatured at 85°C for 5 minutes and separated by electrophoresis in a 5% acrylamide/7 mol/L urea gel at 300 V. After electrophoresis, the gel was fixed for 15 minutes in 10% methanol/5% acetic acid and dried for 1 hour. The dried gel was exposed to x-ray film (Kodak X-Omat AR5) with intensifying screens at −80°C for 1 to 7 days. Relative intensities of AT₁, AT₂, and β-actin bands were determined by analysis of the gel with Scion Image computer software (Scion Corporation, National Institutes of Health). The steady state mRNA levels of AT₁ and AT₂ were expressed in arbitrary units and standardized by comparison with results of the housekeeper gene β-actin.

Data Analysis
Concentration-response curves were analyzed by computer fitting to a 4-parameter logistic equation (Prism3, GraphPad) to evaluate the EC₅₀ (the concentration of Ang II required to produce 50% maximum response) and maximum response (E₅₀). The potency of the AT₁ receptor antagonist losartan was evaluated by the method of Furchgott and is expressed as apparent dissociation constant Kᵣ (pKᵣ). Sensitivity (pD₂, −log EC₅₀) and E₅₀ were compared by ANOVA followed by Dunnett’s test (with nonpregnant rats used as reference); except when effects of losartan were evaluated, a paired Student’s t test was used. Values were considered statistically significant when they reached at least P<0.05. Data are reported as mean±SEM along with the best-fitted curve to the mean data points.

Drugs and Chemicals
All salts used in these experiments were of analytical grade obtained from Fisher Scientific. Ang II ([Asp¹, Ile⁵]Ang II) was purchased from Peninsula Laboratories, phenylephrine hydrochloride and carbachol hydrochloride from Sigma Chemical Co, and L-NNAME from Research Biochemical International. Losartan and PD 123,319 were generously provided by Merck Frosst Canada and Parke Davis, respectively.

Results
The diameter of the uterine arcuate artery of nonpregnant rats was 118±6 μm (n=20) and increased progressively until term pregnancy to reach 183±10 μm (n=19; P<0.001). The diameter progressively regressed after delivery to reach 154±6 by 12 days. Carbachol (10⁻⁴ mol/L) tested at the plateau response of phenylephrine (1 μmol/L) induced some nonsignificant relaxation that was equivalent in all groups of vessel.

Effects of Losartan on Concentration-Response Curves to Ang II
Effects of losartan were measured in uterine arcuate arteries of nonpregnant, pregnant (14 and 22/23 days), and postpartum (5 and 12 days) rats. Figure 1 shows concentration-response curves to Ang II. Maximum responses were markedly increased in vessels of term-pregnant rats (22 days), as indicated in Figure 2A (1.64±0.09 mN/mm), whereas they were similar to those in nonpregnant animals in all other groups (0.92±0.03 mN/mm). Sensitivity to Ang II (Figure

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2B) was not statistically changed during gestation but was significantly increased in the postpartum period (EC₅₀ decreased from 1.3 to 0.61 and 0.59 nmol/L).

Losartan produced a parallel right shift of the concentration-response curve to Ang II in uterine arcuate artery of nonpregnant, pregnant (14 and 22 days), and postpartum (5 and 12 days) rats. Ordinate depicts the contractile responses (mN/mm axial length), and abscissa shows the logarithmic concentration of Ang II (mol/L). Number of vessels used is 8 to 12 from different animals.

The degree of inhibition of Ang II responses was not similar between groups, as depicted by the decrease in pD₂ of Ang II by losartan (Figure 2B). This inhibition was larger in arteries of nonpregnant and 12 days postpartum animals and smallest in vessels of the 5 days postpartum group. This is confirmed by the calculated apparent affinity constant (Kₐ) of losartan, illustrated as pKₐ in Figure 2C, which decreased in the arteries of pregnant animals to reach a significant low value.
in the early postpartum period (5 days). These results indicate that sensitivity of the AT1 receptors in the uterine arcuate artery for its specific ligand, losartan, decreased in pregnant and postpartum animals but returned toward levels observed in nonpregnant rats by 12 days postpartum. In contrast, sensitivity (pD2) to Ang II was not significantly altered by pregnancy and markedly increased in the postpartum period (Figure 2B). Losartan produced large inhibitions of Ang II responses in all 3 groups of arcuate arteries that were less pronounced in arteries of pregnant rats than in the 2 other groups (Figures 3 and 4). Indeed, the increase in EC50 in the presence of losartan was approximately 65-fold in arteries of nonpregnant and postpartum rats and only 15-fold in vessels of term-pregnant animals. In contrast to the results of Figure 1, the AT1 receptor antagonist did not produce, in the presence of AT2 receptor blockade, a parallel right shift of the concentration-response curve to Ang II in the uterine vessels of the 3 groups of vessels, as shown by significant decreases in the maximum response to Ang II (Figure 4A). Consequently, we could not evaluate any affinity constant for the AT1 antagonist.

Effects of PD 123,319 on the Concentration-Response Curve to Ang II
Figure 5 compares the results of the 2 sets of vessels in the absence and presence of PD 123,319 (Figures 1 and 3, respectively). In all groups, low concentrations of Ang II induced similar contractions of the uterine arteries in the absence and presence of the AT2 antagonist, whereas at higher Ang II concentrations (>3 nmol/L) much larger responses were measured in the presence of PD 123,319. This provides an explanation for the decreased sensitivity (Figure 4B) to Ang II in the presence of PD 123,319 compared with its absence (Figure 2B). This observation suggests that some uncoupling of AT1 stimulation to response, at least to high concentrations of Ang II, could be present in the uterine arteries in the absence of AT2 blockade. Sensitivity to Ang II was not affected in arteries of pregnant rats by PD 123,319; pD2 was 8.77±0.07 and 8.73±0.09 in the absence and presence of the AT1 antagonist, respectively (Figures 2B and 4B).

Ang II Receptor Subtype Expression in Uterine Vasculature
Ang II receptor expression was evaluated by RNase protection assay (RPA). Figure 6B shows an example of the autoradiogram. AT1 receptor mRNA was weakly detected in the RNA extracts of only nonpregnant rats, but the intensity of the signal was not sufficient for quantification. AT2 receptor steady state mRNA level was greater in the uterine vessels of nonpregnant rats than in pregnant rats (Figure 6A). This indicates that the increased responses to Ang II in arcuate arteries of term-pregnant rats cannot be attributed to increased expression of the AT1 receptor subtype.

Discussion
The present study was undertaken to determine the consequences of the structural changes of the uterine circulation
during pregnancy and postpartum on Ang II responses and the functional status of Ang II receptor subtypes. We observed that the contractile responses of the rat arcuate uterine artery are mediated by the AT1 receptors in all groups of animal studied. However, sensitivity of uterine vasculature to both Ang II and losartan appears to be modulated along with the structural changes during pregnancy and postpartum. Indeed, sensitivity to Ang II significantly increased in the arteries of postpartum rats, whereas sensitivity to losartan was significantly decreased in arteries of term-pregnant and 5 days postpartum rats. Moreover, the presence of AT2 blockade potentiated the responses to Ang II in nonpregnant, term-pregnant, and 5 days postpartum rats. In this condition, losartan did not produce a competitive inhibition of the contractile responses to Ang II. AT1 is the receptor subtype most abundantly expressed in the uterine circulation of the rat, but the steady state mRNA level of AT1 was decreased at term pregnancy. These results suggest that Ang II receptor subtypes are modulated, relative to each other, in the uterine arcuate artery during pregnancy and postpartum. Moreover, our results provide evidence that stimulation of the AT2 receptors interferes with the stimulation of AT1 by Ang II in uterine microvessels.

The present results confirm our previous findings of increased reactivity to Ang II in the arcuate arteries of term-pregnant in comparison with nonpregnant rats. Similar results were also reported in the main uterine artery, in which the effects of Ang II were similar, eg, approximately 80% of KCl responses (equimolar NaCl replacement) in uterine arteries of nonpregnant and term-pregnant rats, but KCl responses were increased in the uterine arteries of term-pregnant rats, as we have also reported. This confirms earlier results obtained for alpha-adrenergic agonists in pressurized and wire preparations of the uterine arcuate arteries. Ang II responses were also shown to be increased in the uterine artery of the pregnant rabbit. It is evident, from this study and others, that reactivity to several vasoconstrictors is increased in the uterine vasculature of term-pregnant rats, especially to Ang II. In the uterine vasculature of the sheep, Ang II was shown to be less potent when given in vivo to pregnant compared with nonpregnant animals. In contrast, when studied in vitro, uterine arteries of pregnant sheep generated more active stress and were more sensitive than those of nonpregnant animals on stimulation with phenylephrine and KCl. These observations are in accord with other findings regarding the effect of alpha-adrenergic and depolarizing stimuli on isolated uterine arcuate arteries of the rat. These results also suggest that some physiological regulation occurs in vivo to render the increased reactivity of uterine vasculature observed in vitro as a decreased sensitivity to vasoconstrictors in vivo.

The increased responses to vasoconstrictors in uterine vessels in pregnant animals were linked to remodeling of the vessels. D’Angelo and Osol proposed that this occurs only with agonists coupled to G protein because it was observed with phenylephrine but not KCl. This agrees with our previous reports of a direct relationship between maximum response to phenylephrine and Ang II, but not to KCl, and
diameter of the uterine arcuate arteries. Taken together, these observations suggest that the increased Ang II responses in uterine vessels of term-pregnant animals are linked to remodeling. Such an interpretation is supported by recent work in our laboratory, in which we observed that modulation of uterine vessel size, through in vivo interventions, induced parallel changes in responsiveness to Ang II.20

In agreement with the recent report of Zwart et al,10 the effects of Ang II on uterine vasculature are mediated via the AT1 receptor subtype. These authors observed, as we did, a reduction of the pA2 (−log of apparent affinity constant of the antagonist) value for losartan in the uterine artery of term-pregnant versus nonpregnant rats. In the present report the decreased sensitivity to losartan was even larger in vessels of 5 days postpartum rats and returned toward the level measured in nonpregnant animals by 12 days after parturition. Their measurement of sensitivity to the AT1 antagonist was indeed more precise than ours because it was obtained with several concentrations of losartan, although measured in vessels of different animals. Both sets of results described changes in sensitivity to losartan in the same direction and of similar magnitude. These reports are then conclusive that sensitivity to the AT1 antagonist is decreased at term pregnancy, but our results extend this observation to vessels of postpartum animals.

In labeled ligand-binding experiments, Ang II receptors were not altered in the uterine vasculature of pregnant compared with nonpregnant sheep.16 Moreover, AT2 was reported to be the dominant subtype in uterine artery smooth muscle (endothelium-denuded) of this species, but the ratio of AT1/AT2 (15%/85%), as well as density of binding, was not modified during pregnancy.17 On the contrary, it was reported that total Ang II binding and the AT2-specific sites increase in uterine vasculature of pregnant ewe.18 Moreover, the IC50 of losartan to displace labeled Ang II was increased during pregnancy, supporting the present functional results of decreased AT1 sensitivity (Figure 2C). We observed different results in the uterine vasculature of the rat using RPA. AT1 receptor was the major form expressed in this circulation, with AT2 giving a very faint signal. Both receptor subtypes showed decreased expression in the vasculature of pregnant rats. This raises the question of the localization of these receptors. Except for the study of Cox et al,17 the other studies did not distinguish between smooth muscle and endothelial receptors for Ang II. Endothelial Ang II receptors were associated with increased synthesis of prostaglandins and NO by the uterine artery.19 Ang II–stimulated endothelial-derived prostacyclin production was blocked by losartan but not by PD 123,319.17 These authors also reported that AT2 receptors were specifically localized in the lumina (endothelium) of
myometrial branches of the uterine artery, whereas AT2 receptors were mainly present in the smooth muscle layer.17 Similar studies with the uterine circulation of the rat were never performed to our knowledge, but they could not be confirmed in this species with our RPA results. Moreover, results from this laboratory show that inhibition of prostaglandin synthesis with ibuprofen did not influence the concentration-response curve to Ang II in uterine arcuate arteries of nonpregnant and term-pregnant rats (B. Sicotte and J. St-Louis, unpublished data, 1997). Furthermore, data reported here were obtained under maximal inhibition of NO synthases.

The AT2 antagonist PD 123,319 potentiated the responses to Ang II in the arcuate arteries of the 3 groups of rats tested. This effect was manifested by increased responses to large concentrations of Ang II and a consequent decrease in pD2 for Ang II. These results suggest an inhibitory interaction of the AT2 subtype stimulation with the AT1 subtypes at a site beyond the interaction of Ang II and AT1. Zwart et al10 observed a potentiation of Ang II responses in the presence of PD 123,319, but it was manifested by a leftward shift of the concentration-response curve to Ang II. This can be explained by the presentation of their results as relative responses to KCl responses, whereas we used absolute responses (mN/mm). Furthermore, they also reported that CGP-42112A, considered an AT2 agonist, decreased sensitiveness to Ang II in the uterine artery of both nonpregnant and term-pregnant rats. We have obtained similar results (B. Sicotte and J. St-Louis, unpublished data, 1999). Burrell and Lumbers18 found increased AT2 density in uterine arteries of pregnant ewes and speculated that upregulation of AT2 contributes to the reduced sensitivity of the uterine vasculature in pregnancy. This observation was not supported by the work of Cox et al17 in sheep and by our RPA results in rat. We obtained faint expression of AT2 mRNA in the uterine vessels of nonpregnant rats but none in artery extracts from pregnant rats. Moreover, our functional results do not support their speculation,18 because we observed that only high concentrations of Ang II are potentiated in the presence of PD 123,319 in the 3 groups of rats tested (Figure 5).

In summary, the present study indicates that the growth process of the uterine vasculature, more precisely the arcuate arteries, during pregnancy is accompanied by increased responses to Ang II. This was associated with decreased sensitivity to specific AT1 receptor blockade with losartan and by decreased expression of steady state mRNA for AT1 receptors. This paradoxical association can be explained by an antagonist (perhaps uncoupling) effect of AT2 receptor stimulation on AT1-mediated vasoconstriction in uterine arcuate artery. This interpretation is supported by the absence of measurable (by RPA) expression of AT2 mRNA, as we observed. In such a condition, the decreased influence of AT1 receptors could participate in the increased response of Ang II in uterine arteries of term-pregnant rats. This interpretation remains to be further documented.

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References

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