Impaired Baroreflex Gain During Pregnancy in Conscious Rats
Role of Brain Insulin

Araf S. Azar, Virginia L. Brooks

Abstract—Pregnancy impairs baroreflex gain, but the mechanism is incompletely understood. To test the hypothesis that reductions in brain insulin contribute, we determined whether pregnant rats exhibit lower cerebrospinal fluid (CSF) insulin concentrations and whether intracerebroventricular infusion of insulin normalizes gain of baroreflex control of heart rate in conscious pregnant rats. CSF insulin was lower in pregnant (68±21 pg/mL) compared to virgin (169±25 pg/mL) rats (P<0.05). Pregnancy reduced baroreflex gain (pregnant 2.4±0.2 bpm/mm Hg, virgin 4.6±0.3 bpm/mm Hg; P<0.0001) and the maximum heart rate elicited by hypotension (pregnant 455±15 bpm, virgin 507±12 bpm; P=0.01). Infusion of insulin (100 μU/min) intracerebroventricularly increased baroreflex gain in pregnant (2.4±0.4 to 3.9±0.5 bpm/mm Hg; P<0.01) but not virgin (4.6±0.4 to 4.2±0.4 bpm/mm Hg; NS) rats. Maximum heart rate was not altered by intracerebroventricular insulin in either group. Interestingly, while in pregnant rats the baroreflex was unchanged by intracerebroventricular infusion of the artificial CSF vehicle, in virgin rats, vehicle infusion lowered baroreflex gain (4.7±0.3 to 3.9±0.3 bpm/mm Hg; P<0.05) and the maximum baroreflex heart rate (495±19 to 444±21 bpm; P<0.05). These data support the hypothesis that brain insulin is required to support optimal baroreflex function and that a decrease in brain insulin contributes to the fall in baroreflex gain during pregnancy. (Hypertension. 2011;57:283-288.)

Key Words: heart rate ■ insulin resistance ■ baroreceptor reflex sensitivity ■ pregnant ■ cerebrospinal fluid insulin

Pregnancy impairs function of the baroreceptor reflex (for review, see Refs. 1–3). As a consequence, pregnant individuals exhibit an increased incidence of orthostatic hypotension4 and a reduced ability to maintain arterial pressure during hemorrhage.5–7 Despite the fact that peripartum hemorrhage is a major cause of maternal death,8,9 the mechanisms by which pregnancy causes baroreflex dysfunction remain unclear.

Recent studies suggest that insulin resistance contributes to this impairment. First, in pregnant humans, rabbits, and rats, baroreflex sensitivity or gain and insulin sensitivity decrease in parallel as gestation progresses.3,10,11 Second, treatment of baroreflex sensitivity or gain and insulin sensitivity decrease this impairment. First, in pregnant humans, rabbits, and rats, baroreflex gain has not been identified.

Although insulin is present in the brain, it is not made there.12,13 Instead, pancreatic insulin moves from plasma into the brain across the blood-brain barrier via an active, saturable transendothelial transport mechanism. Insulin resistance is associated with reduced blood-brain barrier insulin transport and brain insulin levels in several states, including obesity, aging, and Alzheimer’s disease.12,13 Interestingly, pregnancy in rabbits also markedly reduces insulin levels in cerebrospinal fluid (CSF).10 Because insulin acts centrally to increase baroreflex gain,14,15 pregnancy-induced falls in brain insulin levels could impair baroreflex function. However, whether brain insulin is the link between insulin resistance and a depressed baroreflex has not been examined directly. To test this hypothesis, it was determined (1) whether late pregnant rats exhibit reduced CSF insulin levels as in rabbits and (2) whether normalization of brain insulin levels, via intracerebroventricular insulin infusion, improves baroreflex gain in conscious late pregnant rats.

Methods

Animals

Female Sprague Dawley rats (n=35; Charles River Laboratories), 12 weeks old, were acclimated to the laboratory for at least 1 week prior to any experimentation. Animals were housed with a 12-hour light/dark cycle, and food (LabDiet 5001) and water were provided ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Oregon Health & Science University animal care and use committee.

CSF Collection

Late pregnant (gestational day 20; n=4) and nonpregnant (n=3) rats were anesthetized with pentobarbital (15 mg, IP) and positioned in a Kopf stereotaxic instrument, with the head flexed downward. A...
Animal Survival Surgery
Several small groups of pregnant (2 pregnant and 2 age-matched virgin rats) were studied. To induce pregnancy, each female rat was placed in a male rat’s home cage. Vaginal epithelial cytology was examined daily, and the presence of sperm indicated pregnancy day 0; the female rat was then placed back in its home cage. All surgeries were performed using aseptic technique. Rats received a single intramuscular injection of 30,000 U of Penicillin G (Hanford’s United States Veterinary Products) 10 to 15 minutes prior to incision and codeine (1 mg/100 mL) in their drinking water for 2 to 3 days after surgery.

Intracerebroventricular Cannulae Insertion
The first surgery was performed when the pregnant rats were at 7 to 9 days of gestation. Anesthesia was induced with 5% isoflurane in 100% oxygen and was maintained with 1.5% to 2% isoflurane. The rats were then placed in a Kopf stereotaxic apparatus with the skull flattened between bregma and lambda. After making a midline skin incision, clearing tissue on top of the skull, and drilling a hole through the skull, the tip of the intracerebroventricular guide cannula (23 gauge; Plastics One) was positioned using the following coordinates (in millimeters relative to bregma): 1 caudal, 1 to 1.4 lateral, and 3.8 to 4.1 ventral. The cannula was secured to the skull using dental acrylic and 3 small screws. When not in use, the guide cannula was plugged with an obturator. For experiments, the obturator was replaced with a 30 gauge inner cannula that protruded 0.5 mm beyond the tip of the guide cannula.

Femoral Catheter Implantation
Seven to 9 days following the first surgery, the rats were again anesthetized, and an arterial catheter (PE10 or PE50) was inserted through a small inguinal incision into the femoral artery and advanced into the distal abdominal aorta. In addition, 2 venous catheters (PE10) were inserted into the femoral vein and advanced into the distal inferior vena cava. The catheters were tunneled subcutaneously and were exteriorized between the scapulae. Catheter patency was maintained by flushing with sterile heparin saline (100 U/mL) 3 times per week. At least 3 days of recovery were allowed before experiments were conducted.

Experimental Protocol
Experiments were performed in pregnant rats on gestational days 19 to 21 and in aged-matched virgins while the rats remained in their home cage. Femoral and intracerebroventricular cannulae were attached to infusion pumps and the pressure transducer using sterile PE tubing. After a 1- to 2-hour equilibration period, a control baroreflex curve was produced using well-established, previously published methodology.16,17 Briefly, arterial pressure was gradually and smoothly increased and decreased using slow intravenous infusions of increasing doses of phenylephrine (1 mg/mL; 0.7 to 27 μg/L/min) and nitroprusside (1 mg/mL; 1.35 to 68 μg/L/min), respectively, with each ramp in pressure completed in ~3 to 5 minutes. Blood pressure and heart rate (HR) were allowed to return to basal levels before another ramp was initiated. After completion of the first curve, an intracerebroventricular infusion of insulin (100 μU/mL; Regular Human Insulin; Novo Nordisk) or the artificial CSF (aCSF) vehicle (0.6 μU/mL) commenced. The aCSF contained (in mM/L) 128 NaCl, 2.6 KCl, 1.3 CaCl2, 0.9 MgCl2, 20 NaHCO3, and 1.3 Na2HPO4 (pH 7.4; passed through a 0.2 μm syringe filter immediately before use). After 1 hour of infusion, a second baroreflex curve was generated.

Data were collected using a Biopac MP100 data acquisition and analysis system sampling at 1000 Hz. The sigmoidal baroreflex relationships between mean arterial pressure (MAP) and HR generated in each experiment were fitted and compared (see Figure 4) using the Boltzman equation: \[ HR = A_1 - A_2 / (1 + e^{(MAP - A_3) / A_4}) + A_2, \]
where \( A_1 \) is the maximum HR, \( A_2 \) is the minimum HR, \( A_3 \) is the MAP at the midpoint between the minimum and maximum HR, and \( A_4 \) is the width or operating range. Maximum baroreflex gain was calculated by dividing the HR range (\( A_1 - A_2 \)) by 4 times the width. At the end of the experiment, the rats were anesthetized, and Alcian Blue dye (Sigma) was infused via the intracerebroventricular cannula. After the rats were euthanized, the brain was removed and sectioned to confirm correct cannula placement via visualization of dye in the ventricular system. In addition, in pregnant rats, the abdomen was opened to count the number of viable fetuses (range 10 to 17 pups).

Statistical Analysis
A t test was used to compare CSF insulin levels and baroreflex parameters between pregnant and nonpregnant rats. Three-way ANOVA for repeated measures was used to determine whether intracerebroventricular insulin infusion normalizes baroreflex gain in pregnant rats (factors were group [pregnant, virgin], intracerebroventricular infusion [insulin, aCSF], and time [before and after intracerebroventricular infusion]). If a significant interaction was revealed, two 2-way ANOVAs (factors were group [pregnant, virgin], time [before and after insulin or aCSF]) and the post hoc Neuman-Keuls test were used to identify specific within- and between-group differences. Results of the 3-way ANOVA are provided in the figure or table legends. Baroreflex gain data were log transformed before analysis to normalize variability. Data are expressed as mean±SEM. \( P<0.05 \) was considered statistically significant.

Results

Effects of Pregnancy on CSF Insulin
CSF insulin was lower in pregnant (68±21 pg/mL) compared to virgin (169±25 pg/mL) rats (\( P<0.05 \)).

Effects of Pregnancy on Baroreflex Function
As expected, pregnancy markedly impaired baroreflex gain, attributable both to a reduction in HR range and an increase in width (Figures 1 to 2 and Table 1). The maximal level of HR achieved during hypotension was suppressed, but the minimum HR was not altered (Figure 1 and Table 1). In
addition, MAP and MAP50 were reduced, but basal HR did not differ significantly between pregnant and virgin animals (Table 1).

**Effects of Intracerebroventricular Insulin Infusion**

**Pregnant Rats**
Infusion of insulin intracerebroventricularly in pregnant rats increased baroreflex gain by reducing width, but not by increasing maximum HR and therefore HR range (Figures 2 to 4 and Table 2). MAP, HR, and other baroreflex parameters were not significantly altered (Table 2). On the other hand, intracerebroventricular aCSF infusion did not alter baroreflex gain (Figures 2 to 3 and Table 2). As a result, baroreflex gain was elevated in pregnant rats receiving insulin compared to those treated with the aCSF vehicle (Figure 2) \(P<0.05\).

**Virgin Rats**
In contrast to pregnant rats, intracerebroventricular insulin infusion did not alter baroreflex control of HR in virgin rats (Figure 5 and Table 2). Interestingly, however, intracerebroventricular infusion of the aCSF vehicle shifted the curve downward on the y axis, as reflected by significant decreases in the HR maximum (Figure 5 and Table 2) and in baroreflex gain (Figures 2 and 5).

**Table 1. Effect of Pregnancy on Baroreflex Parameters, MAP, and HR**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pregnant (n=12)</th>
<th>Virgin (n=16)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal Gain, bpm/mm Hg</td>
<td>2.41±0.23</td>
<td>4.64±0.27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maximum, bpm</td>
<td>455±15</td>
<td>507±12</td>
<td>0.01</td>
</tr>
<tr>
<td>Minimum, bpm</td>
<td>263±14</td>
<td>261±7</td>
<td>NS</td>
</tr>
<tr>
<td>Range, bpm</td>
<td>192±19</td>
<td>246±9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Width, mm Hg</td>
<td>20.8±1.6</td>
<td>13.8±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MAP50, mm Hg</td>
<td>89±5</td>
<td>100±3</td>
<td>0.05</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>81±3</td>
<td>102±3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>370±6</td>
<td>366±8</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Discussion**
The purpose of the present study was to test whether low brain insulin contributes to baroreflex dysfunction during pregnancy. The important new findings are (1) pregnancy decreases CSF insulin in rats; (2) intracerebroventricular infusion of insulin normalizes baroreflex gain in conscious pregnant rats without significantly improving other baroreflex parameters; (3) intracerebroventricular infusion of insulin...
pregnancy, or any other insulin-resistant condition. Reduced brain insulin mediates baroreflex impairment during experiments have been performed to directly establish that and insulin sensitivity are temporally correlated during gestation and treatment of pregnant rabbits with the and insulin-sensitizing drug rosiglitazone improves insulin sensitivity and, to a similar extent, the baroreflex. First, the site at which pregnancy impairs the actions of the neurosteroid progesterone, the RVLM is a link in the pathway by which insulin increases the HR maximum. One explanation for this finding is that, during pregnancy, actions of the neurosteroid progesterone, the RVLM decreases maximal baroreflex HR observed in pregnant rats, despite the fact that intracerebroventricular insulin infusion increases baroreflex gain in a rat in late gestation.

While the effect of pregnancy to impair baroreflex function has been known for several decades, it is only recently that insulin resistance has been recognized as a potential mediator. Evidence to support this mechanistic link includes the following: several other insulin-resistant states besides pregnancy, such as obesity and aging, are also associated with dysfunctional baroreflexes; decreases in baroreflex gain and insulin sensitivity are temporally correlated during gestation; and treatment of pregnant rabbits with the insulin-sensitizing drug rosiglitazone improves insulin sensitivity and, to a similar extent, the baroreflex. Further information indirectly suggests that insulin resistance may decrease baroreflex gain by reducing insulin actions in brain. First, the site at which pregnancy impairs the baroreflex within the baroreflex neuronal circuitry is the brain, rather than afferent pathways. Second, pregnancy decreases insulin concentration in CSF in rabbits, and in the present study, in rats. The mechanism that mediates the reductions in CSF insulin levels is currently unidentified, but possibilities include reduced transport of insulin into the brain and CSF, as has been documented in other insulin-resistant states, or increased brain degradation of insulin. Interestingly, in other insulin-resistant states such as obesity, the brain insulin receptor also becomes resistant to insulin, suggesting an additional pathway by which pregnancy could impair the actions of insulin in brain. Third, insulin acts centrally to increase gain of baroreflex control of HR and sympathetic nerve activity. Despite this evidence, no experiments have been performed to directly establish that reduced brain insulin mediates baroreflex impairment during pregnancy, or any other insulin-resistant condition.

The major finding of the present study is that intracerebroventricular insulin infusion normalized maximal gain of baroreflex control of HR in pregnant rats, without altering the baroreflex in virgin animals. This result suggests 2 key conclusions: (1) reductions in brain levels and/or actions of insulin contribute to baroreflex impairment during pregnancy; and (2) insulin levels must be sufficiently high in normal virgin rats to maximally support baroreflex control of HR. Interestingly, insulin did not reverse the depressed maximal baroreflex HR observed in pregnant rats, despite the fact that intracerebroventricular insulin infusion can increase the HR maximum. One explanation for this finding is that other mechanisms underlie this effect of pregnancy. In support of this idea, Heesch and colleagues have suggested that, during pregnancy, actions of the neurosteroid progesterone metabolite allopregnanolone in the rostral ventrolateral medulla (RVLM) decreases maximal levels of renal sympathetic nerve activity during hypotension. While insulin initiates its action in the forebrain, a glutamatergic synapse in the RVLM is a link in the pathway by which insulin increases

<table>
<thead>
<tr>
<th>Variable</th>
<th>Preinsulin</th>
<th>Postinsulin</th>
<th>Pre-aCSF</th>
<th>Post-aCSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum HR, bpm</td>
<td>514±15</td>
<td>530±12</td>
<td>495±19</td>
<td>444±21*</td>
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<tr>
<td>Minimum HR, bpm</td>
<td>261±8</td>
<td>255±14</td>
<td>261±16</td>
<td>238±14</td>
</tr>
<tr>
<td>Range, bpm</td>
<td>252±10</td>
<td>268±10</td>
<td>234±18</td>
<td>206±14</td>
</tr>
<tr>
<td>Width, mm Hg</td>
<td>14.6±1.2</td>
<td>17.4±1.8</td>
<td>12.4±0.7</td>
<td>13.5±0.9</td>
</tr>
<tr>
<td>MAP50, mm Hg</td>
<td>102±5</td>
<td>105±4</td>
<td>98±4</td>
<td>99±4</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>107±3</td>
<td>110±3</td>
<td>95±5</td>
<td>94±4</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>360±12</td>
<td>364±9</td>
<td>376±8</td>
<td>345±12</td>
</tr>
</tbody>
</table>

The results of the three-way ANOVA are as follows: maximum HR indicates significant (P<0.05) group, treatment, and time effects, as well as significant group by treatment, treatment by time, and group by treatment by time interactions; minimum HR, no significant effects; range, significant group and time effects, as well as significant group by treatment and treatment by time interaction; width, significant group and time effects, as well as significant group by treatment, group by time, and group by treatment by time interactions; MAP50, significant group effect, as well as a significant treatment by time interaction; MAP, significant group and group by treatment interaction; HR, significant time effect and time by treatment interaction. See text for definition of baroreflex parameters.

*P<0.05, within-group comparison preinsulin vs postinsulin or aCSF; †P<0.05 compared to virgin rats.
sympathetic nerve activity, and the RVLM is a synaptic relay in brain stem sympathetic baroreflex pathways. Pregnancy decreases gain of baroreflex control of HR primarily by impairing hypotension-induced increases in cardiac sympathetic activity. Therefore, we propose that the effects of acute increases in forebrain insulin to increase maximal responses to baroreceptor unloading are negated during pregnancy by the inhibitory actions of allopregnanolone in the RVLM.

Another interesting finding of the present study was that intracerebroventricular aCSF infusion in virgin, but not pregnant, rats depressed HR baroreflex responses. This result suggests that a CSF/periventricular brain factor in female animals normally supports baroreflex function and that this factor was diluted by the aCSF infusion. Furthermore, it appears that the actions of this factor are muted during pregnancy such that a further reduction in its levels is without effect. The identity of this factor is unknown, but it is tempting to speculate that it may be insulin. In support of this hypothesis, while infusion of aCSF alone attenuated the baroreflex, infusion of aCSF with insulin had no effect in virgin animals. Moreover, addition of insulin to aCSF in pregnant animals improved the baroreflex, while aCSF alone had no effect.

The present results are the first to suggest that brain insulin normally supports baroreflex function and that a decrease in the central actions of insulin, as during pregnancy, can contribute to baroreflex dysfunction; however, some limitations must be acknowledged. First, while insulin was infused intracerebroventricularly to localize its actions to the brain, the concentrations of insulin in CSF produced by the infusion likely are well above the physiological range. Indeed, CSF insulin levels are considerably lower than levels in plasma. However, it is important to emphasize that insulin normally enters the brain via transport across the blood-brain barrier from the plasma, and only a small fraction reaches the CSF. Moreover, insulin in CSF only slowly penetrates brain tissue across the periventricular border; therefore, high levels are required to drive sufficient insulin movement from CSF into distant brain regions. Indeed, it takes ≈60 minutes for intracerebroventricular insulin infusion at the present dose to significantly improve baroreflex function. Thus, whether considering endogenous insulin that enters the brain via the plasma or exogenous insulin that enters the brain via the CSF, the CSF insulin levels do not reflect the concentration of insulin at its receptors. Second, the present results do not identify the site of action of insulin in brain to improve baroreflex function, though previously we demonstrated that insulin acts proximal to the fourth ventricle. Several forebrain sites that influence cardiovascular autonomic function, in particular in the hypothalamus, are enriched with insulin receptors. Given that insulin improves baroreflex function within a relatively short period and its slow rate of penetration, we speculate that the site of action is periventricular, such as the paraventricular nucleus (PVN) or the arcuate nucleus. Indeed, recent work suggests that the PVN is involved. Finally, while our studies indicate that normal brain insulin levels are required for optimal regulation of baroreflex control of HR, whether a similar role for insulin can be documented in the baroreflex control of sympathetic outflow remains to be investigated.

**Perspectives**

The major conclusion of the present study is that insulin resistance contributes to baroreflex impairment during pregnancy by reducing brain insulin. Insulin resistance and reduced baroreflex gain are associated in several other pathophysiological states, such as obesity, type 2 diabetes, hypertension, heart failure, and aging. In women with preeclampsia, further reductions in both insulin sensitivity and baroreflex function have been observed. Moreover, a recent study by Young et al demonstrated for the first time in healthy humans that (1) physiological increments in plasma insulin produce nearly a doubling of gain of arterial baroreflex control of muscle sympathetic nerve activity and (2) in these male subjects, the ability of insulin to enhance the baroreflex was greatest in individuals with higher insulin sensitivity; baroreflex gain and insulin sensitivity were highly correlated. These timely findings in humans coupled with evidence that reduced baroreflex sensitivity or decreased HR variability are risk factors for subsequent serious cardiovascular events increases the need for determining whether insulin resistance, via reductions in brain insulin, contribute to baroreflex dysfunction in these several other diseases as well.
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Disclosures
None.

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