Calcitonin Gene−Related Peptide Is a Depressor in \(N^G\)-Nitro-\(L\)-Arginine Methyl Ester−Induced Hypertension During Pregnancy

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Abstract  Inhibition of nitric oxide production with \(N^G\)-nitro-\(L\)-arginine methyl ester (L-NAME) increases blood pressure and fetal mortality in pregnant rats. We previously reported that administration of calcitonin gene related peptide (CGRP) reduces the blood pressure and fetal death produced by L-NAME. To determine the hemodynamic role of endogenous CGRP in this setting, CGRP\(_{37}\), a CGRP receptor antagonist, was used. In addition, CGRP mRNA and peptide levels were determined in dorsal root ganglia. L-NAME or control rats had intravenous (for drug administration) and arterial (for continuous mean blood pressure monitoring) catheters surgically placed and were studied in the conscious unrestrained state. Baseline blood pressure was higher in the L-NAME than the control rats on days 19, 20, and 21 or pregnancy and postpartum day 1. Vehicle administration did not change blood pressure in any group, and CGRP\(_{37}\) (100 μg) did not change blood pressure in control groups. However, CGRP\(_{37}\) administration to the L-NAME rats further increased blood pressure (\(P<0.05\)) on days 19 (8±1), 20 (12±2), and 21 (7±1) of gestation but was without effect on postpartum day 1. Furthermore, CGRP mRNA or peptide levels in dorsal root ganglia were not different between the L-NAME and control rats at any of the time points studied. These data indicate that in experimental preeclampsia, CGRP is playing a compensatory vasodilator role to attenuate the elevated blood pressure. The mechanism of this effect appears to be an enhanced vascular responsiveness to CGRP that is attenuated after the birth of pups.

Key Words  preeclampsia  • calcitonin gene−related peptide  • L-NAME  • gene regulation

Preeclampsia is one of the most significant health problems in human pregnancy. It is the leading cause of fetal growth retardation and infant morbidity and mortality associated with premature delivery and maternal death. Hypertension, decreased fetal growth, and proteinuria are the hallmark features of preeclampsia. Although the pathophysiology of this condition remains unclear, the role of NO in the etiology of preeclampsia appears to have been the subject of many recent studies. The evidence that there is an impaired function of the NO-cGMP system in women with preeclampsia is the subject of much current study, and the data is uncertain. There is, however, accumulating evidence that there is an impaired function of the NO-cGMP system in women with preeclampsia. We and others have reported that in pregnant rats the inhibition of NO synthesis with analogues of L-arginine such as L-NAME causes hypertension, proteinuria, fetal growth retardation, and increased fetal mortality without affecting gestational length. We have used this model of preeclampsia to assess the role of several regulatory agents on the pathophysiology of this disorder, including the NO-cGMP system. The female sex steroid hormones, and most recently, the potent vasodilator neuropeptide CGRP.

CGRP is produced by the tissue-specific alternative splicing of the primary transcript of the calcitonin/CGRP gene. This peptide is distributed throughout the central and peripheral nervous systems and is located in areas involved in cardiovascular function. A prominent site of CGRP synthesis is the DRG. DRG contain the cell bodies of primary afferent neurons that extend CGRP-containing nerves to peripheral sites such as blood vessels and the central spinal cord. A dense perivascular CGRP neural network is seen around the blood vessels in virtually all vascular beds. Systemic administration of CGRP decreases BP in a dose-dependent manner in both normotensive animals and humans, as well as in SHR. The primary mechanism responsible for this BP reduction is peripheral arterial dilation.

We have previously reported that the neuronal expression of CGRP is differentially regulated in two nonpregnant models of hypertension. In SHR, CGRP content was decreased in lamina I and II of the dorsal horn of the spinal cord, and CGRP mRNA levels were reduced in DRG compared with normotensive Wistar-Kyoto control rats. In contrast, in the mineralocorticoid-salt (DOC-salt)−induced hypertensive rat, CGRP levels were elevated in the spinal cord, and CGRP mRNA accumulation was increased in DRG compared with normotensive controls. These results suggest that a decrease in CGRP expression, as observed in the SHR, could contribute to the high BP by the relative reduction of vasodilator activity, while an increase in CGRP, as seen in DOC−salt hypertension, could attenuate the high BP by the compensatory augmentation of vasodilator activity. In support of the latter, we recently demonstrated that the intravenous administration of CGRP\(_{37}\), a potent and specific CGRP receptor antagonist, resulted in a significant increase in the already elevated BP in DOC-salt hyper-

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tension rats but was without effect in normotensive controls. Several other lines of evidence suggest that CGRP participates in the regulation of vascular adaptations that occur during normal pregnancy and also in the pathophysiology of preeclampsia. During pregnancy in humans, the circulating levels of iCGRP increase up to the time of delivery, with a sharp reduction in the postpartum period. Also, in pregnancy the sensitivity of uterine arteries to the vasodilator effects of endogenous CGRP is higher in comparison with uterine arteries from nonpregnant humans. In addition, we recently demonstrated that coadministration of CGRP and L-NAME to pregnant rats prevented the gestational (but not postpartum) hypertension induced by L-NAME and significantly decreased fetal mortality. These data indicate that CGRP administration has beneficial effects on the hypertension and increased fetal mortality of experimental preeclampsia. Therefore, to determine the hemodynamic role of endogenous CGRP in this setting, we used the specific CGRP receptor antagonist CGRP8.37, in L-NAME treated and control pregnant rats. In addition, we also quantified CGRP mRNA and iCGRP levels in DRG from these same groups of animals.

Methods

Animals

Adult nulliparous pregnant rats (300 to 325 g) were purchased from Harlan Sprague-Dawley (Houston, Tex.) and were given free access to food and water. All procedures were approved by the institutional Animal Care and Use Committee of the University of Texas Medical Branch.

Induction of Preeclampsia Symptoms

Starting on day 17 of pregnancy (day 1 = day of positive sperm smear), osmotic minipumps (Alza Corp, model 2ML1) were implanted subcutaneously under halothane (Halocarbon Laboratories) anesthesia. The osmotic minipumps placed in the experimental groups of rats contained the NO synthase inhibitor L-NAME (Sigma Chemical Co, 50 mg/d per rat) dissolved under halothane anesthesia, the left carotid artery was cannulated and 21 of gestation and postpartum day 1. The rats that were studied postpartum all delivered on day 22, as expected. Hemodynamic determinations were then performed approximately 3 hours after the surgery with the rats in a fully awake and unrestrained state. At the end of each experiment, the animals were deeply anesthetized with ketamine/xylazine (Fort Dodge Laboratories, Inc/Burns Veterinary Supply, Inc) via the cannulated jugular vein, and killed by decapitation. The thoracic and lumbar DRG from each rat were immediately dissected and frozen in liquid nitrogen for subsequent analysis of CGRP mRNA and iCGRP content. All of the dissected DRG from one side of the spinal cord in each animal were pooled and used for the RNA analysis, while the pooled DRG from the opposite side of the cord were used for peptide determination.

Hybridization Probes, RNA Isolation and Analysis, and Radioimmunoassay

The α-CGRP hybridization probe was 1.4-kb Sau3A rat genomic restriction fragment containing CGRP exons 3 and 6. The 18S RNA hybridization probe was 1.5-kb BamHI-EcoRI restriction fragment of the mouse 18S rRNA gene. The DNA inserts were purified by agarose-gel electrophoresis and subsequently labeled with [α-32P]dCTP using a random hexanucleotide DNA labeling kit (Amersham). Total cellular RNA was isolated from the DRG tissue by the guanidine-isothiocyanate method. The RNA samples were fractionated by electrophoresis on denaturing formaldehyde-agarose gels and transferred to nylon membranes. The membranes were autoradiographed with [32P]-labeled CGRP DNA probe. As a control, the CGRP probe was removed from the membrane, which was then rehybridized with the 18S rRNA probe. Following hybridization, the membranes were washed and placed on a phosphor screen. The exposed screen was then placed in a PhosphorImager (Molecular Dynamics), which generates an image of the hybridized RNA and quantifies the radioactivity in each hybridization signal.

To determine iCGRP levels in the DRG from the experimental and control rats, we used a commercially available recombinant CGRP radioimmunoassay kit (Phoenix Pharmaceuticals). This antibody has 100% cross-reactivity with α-CGRP and 79% with rat β-CGRP. There is no cross-reactivity with either rat amylin, calcitonin, somatostatin, or substance P. The total protein content in each sample was determined by the Bradford method (Bio-Rad).

Statistical Analysis

Statistical significance was determined by Student’s t-test. The acceptable level of significance was P< 0.05. Data in the figures are depicted as the mean ± SEM.

Results

Hemodynamic Effects of CGRP8.37 in L-NAME-Induced Hypertension During Pregnancy

On days 19, 20, and 21 of gestation and postpartum day 1, groups of L-NAME-treated or control rats had arterial (for continuous MAP monitoring) and intravenous (for drug administration) catheters surgically placed and were studied in the conscious unrestrained state. As shown in Fig 1, baseline MAP was significantly higher in the L-NAME-treated compared with the control rats on gestational days 19 (153±2 versus 106±6 mm Hg, P<.01) and 21 (139±2 versus 107±11 mm Hg, P<.01) and postpartum day 1 (149±5 versus 118±2 mm Hg, P<.01). Baseline MAP was also higher on day 20 of gestation (131±16 versus 104±9 mm Hg) but did not achieve statistical significance.

Administration of vehicle (0.1 mL saline IV) did not significantly change MAP in either group on days 19 to 21 of pregnancy or postpartum day 1. Similarly, administration of a bolus dose (100 µg in 0.1 mL saline IV) of CGRP8.37 did not significantly increase MAP in the control group at any of the four time points (Fig 2). However,
administration of the CGRP receptor antagonist to the L-NAME-treated rats rapidly (the MAP increase began approximately 15 to 20 seconds after administration of CGRP8-37) induced a further increase of the already elevated MAP on day 19 (8 ± 1 mm Hg, *P < .05), 20 (12 ± 2 mm Hg, *P < .05), and 21 (7 ± 1 mm Hg, *P < .05) of gestation. The duration of the CGRP8-37-induced increase in MAP was relatively short (approximately 90 seconds). This transient effect of CGRP8-37 has been previously observed by us as well as other investigators who have used this antagonist in vivo and most likely reflects the rapid degradation of this peptide in the circulation.20,27,28 In contrast, the pressor activity of CGRP8-37 was not observed in the L-NAME-infused rats on postpartum day 1. These data indicate that in experimental preeclampsia, CGRP is playing a compensatory vasodilator role to lower the elevated BP and that this effect is attenuated after birth of the pups.

Analysis of CGRP mRNA and iCGRP Content in DRG From L-NAME–Treated and Control Pregnant Rats

In the DOC-salt model of hypertension, where CGRP also acts as a compensatory depressor, neuronal CGRP expression is significantly enhanced in the hypertensive rats when compared with the normotensive controls.19 Based on these results, we anticipated that a similar mechanism would be operative in the L-NAME–treated pregnant rats. Therefore, CGRP mRNA and iCGRP levels were quantitated in the DRG taken from the rats used in the hemodynamic experiments described above.

Fig 3A is a representative Northern blot demonstrating the levels of both the 1.2-kb CGRP mRNA species (both α- and β-CGRP) and 18S rRNA present in DRG RNA samples from three to four animals from each group at the four time points studied. The RNA samples from each animal were analyzed in a similar manner. PhosphorImager analysis was then performed to quantify the hybridization signals for CGRP mRNA and 18S rRNA that were used as an internal control for possible differences in loading of the RNA samples between the groups. As Fig 3B shows, when the values for the CGRP mRNA levels were normalized to those for 18S rRNA, there were no significant differences in DRG CGRP mRNA content between the L-NAME–treated and control pregnant rats at any of the time points studied.

A CGRP specific radioimmunoassay was then used to determine iCGRP levels in the DRG from the same groups of rats. The iCGRP concentration in the L-NAME–treated groups during pregnancy and postpartum day 1 (range, 0.32 ± 0.03 to 0.44 ± 0.02 ng/mg protein) showed no significant difference when compared with control groups (0.31 ± 0.07 to 0.37 ± 0.04 ng/mg protein). The results from these experiments (Fig 4) are consistent with those from the RNA analysis and show no significant differences in neuronal iCGRP levels between any of the L-NAME–treated and control pregnant rats on the three consecutive days of pregnancy and postpartum day 1. These results indicate that the depressor effect of CGRP observed on days 19, 20, and 21 of pregnancy in the L-NAME–treated animals does not result from the enhanced neuronal expression of CGRP, as seen in the DOC-salt model, but is instead mediated through a yet unidentified mechanism.

Discussion

In a previous study, we demonstrated that administration of CGRP can reverse the hypertension induced by L-NAME during pregnancy but not during the postpartum period. The results of the present study provide evidence that endogenous CGRP is also playing a hemodynamic role in this model of experimental preeclampsia. The CGRP8-37-induced increase of the already elevated MAP in the L-NAME–treated rats on days 19 to 21 of pregnancy indicates that in this setting, CGRP is acting as a compensatory vasodilator in an attempt to buffer the elevated BP. Interestingly, the pressor effect of the CGRP antagonist was attenuated on postpartum day 1, which is consistent with what we observed in our earlier studies using exogenously administered CGRP.

In light of the rapid onset of the hypertensive effect of CGRP8-37 in the L-NAME–treated pregnant rats, and because the antagonist probably does not penetrate the central nervous system, it is likely that the pressor activ-
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251

CGRP mRNA

E

105 rRNA

Dl0 D20 021 P, D10 D20 021 Pf 0.0

I II I

L.NAME control Dl9 D20 D21 PI

FIG 3. DRG CGRP mRNA content is not significantly altered in the L-NAME-treated pregnant rats. A, Total cellular RNA samples isolated from DRG taken from the L-NAME-treated and control rats on days 19, 20, and 21 (D19-D21) of pregnancy and postpartum day 1 (P1) were fractionated on a denaturing formaldehyde-agarose gel and transferred to a nylon membrane. The membrane was hybridized with the 32P-labeled CGRP genomic DNA insert (top). The CGRP probe was removed from the membrane, which was subsequently hybridized with the 32P-labeled 18S rDNA probe (bottom). Following hybridization with each probe, the membrane was washed and placed on a phosphor screen. The image in this figure was generated from the Phosphorimag analysis of the exposed screen. B, The CGRP mRNA/18S rRNA ratios from the L-NAME-treated (hatched bars) and control (open bars) DRGs were determined by Phosphorimag analysis of the Northern blot assays and are represented here as the fold increase over control (mean±SEM).

ity of CGRP

CGRP receptors (probably CGRP receptor type 1 [CGRP1]).29 Support for this explanation is provided by radioligand binding and functional studies which show that CGRP

CGRP receptors are typified by those present in heart and peripheral blood vessels.29,30 Furthermore, a number of in vivo studies demonstrate that intravenous administration of CGRP results in a significant, reversible inhibition of the hypertensive and vasodilator effects of exogenously administered CGRP in the rat but does not affect the hypertensive action of other vasodilators such as bradykinin, histamine, or substance P.27,30 In other studies designed to investigate the CGRP-evoked increase in skin blood flow, CGRP was able to block the increased blood flow induced by administration of CGRP but had no effect on the vasodilator responses produced by vasoactive intestinal peptide or prostaglandin E1. Importantly, CGRP was also able to inhibit the increase in blood flow in response to capsaicin, an agent which stimulates the release of CGRP from sensory nerve terminals.31 This indicates that the CGRP antagonist can block the vasodilation that is induced in the skin by endogenously released CGRP. Taken together, these reports provide compelling evidence that the CGRP receptor antagonist can inhibit the vasodilation evoked by exogenously administered or endogenously released CGRP in vivo.

CGRP receptors appear to be coupled to G-proteins, and in a number of tissues, including vascular smooth muscle, CGRP acts through increases in cAMP.32,33 There is additional evidence that the vasodilator response evoked by CGRP is mediated in part by NO release and that various vascular beds differ in their degree of dependence on the presence of endothelium for the vasodilator effects of CGRP.36,37 Therefore, under conditions in which the NO generating system is intact, the depressor effects of CGRP appear to be partially mediated by endothelium-derived NO and also involve a direct relaxation of arteries by increasing cAMP. However, because NO synthesis is inhibited in the L-NAME-treated pregnant rats, it appears that the vasodilator effects of CGRP are independent of NO formation.9

As described previously, we recently demonstrated that CGRP plays a compensatory depressor role in DOC-salt-induced hypertension in the rat and that the mechanism underlying this effect was a significant enhancement of neuronal CGRP synthesis. In the present study, however, we did not observe any significant alterations in DRG CGRP mRNA or iCGRP content between the L-NAME-treated and control pregnant rats at any of the time points studied. However, we do not yet know whether neuronal CGRP expression is enhanced in the pregnant versus nonpregnant rats, since it has been shown that there is an increase in circulating iCGRP levels in human pregnancy. Based on these results, we postulate that in pregnancy there is a pro-
gestosterone-mediated increase in the sensitivity of the vasculature to the vasodilator effects of CGRP and that this effect becomes more pronounced in the face of NO synthesis inhibition such that there is a CGRP-dependent decrease in systemic BP. Our reasons for believing this are as follows. First, it is well known that gestosterone levels are significantly elevated during pregnancy and fall at term. In addition, we have found that in the rat, gestosterone, but not estrogen, regulates vascular adaptations that occur during normal pregnancy and that gestosterone can partially counteract the hypertension and fetal growth retardation produced by L-NAME. Second, Nelson et al. showed that during pregnancy the sensitivity of the uterine arteries to endogenous CGRP is higher in comparison with nonpregnant human arteries. Therefore, we suggest that there is an increase in vascular responsiveness to CGRP during pregnancy that appears to be dependent on progesterone. However, the ability of CGRP to increase further the BP in the L-NAME–treated, but not control, rats in the absence of changes in CGRP levels indicate that this increased sensitivity to the vasodilator effects of CGRP is further enhanced in the absence of NO production, perhaps in an attempt to compensate for the loss of a potent depressor. The mechanism(s) that mediate this phenomenon is not known.

The inability of CGRP to alter BP in the control pregnant animals implies that CGRP does not play a major role in the regulation of systemic BP in the normotensive state but does not rule out a role for CGRP in the modulation of regional organ blood flows in this setting. In a recent report from other investigators, CGRP was used in studies of normal nonpregnant rats to show that CGRP is responsible for approximately 30% of basal coronary blood flow. Additional reports suggest that CGRP can modulate regional organ blood flows to critical organs without significant changes in systemic BP. Therefore, the enhanced sensitivity of uterine vessels to CGRP that is observed during pregnancy and the ability of exogenous CGRP to reverse the hypertension and attenuate the fetal death induced by L-NAME suggest that CGRP may play an important role in regulating the blood supply to the utero-placental unit and fetal development. Furthermore, the pressor effects of the CGRP agonist that are seen in the L-NAME–treated rats indicate that in the face of inhibition of the NO-cGMP generating system, which is normally upregulated in the uterus during pregnancy and downregulated during labor, CGRP acts as a compensatory mechanism in an attempt to lower the BP and perhaps increase blood flow to the uterus.

In summary, these studies suggest that endogenous CGRP may play an important role in the regulation of BP and possibly placental perfusion in experimental pre-eclampsia. Further studies are required to clarify the role that CGRP plays in modulating regional organ blood flows in normal pregnancy and pre-eclampsia and to identify the mechanisms involved in the increase in CGRP-dependent vasodilatation that is associated with L-NAME–induced hypertension in pregnancy.

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