Role of Nitric Oxide in Mediating In Vivo Vascular Responses to Calcitonin Gene-Related Peptide in Essential and Peripheral Circulations in the Fetus

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Background—The role of calcitonin gene-related peptide (CGRP) in cardiovascular regulation is gaining clinical and scientific interest. In the adult, in vivo studies have shown that CGRP-stimulated vasodilation in several vascular beds depends, at least in part, on nitric oxide (NO). However, whether CGRP acts as a vasodilator in the fetus in vivo and whether this effect is mediated via NO have been addressed only minimally. This study tested the hypothesis that CGRP has potent NO-dependent vasodilator actions in essential and peripheral vascular beds in the fetus in late gestation.

Methods and Results—Under anesthesia, 5 fetal sheep at 0.8 gestation were instrumented with vascular catheters and Transonic flow probes around an umbilical artery and a femoral artery. Five days later, fetuses received 2- and 5-µg doses of exogenous CGRP intra-arterially in randomized order. Doses were repeated during NO blockade with the NO clamp. This technique permits blockade of de novo synthesis of NO while compensating for tonic production of the gas, thereby maintaining basal cardiovascular function. CGRP resulted in potent and long-lasting NO-dependent dilation in the umbilical and femoral circulations, hypotension, and a positive cardiac chronotropic effect. During NO blockade, the femoral vasodilator response to CGRP was diminished. In contrast, in the umbilical vascular bed, the dilator response was not only prevented but reversed to vasoconstriction.

Conclusions—CGRP has potent NO-dependent vasodilator actions in fetal essential and peripheral vascular beds. CGRP-induced NO-dependent effects in the umbilical vascular bed may provide an important mechanism in the control and maintenance of umbilical blood flow during pregnancy. (Circulation. 2005;112:2510-2516.)

Key Words: calcitonin gene-related peptide • fetus • hemodynamics • nitric oxide • vasodilation

Calcitonin gene-related peptide (CGRP) is a 37–amino acid peptide that was discovered in 1983 by alternative processing of RNA transcripts from the calcitonin gene. It is synthesized primarily in sensory neurones of the dorsal root and trigeminal ganglia, which, in turn, extend axons centrally to the spinal cord and peripherally to various organs, including blood vessels. In the vasculature, terminals from these neurones innervate the smooth muscle layer, and neuronal activation causes the release of CGRP, which induces a powerful nonadrenergic, noncholinergic vasodilation. This potent dilator action of CGRP and the wide distribution of perivascular neurones containing CGRP throughout the cardiovascular system suggest that CGRP is in a prime position to regulate blood flow under physiological conditions.

In the adult, several mechanisms have been proposed for CGRP-induced vasodilation. In vitro studies have suggested either the activation of adenylate cyclase, resulting in the opening of ATP-sensitive K⁺ channels (K⁺ATP) in the vascular smooth muscle, or the release of nitric oxide (NO) from vascular endothelial cells. The finding that CGRP receptors

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are present on both the endothelium and smooth muscle cells of the vasculature further supports the concept that the actions of CGRP are mediated by pathways involving both endothelium-dependent and -independent mechanisms. However, in vivo studies favor the proposal that the vasodilator actions of CGRP are mediated, at least in part, by NO-dependent pathways and not by activation of K⁺ATP channels. The increased maternal plasma levels of CGRP during pregnancy, its presence in human cord and neonatal blood, and the finding that the concentration of CGRP in fetal serum correlates with both fetal weight and gestational age indicate a functional role for CGRP in prenatal life. This is further supported by evidence from Dong and colleagues, who have demonstrated the presence of components of the CGRP receptor in the vascular endothelium and smooth muscle cells of human fetoplacental vessels, and by Terenghi and colleagues, who have detected CGRP within the human nervous system during the first trimester of pregnancy. However, little is known about the actions of CGRP in the fetal vasculature. In the present study, we have tested the hypoth-

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esis that CGRP has important NO-dependent vasodilator actions in fetal essential and peripheral vascular beds. The hypothesis was tested by investigating the in vivo effects of exogenous doses of CGRP before and during NO blockade on changes in umbilical (UBF) and femoral (FBF) blood flow measured by indwelling transonic flow probes in the unanesthetized late-gestation ovine fetus surgically prepared for long-term recording.

Methods

Surgical Preparation

All procedures were performed under the UK Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Committee of the University of Cambridge. Five Welsh Mountain sheep fetuses were surgically instrumented for long-term recording at 120±2 days of gestation (term is ~145 days) under strict aseptic conditions as previously described in detail.16 In brief, food, but not water, was withheld from the pregnant ewes for 24 hours before surgery. After induction with 20 mg/kg IV sodium thiopentone (Intraval Sodium, Merial Animal Health Ltd), general anesthesia (1.5% to 2.0% halothane in 50:50 O2:N2O) was maintained with positive pressure ventilation. Midline abdominal and uterine incisions were made; the fetal hind limbs were exteriorized; and on 1 side, femoral arterial (internal diameter, 0.86 mm; outer diameter, 1.52 mm; Critchly Electrical Products) and venous (internal diameter, 0.56 mm; outer diameter, 0.96 mm) catheters were inserted. The catheter tips were advanced carefully to the descending aorta and inferior vena cava, respectively. Another catheter was anchored onto the fetal hind limb for recording of the reference amniotic pressure. In addition, a transit-time flow transducer was implanted around the contralateral femoral artery (2R or 3S, Transonic Systems Inc) and around one of the umbilical arteries, close to the common umbilical artery inside the fetal abdominal cavity (4SB, Transonic Systems Inc). The uterine incisions were closed in layers; the dead space of the catheters was filled with heparinized saline (1 IU heparin per 1 mL in 0.9% NaCl); and the catheter ends were plugged with sterile brass pins. The catheters and flow probe leads were then exteriorized via a keyhole incision in the maternal flank and kept inside a plastic pouch sewn onto the maternal skin.

Postoperative Care

During recovery, ewes were housed in individual pens in rooms with a 12 hour/12 hour light/dark cycle. Here, they had free access to hay and water and were fed concentrates twice daily (100 g sheep nuts No. 6, H&C Beart Ltd). Antibiotics were administered daily to the ewe (0.20 to 0.25 mg/kg IM Depocillin, Mycofarm), to the fetus (0.3 mL in 0.9% NaCl); and the catheter ends were plugged with sterile butazone. Equipalozone Paste, and venous (internal diameter, 0.56 mm; outer diameter, 0.96 mm) catheters were inserted. The catheter tips were advanced carefully to the descending aorta and inferior vena cava, respectively. Another catheter was anchored onto the fetal hind limb for recording of the reference amniotic pressure.

Experimental Protocol

After at least 5 days of postoperative recovery, all fetuses received 2- and 5-μg bolus doses of CGRP (β-CGRP, C-1044, Sigma Chemicals) dissolved in heparinized saline via the femoral artery catheter in a randomized order. Doses were injected over 2 to 3 seconds and were administered after at least 15 minutes of stable baseline recording. The CGRP doses were chosen from our own pilot experiments and from a previous study by Takahashi and colleagues.10 In that study, treatment of late-gestation fetal sheep with a similar dose of CGRP produced a marked vasodilation in the pulmonary vascular bed. In the present study, exogenous doses of CGRP were first given during a slow intravenous infusion of vehicle (80 IU heparin per 1 mL in 0.9% NaCl) and then repeated during blockade of NO with the NO clamp, a technique that is well established in our laboratory.17,19 The NO clamp permits blockade of de novo synthesis of NO while compensating for the tonic production of the gas, thereby maintaining basal cardiovascular function. In brief, a bolus dose (100 mg/kg bolus dissolved in 2 mL heparinized saline) of Nω-nitro-l-arginine methyl ester (L-NAME; Sigma Chemicals) was injected via the femoral artery catheter. This was immediately followed by fetal intravenous infusion (5.1±2.0 μg·kg⁻¹·min⁻¹, mean±SD, dissolved in heparinized saline) with the NO donor sodium nitroprusside (SNP; Sigma Chemicals, UK) to return fetal arterial blood pressure, heart rate, UBF, and FBF to pretreatment levels. At the end of the experimental protocol, the effectiveness of NO blockade by the clamp and the persistence of L-NAME in the system were tested by withdrawal of the SNP infusion. A day later, the ewes and fetuses were humanely killed with a lethal dose of sodium pentobarbital (200 mg/kg IV Pentoject, Animal Ltd). The positions of the implanted catheters and flow probes were confirmed, and the fetuses were weighed.

Blood Sampling Regimen

Before each experimental protocol, descending aortic blood samples (0.3 mL) were taken using sterile techniques from the fetus to determine arterial blood gas and acid base status (ABL3 Blood Gas Analyzer, Radiometer; measurements corrected to 39.5°C). Values for percentage saturation of hemoglobin with oxygen and blood hemoglobin concentration were determined with a hemoximeter (OSM2, Radiometer). In addition, blood glucose and lactate concentrations were measured by an automated analyzer (Yellow Springs 2300 Stat Plus Glucose/Lactate Analyser, YSI Ltd).

Data and Statistical Analyses

Umbilical vascular conductance (UVC) and femoral vascular conductance (FVC) were calculated by use of Ohm’s principle by dividing mean blood flow (UBF or FBF) by mean corrected arterial pressure. All measured variables were then analyzed for normality of distribution and expressed as mean±SEM. For each dose response of CGRP, baseline values for all cardiovascular variables measured were obtained by averaging the data over the 60 seconds preceding the administration of each bolus dose. The change in all cardiovascular variables to each dose of CGRP was first analyzed for significance in relation to its own baseline, during either saline infusion or NO blockade, using the significance of single mean test. Comparisons between treatments (saline versus NO clamp), dose (2 versus 5 μg), or interaction between treatment and dose were assessed statistically with 2-way repeated-measures ANOVA (Sigma-Stat, SPSS Inc). When a significant effect of time or group was indicated, the post hoc Tukey test was used to isolate the statistical differences. Differences in all cardiovascular variables during and after removal of the NO clamp were analyzed with Student t test for paired data. For all comparisons, statistical significance was accepted at P<0.05.

Results

Fetal Arterial Blood Gas and Metabolic Status

Fetal arterial blood gas and metabolic status measured before each experimental protocol were within normal ranges for
Welsh Mountain sheep at ≈125 days of gestation: pH, 7.33±0.01; ABE, 1.0±1.0 mEq/L; HCO₃⁻, 25.7±0.1 mEq/L; PaCO₂, 54.4±1.2 mm Hg; PaO₂, 22.3±1.2 mm Hg; saturation, 54.8±3.2%; hemoglobin, 10.5±0.6 g/dL; lactate, 0.77±0.06 mmol/L; and glucose, 0.87±0.05 mmol/L. Neither treatment with CGRP nor fetal exposure to the NO clamp affected basal arterial blood gas or metabolic status.

Arterial Blood Pressure and Heart Rate Responses
CGRP (2 and 5 μg) produced a dose-dependent decrease in fetal arterial blood pressure (−7.6±0.8 and −11.8±1.2 mm Hg) and increase in fetal heart rate (66±8 and 106±5 bpm; *P<0.05; Figure 1). During NO blockade, hypotension (−1.9±1.2 and −5.3±1.4 mm Hg) and tachycardia (20±6 and 31±6 bpm) were markedly diminished by both doses of CGRP (*P<0.05; Figure 1), with the alteration in arterial blood pressure to 2 μg CGRP showing no significant change from its own baseline (P>0.05). CGRP also produced a dose-dependent effect on the duration of the hypotensive (10.6±1.3 and 15.6±1.5 minutes) and tachycardic (11.6±1.3 and 21.4±2.0 minutes; *P<0.05; Figure 2) responses, each of which was significantly shortened during NO blockade (*P<0.05; Figure 2).

Umbilical Hemodynamics
CGRP (2 and 5 μg) produced similar increases in UBF (102±8 and 113±7 mL/min) and UVC [1.83±0.17 and 2.26±0.19 (mL · min⁻¹ · mm Hg⁻¹)]. During NO blockade, umbilical vasodilation was reversed to vasoconstriction in response to both doses of exogenous CGRP [UBF, −50±16 and −49±4 mL/min; UVC, −0.78±0.24 and −0.65±0.11 (mL · min⁻¹) · mm Hg⁻¹; *P<0.05; Figure 1]. CGRP also produced a dose-dependent effect on the duration of the umbilical vasodilator response (14.2±2.3 and 19.9±1.4 minutes). This was significantly shortened after reversal to the vasoconstrictor response during NO blockade (6.9±0.8 and 8.6±0.7 minutes; *P<0.05; Figure 2).

Femoral Hemodynamics
CGRP (2 and 5 μg) produced similar increases in FBF (19±2 and 23±3 mL/min) and FVC [0.35±0.05 and 0.47±0.06 (mL · min⁻¹) · mm Hg⁻¹]. During NO blockade, the femoral vasodilation to 2 μg of CGRP was abolished [FBF, 6±2 mL/min; FVC, 0.14±0.07 (mL · min⁻¹) · mm Hg⁻¹; *P<0.05] and markedly diminished to 5 μg CGRP [FBF, 11±1 mL/min; FVC, 0.25±0.04 (mL · min⁻¹) · mm Hg⁻¹; *P<0.05; Figure 1]. CGRP also produced a dose-dependent effect on the duration of the femoral vasodilator response (12.3±1.3 and 17.6±1.5 minutes); this was significantly shortened during NO blockade (8.0±1.5 and 7.9±1.3 minutes; *P<0.05; Figure 2).

An example of the individual cardiovascular responses to 5 μg CGRP before and during NO blockade with the NO clamp is shown in Figure 3.

Removal of the NO Clamp
Withdrawal of the SNP infusion led to a significant increase in fetal arterial blood pressure (Δ12.8±1.4 mm Hg) and to
decreases in fetal heart rate (Δ−38±8 bpm) and vasoconstriction in both the femoral [FVC, Δ−0.32±0.06 (mL · min⁻¹) · mm Hg⁻¹] and umbilical [UVC, Δ−1.65±0.27 (mL · min⁻¹) · mm Hg⁻¹; P<0.05] vascular beds (Figure 4). This provided evidence for the effectiveness of NO blockade by the clamp and the persistence of the action of L-NAME within the system until the end of the experimental protocol.

Discussion
The results of the present study show that fetal treatment with CGRP elicits potent and long-lasting vasodilator actions in the fetal umbilical and femoral vascular beds, hypotension, and a positive cardiac chronotropic effect. During NO blockade, not only were these cardiovascular responses to CGRP significantly reduced, but the umbilical dilator response was

Figure 3. Example of the cardiovascular responses to CGRP before and during NO blockade. An individual example of the response in arterial blood pressure, heart rate, FBF, FVC, UBF, and UVC to 5 μg CGRP before and during NO blockade with the NO clamp.

Figure 4. Effect of NO clamp removal on cardiovascular variables. Values represent the mean±SEM calculated every 30 seconds for arterial blood pressure, heart rate, FBF, FVC, UBF, and UVC in response to removal of the NO clamp (arrow). Significant difference: *P<0.05, during vs removal of the NO clamp (Student t test for paired data).
reversed to vasoconstriction. These data support the hypothesis that CGRP has important NO-dependent vasodilator actions in essential and peripheral vascular beds in the late-gestation ovine fetus.

CGRP is one of the most potent vasodilator peptides known, having a potency of ~10-fold greater than prostanoids and ~100- to 1000-fold greater than classic vasodilators such as acetylcholine, adenosine, and 5-HT. Results from the present study support the current literature and show for the first time that in the fetus, in vivo treatment with CGRP has potent dilator actions in the umbilical and femoral vascular beds. Furthermore, fetal treatment with CGRP resulted in hypotension and a positive cardiac chronotropic effect, which, unlike the vascular responses, showed significant dose-dependent effects. Hypotension is likely to be the result of vasodilation in several vascular beds in the fetus, leading to a reduction in total peripheral vascular resistance and thus a fall in arterial blood pressure at a given cardiac output. The positive cardiac chronotropic effect may be elicited by a baroreflex response to hypotension. However, it is also likely to involve the direct effects of the peptide on CGRP receptors, which have been reported to be present in both adult and fetal hearts. The CGRP receptors have recently been cloned and are characterized as 7-transmembrane G protein–coupled calcitonin receptor-like receptors, which are associated with 1 of 3 single transmembrane spanning receptor activity modifying proteins (RAMP1, RAMP2, RAMP3). The RAMPs interact via their NH2 terminus with the calcitonin receptor-like receptors to produce a different receptor specificity by influencing the formation of the receptor pocket by allosteric modulation. The biological actions of CGRP are mediated predominantly through the CGRP1 receptor, which is made up of the calcitonin receptor-like receptors and RAMP1 proteins, both of which have been demonstrated within the endothelium and underlying smooth muscle cells of human fetoplacental vessels.

Unlike classic vasodilators, CGRP elicited cardiovascular responses, which were particularly long-lasting. CGRP has a half-life of ~7 to 10 minutes in the adult circulation, and its vasodilator activity is removed after clearance by several mechanisms, including those involving mast cell tryptase, neutral endopeptidases, matrix metalloproteinase-2, or reuptake mechanisms into the sensory nerve terminal after repolarization. The duration of all cardiovascular responses showed a significant dose-dependent effect, clearly attributed to the increased circulating concentration of CGRP at higher doses and hence the increased time required for metabolic disposition.

To assess the contribution of NO to the mechanisms of action and clearance of CGRP, each dose of CGRP was repeated during NO blockade with the NO clamp. This technique permits blockade of de novo synthesis of NO while compensating for the tonic production of the gas, thereby maintaining basal cardiovascular function. Results from the present study show that during NO blockade, there were marked reductions in both the magnitude and duration of all cardiovascular responses measured to increasing exogenous doses of CGRP. In peripheral vascular beds in the fetus, the dilator response to low doses of CGRP was totally abolished during NO blockade, suggesting that at low concentrations, the actions of CGRP are completely mediated via mechanisms involving NO-dependent pathways. In contrast, at higher doses of CGRP, NO-independent mechanisms may become recruited to mediate the much reduced but persistent femoral vasodilator actions of the peptide during NO blockade. For example, CGRP has been shown to stimulate adenylate cyclase, which, in turn, will increase cAMP concentrations. This will activate protein kinase A and hence lead to the phosphorylation and opening of K+ATP channels, resulting in hyperpolarization and relaxation of the arterial smooth muscle. Accordingly, an elegant study by Takahashi and colleagues has reported that in another fetal “peripheral” circulation, the pulmonary vascular bed, CGRP increases pulmonary blood flow via its specific receptor in part through NO release and in part through K+ATP channel activation.

In essential fetal vascular beds such as the umbilical circulation, several studies have shown that NO has an important role in the mechanisms regulating blood flow during basal and stimulated conditions. A recent study by Dong and colleagues demonstrated that CGRP antagonism in vitro diminished the dilator actions of exogenous CGRP in the human fetoplacental vasculature. Furthermore, Thakor and Giussani have shown that CGRP antagonism in vivo diminished the umbilical hemodynamic defense response to acute hypoxemia in the ovine fetus. The results of the present study extend these findings to show that the potent dilator action of CGRP in the umbilical vascular bed of the ovine fetus is mediated exclusively by NO because, in contrast to the femoral vascular bed, dilatation was not only diminished but reversed to vasoconstriction during NO blockade. CGRP is known to increase noradrenergic and adrenergic sympathetic outflow after activation of various hypothalamic nuclei. Thus, it is possible that constriction of the umbilical vascular bed after exogenous CGRP during NO blockade may be mediated via activation of adrenergic receptors in the umbilical vasculature; an effect that clearly is overridden by the potent NO-dependent actions of the peptide in this essential vascular bed. Alternatively, constrictor actions of CGRP during NO blockade may be similar to those of acetylcholine in vessels once denuded of the endothelium or in intact vessels after NO synthase blockade, as elegantly demonstrated by Furchgott and Zawadzki and Librizzi and colleagues. These effects of vasodilator agents after NO blockade may be secondary to the release of other vasoactive agents such as thromboxane, as shown for acetylcholine by Tsuji and Cook.

The difference in the pattern of the vascular responses to CGRP after NO blockade between essential and peripheral vascular beds may be attributed to whether these vessels respond to the peptide in an endothelium-dependent or -independent manner. Previous studies have reported that in large conduit vessels such as the aorta, the majority of the actions of CGRP are endothelium dependent, involving the release of NO. Hence, it is no surprise that in the umbilical artery, in essence an extension of the descending aorta in the fetus, the actions of CGRP are largely dependent on NO. In contrast, the femoral vasculature appears to be able...
to recruit other NO-independent mechanisms after NO blockade, consistent with evidence that CGRP has endothelium-independent actions in more peripheral circulations, as reported for the isolated rat caudal artery\(^4\) and rabbit mesenteric artery.\(^5\)

The diminished cardiac positive chronotropic effect of CGRP during NO blockade is likely due to a baroreflex of reduced gain in response to a smaller fall in arterial blood pressure. Alternatively, NO may also be involved in the mechanisms by which CGRP directly affects cardiac pacemaker activity. In the heart, the influx of calcium resulting from the transmembrane calcium current \(I_{\text{Ca}}\) has a fundamental role in pacemaker activity. Furthermore, it has recently been shown that in isolated frog single heart cells, CGRP dramatically increases \(I_{\text{Ca}}\) by mechanisms that cannot be blocked by \(\beta\)-adrenergic antagonism.\(^4\) However, whether this novel CGRP-mediated neurogenic control of cardiac pacemaker activity involves NO-dependent pathways is unknown.

The results of the present study also show that NO blockade reduced the duration of all cardiovascular effects of CGRP, implying that NO may act to inhibit the mechanism mediating the metabolic disposition of the peptide. Although information on the effects of NO on most mechanisms of clearance of CGRP is unavailable, it is known that in adult rats, NO downregulates matrix metalloproteinase-2 in aortic vessels\(^4\) and that inhibition of endogenous NO enhances the release of matrix metalloproteinase-2 in the heart.\(^4\)

In conclusion, the data show that fetal treatment with exogenous CGRP resulted in potent NO-dependent vasodilator actions in the umbilical and femoral vascular beds, hypotension, and a positive cardiac chronotropic effect. CGRP-induced dilatation in peripheral vascular beds involves both NO-dependent and -independent pathways. In contrast, the powerful dilator effects of CGRP in essential vascular beds such as the umbilical circulation appear to be mediated exclusively by NO-dependent pathways. CGRP-induced NO-dependent effects in the umbilical vascular bed may provide an important mechanism in the control and maintenance of UBF during pregnancy.

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References

CLINICAL PERSPECTIVE

Pregnancies complicated by chronic adverse intrauterine conditions show an enhanced umbilical vasodilator response to acute hypoxic stress, of the type that may occur during labor and delivery, which may help to protect the compromised fetus. The nature of this mechanism to enhance dilation in the umbilical circulation appears to involve the actions of NO and calcitonin gene-related peptide. Here, we confirm that the umbilical circulation is highly sensitive to the NO-dependent actions of calcitonin gene-related peptide in such a way that when NO is blocked, the dilator actions of the peptide on the umbilical circulation are not only prevented but are reversed to constriction. Combined, past and present evidence suggests an important role for calcitonin gene-related peptide in the maintenance of umbilical blood flow during late gestation in normal and compromised pregnancies.
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