Mechanisms of calcitonin gene-related peptide-induced increases of pulmonary blood flow in fetal sheep

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1 Department of Pediatrics, Akita University School of Medicine, Akita, 010, Japan; 2 Department of Pediatrics, Leiden University, 2300 RA Leiden, The Netherlands; and 3 Departments of Pediatrics and Obstetrics, Gynecology, and Reproductive Science, and Cardiovascular Research Institute, University of California, San Francisco, California 94143

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Takahashi, Yasushi, Maartje de Vroomen, Christine Roman, and Michael A. Heymann. Mechanisms of calcitonin gene-related peptide-induced increases of pulmonary blood flow in fetal sheep. Am J Physiol Heart Circ Physiol 279: H1654–H1660, 2000.—Fetal pulmonary blood flow is regulated by various vasoactive substances. One, calcitonin gene-related peptide (CGRP), increases pulmonary blood flow. We examined four key physiological mechanisms underlying this response using the blocker drugs CGRP receptor blocker (CGRP8–37), nitric oxide synthase inhibitor [Nω-nitro-L-arginine (L-NNA)], adenosine triphosphate-dependent potassium channel; indomethacin (indomethacin) in 17 near-term fetal sheep. Catheters were placed in the left (LPA) and main pulmonary arteries, and an ultrasonic flow transducer was placed around the LPA to measure flow continuously. CGRP was injected directly into the LPA (mean 1.02 μg/kg) before and after blockade, and responses to CGRP were statistically compared. Before blockade, CGRP increased LPA blood flow from 23 ± 25 to 145 ± 77 ml/min (means ± SD), and these increases were significantly attenuated by CGRP8–37 (n = 6; 91% inhibition), L-NNA (n = 6; 86% inhibition), and glibenclamide (n = 6; 69% inhibition). No significant changes were found with indomethacin (n = 6; 4% inhibition). Thus, in the fetal pulmonary circulation, CGRP increases pulmonary blood flow not only through its specific receptor but also, in part, through nitric oxide release and KATP channel activation.

pulmonary vascular resistance; fetus; nitric oxide; adenosine triphosphate-dependent potassium channel; indomethacin


In adults, several pathways have been proposed for the production of CGRP-induced vasodilation (1, 8, 16, 20–23, 25), e.g., CGRP receptor, adenosine 3’,5’-cyclic monophosphate (cAMP), nitric oxide (NO), or adenosine 5-triphosphate-dependent potassium (KATP) channel-mediated pathways. These reports, however, are not consistent among species or organs and did not comment on all fetal pulmonary vessels, in which the control of vascular tone is different from that in the adult. To evaluate the possible mechanisms responsible, we chose four key blocker agents (CGRP receptor blocker, NO synthase blocker, KATP channel blocker, and cyclooxygenase inhibitor) and examined how these agents affect CGRP-induced increases in PA flow in chronically instrumented fetal sheep.

METHODS

Animals. Seventeen mixed Western pregnant sheep were studied at 125–135 days of gestational age (mean age 128 days; full term ~145 days). Animal husbandry and the study design followed the guidelines of the National Institutes of Health and were approved by the Committee on Animal Research of the University of California, San Francisco.

Surgical preparation. The surgical preparation has been described in detail previously (9, 32). Briefly, under ketamine and diazepam anesthesia, a midline laparotomy was performed on the ewe. The fetus was exposed through a small uterine incision; after local lidocaine anesthesia was administered, a skin incision was made in the fetal forelimb. Polyvinyl catheters were advanced into the ascending aorta and superior vena cava from the brachial artery and brachial vein, respectively. Another incision was made over the left chest. The pericardium was opened, and Teflon-tipped polyvinyl catheters were inserted directly into the main and left pulmonary arteries. The catheter in the left pulmonary artery (LPA) was divided in two with a Y connector 2 cm distal to the LPA. Formalin (10%) colored with a few drops of sterile...
indigo carmine solution was infiltrated into the adventitia and media of the ductus arteriosus to prevent vasoactivity during the subsequent experimental protocols (29). An ultrasonic transit-time Doppler-flow transducer (Transonic Systems, Ithaca, NY) 4–6 mm in diameter was placed around the LPA, and then the thoracotomy was closed. Another polyvinyl catheter was placed in the amniotic cavity. The uterine incision was then closed after antibiotics (100 mg of gentamicin sulfate, \(1 \times 10^6\) units of penicillin G-K) and warm saline were instilled to replace amniotic fluid losses. All vascular catheters were sealed with heparin sodium solution (1,000 U/ml) and exteriorized to the left flank of the ewe with the transducer cable. The laparotomy was closed, and the ewe was returned to the cage for recovery. Antibiotics (100 mg of gentamicin sulfate, \(1 \times 10^6\) units of penicillin G-K) were administered daily, both intravenously to the ewe and directly into the amniotic cavity.

**Experimental protocol.** We performed four protocols based on the same study design: comparison of CGRP-induced hemodynamic changes before and after infusion of a blocker agent (Fig. 1, A–D). Each protocol included 6 studies, for a total of 24 studies performed on 17 fetuses: 1 study in 10 fetuses and 2 studies in 7 fetuses. When we performed two studies in the same fetus, they were conducted 2–3 days apart to avoid interaction between studies. Experiments were performed 1–2 days after surgery with the ewe in a study cage and allowed free access to food and water. After a steady-state period of at least 20 min, a blood gas sample was obtained from the aorta, and the following baseline hemodynamic variables were measured: mean LPA flow, mean aortic (Ao) and PA pressures, and heart rate. These variables were recorded continuously throughout the experiment. In each of the following protocols, CGRP was injected as a 2- to 5-\(\mu\)g bolus (mean 1.02 \(\mu\)g/kg) into the LPA over 5 s. In each

![Fig. 1. Protocols and examples of left pulmonary arterial (LPA) blood flow before and after administration (indicated by horizontal bars) of the calcitonin gene-related peptide (CGRP) receptor blocker CGRP(8–37) (A), \(N\)-nitro-L-arginine (L-NNA; B), glibenclamide (C), and indomethacin (D) (see protocols 1–4 described in METHODS). SVC, superior vena cava.](http://ajpheart.physiology.org/DownloadedFrom)
animal, the same dose was used before and after administration of a specific inhibitor. At the beginning of each protocol, 1 μg of acetylcholine, an agonist for receptor-mediated NO release and a potent vasodilator of fetal pulmonary arteries, was injected as a bolus (over 5 s) into the LPA. This acetylcholine injection produced an increase in LPA flow and was used to check normal reactivity of fetal pulmonary vessels (33). After the experiments, the fetuses were removed from the uterus for measurement of fetal body weight and verification of catheter positions.

Protocol 1: Effects of CGRP receptor blockade by CGRP8–37
After baseline measurements were taken, 1 μg of acetylcholine was injected into the LPA (Fig. 1A). After LPA flow and PA and Ao pressures had returned to baseline values, CGRP was injected. After 120 min of recovery, 1,000 μg of CGRP8–37, a CGRP receptor antagonist, were infused over 30 min into the LPA. After 25 min, while CGRP8–37 was being infused continuously, CGRP was injected again.

Protocol 2: Effects of NO synthesis inhibition by Nω-nitro-
l-arginine
After baseline measurements were taken, 1 μg of acetylcholine was injected as a bolus into the LPA (Fig. 1B). After LPA flow and arterial pressures had returned to baseline values, CGRP was injected. After 120 min of recovery, 30 mg of Nω-nitro-l-arginine (l-NNA) were injected as a slow bolus (over 5 min) into the LPA followed by a 3 mg/min infusion of l-NNA into the superior vena cava. At 60 min of continuous infusion of l-NNA, another bolus of acetylcholine was injected, as before, to check effective blockade of NO-induced increases in PA flow. A second injection of CGRP was then given. If the acetylcholine-induced response was not inhibited, the l-NNA infusion was continued for an additional 30 min until the acetylcholine-induced response was inhibited, and then a second injection of CGRP was injected as before.

Protocol 3: Inhibition of KATP channel by glibenclamide.
After baseline measurements were taken, 1 μg of acetylcholine was injected into the LPA (Fig. 1C). After LPA flow and arterial pressures had returned to baseline values, 200 μg of glibenclamide, an agonist of KATP channels, was injected as a bolus (over 5 s) into the LPA to produce an increase in LPA flow. After LPA flow and pressures had returned to baseline values, CGRP was injected. After 120 min of recovery, 40 mg of glibenclamide were infused over 40 min into the LPA. A second bolus of cromakalim was injected into the LPA, as before, to check effective blockade of NO-induced increases in PA flow. A second injection of CGRP was then given. If LPA flow increased during the infusion of glibenclamide, cromakalim was injected after LPA flow and arterial pressures had returned to baseline values.

Protocol 4: Inhibition of cyclooxygenase pathway by indomethacin.
After baseline measurements were taken, 1 μg of acetylcholine was injected into the LPA (Fig. 10). After LPA flow and arterial pressures had returned to baseline values, CGRP was injected. After 120 min of recovery, a total of 5 mg indomethacin was injected into the superior vena cava as a slow bolus (1 mg over 10 min) followed by an infusion (4 mg over 90 min), a protocol that previously has been shown to effectively block prostaglandin production (36). At the end of the indomethacin infusion, CGRP was injected again.

Drug preparation. Human CGRP (Sigma Chemical, St. Louis, MO) and acetylcholine (Iolab Pharmaceuticals, Clairmont, CA) were dissolved in normal saline at a concentration of 10 μg/ml. Cromakalim (Sigma Chemical) was dissolved in normal saline at a concentration of 400 μg/ml. CGRP8–37 (Sigma Chemical) was dissolved in normal saline at a concentration of 50 μg/ml. l-NNA (Sigma Chemical) was dissolved in normal saline at a concentration of 4 mg/ml. Glubenclamide (Sigma Chemical) was dissolved in distilled water at a concentration of 1 mg/ml and titrated to pH 12–13 by the addition of sodium hydroxide. Indomethacin was dissolved in 100 μl of ethyl alcohol and then suspended with a Tris-buffer solution (pH 8.0) at a concentration of 0.25 mg/ml.

Hemodynamic measurements. LPA flow was measured with ultrasonic flow transducers and a flowmeter (Transonic Systems). Vascular catheters were connected to Statham P23 Db strain-gauge transducers (Statham Instruments, Oxnard, CA), and phasic and mean Ao and PA pressures were measured. Heart rate was obtained from a cardiotachometer triggered from the phasic PA pressure tracing. All variables were recorded continuously on a direct writing polygraph (Gould, Cleveland, OH). Blood samples were obtained from the aorta for determination of pH, PCO2, and PO2 (Corning 158 Blood Gas Analyzer; Corning Medical, Medfield, MA) as well as O2 saturation and hemoglobin concentration (Osm2 hemoximeter; Radiometer, Copenhagen, Denmark).

Data collection and analysis. For every injection of CGRP, hemodynamic data were acquired at two points: before injection (baseline value) and at the point of maximal change. Hemodynamic responses were then calculated as the difference between the maximal change and the baseline value. The differences between the two baseline values and between the baseline value and the maximal change were assessed statistically by using a paired-sample t-test. In addition, the hemodynamic responses were compared statistically before and after administration of each blocker agent by using the Mann-Whitney U test. To assess the effect of blocker agents, the responses in LPA flow induced by the agonists (acetylcholine, cromakalim) were compared statistically before and after administration of the blocker by using a paired-sample t-test and were expressed as inhibition rate: [(response before blocker) – (response after blocker)]/[(response before blocker) × 100. We considered statistical significance to be present when the P value was <0.05. Data are presented as means ± 1 SD except for those presented in a box plot.

RESULTS

Fetal blood gases measured before the experiments were within normal limits for our laboratory (14, 27): hemoglobin, 10.7 ± 2.1 g/dl; pH, 7.39 ± 0.05; O2 saturation, 55.9 ± 9.9%; PO2, 2.51 ± 0.59 kPa (18.8 ± 4.4 mmHg); and PCO2, 7.09 ± 0.68 kPa (53.2 ± 5.1 mmHg).

Fig. 2. Responses to CGRP in LPA blood flow before and after administration of each blocker agent. Box plot shows the 10th, 25th, 50th (median), 75th, and 90th percentiles. Values above the 90th and below the 10th percentile are shown as open circles. Open bars, before blocker agent; filled bars, after blocker agent.
Mean body weight of the fetuses was 3,330 g (range 2,200–4,200 g) at autopsy. Figure 1 shows the protocols and examples of LPA flow measurements. Figure 2 summarizes LPA flow responses to CGRP before and after administration of each blocker agent.

Protocol 1: Effects of CGRP receptor blockade by CGRP8–37. CGRP injection increased LPA flow from 11 ± 16 to 107 ± 48 ml/min, and this response was significantly attenuated (91%) by CGRP8–37 infusion (Table 1, Fig. 1A). CGRP also significantly decreased mean PA and Ao pressures and increased heart rate; these responses also were significantly attenuated by CGRP8–37.

Protocol 2: Effects of NO synthesis blockade by L-NNA. Before L-NNA infusion, acetylcholine increased LPA flow from 18 ± 17 to 164 ± 14 ml/min. This response was significantly inhibited (96%) after the L-NNA infusion (P < 0.01) from 0 ± 0 to 7 ± 16 ml/min. CGRP increased LPA blood flow from 27 ± 33 to 190 ± 84 ml/min before L-NNA, but this response was significantly attenuated (86%) by L-NNA (Table 2, Fig. 1B). The change of mean PA pressure also was significantly attenuated by L-NNA, but Ao pressure and heart rate were not affected (Table 2).

Protocol 3: Effects of K_{ATP} channel blockade by glibenclamide. Before the glibenclamide infusion, cromakalim increased LPA flow from 37 ± 34 to 164 ± 78 ml/min. This response was significantly inhibited (92%) after glibenclamide (P < 0.01) from 26 ± 22 to 36 ± 34 ml/min. CGRP increased LPA flow from 25 ± 24 to 135 ± 78 ml/min before glibenclamide, but this response also was significantly attenuated (69%) by glibenclamide (Table 3, Fig. 1C). The changes in mean PA and Ao pressures also were significantly attenuated by glibenclamide, but heart rate was not affected (Table 3).

Protocol 4: Effects of blockade of cyclooxygenase pathway by indomethacin. Indomethacin infusion produced no significant changes in the CGRP-induced increases in LPA blood flow or in the other variables (Table 4, Fig. 1D).

**DISCUSSION**

In this study, exogenous CGRP significantly decreased PA pressure and increased LPA flow in fetal sheep, consistent with the findings of our previous study (9), in which we demonstrated that CGRP-induced increases of LPA flow were more dependent on its vasodilatory action (decrease in pulmonary vascular resistance) than its positive chronotropic action (increase in heart rate). CGRP-induced vasodilation was significantly attenuated by CGRP8–37, L-NNA, and glibenclamide, but not by indomethacin. Thus the CGRP-induced increase in PA flow in fetal sheep could be mediated, in part, by a CGRP receptor, NO release, and/or K_{ATP} channel activation, but not by a cyclooxygenase-mediated mechanism.

CGRP8–37, a selective CGRP receptor antagonist, caused 91% inhibition of the CGRP-induced increase in LPA flow (Table 1, Fig. 2). This suggests that a CGRP-specific receptor plays a principal role in CGRP-induced vasodilation in fetal sheep. This result is consistent with previous studies in adult animals, e.g., in the pulmonary vascular bed in adult rats and in the isolated left atria in adult guinea pigs (18, 23). There is no information about developmental alterations of expression of a CGRP-specific receptor from prenatal to postnatal.
CGRP in Fetal Pulmonary Circulation

Table 3. Hemodynamic effects of CGRP injection before and after glibenclamide infusion

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<th>Before Glibenclamide</th>
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<td></td>
<td>Baseline</td>
<td>Maximum</td>
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<tr>
<td>LPA blood flow, ml/min</td>
<td>25 ± 24</td>
<td>135 ± 8</td>
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<tr>
<td>Mean pulmonary arterial</td>
<td>57 ± 8</td>
<td>52 ± 6</td>
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<tr>
<td>blood pressure, mmHg</td>
<td>52 ± 12</td>
<td>46 ± 10</td>
</tr>
<tr>
<td>Mean aortic blood pressure,</td>
<td>211 ± 24</td>
<td>240 ± 28</td>
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<td>mmHg</td>
<td>208 ± 49</td>
<td>228 ± 39</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>208 ± 49</td>
<td>228 ± 39</td>
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Values are expressed as means ± SD; n = 6. *P < 0.05 comparing baseline and maximum. †P < 0.05, response to CGRP compared before and after glibenclamide.

nental life, but this study demonstrates that a CGRP-specific receptor works and mediates vasodilation even in prenatal life. This finding is interesting in terms of the dramatic changes in the pulmonary circulation during the transition from fetal to neonatal life.

CGRP has not only a vasodilatory but also a positive chronotropic action (13). Indeed, exogenous CGRP significantly increased heart rate in this and previous studies (9). Interestingly, in this study, the CGRP-induced increase in heart rate was significantly inhibited only by CGRP 8–37, not by L-NNA or glibenclamide (Tables 1–3), even though L-NNA and glibenclamide significantly inhibited the CGRP-induced vasodilatory action quite similarly to CGRP 8–37. These data suggest that NO release and/or K ATP channel activation are involved in the vasodilatory action of CGRP but are not responsible for the positive chronotropic action of CGRP, and that a CGRP-specific receptor plays a role in both actions. This in vivo finding is consistent with those of previous immunohistochemical studies showing that CGRP immunoreactivity is positive in the perivascular nerve fibers in the lungs and the sinoatrial and atrioventricular nodes in the heart (12, 35).

The involvement of NO or guanosine 3’,5’-cyclic monophosphate (cGMP) in CGRP-induced vasodilatory action is still controversial. The NO-mediated mechanism is well demonstrated in the aorta and mesenteric artery in rats (16, 31), but not in the pulmonary artery in guinea pigs (28). In this study, the CGRP-induced increase in LPA flow was attenuated by L-NNA, a specific NO synthase blocker (Table 2, Fig. 1), which suggests some NO-related component in the pulmonary vessels in fetal sheep. The reasons for this inconsistency are not clear but might be explained by differences among species or organs. Adrenomedullin, with 27% structural homology to CGRP, has some cross action with CGRP. Because adrenomedullin responses to NO synthase inhibitors differ among species (26), the same could be true for CGRP. Although L-NNA produced an increase in systemic and PA pressures, probably reflecting overall vasoconstriction, our previous studies (11) showed that vasoconstriction alone, induced in those studies with U-46619, a thromboxane mimetic, had no effect on the responses to further manipulation of pulmonary vascular resistance. Thus the noted effects of L-NNA most likely reflected inhibition of NO production.

The recording of a mean LPA blood flow of 0 ml/min after L-NNA administration and the subsequent assessment of the effects of a vasodilator, in the face of an apparent lack of flow into the lung, require explanation. At low flows, the resolution of the flowmeter system we used does not allow for the precise measurement of the very low flow rates encountered in this model. Furthermore, and more importantly, we have reported mean flows obtained by electrical integration of the phasic blood flow signal. Examination of the phasic flow signal always reveals forward flow during systole and, because of the high pulmonary vascular resistance, no flow, or even some retrograde flow, during diastole. Thus, although the mean flow may register zero, net forward flow occurs.

Indomethacin did not affect the CGRP-induced pulmonary vasodilation (Table 4, Fig. 2). Prostaglandins, particularly prostacyclin, are potent vasodilators of fetal pulmonary arteries, an effect mediated by cAMP (19). In this study, inhibition of prostaglandins did not affect CGRP-induced vasodilation, which might indicate little or no contribution of this cAMP mechanism to the CGRP-induced pulmonary vasodilation in fetal

Table 4. Hemodynamic effects of CGRP injection before and after indomethacin infusion

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<th>Before Indomethacin</th>
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<td></td>
<td>Baseline</td>
<td>Maximum</td>
</tr>
<tr>
<td>LPA blood flow, ml/min</td>
<td>29 ± 27</td>
<td>148 ± 88</td>
</tr>
<tr>
<td>Mean pulmonary arterial</td>
<td>58 ± 8</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>blood pressure, mmHg</td>
<td>50 ± 7</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>Mean aortic blood pressure,</td>
<td>208 ± 49</td>
<td>228 ± 39</td>
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<td>mmHg</td>
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<tr>
<td>Heart rate, beats/min</td>
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Values are expressed as means ± SD; n = 6. *P < 0.05 comparing baseline and maximum.
sheep. Previous investigators demonstrated involvement of both cAMP and cGMP in CGRP-induced vasodilation and suggested dual mechanisms (15, 17). However, our data suggest that, in the pulmonary circulation of fetal sheep, cGMP-mediated mechanisms are dominant.

There is accumulating evidence of involvement of K\textsubscript{ATP} channels in CGRP-induced vasorelaxation, e.g., in rabbit mesenteric artery, pig coronary artery, and rat cerebral artery (21, 25, 37). However, a lesser role of K\textsubscript{ATP} channels also has been shown in other vessels (1, 20). Thus the contribution of K\textsubscript{ATP} channels to CGRP-induced vasorelaxation also may vary among organs or species. In this study, the CGRP-induced increase in LPA flow was significantly attenuated by glibenclamide, a specific K\textsubscript{ATP} channel blocker, which shows involvement of K\textsubscript{ATP} channels. However, glibenclamide inhibited only 69% of the CGRP-induced response in LPA flow, whereas it inhibited 92% of the agonist (cromakalim)-induced response (Table 3). This inhibition of CGRP-induced action is almost 20% lower than that by CGRP\textsubscript{8–37} or L-NNA (91% and 86%, respectively). This indicates that the contribution of K\textsubscript{ATP} channels to CGRP-induced vasorelaxation is only partial in the pulmonary vessels in fetal sheep.

Stimulation of CGRP-specific receptors, NO release, and K\textsubscript{ATP} channel activation all are possible mechanisms, involved to different degrees, in the CGRP-induced increase in PA flow in fetal sheep. This study provides no evidence to establish whether these mechanisms work independently or in concert or which may be more important. Recent studies have reported the linkage of NO release to K\textsubscript{ATP} channels in relaxation of systemic vascular smooth muscle as well as the finding that activation of K\textsubscript{ATP} channels contributes to cGMP-mediated vasodilatation (4, 6, 24). We have previously shown, in fetal sheep, that the pulmonary vasodilation induced by pinacidil, a specific K\textsubscript{ATP} channel agonist, was significantly attenuated by L-NNA, a specific NO synthase blocker (7). Thus there likely is some interaction between NO release and K\textsubscript{ATP} channel-mediated mechanisms in the signal transduction of CGRP stimulation. In addition, more recent studies have demonstrated that both NO release and K\textsubscript{ATP} channel activation are involved in the pulmonary vasodilation during the fetal-to-neonatal transition (2, 34). Further study is required to determine whether endogenous CGRP is involved in pulmonary vasodilation during the transition.

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REFERENCES


