Fetal sheep adrenal blood flow responses to hypoxemia after splanchnicotomy using fluorescent microspheres

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Buchwalder, Lynn F., Michelle Lin, Thomas J. McDonald, and Peter W. Nathanielsz. Fetal sheep adrenal blood flow responses to hypoxemia after splanchnicotomy using fluorescent microspheres. J. Appl. Physiol. 84(1): 82–89, 1998.—Adrenal gland blood flow (ABF) increases during hypoxemia in fetal sheep, but regulation of ABF is poorly understood. The purpose of this study was to determine the effects of splanchnic nerve section on fetal ABF responses to hypoxemia using the fluorescent microsphere (FM) technique. At 125 days of gestation, 14 unanesthetized fetal sheep [bilateral splanchnicotomy (Splx, n = 6) and control (Cont, n = 8)] were injected with FM before and at 60 min of N2-induced hypoxemia (~40% decrease in fetal arterial PO2). Adrenal tissue and reference blood samples were digested and filtered, and FM dye was extracted for spectrometer analysis. Baseline whole, medullary, and cortical ABF for the Cont group were similar to published values using radioactive microspheres and did not differ from Splx values. Hypoxemia increased whole, medullary, and cortical ABF (mean ± SE) from baseline for the Cont group by 281 ± 61, 258 ± 35, and 496 ± 81% (P < 0.05). The increase for the Splx group was attenuated compared with the Cont group (P < 0.05) for whole and medullary ABF (139 ± 27 and 43 ± 27%) but not cortical ABF (326 ± 91%). We conclude that 1) the FM technique is valid for measuring fetal ABF and 2) in fetal sheep the splanchnic nerve is not necessary to maintain basal ABF but plays an important role in regulating the hypoxemia-induced increase in ABF through the medullary, but not cortical, ABF response.

splanchnic nerve section; late gestation; nonradioactive; regional blood flow

HYPOXEMIC STRESS poses a serious challenge to fetal homeostasis and can have long-term health consequences. During fetal hypoxia, blood flow is redistributed to favor the brain, heart, and adrenals (22). Although it is well established that fetal adrenal blood flow increases during O2 deprivation, regulation of adrenal blood flow during hypoxemic stress is not fully understood. Endocrine responses during hypoxemia have been demonstrated to be primarily responsible for changes in cortical, but not medullary, adrenal blood flow (13, 14).

The splanchnic nerve innervates the adrenal gland and, when stimulated, causes adrenal blood flow to increase (8, 20). In anesthetized adult dogs, adrenal denervation by splanchnic nerve section prevents the hemorrhage-induced increase in medullary blood flow (8, 27). Adrenal cortical blood flow changes in response to hemorrhage were unaltered in those studies. Splanchnic innervation may therefore play a role in fetal adrenal blood flow regulation. In fetal sheep, functional innervation of the adrenal gland has been demonstrated after 125 days of gestation (16). We hypothesized that splanchnic nerve section would reduce the increase in adrenal blood flow that occurs in response to hypoxemia in conscious, late-gestation fetal sheep.

Adrenal blood flow has traditionally been measured using radioactive-labeled microspheres. Regional adrenal blood flow was first measured using radioactive microspheres in 1986 in the dog by Breslow et al. (9). The radioactive microsphere technique, which has been used to measure regional blood flow in adult and fetal animals, was first established in 1967 (31). This method is considered the gold standard for other blood flow measurement techniques. Unlike most techniques for measuring blood flow, microspheres can provide measurements for every organ in the body and even specific regions within a tissue or organ. Health hazards and increasing financial costs associated with using radioactive spheres have led to the development of nonradioactive forms of the microsphere method. A fluorescent-labeled microsphere method has become available during the last 3 yr and has been validated in adult animal models (23) but has not been used in any fetal models.

The present study was designed with two specific aims in mind: 1) to establish the validity of the fluorescent microsphere method in measuring adrenal gland blood flow in the fetal sheep and 2) to evaluate the effect of denervation of the adrenal gland on its blood flow response to acute hypoxemia in the unanesthetized fetus by use of the fluorescent microsphere method.

MATERIALS AND METHODS

Animal Use

Fourteen fetuses from 13 pregnant Rambouillet-Columbia ewes (including one set of twins) were brought into the laboratory and placed in individual metabolism cages at least 4 days before surgery. Alfalfa cubes and water were provided ad libitum. At the time of study, fetuses were 124–128 days of gestation (full term = 147 days) and weighed 2.9 ± 0.2 (SE) kg. Four of the fetuses were male. The studies were performed according to protocols approved by the Institutional Care and Use Committee at Cornell University. All facilities were approved by the American Association for the Accreditation of Laboratory Animal Care.

Surgical procedures. Food and water were withheld 24 h before surgery. Between 119 and 122 days of gestation, each ewe was premedicated intramuscularly with 0.8 mg of glycopyrrolate and 1,000 mg of ketamine hydrochloride. General anesthesia was induced and maintained with 2% halothane in O2 via tracheal intubation. Lactated Ringer solution was administered via a maternal jugular vein catheter at 5 ml/min.
Surgery was performed using strict aseptic techniques. The uterus was exposed through a lower midline abdominal incision, and the left fetal hindlimb was exposed by means of hysterotomy. Polyvinyl catheters (Tygon, Norton Performance Plastics; 0.04 in. ID, 0.07 in. OD) were implanted in the femoral artery and pedal vein, and the tips were advanced 12 and 25 cm to the lower descending aorta and inferior vena cava, respectively. A catheter was placed into the amniotic cavity and secured along with the hindlimb catheters to the leg itself. The fetal head was exposed through a separate uterine incision, and catheters were placed into the left carotid artery and jugular vein and advanced 7.5 cm each to the ascending aorta and superior vena cava, respectively. Placement of all catheters was verified after each experiment at the time of necropsy.

In six fetuses, bilateral splanchicotomy (Splx) was performed by the technique we described previously (28). Briefly, the celiac ganglion and adrenal gland were located on each side via a retroperitoneal approach before the splanchic nerves were isolated and severed. Approximately 7 and 3 mm were removed from each of the greater and lesser splanchic nerves, respectively, during this procedure. The other eight fetuses (including the one set of twins) served as controls (Cont). These fetuses underwent similar surgeries, which involved all manipulations of the fetus, but without isolation and dissection of the splanchic nerves to avoid any damage to the nerve rootlets.

Fetal catheters were exteriorized through the ewe's flank using a stainless steel trocar. Similar polyvinyl catheters were placed in the maternal carotid artery and jugular vein and advanced 30 and 25 cm, respectively, toward the heart. A polyvinyl catheter (American Pharmaseal, Valencia, CA) was also implanted in the maternal trachea immediately below the thyroid gland, and the tip was advanced 8 cm caudally.

Postoperative care and maintenance. Beginning on the day of surgery, antibiotics were administered once daily for 5 days: 1 g of ampicillin (Polyflex, Fort Dodge Laboratories, Fort Dodge, IA) intramuscularly to the ewe and 500 mg of ampicillin (Amp-Equine, Smith-Kline Beecham, West Chester, PA) through the amniotic catheter. In addition, 0.1 g of phenylbutazone (Equiphene paste, Luitpold Pharmaceuticals, Shirley, NY) was given twice daily for the first 3 days after surgery as an oral analgesic. After surgery the ewe was returned to the metabolism cage and allowed free access to food and water.

All catheters were maintained patent via a continuous pump infusion of heparin at 25 U/ml in 0.9% NaCl saline solution delivered at 0.5 ml/h by syringes. Daily maternal and fetal arterial blood samples (0.6 ml) were collected for determination of pH, PO2, and PCO2 (ABL600 blood gas analyzer, Radiometer, Copenhagen, Denmark; measurements corrected to 37°C) and hemoglobin concentration and O2 saturation (OSM2 hemoximeter, Radiometer).

Recording of mean arterial pressure. The fetal and maternal carotid artery catheters were connected to pressure transducers (Cobe model CDX III, Argon Medical, Athens, TX). Mean arterial pressure (MAP) data were collected continuously using a computerized data acquisition system. Briefly, pressure signals were sampled and digitized at 32 Hz, averaged over 8 s, and stored with a time signal. To provide immediate display and verify computer-based calculations, the blood pressure tracings were printed using a strip chart recorder.

Fluorescent microsphere injection. Sterile fluorescent-labeled polystyrene microspheres with 15.5 ± 0.3 µm diameter and 1.055 g/cm3 density, suspended in 0.9% NaCl with 0.02% Tween 20 and 0.02% thimerosal, were obtained from Molecular Probes (Eugene, OR). A single color of fluorescent microspheres was injected at 5 min before hypoxemia (baseline) and a different color at 60 min of hypoxemia to determine blood flow at those times. Each fetus received two different colors: yellow-green and red or blue-green and orange. The colors were paired in this manner to minimize spectral wavelength overlap.

Before each injection the fluorescent microspheres were sonicated for 10–15 min, vortexed for at least 2 min, and drawn up into sterile syringes (~2 × 106 microspheres in 2 ml per syringe). The microspheres were then immediately injected into the fetal inferior and superior vena cava over 30 s, then 3.5 ml of normal saline (a total of 4 × 106 microspheres per color) were flushed over 30 s.

Beginning 15–25 s before microsphere injection, reference samples from the ascending and descending aorta were obtained by continuous withdrawal of blood via a syringe pump (model 944, Harvard Apparatus, S. Natick, MA) into heparinized glass syringes at a rate of ~2 ml/min. Sampling continued for 75 s after the microsphere injection was completed. The exact reference blood flow was calculated as the difference between the post-injection and post-withdrawal glass syringe weights, corrected for blood density (divided by 1.05 g/cm3), and divided by the exact collection time obtained using a stopwatch. Each fetus received a total of ~8 × 106 microspheres during the course of experimentation, 4 × 106 microspheres for each of the two flow estimations.

Blood and tissue processing. At the end of the experiment, ewes and their fetuses were euthanized by maternal intravenous injection of pentobarbital sodium solution. The fetus was immediately removed and weighed, then individual organs were dissected and weighed. To separate the adrenal cortex from the medulla, a longitudinal incision was made through the adrenal gland, dividing the gland in half, before the medullary tissue was gently teased away from the cortical tissue in each half of both adrenals.

The reference blood sample syringes were emptied into individual glass test tubes. Each syringe was subsequently rinsed three times with Tween 80 (Aldrich Chemical, Milwaukee, WI) in distilled water (1% final concentration), and the rinse was then added to the corresponding test tube. To digest the blood-rinse solutions, 5 ml of 16 M potassium hydroxide solution were added to each tube. Tubes were then left to sit at room temperature for 24 h. Tissue samples were placed in similar glass test tubes and digested in 7 ml of 4 M potassium hydroxide solution and 1% Tween 80 for 24 h in a 43°C water bath. All the test tubes were protected from light by a dark
cover over the water bath. The test tubes were manually shaken at least every 12 h.

To recover the microspheres after digestion, each sample was vacuum filtered onto a 10-µm-pore polycarbonate filter membrane (Poretics, Livermore, CA) using sink aspiration with a glass microanalytical filtration apparatus (Fisher Scientific, Pittsburgh, PA). The sample tube and filtration funnel were then immediately rinsed three times each with 1% Tween 80, and the filter was allowed to dry for 30–45 s via the vacuum. With use of forceps, the filter was carefully folded several times while still on the filtration screen before being placed into a capped polypropylene microfuge tube.

Fluorescence measurement. To extract the fluorescent dye from the recovered microspheres, 1.25 ml of 2-ethoxyethyl acetate solvent (Cellosolve acetate, Aldrich Chemical) were added to each microfuge tube using a digital Finnpipette (1–5 ml range, Labsystems, Helsinki, Finland). The tubes were then vortexed, visually inspected to ensure that each filter was completely immersed in the solvent, and allowed to sit for 24 h in a light-proof container.

Fluorescence was determined with a luminescence spectrophotometer (model CM1T111, SPEX, Edison, NJ). All fluorescence measurements were made with instrument parameters set at 2.5-mm excitation and emission slit widths with a 0.2-s integration period for each 1-nm interval read over the spectral range of 400–700 nm. A 600-µl aliquot was taken from the microfuge tube and pipetted (200- to 1,000-µl range, Finnpipette) into a 0.7-ml, 10-mm path-length glass cuvette with a Teflon stopper (Hellma, Forest Hills, NY). The sample tube and filtration funnel were then immediately rinsed three times while still on the filtration screen before being placed into a capped polypropylene microfuge tube.

Fluorescence was completely immersed in the solvent, and allowed to sit for 24 h in a light-proof container. The sample was then measured for fluorescence using the appropriate excitation/emission wavelengths in nm: 430/466 (blue-green), 490/506 (yellow-green), 530/553 (orange), and 565/596 (red). Between samples, the cuvette was washed with ~40 ml of 100% ethanol and air dried using a sink aspiration cuvette washer (Kontes, VWR Scientific, Roanoke, NY). Serial dilutions of the sample were made to ensure that quenching of the sample did not occur. In cases in which there was evidence of sample quenching, the fluorescence of the final dilution in the series was used and multiplied by the dilution factor to obtain the "true" fluorescence of the original sample.

"Standard curves" reflecting the fluorescence intensity as a function of microsphere number were generated as follows: with use of a pipette (200- to 1,000-µl range, Finnpipette) a known aliquot from the well-sonicated and vortexed original lot of each color was serially diluted for five different dilutions (400, 800, 1,200, 1,600, and 2,000 fluorescent microspheres/ml solvent). The manufacturer’s specific concentration, as listed on the certificate of analysis for each color’s lot, was used as the concentration of that aliquot. The dilutions chosen were based on the range of fluorescence intensities measured in the study. In a manner similar to the blood and tissue sample filtration, known aliquots of these dilutions were then filtered and rinsed with 1% Tween 80, and the filters were subsequently placed in microfuge tubes before addition of 1.25 ml of ethoxyethyl acetate. Within 24 h the fluorescence was measured as described above. Filters containing no microspheres were also measured for fluorescence. This entire procedure for treatment of standards was performed twice to generate two curves for each fluorescent color, which were then averaged for a single curve per color. The amount of fluorescence intensity per microsphere was thus generated for each color. This process also allowed for testing of the linearity of the fluorescent signal.

Data Analysis

Adrenal blood flow calculations. Because there was minimal, if any, fluorescence spillover of one color into another for each of the pairs used, no spillover correction was needed. The ethoxyethyl acetate exhibited no background fluorescence at any of the excitation and emission wavelengths examined; hence, it was not subtracted from the raw data. For adrenal tissue samples, blood flow (using only the femoral artery reference sample) was calculated using the formula

$$Q_{sample} = \frac{(Q_{ref} \times F_{sample})}{F_{ref}}$$

where $Q_{sample}$ and $Q_{ref}$ are the blood flow (in ml/min) in the specific sample and the exact rate of withdrawal of the reference sample, respectively, and $F_{sample}$ and $F_{ref}$ are the fluorescence intensity in the specific sample and reference blood sample, respectively. The calculated tissue sample blood flow was then divided by the weight of the sample and multiplied by 100 to describe blood flow in milliliters per minute per 100 g. To determine the number of microspheres in each tissue and blood sample, the fluorescence in the sample was divided by the fluorescence per microsphere. In cases where the adrenal had been separated, whole adrenal blood flow was calculated as the sum of the absolute medullary and cortical blood flows divided by the adrenal gland weight.

Vascular resistance calculations. Vascular resistance was determined at 5 min before and at 60 min during hypoxemia with the microsphere-determined blood flow. Resistance (expressed as mmHg · ml⁻¹ · min⁻¹ · 100 g⁻¹) was calculated for each tissue sample using MAP divided by blood flow.

Statistics. All data were analyzed using SigmaStat (version 1.0, Jandel Scientific Software, San Rafael, CA) and are expressed as means ± SE. A simple linear regression was used to characterize the relationship between fluorescence intensity and number of microspheres (as determined from serial dilutions).

For each ewe and fetus the blood gas data for the 20 and 10 min before hypoxemia differed from their mean by <9%; thus they were averaged to give single independent baseline values. Similarly, the five samples obtained during hypoxemia for each animal yielded coefficients of variation of <15%. Thus they were averaged to give single independent hypoxemia values for each animal. The MAP values were averaged in a comparable manner for single independent baseline and hypoxemia values. For the determination of resistance, specific MAP values obtained at the time of microsphere injections, and not the averaged values, were used, since they are more accurate with respect to time.

Baseline and hypoxemia values were compared within Cont and Splx groups using paired Student’s t-tests and between Cont and Splx groups using Student’s nonpaired t-test. In situations where the animal data did not achieve a normal distribution (as in a few instances with the some of the smaller n sizes for blood flows and resistances), nonparametric statistical tests were employed. Specifically, the Wilcoxon signed rank test was used for within-group comparisons, and the Mann-Whitney rank sum test was used for between-group comparisons. Significance was accepted at $P < 0.05$.

RESULTS

Fluorescence Measurements

The correlation between fluorescence intensity and the number of microspheres for each color was $r^2 > 0.99$. Specifically, $r^2$ values were 0.999 (blue-green), 0.994 (yellow-green), 0.997 (orange), and 0.997 (red).

Physiological Measurements

Within the Cont group, three of the maternal and two of the fetal MAP values were not available because of
methodological problems. All baseline and hypoxemia values for carotid arterial pH, blood gases, and MAP are illustrated in Table 1 for the ewe and fetus.

Maternal cardiovascular responses. Maternal blood gas variables and MAP were similar between groups for baseline and hypoxemia (Table 1). No significant alterations in response to hypoxemia were seen in maternal MAP in either group. Hypoxemia produced significant decreases (−50 and 30%, respectively, compared with baseline) in maternal CO2, PO2, and O2 saturation for Cont and Splx groups. There was a slight increase in maternal pH during hypoxemia; however, this increase from baseline was significant only for the Cont group (P < 0.05) and did not differ from maternal Splx pH. In response to hypoxemia, maternal CO2 decreased by ~7% for both groups, but this alteration was significant only for the Cont group (P < 0.01) and was not different from Splx hypoxemia values.

Fetal cardiovascular responses. There were no differences between Cont and Splx groups for any of the fetal baseline MAP or blood gas variables (Table 1). Hypoxemia resulted in significant decreases compared with baseline (P < 0.001; −10, 40, and 50%, respectively) in fetal CO2, PO2, and O2 saturation in Cont and Splx groups, with no differences between the two groups. There was a slight decrease compared with baseline (P < 0.05) in fetal pH in the Splx group; however, this did not significantly differ from Cont fetal pH.

Fetal adrenal blood flow. There was no difference between body weights of Cont (2.91 ± 0.20 kg) and Splx fetuses (2.88 ± 0.14 kg). There was a difference (P < 0.01) in fetal adrenal weights between Cont (0.12 ± 0.01 g) and Splx groups (0.17 ± 0.01 g). When total adrenal weight was expressed as a percentage of body weight, a difference was still present (P < 0.05) between Cont (0.0086 ± 0.0010%) and Splx (0.0118 ± 0.0005%) groups. There were no differences between right and left adrenal gland blood flows within groups at baseline or hypoxemia. All comparisons between groups and between baseline and hypoxemia were performed on combined adrenal data.

Table 2. Left and right whole adrenal blood flow for Cont and Splx fetuses at baseline and 60 min of hypoxemia

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Hypoxemia</th>
<th>Baseline</th>
<th>Hypoxemia</th>
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<tbody>
<tr>
<td>Cont</td>
<td>354.5 ± 54.8</td>
<td>1,312.3 ± 177.5</td>
<td>1,394.4 ± 221.6</td>
<td></td>
</tr>
<tr>
<td>Splx</td>
<td>314.9 ± 64.8</td>
<td>731.8 ± 180.0</td>
<td>781.7 ± 187.5</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE expressed in ml·min−1·100 g−1. There were no differences between left and right adrenal blood flows within groups at baseline or hypoxemia. All comparisons between groups and between baseline and hypoxemia were performed on combined adrenal data.

Whole adrenal blood flow measurements were made using adrenals from all eight of the Cont and all six of the Splx fetuses. For regional adrenal blood flow measurements, adrenals from seven of the eight Cont fetuses were separated into medulla and cortex, whereas adrenals from four of the six Splx fetuses were similarly separated. At least 400 fluorescent microspheres were contained in each reference blood sample and individual fetal adrenal gland, medulla, and cortex. The numbers of microspheres (means ± SE) entrapped per adrenal were 5,758 ± 781 and 4,388 ± 1,038 for Cont and Splx groups, respectively. The paired adrenal medullae and paired cortices also contained at least 400 microspheres, even for baseline microspheres alone.

Baseline whole and regional adrenal blood flows did not differ between Cont and Splx fetuses (Fig. 1). Baseline whole adrenal blood flow was 365.4 ± 56.4 and 318.4 ± 63.4 ml·min−1·100 g−1 for Cont and Splx groups, respectively. Baseline blood flow to the medulla was five to six times higher than that to the adrenal cortex for both groups (P < 0.05). Medullary baseline blood flow was 1,119.5 ± 255.4 and 1,151.8 ± 451.0 ml·min−1·100 g−1 for Cont and Splx groups, respectively. Cortical baseline blood flow was 171.9 ± 32.0 and 213.4 ± 34.3 ml·min−1·100 g−1 for Cont and Splx groups, respectively.

Hypoxemia produced significant increases compared with baseline (P < 0.001) and Splx groups (P < 0.05) in whole adrenal blood flow. This increase was significantly attenuated in the Splx compared with the Cont fetuses (Fig. 1). Adrenal cortical blood flow increased severalfold from baseline during hypoxemia (P < 0.05) in Cont and Splx fetuses (Fig. 1). There was no difference between Cont and Splx cortical blood flow during hypoxemia. Adrenal medullary blood flow increased (P < 0.01) more than threefold from baseline in Cont fetuses, whereas Splx fetuses demonstrated no change in medullary blood flow (Fig. 1). Expressed as percent change from baseline, hypoxemia increased whole, medullary, and cortical blood flow for Cont fetuses by 281 ± 35, 258 ± 31, and 496 ± 81% (P <

Table 1. Fetal and maternal pH, blood gas, and MAP at baseline and during 60 min of acute hypoxemia for Cont and Splx sheep

<table>
<thead>
<tr>
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<th>Baseline</th>
<th>Hypoxemia</th>
<th>Baseline</th>
<th>Hypoxemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.36 ± 0.007</td>
<td>7.34 ± 0.018</td>
<td>7.49 ± 0.009</td>
<td>7.51 ± 0.015*</td>
</tr>
<tr>
<td>Pco2, Torr</td>
<td>7.37 ± 0.005</td>
<td>7.34 ± 0.010*</td>
<td>7.49 ± 0.010</td>
<td>7.51 ± 0.014</td>
</tr>
<tr>
<td>PaO2, Torr</td>
<td>50.7 ± 1.7</td>
<td>45.0 ± 1.8*</td>
<td>36.6 ± 0.8</td>
<td>31.9 ± 0.5*</td>
</tr>
<tr>
<td>Pao2, Torr</td>
<td>51.1 ± 1.5</td>
<td>47.2 ± 1.4</td>
<td>38.3 ± 0.8</td>
<td>33.8 ± 0.8</td>
</tr>
<tr>
<td>So2, %</td>
<td>54.3 ± 3.2</td>
<td>23.9 ± 2.9*</td>
<td>39.3 ± 3.4</td>
<td>57.1 ± 4.1</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>57.1 ± 4.1</td>
<td>27.3 ± 1.4*</td>
<td>70.5 ± 4.5*</td>
<td>70.7 ± 5.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 fetal and 7 maternal control (Cont) sheep for pH and blood gases and 6 fetal and 4 maternal sheep for mean arterial pressure (MAP); for splanchic nerve-sectioned (Splx) sheep, n = 6 for all. Pao2, and Pco2, arterial PO2, and Pco2, O2 saturation. Significantly different from baseline: *P < 0.05; †P < 0.01; ‡P < 0.001. §Significantly different from Cont: P < 0.05.
Within the Cont group, whole and regional adrenal resistances were available from six and five fetuses, respectively. Within the Splx group, whole and regional adrenal resistances were obtained from six and four fetuses, respectively.

There were no differences between Cont and Splx baseline vascular resistances (mmHg·ml⁻¹·min⁻¹·100 g⁻¹) for the whole or regional adrenal gland or cortex and medulla separately. Baseline whole adrenal resistance was 0.170 ± 0.042 and 0.185 ± 0.031 for Cont and Splx fetuses, respectively. Baseline cortical resistance was four to five times higher than medullary resistance for both groups (P < 0.01). Medullary resistance was 0.068 ± 0.021 and 0.060 ± 0.020 for Cont and Splx fetuses, respectively. Cortical resistance was 0.339 ± 0.053 and 0.234 ± 0.052 for Cont and Splx fetuses, respectively.

Hypoxemia decreased (P < 0.05) whole adrenal vascular resistance from baseline for Cont and Splx groups to 0.044 ± 0.012 and 0.087 ± 0.014, respectively, and this response was significantly attenuated in the Splx group compared with the Cont group (P < 0.05). In response to hypoxemia, medullary and cortical resistances within the Cont group significantly decreased compared with baseline. In the Splx group only cortical resistance was decreased, whereas medullary resistance did not change from baseline (Fig. 2). Cortical resistance fell to 0.054 ± 0.007 and 0.064 ± 0.012 for Cont and Splx groups, respectively. Cortical resistances were not different between Cont andSplx groups during hypoxemia. The medullary resistance during hypoxemia for the Splx group (0.049 ± 0.014) was marginally different (P = 0.06) from that for the Cont group (0.018 ± 0.006); however, a difference was apparent when resistance was expressed as percent change from baseline (Fig. 2).

**DISCUSSION**

Adrenals of Splx fetuses weighed more than Cont adrenals, whereas body weights were similar. We have
no clear explanation for this finding. However, several studies have demonstrated that surgical manipulation of the adrenal is accompanied by variable unilateral or bilateral increases in adrenal weight and DNA, RNA, and protein content (18, 19). For this reason we avoided adrenal manipulation in our control surgeries. The mechanism responsible for compensatory hypertrophy is unlikely to be blood borne and is considered to be neurally mediated (18, 19). Additionally, it is important to note that, when adrenal blood flow was expressed per 100 g of adrenal weight, as is standard in adrenal blood flow studies, there were no differences between Cont and Splx groups at baseline. Accordingly, adrenal blood flow was analyzed in terms of adrenal gland weight.

The intent of the present study was to determine regional differences in the regulation of adrenal blood flow in the cortex and medulla during hypoxic stress; hence, separate measurements were made of the adrenal cortex and medulla. It is recognized that because of the interdigitation between the fetal medullary and cortical zones it is not possible to obtain a complete separation of medulla and cortex during dissection, and therefore a small degree of cross contamination is likely to have occurred.

Baseline whole adrenal blood flows were the same for Cont and Splx fetuses (Fig. 1). Baseline regional adrenal blood flows were similar between groups, with the adrenal medulla receiving about five times more blood flow per gram than the cortex, as expected. The observed regional difference in baseline blood flow allocation agrees with the observations in intact, unanesthetized fetal sheep by other investigators (13, 14). These results indicate that splanchnic innervation is not necessary in maintaining whole or regional adrenal blood flows in the resting state. Although no published data exist regarding the effects of splanchnic nerve section on adrenal blood flow in any fetal models, similar findings were reported in the anesthetized adult dog (21, 27).

During hypoxemia, whole adrenal blood flow increased in Cont and Splx fetuses compared with baseline. However, the response in the Splx group (~150% increase) was blunted compared with Cont fetal responses (~300% increase), confirming our hypothesis that splanchnicotomy reduces the adrenal blood flow response to hypoxemia. The increased adrenal blood flow was attributed in large part to a significant decrease from baseline in adrenal vascular resistance for Cont and Splx fetuses (Fig. 2). These observations demonstrate that the adrenal blood flow response to hypoxemia that is splanchnic nerve dependent is primarily a vasodilatory response.

During hypoxemia, cortical adrenal blood flow increased similarly (~300%) for Cont and Splx groups. However, whereas Cont medullary blood flow increased from baseline by ~250%, Splx medullary blood flow did not change during hypoxemia. The medullary and cortical vascular resistances inversely paralleled the blood flow results, demonstrating the occurrence of splanchnic nerve-independent vasodilatory effects in the cortex, but not in the medulla. The medullary, but not cortical, blood flow response is abolished in unilaterally splanchnicotomized anesthetized dogs during hemorrhage (27). That report and the present study examined different species (fetal sheep vs. adult dogs) and utilized different challenges (gas-induced hypoxemia vs. hemorrhage) and different states of consciousness (conscious vs. anesthetized). The similar findings between the studies indicate that the splanchnic nerve plays an important modulatory role in adrenal medullary blood flow regulation across species.

Current theories hold that adrenal cortical blood flow is regulated via endocrine factors, particularly adrenocorticotropic hormone, and the medullary component is mediated through some nonendocrine mechanism (14). Our investigation demonstrates that fetal medullary blood flow is neurally regulated. It has been shown in fetal sheep at this gestational age that hypoxemia-induced increases in cortical blood flow are adrenocorticotropic hormone dependent (13, 14) and arginine vasopressin independent (12). These investigators also reported that this response occurs as a result of decreased adrenal vascular resistance. Administration of an arginine vasopressin V1 receptor antagonist during hypoxemia did not reduce the hypoxemia-induced increase in adrenal blood flow (29).

Splanchnic nerve stimulation increases adrenal vasoactive intestinal polypeptide (VIP) output in the absence of any increase in peripheral plasma VIP concentrations (5). Exogenous VIP increases whole adrenal blood flow in the intact in situ rat adrenal gland (24); thus splanchnic nerve section in fetal sheep may reduce adrenal VIP secretion and blunt adrenal medullary blood flow responses. The recent report of diminished VIP content in the medulla and inner cortical zones of rat adrenals 10 days after splanchnic nerve section supports this view (25). It remains to be established whether the splanchnic nerve directly regulates these neuropeptides in the adrenal. Although splanchnic nerve stimulation also increases the secretion of cate-

### Table 3. Comparison of Cont adrenal blood flow in the present study and in literature determined by radiolabeled microspheres

<table>
<thead>
<tr>
<th>Fluor</th>
<th>Cont (Baseline)</th>
<th>Hypoxemia</th>
<th>Whole</th>
<th>Medulla</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref. 1</td>
<td>365 ± 56</td>
<td>1,119 ± 255</td>
<td>172 ± 32</td>
<td>1,339 ± 197</td>
<td></td>
</tr>
<tr>
<td>Ref. 2</td>
<td>379 ± 9</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Ref. 3</td>
<td>221 ± 28</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Ref. 4</td>
<td>314 ± 52</td>
<td>*</td>
<td>*</td>
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<tr>
<td>Ref. 6</td>
<td>393 ± 53</td>
<td>*</td>
<td>*</td>
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<tr>
<td>Ref. 12</td>
<td>484 ± 124</td>
<td>1,159 ± 305</td>
<td>181 ± 50</td>
<td>*</td>
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<tr>
<td>Ref. 13</td>
<td>308 ± 57</td>
<td>762 ± 166</td>
<td>107 ± 27</td>
<td>762 ± 71</td>
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<tr>
<td>Ref. 14</td>
<td>286 ± 65</td>
<td>649 ± 61</td>
<td>138 ± 30</td>
<td>*</td>
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<tr>
<td>Ref. 15</td>
<td>271 ± 34</td>
<td>*</td>
<td>*</td>
<td>828 ± 82</td>
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<tr>
<td>Ref. 17</td>
<td>234 ± 66</td>
<td>*</td>
<td>*</td>
<td>1,417 ± 467</td>
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<tr>
<td>Ref. 27</td>
<td>455 ± 140</td>
<td>*</td>
<td>*</td>
<td>1,711 ± 140</td>
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</table>

Values are means ± SE expressed in ml·min⁻¹·100 g⁻¹. Values are for fetal sheep of ~125 days gestation at baseline and during similar hypoxic challenges. *Data not available.
cholamines (20), we previously showed that splanchnicotomcy abolishes this increase when fetal sheep experience hypotension (28). Catecholamines were not measured in the present study, but it would be expected that their secretion would be similarly suppressed. Moreover, catecholamine secretion does not appear to directly alter adrenal blood flow. Administration of nicotinic receptor antagonists was reported to eliminate most catecholamine secretion during nerve stimulation without altering the vasodilatory response (7, 10).

The present study evaluated the fluorescent microsphere technique for measuring adrenal blood flow in fetal sheep. All four fluorescent colors yielded linear correlation coefficients >0.99 for the relationship between microsphere number and fluorescence intensity, confirming previous findings (23). Comparison of the number of microspheres in left and right paired organs is an index of mixing sufficiency and uniform microsphere distribution (31). In the present study, blood flows to the right and left adrenals were identical at baseline and during hypoxemia for Cont and Spnx fetuses (Table 2), confirming uniform distribution of fluorescent microspheres in the blood. This consideration is particularly important in relation to the various shunts present in the fetal, but not adult, circulation.

Stochastic analysis and Poisson distribution considerations indicate that a sample must contain at least 400 microspheres to achieve a coefficient of variation <5% (11). Reference blood samples and whole adrenals contained 4,000–5,800 microspheres, resulting in an error coefficient (associated with microsphere number) for whole adrenal blood flow of ~1.5%. Several radioactive microsphere investigations (1, 11) have reported that the most important determinant of variability is the number of microspheres injected; however, it is important that potential methodological limitations be considered for each form of microsphere label. The fluorescent microsphere technique involves a potential loss of microspheres due to extensive tissue processing and filtration, which could increase this variability (23, 32). Recently, a simplified tissue-processing technique has been described that yielded microsphere recovery of ~100%, thereby eliminating that potential source of error (32). Other sources of variability exist with all microsphere techniques and require appropriate consideration to reduce their extent (30). Nonetheless, fluorescent microspheres offer several advantages over their radioactive counterpart, including avoiding the use of radioactivity (this is both financially and environmentally beneficial), having labels with longer shelf lives, and providing superior spectral separation of signals (23, 30).

The range of published whole adrenal blood flow values measured with radioactive microspheres for fetal sheep of a similar gestational age (~125 days) at baseline is shown in Table 3. Fewer data exist for comparison with regional adrenal blood flow in fetal sheep at baseline (Table 3). The observation that whole and regional adrenal blood flows fall within the range of those determined with radioactive microspheres is yet another indication that the fluorescent microsphere technique is a valid means of measuring blood flow in the fetal sheep adrenal. Additionally, five studies were found in which whole adrenal blood flow was measured with radioactive microspheres under conditions of hypoxemia equivalent to those in the present investigation (Table 3). Thus, during an experimental challenge that physiologically and significantly altered adrenal blood flow, the fluorescent microsphere technique yielded values well within the range of values obtained using radioactive microspheres.

In conclusion, the present study demonstrates for the first time that 1) the fluorescent microsphere technique is valid for measuring adrenal blood flow in the fetus and 2) the splanchnic nerve plays an important role in adrenal blood flow responses to hypoxemia by selectively regulating medullary blood flow in unanesthetized fetal sheep. Fluorescent microsphere measurements of fetal adrenal blood flow agreed well with radioactive microsphere-determined values. Splanchnic denervation had no effect on whole or regional adrenal blood flow in the resting state. In response to hypoxemia, however, splanchnic denervation prevented the increase in medullary blood flow but had no effect on cortical blood flow in late-gestation fetal sheep. Further studies, particularly those that would elucidate the role of neuropeptides, are needed to more completely establish the factors that mediate this neuronal regulation of the fetal adrenal gland blood flow responses to hypoxemia.

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