Effects of nociceptive stimuli on the pulmonary circulation in the ovine fetus

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Houfflin Debarge, V., A. Delelis, S. Jaillard, B. Larrue, P. Deruelle, A. S. Ducloy, F. Puech, and L. Storme,. Effects of nociceptive stimuli on the pulmonary circulation in the ovine fetus. Am J Physiol Regul Integr Comp Physiol 288: R547–R553, 2005; doi:10.1152/ajpregu.00433.2004.—The fetus is able to exhibit a stress response to painful events, and stress hormones have been shown to modulate pulmonary vascular tone. At birth, the increased level of stress hormones plays a significant role in the adaptation to postnatal life. We therefore hypothesized that pain may alter pulmonary circulation in the perinatal period. The hemodynamic response to subcutaneous injection of formalin, which is used in experimental studies as nociceptive stimulus, was evaluated in chronically prepared, fetal lambs. Fetal lambs were operated on at 128 days gestation. Catheters were placed into the ascending aorta, superior vena cava, and main pulmonary artery. An ultrasonic flow transducer was placed around the left pulmonary artery. Three subcutaneous catheters were placed in the lambs’ limb. The hemodynamic responses to subcutaneous injection of formalin, to formalin after fetal analgesia by sufentanil, and to sufentanil alone were recorded. Cortisol and catecholamine concentrations were also measured. Pulmonary vascular resistances (PVR) increased by 42% (P < 0.0001) after formalin injection. Cortisol increased by 54% (P = 0.05). During sufentanil infusion, PVR did not change significantly after formalin. Cortisol increased by 56% (P < 0.05). PVR did not change during sufentanil infusion. Norepinephrine levels did not change during any of the protocols. Our results indicate that nociceptive stimuli may increase the pulmonary vascular tone. This response is not mediated by an increase in circulating catecholamine levels. Analgesia prevents this effect. We speculate that this pulmonary vascular response to nociceptive stimulation may explain some hypoxemic events observed in newborn infants during painful intensive care procedures.

Animal Preparation

All animal procedures and protocols used in this study were reviewed and approved by the French Ministère de l’Agriculture, de la Pêche et de l’Alimentation before the studies were conducted. Mixed-breed (Columbia-Rambouillet) pregnant ewes at 128 days of gestation (term = 147 days) were fasted for 24 h before surgery. Ewes were sedated with intravenous pentobarbital sodium (total dose: 2–4 g) and anesthetized with 1% buvacaine hydrochloride (4 mg) by lumbar puncture. Ewes were kept sedated but breathed spontaneously throughout the surgery. Under sterile conditions, the fetal lamb’s left forelimb was delivered through a uterine incision. A skin incision was made under the left forelimb after local infiltration with lidocaine (2 ml, 1% solution). Polyvinyl catheters (20 gauge) were advanced into the ascending aorta and the superior vena cava after insertion in the axillary artery and vein. A left thoracotomy exposed the heart and great vessels. A catheter (20 gauge) was inserted into the main pulmonary artery by direct puncture through purse string suture. An ultrasonic flow transducer (size 6; Transonic Systems, Ithaca, NY) was placed around the left pulmonary artery to measure norepinephrine, cortisol, β-endorphin) from as early as 18 wk of gestation (17, 18).

At birth, the increase in stress hormones plays a significant role in the adaptation to postnatal life, including increased left ventricular contractility, lung fluid reabsorption, and surfactant release (11, 25, 35, 47, 49, 60). Stress hormones have also been shown to modulate the pulmonary vascular tone and reactivity during the perinatal life (12, 26, 29). These data suggest that perinatal stress may alter the pulmonary circulation. Furthermore, hypoxemia is usually observed during stressful intensive care procedures in newborn infants with respiratory failure (38). Duration of hypoxemia is reduced by opioid analgesia (6, 38). However, the mechanism of stress-induced hypoxic response is presently unknown but may include changes in pulmonary vascular tone. Therefore, we hypothesized that pain and/or stress could alter pulmonary circulation. To test this hypothesis, we studied the pulmonary vascular response to nociceptive stimulus in chronically prepared, late-gestation fetal lambs. Subcutaneous formalin injection was used as nociceptive stimulus (27, 55).

MODEL AND METHODS

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blood flow. Three other catheters (22 gauge) were placed subcutaneously into the left hind paw for formalin injection. The uteroplacental circulation was kept intact, and the fetus was gently replaced into the uterus. An additional catheter was placed in the amniotic cavity to measure pressure. Ampicillin (500 mg) was added to the amniotic cavity before closure of the hysterotomy. The flow transducer and catheters were exteriorized through a subcutaneous tunnel to an external flank pouch. Food and water were provided ad libitum after surgery. Vascular catheters were maintained by daily infusions of 2 ml of heparinized saline (10 U/ml). Catheter positions were checked at autopsy. Studies were performed after a minimum recovery time of 96 h. Estimated weight of the fetal lambs was 3000 g.

Physiological Measurements

The flow transducer was connected to an internally calibrated flowmeter (T201, Transonic Systems) for continuous measurements of left pulmonary artery blood flow (Qp). Output filter of the flowmeter was set at 30 Hz. The absolute value of flow was determined from the mean of phasic blood flow signals (at least 30 cardiac cycles), with zero blood flow defined as the measured flow value immediately before the beginning of systole. Main pulmonary artery, aortic, and amniotic catheters were connected to a blood pressure transducer (Merlin monitor, Hewlett-Packard, Palo Alto, CA). Pressures were referenced to the amniotic cavity pressure. Heart rate was determined from the phasic Qp signal. Pulmonary vascular resistance (PVR) in the left lung was calculated as the difference between mean pulmonary artery pressure (PAP) and left atrial pressure divided by mean left Qp. From earlier observations (12, 26) in which left atrial pressure was found constantly close to 2 cmH$_2$O, a value of 2 cmH$_2$O was used as an estimate of left atrial pressure.

Blood samples from the aorta were used for blood-gas analysis, lactate, and oxygen saturation measurements (OSM 3 hemoximeter and ABL 250, Radiometer, Copenhagen, Denmark; EC$_2$, iSTAT, Abbott) and for cortisol and catecholamine (norepinephrine, epinephrine, and dopamine) plasma concentration measurements. All blood samples were replaced with an equal volume of sterile 0.9% saline to prevent hypovolemic stress. Samples were immediately centrifuged and stored at $-80^\circ$C until further analyses. Plasma cortisol concentration was measured by chemiluminescence (cortisol chemiluminescence assay, Nichols Advantage, Nichols Institute Diagnostics, San Juan Capistrano, CA). The intra-assay and interassay coefficients of variation were 9.1% and 12.2%, respectively. Plasma catecholamine levels were measured by HPLC (Alumina from Chromsystems and Coulochem II from ESA). The interassay and intra-assay coefficients of variation were 6% and <5%, respectively.

Drug Preparation

Formaldehyde and 0.9% saline were used to prepare formalin solution. Two milliliters of 1% formalin were injected subcutaneously. Sufentanil (10 ml = 50 µg) was diluted with sterile 0.9% saline to a final concentration of 1 µg/ml.

Experimental Design

Three different experimental protocols were included in this study: 1) pulmonary hemodynamic response to formalin injection, 2) pulmonary hemodynamic response to formalin after fetal analgesia by sufentanil, and 3) pulmonary hemodynamic response to sufentanil infusion alone. All the protocols were applied in a random order. A minimum recovery period of 24 h was required between each protocol. To ensure that complete recovery was achieved before starting a protocol, we checked that the measured hemodynamic parameters and arterial blood gases returned to the baseline values.

**Protocol 1: effects of the formalin test on pulmonary circulation.** To investigate the effects of nociceptive stimulus on fetal pulmonary circulation, we studied the hemodynamic response to the formalin test. Mean PAP, mean aortic pressure (MAP), amniotic pressure, left Qp, and heart rate were recorded at 5-min intervals throughout the study period. PVR in the left lung was calculated. After 30 min of stable baseline measurements, formalin was injected in one of the subcutaneous catheters. Duration of each experiment was at least 140 min. Fetal blood gases, lactate, pH, cortisol, and catecholamine levels were measured before and 30 min after the formalin test.

**Protocol 2: effects of the formalin test on pulmonary circulation after fetal opioid analgesia.** To ensure that the pulmonary vascular response to formalin was due to nociceptive stimulus and not to formalin toxicity, we studied this response after fetal analgesia by sufentanil. Hemodynamic measurements were performed as in protocol 1. After 30 min of stable baseline measurements, 6 µg of sufentanil were injected into the venous catheter. This injection was followed by a continuous sufentanil infusion at a rate of 6 µg/h during the whole study period. Formalin was injected 20 min after the beginning of sufentanil infusion.

Fetal blood gases, lactate, pH, and plasma cortisol and catecholamine levels were measured before and 30 min after the formalin test.

**Protocol 3: effects of sufentanil on pulmonary circulation.** To investigate the effects of sufentanil infusion on pulmonary circulation, the same hemodynamic measurements as in protocol 1 were conducted during fetal opioid analgesia by sufentanil. Bolus of 6 µg followed by continuous infusion (6 µg/h) during 90 min. Fetal blood gases, lactate, and pH were measured before and 30 min after sufentanil injection.

Data Analysis

Results are presented as means ± SE. The data were analyzed using repeated-measures and factorial ANOVA. Intergroup differences were analyzed with Fisher’s, Scheffe’s, and Bonferroni/Dunn’s least significant test (Stat View for PC, Abacus Concepts, Berkeley, CA). Mann-Whitney test (independent values) was also performed on the quantitative data to test for statistical differences between the groups. A $P < 0.05$ was considered statistically significant. In each experiment, $n$ represents the number of studied fetuses.

RESULTS

Eight pregnant ewes were operated at 128 days of gestation (term 147 days). Baseline values (mean PAP, MAP, Qp, PVR,
blood gas, pH, lactate, and plasma cortisol and norepinephrine levels) were similar in the three protocols. Epinephrine and dopamine were only found as traces; thus statistical analysis was not possible.

**Protocol 1: Effects of the Formalin Test on Pulmonary Circulation (n = 8)**

Mean PAP increased by 12% (from 49 ± 0.9 to 55 ± 0.7 mmHg) after formalin injection (P < 0.01) (Fig. 1). Despite a slight decrease, Qp did not change significantly (Fig. 1). Mean PVR increased from 0.76 ± 0.01 to 1.08 ± 0.05 mmHg·ml⁻¹·min (P < 0.0001) (Fig. 1). However, the response was biphasic. After a transient increase in PVR starting immediately after formalin injection, PVR returned to the baseline level. Twenty-five minutes after administration of formalin, PVR increased by 42% for more than 1 h (P < 0.0001) (Fig. 1).

MAP increased by 15% (P < 0.01). Changes were parallel to mean PAP. Fetal heart rate after formalin injection increased by 10% (P < 0.01) (Fig. 2). A biphasic pattern of response was observed with a maximum at 10 and 50 min (Fig. 2).

Formalin injection did not alter fetal blood-gas, pH, lactate, or plasma norepinephrine levels (Tables 1 and 2). Cortisol increased from 2.23 to 3.45 µg/dl (54%) after formalin injection (P = 0.05) (Fig. 3).

**Fig. 1.** Hemodynamic response to formalin test. Formalin increased mean aortic pressure (MAP) (P < 0.01), mean pulmonary artery pressure (PAP) (P < 0.01), and mean pulmonary vascular resistance (PVR) (P < 0.0001). Left pulmonary artery blood flow (Flow) did not change significantly. Values are expressed as means ± SE.

**Fig. 2.** Top: fetal heart rate increased after formalin test (P < 0.01). Middle: sufentanil infusion increased fetal heart rate from the beginning and during all of the infusion (P < 0.0001). No additional increase in fetal heart rate was observed after formalin test. Bottom: sufentanil infusion alone increased fetal heart rate (P < 0.001). Values are expressed as means ± SE. bpm, Beats per minute.
**Protocol 2: Effects of the Formalin Test on Pulmonary Circulation After Fetal Opioid Analgesia (n = 6)**

Mean PAP, MAP, Qp, and PVR did not change after formalin injection in the sufentanil-treated fetuses (Fig. 4). Fetal heart rate increased by 34% after sufentanil infusion was started (P < 0.0001) (Fig. 2). No further increase in fetal heart rate was observed after formalin injection (Fig. 2).

Blood-gas, pH, lactate, and norepinephrine levels did not change during the study period (Tables 1 and 2). Cortisol increased by 56% (from 1.7 to 2.66 μg/dl) after formalin injection with fetal analgesia (P < 0.05) (Fig. 3).

**Protocol 3: Effects of Sufentanil on Pulmonary Circulation (n = 6)**

Mean PAP, MAP, Qp, blood-gas, pH, and lactate levels did not change during sufentanil infusion (Fig. 5, Table 1). Heart rate increased by 21% after sufentanil infusion (P < 0.001) (Fig. 2).

**DISCUSSION**

In this in vivo experimental study, we tested the hypothesis that nociceptive stimuli may modulate pulmonary vascular tone during the perinatal life. To test this hypothesis, the pulmonary vascular response to subcutaneous formalin injection was studied in chronically prepared, late-gestation fetal lambs. We found that formalin injection increased PVR. The addition of sufentanil abolished this response, but sufentanil alone did not alter the basal pulmonary vascular tone. These results support the hypothesis that nociceptive stimuli increase PVR during the perinatal period.

The fetal lamb has been extensively used to study the perinatal pulmonary circulation (1). Chronically prepared, late-gestation fetal lambs provide reproducible and reliable measurements of basal pulmonary vascular tone and reactivity to various vasoactive stimuli (1, 2). The formalin test is commonly used in animal models to study the effects of nociceptive stimuli (14). Subcutaneous formalin injection produces mild pain generated by tissue injuries. The systemic circulatory responses to formalin are highly reproducible. An increase in aortic pressure and an increase in heart rate are observed immediately after formalin injection for ~1 h (8, 52, 55). Furthermore, hormonal responses to stressful events, such as increased plasma cortisol levels, have been demonstrated both in experimental studies and in human fetuses (15, 17–19, 32). In accordance with previous studies performed in other species, we found that subcutaneous formalin injection elevates aortic pressure, heart rate, and plasma cortisol concentration. Thus the usual autonomic and hormonal response to formalin test also exists in our model. To our knowledge, this test has never been performed in fetal lambs.

The behavioral and cardiovascular responses to formalin injection in adult animals present 2 phases: 1) an early phase that lasts no longer than 10 min followed by a period of quiescence and 2) a late phase, ~20–30 min after formalin injection, that matches or exceeds the initial response (8, 14, 55). The biphasic cardiovascular response is not observed in newborn rats and occurs substantially later when the autonomic nervous system is mature (8). In our study, the cardiovascular responses to formalin test (heart rate, MAP, PAP, PVR) were biphasic, suggesting that the maturation of the regulatory mechanisms might be achieved earlier in fetal lambs.

Our study provides new information regarding the hemodynamic effects of nociceptive stimuli. Whereas systemic effects of pain have largely been studied (8, 52, 55), little is known about the lung vascular response to stressful events. Data suggest that endotracheal suctioning may elevate PAP in infants with congenital heart defect (22). Analgesia improves the outcome in infants with pulmonary hypertension (57). Furthermore, stressful intensive care procedures may cause hypoxemia in newborn infants with respiratory failure (38). This

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**Table 1. Fetal blood gases, pH, and lactate before and after formalin and/or sufentanil injection**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before</th>
<th>After</th>
<th>Before</th>
<th>After</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
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<td>7.39±0.01</td>
<td>7.4±0.01</td>
<td>7.37±0.01</td>
<td>7.36±0.01</td>
<td>7.31±0.01</td>
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<tr>
<td>PO2, Torr</td>
<td>19.2±0.7</td>
<td>21.4±0.5</td>
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<td>19.8±0.01</td>
<td>15±0.8</td>
<td>13±0.8</td>
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<tr>
<td>PCO2, Torr</td>
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<td>47±0.2</td>
<td>41.2±2.8</td>
<td>47.7±0.3</td>
<td>46±2.3</td>
<td>46±0.8</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>1.15±0.05</td>
<td>1.13±0.04</td>
<td>1.26±0.06</td>
<td>1.74±0.15</td>
<td>1.60±0.1</td>
<td>1.28±0.1</td>
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</table>

Values are means ± SE. No difference was observed between the three protocols.

**Table 2. Mean plasmatic values of norepinephrine (µg/l) during baseline (before) and at 30 min after formalin injection with or without fetal analgesia by sufentanil**

<table>
<thead>
<tr>
<th>Time</th>
<th>Parameter</th>
<th>Formalin (n = 6)</th>
<th>Sufentanil + formalin (n = 5)</th>
<th>Sufentanil (n = 5)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Before</td>
<td>0.54±0.07</td>
<td>0.77±0.09</td>
<td>0.85±0.07</td>
</tr>
<tr>
<td></td>
<td>At 30 min</td>
<td>0.61±0.07</td>
<td>0.85±0.07</td>
<td>0.85±0.07</td>
</tr>
</tbody>
</table>

Values are means ± SE. No difference was observed between the 2 protocols.

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**Fig. 3. Hormonal response (cortisol) to formalin test with and without fetal analgesia. Basal cortisol levels were similar in the two groups (formalin and sufentanil + formalin). Cortisol levels increased after formalin test both without and with fetal analgesia by sufentanil. No difference was observed between the 2 groups.**
The hypoxemic response to stress is reduced by opioid analgesia (4, 5, 38). Although these studies support the hypothesis that stressful events may alter lung circulation, the effects of pain on the pulmonary vascular tone have not been fully investigated. Our results clearly show that noxious stimuli may elevate the basal pulmonary vascular tone. Indeed, the formalin-induced increase in PVR was completely abolished by sufentanil, indicating that this pulmonary vascular response was related to formalin-mediated nociception.

However, sufentanil did not decrease the autonomic and hormonal responses to formalin test but caused a prolonged rise in fetal heart rate and an increase of plasma cortisol concentration. Tachycardia has previously been reported after opioid injection in fetal lambs (23, 46, 61). In human fetuses undergoing invasive procedures, fentanyl attenuates the fetal β-endorphin stress responses and the brain-sparing effect to needling but has no significant effect on fetal heart rate (15). Opioid drugs are also known to have significant effects on the hypothalamic-pituitary-adrenal axis in sheep and humans (37, 53). Therefore, fetal heart rate or cortisol increases cannot be used in fetal lambs as indexes of analgesia induced by opioids.

Fig. 4. Hemodynamic response to formalin test after fetal opioid analgesia \((n = 6)\). MAP, mean PAP, mean left pulmonary artery blood flow, and mean PVR did not change after formalin test in fetuses pretreated with sufentanil. Values are expressed as means ± SE.

Fig. 5. Hemodynamic response to sufentanil. MAP, mean PAP, mean left pulmonary artery blood flow, and PVR did not change during fetal sufentanil infusion. Values are expressed as means ± SE.
The mechanisms by which nociceptive stimuli elevate basal pulmonary vascular tone remain speculative. When the ductus arteriosus is widely opened, as in the fetus, the pressure gradient between pulmonary artery and aorta is low. In our study, the pressure gradient remains constant after formalin injection, indicating that ductal flow and resistance did not change. Thus it is likely that the increase in PAP after formalin resulted from increase in aortic pressure. Usually, an increase in PAP in the fetus causes a pulmonary vasodilator response due to a mechanical increase in shear stress (1, 50). Therefore, formalin-mediated passive increases in PAP should have increased Qp and decreased PVR. At the opposite, we found that formalin elevated pulmonary vascular tone. Therefore, increased aortic pressure cannot be responsible for pulmonary vasoconstriction.

Nociceptive stimuli may have enhanced the release of pulmonary vasoconstrictors or decreased the release of pulmonary vasodilators. Numerous mediators have been associated with formalin responses, including histamine, serotonin, substance P, bradykinin, cortisol, and catecholamines (8, 24, 30, 36, 39, 45, 55). Evidence exists that each of these mediators may modulate pulmonary vascular tone both in vitro and in vivo (10, 41, 48). The pulmonary vascular responses to histamine and serotonin are receptor- and tone-dependent with developmental changes (9, 21, 34). In mature animals, histamine and serotonin typically cause an H1 and 4-HT2a receptor-mediated increase in PVR (9, 21). At the opposite, histamine was found to decrease pulmonary vascular tone in the ovine fetus, probably because of high basal PVR (56). To our knowledge, the effect of serotonin in the ovine fetus has not been evaluated. However, a relaxation response was found in preconstricted pulmonary vessels (33, 42). In the same way, substance P has a potent pulmonary vasodilator effect when vascular tone is elevated as in the fetus (3, 31). In the lung, substance P also causes mast cell degranulation and histamine release, inducing a vasodilator response (43). Bradykinin is released during the two phases of the formalin test and may modulate pulmonary vascular tone (9, 45). However, bradykinin is also known as a potent pulmonary vasodilator during the perinatal period through release of nitric oxide and prostacyclin (7, 13, 16). Plasma concentrations of histamine, serotonin, substance P, and bradykinin were not measured in our study. However, none of these mediators seems to be able to cause the pulmonary vasoconstriction observed during the formalin test. We found that plasma cortisol concentrations increased after the formalin test. However, glucocorticoids do not alter basal pulmonary vascular tone in fetal lambs (12). No change in circulating catecholamine levels was found after the formalin test. Catecholamines can be released directly at the level of the vascular smooth muscle cells through bradykinin and substance P stimulation (44). Furthermore, sympathetic nerve stimulation can cause direct pulmonary vasoconstriction related to local noradrenaline release (59). Thus we cannot rule out the hypothesis that catecholamines are involved in the formalin-induced pulmonary vasoconstriction.

In conclusion, our study demonstrated that nociceptive stimuli may increase PAP and PVR. It is further evidence that fetuses can respond to painful events and highlights the need for studies in the field of fetal analgesia.

The postnatal circulatory adaptation is highly dependent on the decrease in PVR (40). Failure of the pulmonary circulation to dilate at birth contributes to significant morbidity and mortality (58). This syndrome, named persistent pulmonary hypertension of the newborn (PPHN), is characterized by sustained elevation of PVR, causing extrapulmonary right-to-left shunting of blood across the ductus arteriosus and foramen ovale and severe hypoxemia (28). We speculate that stress-induced hypoxemia observed in newborn infants with respiratory failure may result from increased PAP. Whether nociceptive stimuli could have long-term consequences on the pulmonary circulation is presently unknown. However, brief pulmonary hypertension can alter pulmonary reactivity (51). Our study further supports the need for analgesia in newborn infants with PPHN to protect from stressful event-mediated pulmonary hypertension and hypoxemia.

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