Suppressive action of endogenous adenosine on ovine fetal nonshivering thermogenesis

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The stimulus, or combination of stimuli, that may account for the initiation of nonshivering thermogenesis in minutes of birth is unknown. Although a number of potential stimuli have been tested and found to be necessary, none has proved sufficient alone. For example, cooling and increased oxygenation of the fetal sheep (16) and an intact sympathetic nervous system (2) are necessary for thermogenesis but fail to evoke responses comparable to those seen after birth. Only after the umbilical cord has been occluded do the thermogenic responses increase appreciably (17). Such observations suggest that the placenta produces a thermogenic inhibitor(s) that circulates before birth and tonically suppresses nonshivering thermogenesis. Once the umbilical cord is occluded, the inhibitor can no longer enter the fetal circulation, and nonshivering thermogenesis commences.

Our previous observations with prostaglandin E2 and indomethacin during the simulation of birth in the fetal sheep clearly show an inhibitory role for placental prostaglandins in nonshivering thermogenesis (9). Indomethacin administered before cord occlusion enabled prompt responses to increased oxygenation and cooling alone; the rise in nonshivering thermogenesis that would generally occur on umbilical cord occlusion was minimized. Furthermore, this response was inhibited by infusion of prostaglandin E2 (9). Because cord occlusion induced a further small rise in thermogenic indexes after indomethacin treatment, however, there was the suggestion of multiple intrauterine inhibitors of nonshivering thermogenesis in the fetal sheep.

Adenosine is a compound that is known to be released by the placenta (24), to be present in high concentrations in fetal plasma (19), to have a short half-life in circulating blood (5), and to inhibit catecholamine-stimulated lipolysis in brown adipose tissue in vitro (29). That adenosine is a potential candidate for one of the placental inhibitors of nonshivering thermogenesis (16, 17) is suggested by the effects of the infusion of N6-(2-phenylisopropyl)-adenosine (PIA), a stable, long-acting, and extremely potent agonist, selective for adenosine A1-receptors on fat cells during the simulation of birth in utero. Low and medium doses of PIA administered after umbilical cord occlusion and during maximal nonshivering thermogenesis induced a dose-dependent fall in fetal core temperature, FFA and glycerol concentrations, and oxygen consumption (4). Thus we hypothesized that the high intrauterine levels of adenosine would inhibit nonshivering thermogenesis in utero, but the decreased adenosine levels on removal of the umbilical circulation would therefore enable the initiation of thermogenesis. In these experiments, we examined this hypothesis by simulating the birth of fetal sheep in utero and administering the adenosine antagonist theophylline before and during umbilical cord occlusion. Results are compared with a control study undergoing simulated birth without adenosine antagonist administration.
ADENOSINE AND OVINE FETAL NONSHIVERING THERMOGENESIS

METHODS

Surgical and Postoperative Procedures

The surgical procedure was performed under general anesthesia in 14 pregnant Western ewes (135-144 days gestation) mated to Suffolk rams. As previously described (16), an irreversible umbilical cord occluder was placed loosely around the umbilical cord (protocol 1 only), and an external cooling coil was placed around the fetal thorax. A tracheal cannula was inserted and connected to a length of tubing to allow subsequent ventilation of the fetus. Polyvinyl catheters were implanted into the fetal common carotid artery, external jugular vein, and amniotic fluid cavity, and two calibrated thermistors were advanced nasally into the stomach to record core body temperature. The fetal thorax and head were then eased back into the uterus. The occluder, afferent and efferent limbs of the cooling coil, tracheal tube, catheters, and thermistor leads were exteriorized through the maternal flank.

After the surgery, the ewes were housed in metabolic cages at a constant temperature (18°C) and humidity (50%) and given free access to concentrates and water. The experiments were performed while the ewe stood quietly in the cage. All studies were performed 24-72 h after surgery on fetuses with normal arterial blood pH (7.34), arterial PO2 (PaO2), arterial PCO2 (PaCO2), and arterial PO2 (PaO2; >18 Torr), and arterial PCO2 (PaCO2; <55 Torr). The thermistors were advanced nasally into the stomach to record core body temperature. The fetal thorax and head were then eased back into the uterus. The occluder, afferent and efferent limbs of the cooling coil, tracheal tube, catheters, and thermistor leads were exteriorized through the maternal flank.

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Experimental Procedures

Fetal arterial heparinized blood samples (2.3 ml) were collected anaerobically at 15-min intervals and were replaced isovolumetrically with isotonic saline. When a small fetus was studied, the erythrocytes removed during sampling were returned to the fetal circulation. Blood samples were promptly analyzed for pH, PaO2, and PaCO2 with measured values corrected to core temperature (Radiometer ABL 3, Copenhagen), and for hemoglobin levels (Radiometer OSM2 hemoximeter, Copenhagen). Plasma was separated after centrifugation and stored at -20 to -70°C for later analysis.

Fetal temperature measurements were made by connecting the thermistors to a unit capable of linear temperature measurement from 32-43°C with a resolution of 0.01°C. Recordings of fetal arterial blood pressure, tracheal pressure (both corrected for amniotic pressure), amniotic pressure, and heart rate were monitored by using calibrated transducers and a Gould 200 recorder (Gould Instruments). Recordings were begun 1 h before experimentation and continued until the termination of the study.

Simulation of birth in utero. The simulation of birth in utero was undertaken as described previously (16). Briefly, cooling was induced by passing iced water through the fetal thoracic cooling coil at a rate of 20-40 ml/min. This rate was adjusted to cause a fall in fetal core temperature of 2°C in the first 60 min of cooling. The rate of cooling was adjusted to cause a fall in fetal core temperature of ~2°C in the first 60 min of cooling, and this rate of cooling was sustained throughout the rest of the study. The different methods of cooling in the experimental and control protocols resulted in comparable responses of fetal core temperature in the two protocols. Sixty minutes after cooling commenced, the fetus was ventilated with oxygen (60-300 min). The nonspecific adenosine antagonist theophylline (1,3-dimethylxanthine, 18 mg/kg bolus, then 0.6 mg·kg·1·min⁻¹) was administered intravenously to the fetus 60 min later and continued until the end of the study (120-300 min). The umbilical cord was occluded 90 min after commencing the theophylline infusion, and the responses were followed for a further 90-min period (210-300 min), with the fetus isolated from the placenta.

Protocol 2: birth simulation control (n = 8). One to three days after surgery, after a 30-min control period (from -30 to 0 min), the fetus was cooled (0-300 min). The rate of cooling was adjusted to maintain the fetal core temperature at the level reached after 1 h of cooling. Sixty minutes after cooling commenced, the fetus was ventilated with oxygen (60-300 min), and the responses were followed for a 240-min period.

After completion of the birth-simulation study, the fetuses were euthanized. In all experiments a standard dose of pentobarbital sodium (Eutha-6 CII, Western Medical) was injected intravenously to the fetus isolated from the placenta. The thermistors were then recalibrated to establish that their response characteristics had not changed during their implantation.

All these procedures were approved by the Animal Ethics Committee of Loma Linda University.

Analytical Procedures

The colorimetric method of Falholt et al. (6) was used to measure plasma FFA concentrations. Palmitic acid was used as the standard, and a sample size of 0.1 ml was used to increase the sensitivity. The within-assay coefficient of variation was 14.9%, the between-assay coefficient of variation was 23.3%, and the assay sensitivity was 40 µeq/l. Plasma glycerol levels were determined after enzymatic conversion with glycerokinase by using the method of Pinter et al. (15). The within- and between-assay coefficients of variation were 14.3 and 20.2%, respectively, and the assay sensitivity was 50 µmol/l. Plasma glucose concentrations were measured by using an immobilized enzyme (2700 Select analyzer, YSI). Plasma theophylline levels were measured by the Emit theophylline assay (Syva). The between-assay coefficient of variation was 7.3%, and the assay sensitivity was 5 µmol/l.
The results are given as means ± SE. Two-way analysis of variance with repeated measures was employed to determine the significance of change in response to various stimuli in each protocol. The significance of each added stimulus was tested post hoc by Fisher’s least significant difference test for multiple comparisons between the value immediately before and at the end of the experimental period. Protocol 2, the birth simulation control study, was undertaken to assess the effect of theophylline by comparing the response to the adenosine antagonist administration to the response in the absence of the drug. The response was calculated by subtracting the mean of each animal in the initial 60 min of ventilation from the mean of each animal in the subsequent 90 min of adenosine antagonist administration (experimental) or continuing ventilation (control). The response mean and SE values for all animals in the experimental and control groups were then calculated. The significance of the response difference between the antagonist-treated and the untreated control groups was assessed with the t-test. P < 0.05 was considered significant.

RESULTS

Protocol 1: Administration of Theophylline During Birth Simulation

To examine whether the removal of the inhibitory effects of endogenous adenosine initiated nonshivering thermogenesis after umbilical cord occlusion, the frequently used adenosine antagonist theophylline was administered before and continued during cord occlusion. In this study, six near-term fetal sheep with an average body weight of 4.16 ± 0.67 kg were examined. The effects of the simulation of birth in utero on fetal core temperature, arterial hemoglobin, pH, and PaO₂, and plasma glucose and theophylline measurements are summarized in Table 1. The time course of the fetal temperature and indexes of nonshivering thermogenic activity are illustrated in Fig. 1.

To assess the well-being of the fetus before and during the simulation of birth, arterial hemoglobin, PaO₂, and plasma glucose levels were monitored. All indexes came within the normal range during the control period. Fetal hemoglobin levels did not decrease significantly during the birth simulation despite repeated blood sampling. Upon ventilation, the fetal PaO₂ rose 469% (P < 0.05) and remained elevated thereafter. Such levels of oxygenation are adequate in the newborn. Fetal glucose concentrations increased 43% during ventilation (P < 0.05) and remained high, indicating an abundance of available metabolic substrate during the simulation.

Table 1. Effect of administration of theophylline during birth simulation on fetal core temperature, arterial hemoglobin, pH and PaO₂, and plasma glucose and theophylline levels

<table>
<thead>
<tr>
<th>Expt Period</th>
<th>Fetal Core Temperature, °C</th>
<th>Hemoglobin, g/dl</th>
<th>Fetal Arterial Blood</th>
<th>T, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>39.6 ± 0.2</td>
<td>12.3 ± 0.7</td>
<td>7.34 ± 0.01</td>
<td>21.2 ± 2.5</td>
</tr>
<tr>
<td>Cool</td>
<td>37.8 ± 0.1</td>
<td>13.5 ± 0.9</td>
<td>7.31 ± 0.02</td>
<td>14.9 ± 1.9</td>
</tr>
<tr>
<td>Ventilate</td>
<td>37.8 ± 0.1</td>
<td>12.2 ± 0.8</td>
<td>7.26 ± 0.02</td>
<td>84.9 ± 28.6</td>
</tr>
<tr>
<td>Theophylline</td>
<td>37.9 ± 0.2</td>
<td>12.3 ± 0.6</td>
<td>7.28 ± 0.02</td>
<td>124 ± 5</td>
</tr>
<tr>
<td>Occlude</td>
<td>37.6 ± 0.3</td>
<td>12.7 ± 0.5</td>
<td>7.06 ± 0.13</td>
<td>110 ± 39</td>
</tr>
<tr>
<td>+ 210 min</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Values are means ± SE of 6 fetal sheep. PaO₂, arterial Po₂; T, plasma theophylline concentration.

Fig. 1. Effect of control (Ctrl) birth simulation (○, n = 8 sheep) and administration of theophylline during birth simulation (●, n = 6 sheep) on fetal core temperature, plasma free fatty acid, and glycerol concentrations. Administration of theophylline and cord occlusion were omitted in control experiments. Results are means ± SE.
Control period. During the control period, the mean values for fetal core temperature, plasma FFA, and glycerol were within the normal range.

Cold exposure. During cooling, the fetal core temperature fell over a 60-min interval to 37.81 ± 0.13°C, averaging 1.78 ± 0.11°C less than the mean of the control period (P < 0.001). Plasma FFA (75.4 ± 6.7 µeq/l) and glycerol (151 ± 42 µmol/l) concentrations remained at low levels.

Ventilation with oxygen. During the subsequent ventilation with oxygen, theophylline administration, and umbilical cord occlusion phases, adjustment of the rate of cooling maintained the fetal core temperature at the level reached after 60 min of cooling. Upon elevation of arterial oxygen levels of >100 Torr, the fetal nonshivering thermogenic responses became apparent. Plasma levels of FFA and glycerol rose 176% to 208 ± 40 µeq/l (P < 0.05) and 87% to 282 ± 51 µmol/l (not significant), respectively, after 60 min of supplemental oxygen.

Theophylline administration. Within minutes of the theophylline infusion, an additional effect on fetal thermogenic responses became apparent. After 90 min of theophylline administration, plasma concentrations of FFA rose 99% (P < 0.01) to 415 ± 60 µeq/l, and glycerol levels rose 87% to 526 ± 135 µmol/l (P < 0.05).

Umbilical cord occlusion. Plasma FFA concentrations did not significantly change with umbilical cord occlusion. In contrast, cord occlusion increased glycerol concentrations 41% (P < 0.05) to 774 ± 203 µmol/l.

Protocol 2: Birth-Simulation Control

To examine the effect of theophylline, the administration of the drug and umbilical cord occlusion were omitted and the responses compared with those during antagonist administration in protocol 1. In this study, eight fetal sheep at 135–144 days gestation with an average body weight of 2.96 ± 0.16 kg were examined. The effects of the control simulation of birth in utero of fetal core temperature, arterial hemoglobin, pH and PaO2, and plasma glucose levels are summarized in Table 2. The time course of the fetal temperature and indexes of nonshivering thermogenic activity are illustrated in Fig. 1.

As in protocol 1, during the control period, all indexes of fetal well-being were within the normal range. Fetal hemoglobin concentrations remained stable until the end of the birth simulation. Upon ventilation, the PaO2 rose (P < 0.005) above the levels commonly seen in the newborn, and increased (P < 0.05) glucose concentrations ensured an adequate availability of metabolic substrate.

Control period. During the control period, the mean values for fetal core temperature, plasma FFA, and glycerol were within the normal range.

Cold exposure. During cooling, the fetal core temperature fell over a 60-min interval to 38.2 ± 0.3°C, averaging 1.65 ± 0.28°C less than the mean of the control period (P < 0.001). Plasma FFA (78.4 ± 16.5 µeq/l) and glycerol (83.7 ± 18.4 µmol/l) remained at low levels.

Ventilation with oxygen. During the subsequent ventilation with oxygen phase, the rate of cooling continued unchanged from that achieved during the cooling phase. When arterial oxygen levels rose above 200 Torr, the fetal nonshivering thermogenic responses became apparent. Plasma levels of FFA and glycerol rose 112% to 166 ± 34 µeq/l (P < 0.01) and 54% to 128 ± 32 µmol/l (not significant), respectively, after 60 min of supplemental oxygen. There was no further significant change in FFA concentrations. Glycerol levels, on the other hand, increased 62% (P < 0.05) after 150 min of supplemental oxygen. There was no further significant change in FFA concentrations. Glycerol levels, on the other hand, increased 62% (P < 0.05) after 150 min of supplemental oxygen. There was no further significant change in FFA concentrations. Glycerol levels, on the other hand, increased 62% (P < 0.05) after 150 min of supplemental oxygen. There was no further significant change in FFA concentrations. Glycerol levels, on the other hand, increased 62% (P < 0.05) after 150 min of supplemental oxygen. There was no further significant change in FFA concentrations. Glycerol levels, on the other hand, increased 62% (P < 0.05) after 150 min of supplemental oxygen. There was no further significant change in FFA concentrations. Glycerol levels, on the other hand, increased 62% (P < 0.05) after 150 min of supplemental oxygen.

DISCUSSION

Circulating plasma adenosine concentrations are three- to fivefold higher in the fetal sheep than in the adult (19). During the simulation of birth in utero, adenosine levels decline simultaneously with the initiation of nonshivering thermogenesis (19). Fetal cooling is followed by an increase in plasma adenosine and supplemental oxygenation is followed by a subsequent fall. Of most relevance are the responses to cord occlusion. At this time, plasma adenosine levels decline ~60% within 60 min (19), suggesting the placenta or umbilical vasculature as a likely source of a significant fraction of circulating adenosine. Similarly, in the human, adenosine has been reported to be released from isolated perfused cotyledons into the umbilical vein (24). In the fetus, the liver is another potential source of circulating adenosine (3), the contribution of which might change after cord occlusion with redirection of blood flow away from the placenta and liver; this remains to be tested experimentally. This series of studies tested the possible suppressive action of endogenous adenosine on ovine fetal nonshivering thermogenesis during the simulation of birth in utero.

Table 2. Effect of control birth simulation on fetal core temperature, arterial hemoglobin, pH and PaO2, and plasma glucose levels

<table>
<thead>
<tr>
<th>Expt Period</th>
<th>Fetal Core Temperature, °C</th>
<th>Hemoglobin, g/dl</th>
<th>Fetal Arterial Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PaO2, Torr</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>39.8 ± 0.1</td>
<td>11.6 ± 0.5</td>
<td>7.34 ± 0.01</td>
</tr>
<tr>
<td>Cool 60 min</td>
<td>38.2 ± 0.3</td>
<td>11.9 ± 0.6</td>
<td>7.34 ± 0.01</td>
</tr>
<tr>
<td>Ventilate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 120 min</td>
<td>38.0 ± 0.3</td>
<td>11.1 ± 0.5</td>
<td>7.31 ± 0.03</td>
</tr>
<tr>
<td>+ 210 min</td>
<td>37.9 ± 0.2</td>
<td>11.0 ± 0.5</td>
<td>7.33 ± 0.03</td>
</tr>
<tr>
<td>+ 300 min</td>
<td>37.9 ± 0.2</td>
<td>10.9 ± 0.7</td>
<td>7.34 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 fetal sheep.
Adenosine suppresses many metabolically dependent cellular activities by preventing the intracellular accumulation of adenosine 3’,5’-cyclic monophosphate (cAMP) (14). Direct evidence for this metabolic control has been found in the adult brain, heart, and white and brown adipose tissue of several species (14, 27). The regulatory role of adenosine as an inhibitor of β- and α-adrenergic-stimulated lipolysis in brown adipocytes is well documented (20, 29).

In brown adipose tissue, adenosine inhibits β-adrenergic activation of adenyl cyclase activity (22) through its interaction with specific cell-surface A1 receptors. The α-adrenergic nonshivering thermogenic component is less responsive to the inhibitory actions of adenosine than is the β-adrenergic component (21) and is not related to adenylate cyclase activity (23). This suggests the presence of a second mechanism, unrelated to adenylate cyclase, elicited by activation of adenosine receptors on brown adipose cells (21). Plasma levels of adenosine in the ovine fetus are well into the range that suppresses lipolysis in adult fat (27).

The administration of theophylline during protocol 1 was undertaken to investigate whether removal of the inhibitory effects of endogenous adenosine initiates nonshivering thermogenesis before umbilical cord occlusion in the fetal sheep. The infusion of theophylline began 90 min before cord occlusion and continued throughout the study. Theophylline significantly enhanced the nonshivering thermogenic responses before umbilical cord occlusion. The FFA and glycerol responses to oxygenation in the presence of theophylline were twofold greater than normal. Subsequent cord occlusion did not alter FFA concentrations but did induce a significant rise in glycerol levels. The failure of plasma FFA levels to rise and the smaller increase of glycerol concentrations on umbilical cord occlusion in the presence of theophylline suggest that the removal of the inhibitory effects of endogenous adenosine before cord occlusion preempts the rise in the indexes of thermogenesis that occurs in the absence of theophylline.

Theophylline has been reported to increase cold resistance, possibly due to enhanced endogenous substrate mobilization (28). It may also do so through inhibition of adenosine 3’,5’-cyclic monophosphate phosphodiesterase and increasing intracellular cAMP concentrations (26). However, a 5–10% inhibition of phosphodiesterase requires a theophylline concentration of 560 µmol/l (18), so this action is unlikely in these studies, since the theophylline levels peaked at lower concentrations. Rather, it seems more likely that theophylline acts by blocking adenosine-mediated antilipolysis, an action that is fully exemplified at a theophylline level of <110 µmol/l and is particularly prominent under sympathetic-stimulated lipolysis (8). It has also been demonstrated that theophylline at low plasma concentrations can increase adrenal-medullary release of epinephrine and norepinephrine (10) and that theophylline may enhance peripheral sympathetic discharge of epinephrine by counteracting the inhibitory action of adenosine on such neural transmission (7). Furthermore, elevated plasma levels of free thyroxine and triiodothyronine have been reported after theophylline treatment (28). However, enhanced catecholamine and thyroid hormone levels induced by theophylline are likely to have only a minor effect on nonshivering thermogenesis before umbilical cord occlusion in the fetal sheep, in view of the minimal effect of administration of norepinephrine (11) and triiodothyronine (17) in previous studies.

In summary, it may be concluded that there are increases in the indexes of nonshivering thermogenesis due to treatment with an adenosine-receptor antagonist. Furthermore, the inhibition of nonshivering thermogenesis by an adenosine analogue reported earlier suggests that cord occlusion may act via changes in circulating adenosine. Adenosine must thus be given serious consideration as one inhibitor of intrauterine thermogenesis. The further and continuing rise in plasma glycerol after cord occlusion, however, suggests that there is at least one other intrauterine thermogenic inhibitor physiologically linked to the onset of nonshivering thermogenesis at birth. Another intrauterine inhibitor candidate is an eicosanoid, and this possibility has been investigated elsewhere (9).

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