PERINATAL CHANGES IN MYOCARDIAL METABOLISM IN LAMBS

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**Methods**

We used sheep of mixed western breed with documented gestational age or dates of birth. The sheep were obtained from the Central Animal Laboratory of the University of Groningen, where they were also kept. Throughout the study the lambs remained with their mother. Three lambs were rejected by the mother and were bottle-fed. Their weight gain throughout the study period was comparable to those who were nursed by the ewe. Surgical and experimental procedures were approved by the animal research committee of the University of Groningen.

**Instrumentation**

The *fetal lambs* were instrumented at 124-126 days of gestation (term is 145 days). After an overnight fast, maternal anesthesia was induced with 1g ketamine, 0.4 mg robinal, and 100 mg diazepam i.v. The ewe was intubated and ventilated with oxygen. Anesthesia was maintained by ventilation with 0.5-1% halothane and 1.5 g ketamine per hour i.v. The ewe was placed at her back on a heating pad. Via a distal artery and vein of the hind limb, we advanced catheters (ID 1.3 mm, OD 2.3 mm) into the maternal descending aorta and caval vein. Via a maternal midline laparotomy, we exposed the pregnant horn of the uterus. The uterus was opened, thereby carefully avoiding the cotyledons, which are the placental units of the sheep. The head and forelimb of the fetal lamb were exposed. The uterus was covered with wetted cloth to prevent loss of fluid via evaporation. Via a brachial artery and vein, we inserted polyvinyl catheters (ID 0.3 mm, OD 0.5 mm) into the ascending aorta and superior caval vein.

Via a left thoracotomy through the 4th intercostal space, we identified the azygos vein, running from the paravertebral space through the pericardium to the coronary groove. In the sheep, the left azygos vein confluences with the great cardiac vein to form the coronary sinus, into which blood drains predominantly from the left ventricular free wall (53). After placement of two sutures around the azygos vein, the vessel wall was incised and a catheter inserted just upstream from the site where it enters the pericardium, and the catheter tip advanced into the coronary sinus, two cm downstream from the insertion point (64). The left azygos vein was then ligated in order to prevent admixture of blood from sources other than the coronary sinus. Subsequently, the pericardium was opened, the left auricle was clamped and a loose suture was placed around the auricle under the clamp. After a hole had been cut in the auricle, a catheter was inserted into the body of the atrium and fixed with the suture. The catheters were exteriorized from the thorax and the ribs were tied with two sutures. The thorax was closed in layers. Via a lateral incision in the neck, we also inserted catheters into a carotid artery and a jugular vein. A catheter was placed into the amniotic cavity for zero pressure reference. All vascular catheters were filled with heparin sodium solution (500 IU/ml) and sealed. The catheters were exteriorized.
to the maternal flank, where they were stored in a pouch sewn to the flank. The lamb was returned to the uterus. Warm 0.9% NaCl solution, to replace the lost amniotic fluid, was instilled into the amniotic cavity. The uterus was closed in two layers in order to prevent leakage of amniotic fluid. The peritoneum and the skin were closed. After the surgery, the ewe received 0.3 mg temgesic i.m. for analgesia. Antibiotics (gentamicin 80 mg, 10⁶ IU penicillin G) were given daily for 5 days into the amniotic cavity and into the maternal vein, starting at the day of the surgery.

In order to limit the interanimal variation, we attempted to study the lambs in utero, to deliver them via a cesarean section, and to study them again after birth. However, we were faced with a substantial drop-out throughout the study. Of the 46 lambs instrumented in utero, only ten survived until the day of the cesarean section (day 140 of gestation). There were three major causes of death in the fetal lambs in utero: development of fetal hypoxia (47%), bleeding (19%), and premature delivery (11%). Of the ten lambs delivered via a cesarean section, only six lambs survived breathing spontaneously. The others died because of pulmonary dysfunction. Four of the surviving lambs had clogged catheters, two of the lambs were studied after birth. Because so few lambs survived the intended study period, we were forced to instrument a group of newborn lambs separately.

The newborn lambs were instrumented within the first 2 days after birth. Anesthesia was induced by inhalation of 2-3% halothane via a mouth/nose mask. The lambs were intubated and ventilated with 0.5-1% halothane in oxygen. The lambs were placed on a heating pad. To prevent hypoglycemia and dehydration, the lambs received 30 mg glucose per kg per hour in 0.9% NaCl during the surgery. Via a left thoracotomy through the 4th intercostal space, we exposed an internal thoracic artery and vein and inserted polyvinyl catheters (ID 0.3 mm, OD 0.5 mm) into the aorta and caval vein. We also inserted catheters into the coronary sinus and left atrium using the same procedure as described for the fetal lambs. The catheters were filled with heparin sodium solution (500 IU/ml), sealed, and exteriorized to the flank via a subdermal tunnel in order to prevent infection via the catheters. The ribs were tied with two sutures. The incision was closed in layers. The lamb was allowed to recover from the surgery and was returned to its mother when it was able to stand firmly on its feet. Antibiotics (ampicillan 50 mg.kg⁻¹ i.m.) were given daily for 5 days, starting at the day of the surgery. All animals were allowed two to three days to recover from surgery (161), before experiments were performed.

Labeled substrates
We used substrates labeled with the stable isotope ¹³C. The number of labeled ¹³C atoms within a substrate is indicated by the subscript, i.e. ¹³C₃ means three ¹³C atoms. The position of the ¹³C atoms within the molecule is indicated by
conventional chemical numbering, hence [1-13C]lactate is a lactate molecule that is labeled with 13C at the first carbon atom. The symbol U is used when all carbon atoms of a substrate are 13C atoms, i.e. [U-13C]lactate means that all three carbon atoms are 13C.

[1-13C]Lactate (99% enriched) was obtained as its sodium salt from Tracer Technologies (Somerville, MA, USA). Before infusion, [1-13C]lactate was dissolved in 0.9% NaCl. We calculated the amount of [1-13C]lactate needed to obtain a 2.5% enrichment in the arterial blood assuming a turnover of 6.0 mg.kg\(^{-1}\).min\(^{-1}\) (164).

D-[U-13C]Glucose (99% enriched) was obtained from Mateson (Miamisburg, OH, USA). Before infusion, [U-13C]glucose was dissolved in 0.9% NaCl. We calculated the amount [U-13C]glucose needed to obtain a 1.5% enrichment of the arterial blood assuming a turnover of 5 mg.kg\(^{-1}\).min\(^{-1}\).

[U-13C]\(\beta\)-Hydroxybutyrate (99% enriched) was obtained from Masstrace (Somerville, MA, USA). Before infusion, [U-13C]\(\beta\)-hydroxybutyrate was dissolved in 0.9% NaCl. We calculated the amount [U-13C]\(\beta\)-hydroxybutyrate needed to obtain a 2.5% enrichment of the arterial blood assuming a turnover of 2.6 mg.kg\(^{-1}\).min\(^{-1}\) (26).

[1-13C]Palmitate (99% enriched) was obtained from Masstrace (Woburn, MA, USA). In order to infuse [1-13C]palmitate it must be bound to albumin. For that purpose a weighed quantity of [1-13C]palmitate was dissolved in ethanol. We calculated the amount of [1-13C] palmitate needed to obtain a 5% enrichment of the arterial blood assuming a turnover of 1.36 mg.kg\(^{-1}\).min\(^{-1}\) (25). Albumin, dissolved in H\(_2\)O, was added to the palmitate at a 1 to 7 ratio (33). This solution was frozen at -80 °C for 24 hours and subsequently lyophilized to remove the ethanol from the solution (33). The dried albumin-13C-palmitate-complex was stored at -80 °C until the day of the study. On the day of the study the complex was dissolved in 0.9% NaCl.

The dose needed for the fetal lambs was calculated assuming a body weight of 3 kg. The fetal lambs were weighed at autopsy. All solutions were passed through a 0.20 \(\mu\)m bacteriologic filter (Schleicher/Schuell, Den Bosch, The Netherlands) before they were infused.

**Experimental protocol**

On the day of the study, the ewes were brought to the study room and placed in a cart with free access to food and water. The newborn lambs were weighed and placed in a sling; food was withheld from that timepoint. The animals were allowed 60 to 90 min to get accustomed to the study room. To prevent interference with free fatty acid metabolism, we removed heparin from the catheters at least one hour before blood samples were taken. The catheters were connected to a pressure transducer, which was connected to a thermal array recorder. Heart rate and blood pressures were recorded every 5 min throughout the experiment. An outline of the time-frame of the experimental protocol is given in figure 2.1 and
of the sites for blood sampling or infusion of substrates is given in figure 2.2. Before the start of the infusion with the labeled substrates, heart rate and blood pressures were recorded every 5 min for 30 min. After 30 min, an arterial blood sample was withdrawn (Fig. 2.1) for the determination of oxygen saturation, hemoglobin concentration, hematocrit, pH, pCO₂, pO₂, and HCO₃⁻ concentration and the ¹³C-enrichment of the labeled substrate. Before the start of the infusion, there were no differences in natural abundance of ¹³C-labeled substrates between the studies. When the lambs were calm and resting, the infusion was started. We infused one of the four labeled substrate in each lamb per experiment. If more than one experiment was performed in a lamb there was always a day between the experiments.

We infused [1-¹³C]lactate into the caval vein at a priming dose rate of 15 mg.kg⁻¹ in 10 min and at a continuous rate of 0.15 mg.kg⁻¹.min⁻¹ thereafter (Fig. 2.2). Previous experiments had shown that a steady state of arterial ¹³C-enrichment of lactate is reached after 30 min of infusion (17). We infused [U-¹³C]glucose into the caval vein at a priming dose rate of 36 mg.kg⁻¹ in 10 min and at a continuous rate of 0.36 mg.kg⁻¹.min⁻¹ thereafter. We infused [U-¹³C]β-hydroxybutyrate into the fetal caval vein at a priming dose rate of 6.5 mg.kg⁻¹ in 10 min and at a continuous rate of 0.065 mg.kg⁻¹.min⁻¹ thereafter. We infused [1-¹³C]palmitate in fetal lambs into the superior fetal caval vein at a continuous dose.
rate of 0.081 mg.kg\textsuperscript{-1}min\textsuperscript{-1}. Studies in dogs have shown that no priming dose was needed (210). In previous experiments it was shown that after 30 min of infusion a steady state of arterial [1-\textsuperscript{13}C]palmitate had been reached. After 30 and 45 min of infusion (Fig. 2.1A), we withdrew blood samples simultaneously from the ascending aorta and coronary sinus for the determination of 13C-enrichment of the substrate, CO\textsubscript{2}-concentration, \textsuperscript{13}C-enrichment of CO\textsubscript{2}, oxygen saturation, hemoglobin concentration, hematocrit, pH, pCO\textsubscript{2}, pO\textsubscript{2}, and HCO\textsubscript{3}\textsuperscript{-} concentration and concentrations of lactate, glucose, free fatty acids, triglycerides, \(\beta\)-hydroxybutyrate, and aceto-acetate.

To test whether the myocardium of near-term fetal lambs has the capacity to use LC-FA as an energy substrate, we increased the arterial supply of LC-FA in near-term fetal lambs with the use of a fat infusion and measured palmitate metabolism. For that purpose, we infused a fat emulsion, Lipofundin 20\% (B.Braun Melsungen AG, Melsungen, Germany), before we infused [1-\textsuperscript{13}C]palmitate (Fig. 2.1B). Lipofundin is composed of soya bean oil (20\%), phosphatidyl-choline (1.2\%) and glycerine (2.5\%) and has a pH between 7 and 8.5. The osmolarity is 360 mosmol.kg H\textsubscript{2}O\textsuperscript{-1}. Lipofundin was infused into the fetal jugular vein at a con

**Fig. 2.2** Sites for blood sampling or infusion of substrates in the fetal circulation

This figure is composed from [45] and [46]. The arrows point to the sites for infusion of substrates or blood sampling.

Ao = Aorta, PA = Pulmonary Artery, DA = Ductus Arteriosus, PV = Pulmonary Vein, UV = Umbilical Vein, DV = Ductus Venosus, IVC = Inferior Caval Vein, RA = Right Atrium, LA = Left Atrium, SVC = Superior Caval Vein, RV = Right Ventricle, LV = Left Ventricle.
stant rate of 4.86 ml.h\(^{-1}\) after a bolus injection of 4 ml. In pilot studies we found that after 30 min a steady state of arterial free fatty acid concentration had been reached, which was similar to that seen in newborn lambs. After 30 min of fat infusion, \([1-\text{13C}]\text{palmitate}\) was infused into the fetal caval vein, as in the fetal lambs without fat infusion (Fig. 2.1B). After 45 min of \([1-\text{13C}]\text{palmitate}\) infusion, that is after 75 min of fat infusion, blood samples were withdrawn simultaneously from the ascending aorta and coronary sinus. A second, and in five lambs a third, set of blood samples was withdrawn after 100 and 115 min of fat infusion.

In newborn lambs, the experimental protocol was the same as described for the fetal lambs studied under physiological conditions (Fig. 2.1A). In the experiments with \([1-\text{13C}]\text{lactate}\), \([\text{U-13C}]\text{glucose}\), and \([\text{U-13C}]\text{\beta-hydroxybutyrate}\), blood samples were withdrawn after 30 min and 45 min. In the experiments with \([1-\text{13C}]\text{palmitate}\) blood samples were withdrawn after 30 min and 45 min in five lambs and after 45 min, 60 min and 75 min in seven lambs.

Immediately after collection of the last blood samples radioactive microspheres labeled with either \(^{141}\text{Ce}\), \(^{113}\text{Sn}\), \(^{103}\text{Ru}\), or \(^{95}\text{Nb}\) (New England Nuclear-Trac, DuPont Biotechnology Systems, Wilmington, DE) were injected into the left atrium. Simultaneously a reference sample was withdrawn with a pump (Harvard Apparatus, Millis, MA) from the aortic catheter into a preweighed heparinized syringe for 1.25 min at a rate of 4.5-6 ml.min\(^{-1}\). (96). At the end of the experiment the volume of blood withdrawn was replaced with maternal blood.

**Autopsy**

After the last experiment the ewes and lambs were killed with an overdose of pentobarbital sodium. In the fetal lambs that were still alive at the day of autopsy, the heart was excised quickly, with the catheters in situ. Left ventricular biopsies, approximately 100 mg, were excised, transferred to RNase-free vials, and immediately frozen in liquid nitrogen. Thereafter, biopsies from the liver and skeletal muscle (the hind limb) were also transferred to RNase-free vials and frozen in liquid nitrogen. The biopsies were transported in liquid nitrogen and kept at -80°C until the day of analysis. The time from death to the collection of the last sample was 15-20 min. After excision of the biopsies, the position of the coronary sinus catheter was verified. Then, the heart and cerebral hemispheres were removed and weighed. The left ventricular wall was separated from the rest of the heart, and was weighed. The fetal lambs were weighed and this weight was used as their body weight throughout the experiments. In the fetal lambs that had already died before the ewe was killed, no biopsies were obtained.

**Measurements**

Heart rate was obtained from the blood pressure signal. Aortic, atrial, and amniotic pressures were measured with Baxter pressure transducers (Baxter Medical, Uden, The Netherlands) and recorded on a thermal array recorder (Nihon
Kohden, Tokio, Japan). Fetal blood pressures were corrected to amniotic fluid pressure as zero pressure. Oxygen saturation was determined in duplicate with a hemoxymeter (OSM2, Radiometer, Copenhagen, Denmark). Hemoglobin concentration was determined with a hemoglobin photometer (Hemocue AB, Helsingborg, Sweden). Hematocrit was determined in duplicate by the microcapillary method. pH, pCO₂, pO₂, HCO₃⁻ concentrations were determined with a blood gas analyzer (ABL-2, Radiometer). Blood flow to the myocardium was determined with radionuclide-labeled microspheres. Radioactivity in the left ventricular free wall was determined in a Packard 5550 gamma counter (Packard Instrument Co., Meriden, CI, USA). Organ blood flows were calculated with the aid of a special computer program (156). Blood flows are expressed in milliliter per minute per 100g wet weight. Adequate mixing of microspheres was checked by ascertaining that blood flow per 100g of tissue of the two cerebral hemispheres did not differ by more than 10% (96).

The concentrations of glucose, lactate, pyruvate, β-hydroxybutyrate, and acetoacetate were determined in duplicate in whole blood by enzymatic methods (20). For that purpose, the blood collected for the determination of substrate concentrations was transferred immediately to a tube containing a dash of NaF to stop glycolysis, mixed, and kept in ice. The blood was deproteinized with 18% perchloric acid. After subsequent neutralization to pH 7 with KOH and morpholinosulfonic acid, the mixture was centrifuged and the supernatant removed to determine the substrate concentrations. The concentrations of free fatty acids, total glycerol, and free glycerol were determined in duplicate in plasma. For these assays blood was mixed with NaF, centrifuged, and the plasma was removed and stored at -80 °C, pending determination of the substrate concentration. The concentrations of free fatty acids and total glycerol were determined enzymatically with commercial kits (NEFAC, Wako Chemical, Neuss, Germany and Triglycerides GPO-PAP, Boehringer Mannheim, Mannheim, Germany, respectively). Free glycerol was also determined enzymatically (20). Plasma concentrations were converted to blood concentrations through multiplication with the following factor: [100-hematocrit (%)]/100. The triglyceride kit measures total glycerol concentration after hydrolysis of triglycerides. To obtain the triglyceride concentration we subtracted free glycerol from total glycerol concentration. The coefficients of variation for the assays of free glycerol, total glycerol, and free fatty acids were 0.66%, 0.55%, and 0.52% (n=15) respectively.

For the determination of the ¹³C-enrichment of lactate, fatty acids were removed from the plasma by extraction with chloroform. The lactate was extracted with diethyl ether-ethyl acetate and dried under nitrogen. Butylamine was added and allowed to react for 30 min at 100°C. Subsequently, n-heptfluorobutyrate anhydride (17) was added and allowed to react for 5 min at room temperature. We determined the isotope ratio of the lactate derivative by gas chromatography mass spectrometry (GCMS), using a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) interfaced to a VG Trio-2 qua
drupole mass spectrometer (Fisons Instr., Manchester, UK). The mass spectrometer was used in the chemical ionization mode. Ammonia was added as reactant gas. Selective Ion Monitoring (SIM) was carried out at m/z 359 (m+0) and 360 (m+1), corresponding to [m+NH4]+ of the unlabeled and labeled lactate, respectively. To obtain a calibration graph, we prepared standards containing 0.0, 2.5, 5.0, and 7.5% [1-13C]lactate by dilution with natural lactate. A calibration graph was obtained by plotting isotope ratio vs molar fraction. The molar fraction of [1-13C]lactate (F) of the blood samples was calculated from this calibration graph. The coefficient of variation was 3.7% (n=5) (17).

For the determination of the 13C-enrichment of glucose, we deproteinized the plasma by incubation with ethanol for 30 min at 4°C. After centrifugation, the supernatant was transferred and dried under nitrogen at 60°C. Pyridine / acetic anhydride (1:2 vol/vol) was added and the mixture was allowed to incubate at room temperature for at least 24 h in order to form a penta-acetate derivative. Subsequently, the samples were dried under nitrogen and dissolved in ethylacetate. We determined the isotope ratio of the glucose-derivative by GCMS. The mass spectrometer was used in the chemical ionization mode. Ammonia was added as reactant gas. SIM was carried out at m/z 408, 411, and 414, corresponding to m+0, m+3, and m+6, respectively. To obtain a calibration graph, we prepared standards containing 0, 2, 4, and 6% [U-13C]glucose by dilution with natural glucose. A calibration graph was obtained by plotting isotope ratio vs molar fraction. The molar fraction of [U-13C]glucose of the blood samples was calculated from this calibration graph. The coefficient of variation was 3.1% (n=5).

For the determination of 13C-enrichment of β-hydroxybutyrate, we removed fatty acids from the plasma by extraction with chloroform. The β-hydroxybutyrate was extracted with diethyl ether-ethyl acetate and dried under nitrogen. Acetonitril and acetic anhydride were added and allowed to react for 60 min at room temperature. Subsequently, penta-fluorobenzyl-bromide and triethylamine were added and allowed to react for 10 min at room temperature. The β-hydroxybutyrate-derivative was washed with ethylacetate and HCl. The upper layer was extracted and dried under nitrogen and solved in ethylacetate. We determined the isotope ratio of the β-hydroxybutyrate-derivative by GCMS. The mass spectrometer was used in the chemical ionization mode. Ammonia was added as reactant gas. SIM was carried out at m/z 145 (m+0) and 149 (m+4), corresponding to [m+NH4]+ of the unlabeled and labeled β-hydroxybutyrate, respectively. To obtain a calibration graph, we prepared standards containing 0, 2, 4, and 6% [U-13C]β-hydroxybutyrate by dilution with natural β-hydroxybutyrate. A calibration graph was obtained by plotting isotope ratio vs molar fraction. The molar fraction of [U-13C]β-hydroxybutyrate of the blood samples was calculated from this calibration graph. The coefficient of variation for [U-13C]β-hydroxybutyrate was 3.66% (n=20).
For the determination of the \( ^{13}\text{C}\)-enrichment of palmitate, we extracted free fatty acids from the plasma by adding a chloroform/heptane/methanol mixture (49:49:2) to the plasma, which was buffered with phosphate. This mixture was shaken vigorously and subsequently centrifuged for 15 min at 5000 rpm at 4°C. The bottom layer, which contained the free fatty acids, was extracted, dried under nitrogen, and dissolved in methanol-ether (10%). The free fatty acids were treated with n-methyl-n-nitroso-p-toluolsulfonamid and subsequently derivatised with diazomethane. The derivative was dried under nitrogen and dissolved in hexane. The concentration of palmitate was determined with a gas chromatograph (HP 6890, Hewlett Packard, Palo Alto, CA, USA) using heptadecanoic acid (C17) as internal standard. The gas chromatograph was equipped with a 25 m x 0.2 mm i.d. x 0.1 µm Ultra 1 971 A column (Hewlett-Packard, Palo Alto, CA, USA).

The isotopic enrichment of palmitate was determined with GCMS. The mass spectrometer was used in the electrical impact mode. SIM was carried out at \( m/z \) 270 en 271, corresponding to \( m+0 \) and \( m+1 \) of the unlabeled and labeled palmitate, respectively. To obtain a calibration graph, we prepared standards containing 0.0, 2.5, 5.0, and 7.5% \( [1-^{13}\text{C}] \)palmitate by dilution with natural palmitate. A calibration graph was obtained by plotting isotope ratio vs molar fraction. The molar fraction of \( [1-^{13}\text{C}] \)palmitate of the blood samples was calculated from this calibration graph. The coefficient of variation was 3.7% (n=13).

The blood samples for determination of CO\(_2\) were withdrawn in heparinized vacutainer tubes (Becton Dickenson, Rutherford, NJ) and stored at -20°C until further analysis. For the determination of total CO\(_2\) concentration the titrimetric method described by Dijkhuizen et al was used (52). The titration was carried out as described previously (15). Briefly, in an extraction vessel the CO\(_2\) is released from 0.5 mL of blood by lactic acid and carried by a stream of CO\(_2\)-free air to a titration vessel which contains a solution of 0.5 mol.L\(^{-1}\) BaCl\(_2\). The pH of the BaCl\(_2\)-solution was maintained at 10 by titration with 0.05 mol.L\(^{-1}\) NaOH. The amount of CO\(_2\) released was calculated from the amount of NaOH used in the titration. The coefficient of variation was 0.8% (n=7). For the determination of the isotope ratio of CO\(_2\), the same extraction procedure in the same blood sample was used (15). After extraction, the CO\(_2\) was carried by a stream of CO\(_2\)-free air through a trap immersed in liquid air wherein the CO\(_2\) was frozen. Thereafter, the sample was purified from water vapor and other gases by leading it through a trap immersed in a mixture of acetone/dry-ice. Finally, it was transferred to a sample vial immersed in liquid air. For measurement of the isotope ratio, the sample vial was connected to an IRMS (VG Sira 9 Isotope Ratio Mass Spectrometer, VG, Manchester, UK). Two masses (m/z 44 = mass of \( ^{12}\text{CO}_2 \), m/z 45 = mass of \( ^{13}\text{CO}_2 \)) were measured. The molar fraction of \( ^{13}\text{CO}_2 \) (\( F_{\text{CO}_2} \)) was calculated from the isotope ratio (\( R_{\text{CO}_2} \)) as follows:
The molar fraction was used to calculate the concentration (C) of $^{13}$CO$_2$:

$$C_{^{13}CO_2} = F_{CO_2} \cdot C_{CO_2}$$  \hspace{1cm} (2.2)$$

where $C_{CO_2}$ is the total CO$_2$ concentration.

**Calculations**

The O$_2$ concentration in blood (in µmol.L$^{-1}$) was calculated as follows:

$$C_{O_2} = S_{O_2} \cdot C_{Hb} \cdot \beta_{O_2} \cdot a$$  \hspace{1cm} (2.3)$$

where $S_{O_2}$ is the oxygen saturation, $C_{Hb}$ is the hemoglobin concentration (g.L$^{-1}$), $\beta_{O_2}$ is the hemoglobin binding capacity for oxygen (1.36 mL.g$^{-1}$, (113)), and a is the factor for conversion from mL to µmol of oxygen (1 mmol O$_2$ = 22.4 mL O$_2$).

Left ventricular oxygen supply (expressed in µmol.min$^{-1}$.100g$^{-1}$) was calculated as:

$$\dot{n}_{O_2} (su) = C_{O_2} \cdot \dot{Q}$$  \hspace{1cm} (2.4)$$

where $\dot{Q}$ is the left ventricular free wall blood flow obtained with radionuclide labeled microspheres in L.min$^{-1}$.100g$^{-1}$. Because coronary sinus blood of lambs consists predominantly of venous blood from the left ventricle (53), we calculated oxygen consumption of the left ventricular free wall as follows (expressed in µmol. min$^{-1}$.100g$^{-1}$):

$$\dot{V}_{O_2} = [C_{O_2}(ao) - C_{O_2}(cs)] \cdot \dot{Q}$$  \hspace{1cm} (2.5)$$

where ao and cs are the aorta and coronary sinus, respectively. The left ventricular flux (fl), uptake (up), release (re), and oxidation (ox) of a substrate (expressed in µmol.min$^{-1}$.100g$^{-1}$) were calculated as follows:

$$\dot{n}_s (fl) = [C_s (ao) - C_s (cs)] \cdot \dot{Q}$$  \hspace{1cm} (2.6)$$
where \( C_s \) is the concentration of the substrate in \( \mu \text{mol.L}^{-1} \). The concentrations of lactate, \( \beta \)-hydroxybutyrate, and glucose were determined enzymatically, the concentration of palmitate was determined by gas chromatography.

\[
\dot{n}_s(\text{up}) = \left[ F(\text{ao}) \cdot C_s(\text{ao}) - F(\text{cs}) \cdot C_s(\text{cs}) \right] \cdot \frac{1}{F(\text{ao})} \cdot \dot{Q} \quad (2.7)
\]

where \( F(\text{ao}) \) and \( F(\text{cs}) \) are the molar fractions of the labeled substrate in the aorta and coronary sinus, respectively.

\[
\dot{n}_s(\text{re}) = \dot{n}_s(\text{up}) - \dot{n}_s(\text{fl}) \quad (2.8)
\]

\[
\dot{n}_s(\text{ox}) = \left[ C_{13\text{CO}_2}(\text{ao}) - C_{13\text{CO}_2}(\text{cs}) \right] \cdot \frac{1}{k} \cdot \frac{1}{F(\text{ao})} \cdot \dot{Q} \quad (2.9)
\]

where \( C_{13\text{CO}_2} \) is the concentration of \( ^{13}\text{CO}_2 \), which is corrected for the natural abundance of \( ^{13}\text{CO}_2 \), and \( k \) is the number of labeled carbon atoms per substrate. For lactate and palmitate \( k=1 \), for \( \beta \)-hydroxybutyrate \( k=4 \), and for glucose \( k=6 \).

The oxidation of LC-FA was calculated from the oxidation of palmitate as

\[
\dot{n}_{\text{lc-fa (ox)}} = \dot{n}_p(\text{ox}) \cdot \frac{C_{\text{fa}}(\text{ao})}{C_p(\text{ao})} \quad (2.10)
\]

where \( C_{\text{fa}} \) is the concentration of free fatty acids determined enzymatically and \( C_p \) is the concentration of palmitate determined by gas chromatography. The oxidation of ketone bodies (kb) was calculated from the oxidation of \( \beta \)-hydroxybutyrate (bob) as

\[
\dot{n}_{\text{kb (ox)}} = \dot{n}_{\text{bob (ox)}} \cdot \frac{C_{\text{kb}}(\text{ao})}{C_{\text{bob}}(\text{ao})} \quad (2.11)
\]

where \( C_{\text{bob}} \) is the concentration of \( \beta \)-hydroxybutyrate, and \( C_{\text{kb}} \) is the concen
tration of ketone bodies, which is the sum of the concentrations of β-hydroxybutyrate and acetoacetate. The contribution of the oxidation of a substrate to left ventricular oxygen consumption (f_s) was calculated as

\[ f_s = \frac{\dot{n}_s(\text{ox})}{V_{O_2}(lv)} \cdot n \]  

(2.12)

where n is the amount of oxygen which is used for the oxidation of one mol of that particular substrate. For lactate n=3, for palmitate n=23, for β-hydroxybutyrate n=4, for glucose n=6, and for LC-FA n=25. This equation differs from the previously used oxygen extraction ratio (14, 63, 64, 83, 84), which was calculated with the use of the arterio-coronary sinus concentration difference as

\[ \frac{[C_s(ao) - C_s(cs)]}{[C_{O_2}(ao) - C_{O_2}(cs)]]} \cdot n \]  

(2.13)

The ATP produced by oxidation of a substrate was calculated as

\[ \dot{n}_s(\text{atp}) = \dot{n}_s(\text{ox}) \cdot m \]  

(2.14)

where m is the amount of ATP produced by the oxidation of one mol of that particular substrate. For lactate m=15, for palmitate m=106, for β-hydroxybutyrate m=22.5, for glucose m=32, and for LC-FA m=318.

Part of the [U-13C]glucose infused is metabolized to lactate by glycolysis in peripheral tissues and will thereby produce [U-13C]lactate (54, 204). The peripherally produced lactate might add to the production of 13CO2 by the myocardium because lactate may be used as an energy substrate by the myocardium before and after birth. To correct for the possible contribution of oxidation of [U-13C]lactate to 13CO2 production, we measured the enrichment of [U-13C]lactate in the aorta and coronary sinus in four fetal and six newborn lambs and compared this with the actual 13CO2 production. The enrichment of [U-13C]lactate was obtained with the use of the calibration graph made with [1-13C]lactate. We used the slope of the calibration graph made with [1-13C]lactate and assumed that the y-intercept was represented by the isotope ratio of [U-13C]lactate in the arterial sample obtained prior to infusion of the label. The actual 13CO2 production was calculated as
The possible production of $^{13}$CO$_2$ from [U-^{13}C]lactate was calculated as

$$\dot{n}_{^{13}CO_2} = [C_{^{13}CO_2}(ao) - C_{^{13}CO_2}(cs)] \cdot Q$$

(2.16)

The possible production of $^{13}$CO$_2$ from [U-^{13}C]lactate was calculated as

$$\dot{n}_{^{13}CO_2}(L_3) = [F_{L_3}(ao) \cdot C_L(ao) - F_{L_3}(cs) \cdot C_L(ao)] \cdot k \cdot \dot{Q}$$

(2.17)

where $F_{L_3}$ is the molar fraction of [U-^{13}C]lactate, respectively, $C_L$ is the concentration of lactate determined enzymatically, and k, the number of labeled carbon atoms, is three. When the peripherally produced [U-^{13}C]lactate is used for gluconeogenesis, $^{13}$C$_3$-glucose will be produced (178), which can also add to myocardial $^{13}$CO$_2$ production. Therefore, we also measured the enrichment of $^{13}$C$_3$-glucose and calculated the possible contribution of oxidation of $^{13}$C$_3$-glucose to $^{13}$CO$_2$ production. We used the calibration graph made with [U-^{13}C]glucose to calculate the molar fraction of $^{13}$C$_3$-glucose, similar to that of lactate.

To further evaluate the possible limitation in myocardial long-chain fatty acid metabolism, we characterized the supposed rate-limiting enzyme-system, carnitine palmitoyltransferase. Of the enzyme carnitine palmitoyltransferase I (CPT I) two isoforms are known, a liver-type (L-CPT I) and a muscle-type (M-CPT I). These isoforms are encoded by two different genes, CPT1A and CPT1B, respectively. To characterize the contribution of the carnitine palmitoyltransferase system to perinatal myocardial metabolism we measured the steady-state level of transcripts of the genes encoding the specific enzymes, the amount of enzyme present in the myocardium, and the activity of the enzymes in left ventricular tissue from fetal and newborn lambs.

**Carnitine palmitoyltransferase expression**

The expression of the genes encoding the isoforms of carnitine palmitoyltransferase were determined in tissue biopsies obtained from the fetal and newborn lambs. We isolated RNA from the tissue samples with RNAzol (Campro Scientific, Veenendaal, The Netherlands), using a standard isolation procedure (154). The isolated RNA was dissolved in autoclaved demineralized water that was treated with DEPC (di-ethyl-pyro-carbonaat) to inactivate RNases. The concentrations of RNA obtained were determined by spectrophotometry (A 260/280) and the integrity was checked by gel electrophoresis.

Several attempts to quantify the amount of RNA of CPT1A and CPT1B by heterologous Northern hybridizations with probes derived from human genes
(102, 187) resulted in signals that were too faint to reliably quantify. Because a probe for human subunit 1 of cytochrome c oxidase, added as control, gave good signals in all samples, we concluded that the expression of the CPT genes may be too low to be detected by heterologous Northern hybridization. These results were in accordance with homologous hybridization attempts reported for CPT expression analysis in mice (99). Therefore, we decided to switch to the reverse transcriptase-polymerase chain reaction (RT-PCR), a sensitive method to detect small amounts of mRNA (183). For that, we needed the ovine gene sequences of \textit{CPT1A} and \textit{CPT1B}. To obtain these genes, we first compared the amino-acid sequences of the human and rat isoforms of CPT I. We selected two parts of the amino-acid sequence that were closely similar to each other (black boxes in Fig. 2.3). Subsequently, we developed primers based upon the degenerate sequence of these regions of interest, so that we were able to screen both liver and muscle from sheep with the same primer set. The sequence of these primers is given in the appendix. To screen for these sequences in sheep tissue, we isolated RNA from liver and muscle of an adult sheep of Texels breed. This RNA was reverse transcribed to its complementary DNA (cDNA) with the enzyme reverse transcriptase. Next, we performed a PCR with these primers. The PCR products were separated by gel-electrophoresis. With this method we obtained several fragments of genes, which were subsequently isolated, ligated into a vector, and transformed into bacteria to be multiplied for sequencing. Sequence analysis revealed a deduced protein sequence for ovine CPT1A (EMBL/Genbank accession number Y18831) that shows 89.0% identity with human CPT1A (Table 2.1). The sequence of CPT1B shows 88.1% identity with human CPT1B.

Based upon these sequences we developed two primer sets with the use of a computer program (Lasergene Navigator, DNASTAR): one for CPT1A and one for CPT1B. We also developed a primer set for \(\beta\)-actin as control. Since \(\beta\)-actin is a so-called house-keeping gene, it can be used to distinguish a specific change in gene expression from a change parallel to the general postnatal increase in gene expression. Thus, we normalized the amount of CPT1A and CPT1B expressed to the amount of \(\beta\)-actin expressed. The sequences of the primer sets are given in the appendix.

Next, we needed a fragment, that would be amplified by the same primers as

<table>
<thead>
<tr>
<th>Table 2.1</th>
<th>Percentage identity of the two isoforms of CPT I in several species, as deduced from the corresponding genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>vs Sheep</td>
<td>Liver-type</td>
</tr>
<tr>
<td>Human</td>
<td>89.0</td>
</tr>
<tr>
<td>Rat</td>
<td>86.8</td>
</tr>
<tr>
<td>vs Human</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>86.4</td>
</tr>
</tbody>
</table>
our template, but would be different in size, so that competitive PCR could be applied and the two fragments could be separated by gel-electrophoresis. Therefore, we constructed two control fragments by the MIMIC method (see Fig. 2.3, (162)). For this purpose, we developed a MIMIC-primer (Fig. 2.3), of which the first part would anneal to the fragment at approximately 30-50 basepairs from the end. The second part of the MIMIC-primer is identical to one of the primers (primer 2 in Fig.2.3). We performed one PCR with the MIMIC-primer and primer 1 and obtained a fragment which was approximately 30-50 basepairs shorter than the fragment of interest and flanked by annealing sites for the original primer set. This fragment was isolated, ligated into a vector, and transformed into bacteria to be multiplied and used as control fragment in the RT-PCR.

For the development of a control fragment for b-actin we used an alternative method. We performed a PCR with human genome DNA using the primers based upon the gene sequence obtained from sheep (EMBL/Genbank accession number U39357). The obtained fragment was isolated, ligated into a vector, and transformed into bacteria to be multiplied for sequencing. Sequencing revealed a human beta-actin pseudo-gene with a length of 426 basepairs. The length of this fragment is used as control fragment in the RT-PCR.

**Fig. 2.3** Overview of experimental strategy for competitive RT-PCR

The dashed lines at the top represent the amino acid sequences of the isoforms of the two species. The one-letter code of the amino-acids of the two identical regions are given in the boxes. The number at the top of the box is the position of the amino-acids. From these amino-acid sequences a degenerated sequence was obtained and two primers were developed based upon that sequence. With these primers, we performed a PCR with cDNA of sheep liver and muscle. The fragments obtained were sequenced and this sequence was used to develop primers for the RT-PCR. One of these fragments obtained was isolated and a PCR was performed with a MIMIC-primer, of which the first part anneals to a region approximately 30-50 basepairs from the original primer (region 4) and the latter part is the original primer annealing to region 2. With this primer a fragment was obtained approximately 30-50 basepairs shorter than the fragment of interest. This fragment is used as control fragment in the RT-PCR.
fragment was so close to that obtained from sheep (428 bp) that these two could not be separated by gel electrophoresis. Therefore, we removed an internal part of the human pseudo-gene with the use of a commercial kit (Erase-a-Base, Promega Biotech, USA). With this method we obtained a control fragment of approximately 350 basepairs that was flanked by sites that would anneal to the original primer set. Finally, we performed a competitive PCR with cDNA of the sheep tissue, the appropriate control fragment and the appropriate primer set (for CPT1A, CPT1B, and β-actin, respectively). For that purpose, isolated RNA from sheep tissue was reverse transcribed to its complementary DNA (cDNA) with the enzyme reverse transcriptase using the lower primers. Reverse transcription was carried out with 4 μg RNA, 0.02 nmol of the lower primer, 40 U reverse transcriptase, 0.1 mol.l⁻¹ DTT, 5 nmol of each dNTP, and 32 U Rnasin in a final volume of 20 μL at 42°C for 60 min.

The concentrations in the PCR reaction were: 0.8 μg cDNA, 5 nmol dNTPs, 50 pmol lower primer, 50 pmol upper primer, 4% DMSO, and 1.7 U DNA polymerase in a final volume of 50 μL. After 5 min of denaturation at 95°C, the three steps in the PCR were carried out under the following conditions: denaturation at 95°C for 30 s, annealing at 62°C for 1 min, and elongation at 72°C for 30 s. The steps were repeated 30 times, followed by a final elongation step of 5 min.

The concentration of the control fragment in the PCR was varied, in order to measure the amount of cDNA of the fragment of interest. When both signals on the gel appeared to be approximately equal in density, we assumed a similar amount of cDNA had been present in the assay. These lanes were scanned using a Pharmacia scanner and the density was measured. The concentration of cDNA of the fragment of interest was calculated as:

\[
\text{concentration of control fragment} \times \frac{\text{density of fragment of interest}}{\text{density of control fragment}}
\]

**Amount of carnitine palmitoyltransferase**

The amount of L-CPT I present in the left ventricular tissue from fetal and newborn lambs was determined in tissue homogenates and in total membrane fractions. The proteins were separated by SDS/PAGE [7.5% (w/v) gel]. Aliquots (40 μg) of protein were solubilized in a buffer containing 1.5% (w/v) SDS. Gels were run with a prestained molecular mass marker (Bio-Rad, Veenendaal, The Netherlands) yielding bands of 20.0, 28.5, 36.4, 49.3, 74, and 107 kDa. Blotting and detection was by standard methods, with rabbit anti-(rat L-CPT I) (141) as primary antibody and pig anti-(rabbit IgG) conjugated to horseradish peroxidase as secondary antibody and with the use of an enhanced chemiluminescence method (Luminaol, Sigma) and exposure times of 0.5-5 min.
Carnitine palmitoyltransferase activity

The activity of carnitine palmitoyltransferase (CPT) was first measured in homogenated tissue. For that purpose, the tissue samples obtained at autopsy were homogenized in a buffer (KCl 1 mol.L⁻¹, Hepes 1 mol.L⁻¹, pH 7.0). The protein content of the homogenate was measured with the use of a commercial kit (BCA protein assay, Pierce, Omniflabo, Breda, The Netherlands). The CPT activity was measured in a forward assay (124), which measures the conversion of ¹⁴C-carnitine to ¹⁴C-palmitoylcarnitine. In pilot experiments, we had shown that the conversion rate of ¹⁴C-carnitine to ¹⁴C-palmitoylcarnitine in the assay was linear up to 10 min. The temperature of the assay was 30°C and the final volume 0.5 mL. The reaction was started by adding a mixture of substrates to the homogenate (2 mg protein.mL⁻¹). This mixture contained: ¹⁴C-carnitine 50 µCi, carnitine 500 µ mol.L⁻¹, palmitoylcarnitine 100 m mol.L⁻¹, BSA 1.3 g.L⁻¹, DTT 154 mg.L⁻¹, EDTA 1 mol.L⁻¹, KCl 15 mol.L⁻¹, 50 mol.L⁻¹. The reaction was stopped by adding 0.5 mL HCl (1.2 mol.L⁻¹) after 10 min. The ¹⁴C-palmitoylcarnitine was extracted from the mixture with the use of a butanol extraction procedure. For that purpose, 0.5 mL of N-butanol saturated with water was added, the mixture was shaken vigorously for 20 s and centrifuged at 3000 rpm for 10 min. The bottom layer, which contains unincorporated ¹⁴C-carnitine, was removed. Subsequently, 0.75 mL of water saturated with butanol was added to the upper layer. The mixture was shaken vigorously and centrifuged at 3000 rpm for 10 min. Again the bottom layer was removed and 0.75 mL of water saturated with butanol was added to the mixture. This mixture was shaken and centrifuged once more. Thereafter 0.2-0.3 mL of the upper layer was transferred to a separate vial, which contained 3 mL of scintillation fluid (UltimaGoldXR, Packard, Meriden, CT, USA). The radioactivity in this vial was counted with a liquid scintillation counter (Packard Tricarb 2500TR). The total CPT activity, expressed in nmol.min⁻¹.mg protein⁻¹, was calculated as follows:

\[
\frac{\text{dps} - \text{dps}_0}{\text{dps}_m - \text{dps}_0} \cdot C \cdot V \cdot \frac{1}{T} \cdot \frac{1}{P} \cdot 1000
\]

where dps is disintegrations per second, the subscript 0 represents the background dps, the subscript m represents the dps added to the mixture in the form of ¹⁴C-carnitine, C is the concentration of carnitine (in mol.mL⁻¹), V is the ratio of the assay volume and the volume counted in the vial, T is the assay time (in min), and P is the protein concentration (in mg.mL⁻¹).

The tissue homogenate contains fractions of mitochondrial membranes. Therefore, the activity in these samples was the result of CPT I (located in the outer mitochondrial membrane) as well as CPT II (located in the inner mitochondrial membrane). To distinguish the activity of CPT I from CPT II, Triton X-100 (2%) was added to the homogenate before the reaction was started. Triton X-100
dissolves the mitochondrial membranes and deactivates CPT I (208). The activity of CPT II was the CPT activity measured after inhibition by Triton X-100. The activity of CPT I was calculated as:

$$\text{CPT}_{\text{total}} - \text{CPT II},$$

Next, we attempted to determine the contribution of the two isoforms of CPT-I, the liver-type (L-CPT I) and the muscle-type (M-CPT I) to the CPT I activity. For that purpose we have performed several experiments in isolated mitochondria, of which the experimental design is described below. Unfortunately, we have not been able to separate the contribution of each of the isoforms of CPT I in the heart until now.

To determine the contribution of L-CPT I and M-CPT I to the CPT I activity a selective inhibitor was added to the reaction. Etomoxir (2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylic acid) inhibits both L-CPT I and M-CPT I, whereas DNP-etomoxir (2-[6-(2,4-dinitrophenoxy)hexyl]oxirane-2-carboxylic acid) was shown to preferentially inhibit L-CPT I in the rat (196). Unexpectedly, the suggested liver-type specific inhibitor DNP-etomoxir-CoA also inhibited the activity of CPT I in muscular tissue of sheep and mice. Although DNP-etomoxir-CoA inhibited CPT I in muscle to a lesser extent than etomoxir-CoA (29±5 vs 65±3%), it was not possible to quantify the activity of L-CPT I and M-CPT I by this approach.

To separate the contribution of L-CPT I and M-CPT I to total CPT I activity in the heart, we used another feature that distinguishes these isoforms, that is their sensitivity for inhibition by malonyl-CoA. The IC50 for malonyl-CoA, that is the concentration of malonyl-CoA at which 50% of the activity of CPT I is inhibited, is 2.7 µM in rat liver versus 0.03 µM in rat muscle (124). Therefore, theoretically the amount of inhibition obtained at low malonyl-CoA concentrations can only be due to inhibition of M-CPT I. For these assays it was necessary to determine the IC50 for CPT I muscle and liver from sheep. However, tissue homogenates did not show malonyl-CoA sensitivity. The most likely explanation for this is that the homogenate contains enzymatic activities that digest malonyl-CoA (4, 56). Therefore, we isolated mitochondria from the sheep and liver using a method described by Maeda et al (118). In these mitochondria we determined the IC50 for malonyl-CoA. These experiments were performed in fresh intact mitochondria, so that only CPT I activity is measured.

**Statistics**

Data are presented as mean±SE. Differences in baseline values between the fetal and newborn lambs were tested by unpaired t-test. Differences within groups were tested by paired t-test when possible. Differences between more than two groups ( e.g. fetal lambs under physiological conditions, fetal lambs during fat infusion, and newborn lambs) were tested by one-way analysis of variance, using post hoc Newman-Keuls test to detect the differences. A p-value <0.05 was considered significant.
Appendix

Sequence of primers

Primers based upon the degenerated sequence from the amino-acid analysis

ODC 1    GCN GTN GCN TTY CAG TTC AC
ODC 2    AGG TAG AYR TAY TCY TCC CAC CAG TC

All sequences are given from 5’ to 3’. G, A, T, and C are the four nucleotides, Y is a pyrimidine, R is a purine, and N is any of the four nucleotides. ODC is an acronym for Ovine Degenerate sequence for CPT. ODC1 is located between the amino acids at position 7 and 13 and ODC2 between those at 234 and 242.

MIMIC Primers

CPT1A    CGG CGT GTA CCC AGC GAG TCG ACC AGT ACG
CPT1B    CCG GCA GCT TGG GCA GAG ACA GAA GGC GGA C

Primers for competitive RT-PCR:

CPT1A    CGC CGT GTA CCC AGC GAG TC
         GCA GGC GCG GCA GAG ATG T
CPT1B    GCT GCG TGG TCG TGG TTA TGA
         CCG GCA GCT TGG GCA GAG AC
β-actin    ATA TTG CTG CGC TCG TGG TTG ACA
          GCT GTG CTG TCC CTG TAC GCC TCT

Length of the fragments obtained with RT-PCR (in basepairs):

<table>
<thead>
<tr>
<th>Fragment of interest</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT1A</td>
<td>349</td>
</tr>
<tr>
<td>CPT1B</td>
<td>372</td>
</tr>
<tr>
<td>β-actin</td>
<td>428</td>
</tr>
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</table>