Metabolism in preterm infants on the first days of life
The effect of corticosteroids

Het metabolisme van de te vroeg geborenen gedurende de eerste levensdagen
Het effect van corticosteroiden

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Chapter 1

Introduction

INTRODUCTION

During pregnancy, the fetus receives from its mother a continuous intravenous supply of energy substrates via the placenta. In most species, plasma lipids do not readily cross the placenta. In the human, where the placental transfer of lipids is limited, these substrates do not contribute to fetal oxidative metabolism since fetal tissues have a very low capacity for free fatty acids (FFA) oxidation [1]. The FFA that cross the placenta are stored in adipose tissue and liver. When the mother is well fed, the supply of glucose and amino acids to the fetus is sufficient to cover its needs for oxidative metabolism and growth, and the fetus has no need to produce endogenous glucose. Excess of glucose is stored as glycogen in many tissues, particularly in the liver and as lipids in adipose tissue. After birth, the continuous intravenous high-carbohydrate low-fat diet of the fetus is replaced by a discontinuous enteral high-fat low-carbohydrate diet [2].

GLUCOSE METABOLISM

Immediately after birth, the newborn is entirely dependent on the mobilisation of his glycogen and fat stores during a transient period of starvation. Liver glycogen stores are rapidly mobilised to provide an immediate source of glucose for tissues such as the erythrocytes, the brain and kidney, that are entirely or partially dependent upon glucose to maintain their functional activity [3].

As a response to birth, plasma glucagon and catecholamines increase and plasma insulin levels fall [4]. These changes in hormonal levels result from increased activity of the sympathetic nervous system as cold exposure and cord cutting stimulate glucagon secretion and inhibit insulin secretion [5].

The ability to synthesise glucose from lactate, pyruvate and amino acids is very low in fetal liver and develops after birth. Most of the enzymes involved in the pathway of gluconeogenesis have a substantial activity in the liver of near term fetuses, while the activity of cytosolic phosphoenolpyruvate carboxykinase (PEPCK) is low. After birth, the PEPCK activity is markedly elevated immediately after birth, also in premature infants. The fall in plasma insulin/glucagon ratio that occurs after birth triggers the induction of liver PEPCK. Premature infants are at risk for hypoglycaemia as the glycogen that is normally stored in the last trimester of the normal pregnancy is very limited while the glucose demands for thermo regulation and illness are high. For this reason, most preterm infants receive intravenous glucose shortly after birth.

Glucose metabolism can well be studied using the stable isotope dilution technique. In steady state, the glucose turnover is the resultant of several factors, best summarised by the formula: Rate of glucose appearance (Ra) = glucose intake + endogenous glucose production = glucose oxidation + glucose storage, either as glucose or in other metabolites. Under conditions of stable plasma glucose levels is Ra =Rd. Rd the rate of disappearance.

Kalhan has addressed the glucose turnover in term infants on the first day of life [6]. Endogenous glucose production rates range from 4.2-5.4 mg/kg/min in term infants. It was shown that gluconeogenesis is initiated soon after birth. In preterm infants glucose turnover rates of 5.3-6.0 mg/kg/min were found [7] and with a low glucose infusion (1.4 mg/kg/min) glucose production rates of 6.1 mg/kg/min are measured [8].

The control of glucose production by the neonate is a complex process, partially controlled by insulin and blood-glucose concentration. The endogenous glucose production of the term and preterm infant when not given exogenous glucose is 5 - 6 mg/kg/min. In contrast to adults, endogenous glucose production in preterm infants, is not completely suppressed by glucose intake higher than the endogenous production [9,10,11]. This may well be in order to ensure adequate glucose delivery to the brain under different metabolic circumstances. Until now, no data exist on glucose turnover over the whole period of the first 24 hours after birth, when major clinical and hormonal changes take place.

LIPID METABOLISM

The capacity for long chain fatty acid oxidation and ketone body production is very low in fetal liver and increases in the first 24-hours after birth. Studies in rabbits indicate that the increase in fatty acid oxidation capacity is the result of decreased lipogenesis and a decreased malonyl-CoA concentration [12] These effects are induced by glucagon and antagonised by insulin [13].

Lipids are not only an important energy source, but they are also structural components of all tissues and are indispensable for cell membrane synthesis. Lipids can be divided in essential- and non essential. Essential lipids can not be synthesised by the human and have to be taken in with the diet. For newborn infants, not only linoleic and linolenic acid but especially their elongation products like arachidonic (AA) and docosahexaenoic acid (DHA) are essential.

Lipid intake with food provides a major source of energy. In the last trimester, the fetus stores lipids in brown adipose tissue. In this tissue, lipids can be converted to heat because of the high concentration of mitochondria and uncoupling protein in these fat cells [14]

In very low birth weight infants, intravenous lipid emulsions are important constituents for total parenteral nutrition. They provide essential fatty acids and allow high caloric intake in a small volume.

All organisms are capable of de novo lipogenesis (DNL) of non-essential lipids. In adults, DNL is presumed to function as a means to store excess carbohydrate (CHO) and for synthesis of structural, non essential lipids. In infants, and even more in the foetus, lipogenesis might well be indispensable to provide lipids for storage in subcutaneous fat as well as for myelination [15]. Measurement of lipogenesis posed a problem for a long time.

Lipids are important sources of energy in the preterm infant also on the first day of life. Previous studies we performed on glucose oxidation [16-19] on the first days of life have shown that glucose oxidation is supplying only approximately 50% of the energy consumption at this age. Indirect calorimetry indicated a higher glucose utilisation than glucose oxidation measured by stable isotopes. We hypothesised therefore that glucose was converted to lipids while at the same time lipid was oxidised.

PROTEIN METABOLISM

Protein is an essential component of new tissue. At the same time, protein is continuously synthesised from, and broken down to amino acids. [20].

Studies in preterm neonates examining protein metabolism have shown that rates of protein turnover and protein breakdown are higher compared to term neonates and adults. The hormonal changes during the perinatal period in many preterm infants resemble the hormonal situation caused by stress. Several hormones are known to have an effect on protein turnover. Glucocorticoids for instance, cause an increase in protein breakdown.[21-25]. In the past decade, antenatal steroids are used frequently with the purpose of enhancing lung maturation when a delivery is expected at a gestational age less then 34 weeks [25]. The possible negative effect of antenatal steroids on protein turnover remains to be further elucidated. The negative effect of high doses corticosteroids in preterm infants with broncho pulmonary dysplasia are well known[26]. Protein synthesis is not affected, but protein breakdown is significantly increased by dexamethasone.

Insulin is an anabolic hormone. During the infusion of insulin, a decrease in the rate of proteolysis is found, as measured by the rate of appearance of leucine [27,28]. Since corticosteroids are used in an attempt to wean infants with chronic lung disease from the ventilator, studies on the effects of insulin infusion during corticosteroid administration on protein metabolism in preterm neonates are needed.

ENTERAL FEEDING

Very preterm infants can not be enterally fed in adequate amounts during the first days of life due to immaturity of the GI tract. Moreover, these infants are at risk for necrotising enterocolitis when fed too soon, too large amounts of enteral feeding. For this reason, many neonatal centers have a feeding policy that consists of a period of total parenteral nutrition and withholding enteral nutrition. The duration of this period varies from 3 to approximately 10 days. In recent years, it was recognised that small volumes of milk might promote maturation of gut function while avoiding the disadvantages of full-volume enteral feeding [29,30]. The administration of nutritionally inconsequential quantities of feed while the main route for nutrition is parenteral is called minimal enteral feeding or trophic feeding.

Recent studies have shown that median whole gut transit time is reduced in infants receiving minimal enteral feeding. Tolerance of enteral feeding is increased, reducing hospital stay and decreasing the need for parenteral nutrition. The frequency of sepsis is reduced, possibly because of a decreased mucosal permeability after feeds. In animal studies, minimal enteral feeding resulted in an increase in weight of the small intestine [31,32]. Minimal enteral feeding therefore may serve as nutrition for the gastro intestinal tract itself. The effect of minimal enteral feeding on protein metabolism of splanchnic tissue is unknown.

CORTICOSTEROIDS IN THE FETUS AND INFANT

Glucocorticoids are synthesised in the human adrenal cortex. Although glucocorticoids are highly lipophylic and rapidly cross the placenta, the normal fetus has much lower levels of physiologic glucocorticoids than its mother [33]. This is thought to be caused by rapid conversion of glucocorticoids to the inert 11-keto glucocorticoid by placental 11ß-hydroxysteroid dehydrogenase type 2(11ß-HSD-2) [34]. This placental enzyme is quite efficient and appears to ensure that most, but not all, maternal cortisol is inactivated before reaching the fetal blood. Dexamethasone and betamethasone, synthetic corticosteroids, on the other hand cross the placenta readily.

ANTENATAL STEROIDS

In the last decade, steroids are administered in increasing frequency to pregnant women. The history of antenatal steroids goes back to 1969, when Liggins observed that lambs, delivered prematurely after intra-foetal infusion of ACTH, cortisol or dexamethasone, were viable and, when sacrificed, their lungs remained partially expanded [35]. He suggested that glucocorticoids caused enhanced excretion of surfactant into the alveoli.

In 1972, Liggins and Howie reported the results of a controlled trial of antenatal betamethasone versus placebo for prevention of the respiratory distress syndrome in 282 mothers in whom premature delivery was planned or imminent [36]. The incidence of respiratory distress syndrome (RDS) in the betamethasone treated infants was only one-third that of the controls. The difference was confined to infants delivered at less than 32 weeks. There was a five-fold reduction in neonatal mortality among preterm infants born after corticosteroid administration compared to placebo. In the infants who died, hyaline membrane disease was present in none of the betamethasone treated infants versus 6.7% in the control group. This was the first study in humans that reported a beneficial effect on lung maturation of antenatal steroids in anticipated premature delivery.

Since these classic studies, several further randomised controlled investigations have suggested the advantage of antenatal steroid treatment [37-40], but did not result in common use of antenatal steroids in daily practice until 1995. In that year, the NIH consensus conference was published [41] stating that the benefits of antenatal corticosteroids to fetuses at risk for preterm delivery vastly outweighs the potential risks. All fetuses between 24 and 34 weeks gestation at risk of premature delivery should be considered candidates for antenatal treatment with corticosteroids. Since then, the use of antenatal steroids appears to have increased substantially.

The initial trials suggested that the beneficial effects of corticosteroids was absent if there was an interval of over seven days between treatment and delivery. This finding persuaded an increasing number of obstetricians to repeat the course of steroids after seven days if the pregnant woman at risk for preterm delivery had not yet given birth. Later studies have not shown a positive effect of repeated steroid courses on pulmonary function, while negative side effects have been reported. French [42] showed in a cohort study that birth weight reduction was independently associated with increasing corticosteroid courses. Head circumference is significantly reduced up to 4% in cases in which 3 courses are given. There are no significant clinical gains for those infants whose mothers had received additional courses of corticosteroids beyond the first. At 3-year follow-up, no adverse effects regarding growth are seen. Smith on the other hand did not find harmful effects on growth after multiple steroid courses. This might be due to the fact that they included infants from mothers with preeclampsia [43].

Many studies investigated the effect of antenatal steroids on lung metabolism and physiology, in animals as well as in humans. Corticosteroids have an effect on several known and probably a number of yet unknown metabolic and physiologic processes, which is expressed in a variety of clinical findings. In the following paragraphs, the theoretical advantages and disadvantages of antenatal steroids will be discussed briefly.

PULMONARY EFFECTS

As discussed before, in fetal lambs, betamethasone administration results in a reduction of RDS. Brehier [44] reported that maternal betamethasone treatment in rats, resulted in a significant increase in phosphatidic acid phosphatase, an enzyme controlling the supply of diglycerides which are utilised as substrate by choline phosphotransferase.

In cultured human fetal lung, dexamethasone treatment increases the content of surfactant proteins A,B,C and D; stimulates activity of fatty-acid synthetase, choline phosphate cytidyltransferase and lysophosphatidylcholine acyl coenzyme A, key enzymes of phospholipid synthesis, increases the content of saturated phophatidylcholine, and promotes development of lamellar bodies that are secreted into the airspace lumen. [45,46] After antenatal steroids, an important reduction in the risk of respiratory morbidity among neonates born at less than 31 weeks is observed. Significantly fewer neonates require surfactant treatment [47]. The incidence of broncho pulmonary dysplasia is not influenced. The explanation is thought to be a shift to younger and smaller surviving premature infants that acquire broncho pulmonary dysplasia compared to infants of mothers not receiving antenatal corticosteroids.

ANTENATAL STEROIDS: CENTRAL NERVOUS SYSTEM AND FOETAL BEHAVIOUR

Antenatal administration of betamethasone in a dose comparable to that used in humans, to foetal rabbits results in a cessation of growth, characterised also by a reduction in brain weight, but with a significant higher brain weight:body weight ratio [48]. Brain DNA concentration, number of cells and brain cell phospholipid are however not affected. This suggests a growth retardation with brain sparing effect. Though not significant, the brain phospholipid content shows even a tendency to increase, suggesting an acceleration of myelination.

Uno showed that antenatal administration of dexamethasone to preterm monkeys induces irreversible damage to the neurones, especially the pyramidal neurones in the hippocampal region [49].

In humans, maternal betamethasone administration is followed by an almost immediate disappearance of fetal breathing movements, whereas a slight nonsignificant increase was observed after dexamethasone [50]. Also, fetal heart rate variability decreased significant after betamethasone administration. Though fetal biophysical profile scores decrease in one third of the cases, there is no difference in apgar score, mortality and gestational age at birth between infants whose biophysical profile score decrease after steroid administration and those whose do not [51].

In a retrospective study, antenatal betamethasone but not dexamethasone was associated with a decreased risk of cystic periventricular leucomalacia [52]. The incidence in intracerebral haemorrhage decreases after antenatal steroids [40,47,53]. In a study where betamethasone was used, no effect of antenatal betamethasone on school related or developmental problems in a follow-up study were seen in spite of the greater number of "at risk" infants surviving in the betamethasone-treated group [54].

From these observations it seems that there is a difference in (side) effects when either betamethasone or dexamethasone is used. Since both steroids are stereo-isomers with the only difference being the C-16 methyl group in the α-position (dexamethasone) or the ß-position (betamethasone), the higher affinity for the fetal brain glucocorticoid receptor and the longer plasma half life of betamethasone might explain the differences in effect on the foetus.

EFFECTS ON GROWTH

Antenatal betamethasone given to pregnant sheep in a manner similar to that used in human obstetric practice results in reduced weight at birth at 125 and 146 days [55].

In human studies, a reduction in birth weight of as much as 9% is observed in infants whose mothers received multiple courses of steroid. At 3- and 11year follow-up, despite a smaller neonatal size, no adverse growth effects are seen [42,56,57].

HORMONE LEVELS AND THE PITUITARY ADRENAL AXIS

Uno showed that levels of plasma cortisol levels are higher post natally in primate infants, both at baseline and in post stress situations after antenatal dexamethasone compared to controls [49]. In a study in fetal sheep of Sloboda, cord plasma ACTH and cortisol binding capacity was significantly increased after antenatal glucocorticoid [55]. Ballard showed in humans that prenatal glucocorticoid administration results in fetal glucocorticoid levels in the physiologic stress range. Therefore, this antenatal treatment does not seem to expose the infant to potentially harmful pharmacological levels of betamethasone [58]. On day 7 and 14 after birth, the corticotropin-releasing-hormone test for pituitary-adrenal axis is not different between infants whose mothers did not receive antepartum steroids and those who received 1-2 or more than 2 doses of steroids [59]. Mean arterial blood pressure is significantly higher after antenatal steroids [47].

IMMUNOLOGY

Antenatal glucocorticoids accelerate natural killer cell activity in very preterm infants which may be beneficial. In contrast, it also results in decreased T cell proliferative responses [60]. Antenatal steroids are associated with a decrease in the absolute lymphocyte count, the number of CD4+ lymphocytes and in the number of CD25+ lymphocytes [61]. In a randomised controlled study, the number of hospital admissions due to infectious diseases in early childhood was significantly greater in the group that received antenatal steroids [56]. The course of these infectious diseases was not serious. It appeared that there was a temporarily heightened sensitivity for infections in the group of children who were treated antenatally with corticosteroids.

POSTNATAL STEROID TREATMENT

Several studies have shown that steroids given post natally to infants that can not be weaned from the ventilator is associated with a reduced duration of oxygen requirements and mechanical ventilation. Mortality rates are reduced, especially in the group of infants treated between 7 and 14 days of age. Today, more than 50% of infants with a birth weight between 500 and 750 gram are given post natal steroids in the United States. There is however emerging evidence that post natal steroids are associated with increased short-term neonatal complications and long-term adverse effects. In the short-term, gastrointestinal hemorrhage and perforation can occur. Infants may suffer from hypertension, hyperglycaemia, increased protein breakdown, poor weight gain and cardiac hypertrophy. Tschanz described suppression of outgrow of new alveolar septa and reduced interstitial tissue mass in rats. The late sequelae of post natal steroids in rats are manifested as an emphysematous condition of the lungs [62] Postnatal treatment with corticosteroids in infant-rats and -mice results in a decreased cerebral weight gain and reduction of cerebral DNA content that endures for virtually the entire lifespan [63,64]. A significant delay in myelination in the developing rat brain follows early corticosteroid administration [65]. This correlates well with the observation of an irreversible decrease in cerebral cholesterol after neonatal steroid treatment [63].

In human follow-up studies, increased neurological deficits, cerebral palsy and lower psychomotor development is seen after post natal steroid administration. It appears that for every 3 to 4 surviving treated infants, one infant would experience an adverse neurodevelopmental outcome [66]. In view of the effects of steroids on the central nervous system with a reduction in cerebral DNA content and delayed myelination in animals as mentioned before, this is no surprise.

The question remains whether we should continue to use postnatal steroids, and if so, what the appropriate dose of dexamethasone used in postnatal treatment should be [67]. Usually, 0.25 –0.5 mg/kg/day is used which is almost 10 times the endogenous production of corticosteroids during stress. Secondly, the choice for dexamethasone instead of betamethasone needs to be re-evaluated, as we have seen the difference in effect between the two steroids in antenatal use with regard to the development of periventricular leucomalacia [52].

To conclude: The use of corticosteroids antenatally has proven to be the most cost reducing medical treatment thus far, reducing neonatal mortality and morbidity. Side effects on the central nervous system, growth and hormonal homeostasis in animal studies however should prompt us to consider the use of antenatal steroids with care. Since no beneficial effects of repeated antenatal doses is seen, antenatal steroids should be used in one treatment course only .

Post natal steroids are also used frequently, especially in extremely low birth weight infants for purposes of improving pulmonary physiology. The side effects of post natal steroids are much more pronounced than with antenatal steroids. In the short-term gastrointestinal problems, hypertension, hyperglycaemia, increased protein breakdown, poor weight gain and cardiac hypertrophy is seen. In the long-term, increased neurological deficits, cerebral palsy and lower psychomotor development is found. Future studies need to focus on finding the minimum dose and duration of treatment that will be effective.

AIM OF THIS THESIS

The aim of the work reported in this thesis is to further elucidate the neonatal glucose-, lipid- and protein metabolism of neonates as part of the studies performed in the neonatal unit of the Sophia Childrens Hospital, and to evaluate a possible effect of antenatal steroids on these substrates.

The first aim is to measure glucose turnover, endogenous glucose production and glucose carbon recycling during the first 24 hours of life in ventilated preterm neonates, a period with extensive changes in nutrient supply and hormone levels.

The second aim is to measure the protein turnover on the first day of life and to evaluate the effect of antenatally administered corticosteroids on protein turnover.

Lipogenesis has not been studied in preterm infants on the first day of life. The third aim therefore is to evaluate if ventilated preterm infants show lipogenesis on the first day of life while receiving a hypocaloric intake and if lipogenesis is affected by antenatal corticosteroids.

With the use of a recently developed method using deuterium labelled water for gluconeogenesis measurement we wanted to answer aim number four, what is the contribution of gluconeogenesis via pyruvate to total glucose production in healthy, normal full term and in premature infants.

It is impossible to supply the preterm infant adequate nutrition via the gastro intestinal route on the first day of life. Moreover, introducing enteral feeding in very preterm infants is related to diseases like necrotising enterocolitis. Parenteral nutrition to provide adequate amounts of fluid and nutrients is used therefore in these infants. The administration of small amounts of enteral feeding to promote the maturation of the gastro enteral tract has recently been advocated. Aim number five regards the possible effect of minimal enteral feeding during total parenteral nutrition on splanchnic protein uptake.

Corticosteroids are not only used before birth, but also in the postnatal period to improve lung function. In a previous study we showed an increased protein breakdown in preterm infants receiving corticosteroids accompanied by a diminished growth.

The sixths aim in this thesis is to answer the question whether insulin administration simultaneously with corticosteroids would prevent the catabolic effect of dexamethasone given to preterm infants with chronic lung disease.

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Chapter 2

Glucose kinetics during the first day of life in ventilated preterm infants

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ABSTRACT

The aim of this study was to evaluate glucose turnover and endogenous glucose production (GPR) during the first 24 hours of life in preterm infants with RDS. We also estimated the glucose carbon recycling and evaluated the effect of antenatal corticosteroids on GPR and recycling.Twenty-seven premature infants, birth weight 1091 ± 377 gm and gestational age 28.5 ± 2.1 wk, were studied immediately after birth. 11 Infants had not received antenatal steroids, 6 infants received only one dose steroid and 10 patients received two antenatal steroid doses. Mean glucose infusion rate was 5.3 ± 0.7 mg/kg/min. Glucose turnover and endogenous glucose production remained constant during the period from 11 to 29 hours after birth. Mean Ra-true was significantly higher (8.37 ± 1.5 mg/kg/min) than mean Ra-apparent (6.78 ± 1.4 mg/kg/min $p<0.001$). The mean GPR was 2.56 ± 1.5 mg/kg/min. The glucose recycling calculated from the difference between Ra-true and Ra-apparent was 1.45 ± 0.8 mg/kg/min. The mean glucose carbon recycling estimated by the method of Tayek and Katz was 0.67 ± 0.5 mg/kg/min. Glucose Production in infants who had been exposed to 2 doses of steroids was significantly higher (two dose group versus no steroid group: $p = 0.05$). Conclusion: in VLBW infants glucose turnover and endogenous glucose production remain constant during the period from 11 to 29 hours after birth. VLBW infants are capable of glucose recycling and this is constant over time. Antenatal steroids increase postnatal endogenous glucose production.

INTRODUCTION

The transition of intrauterine to extrauterine life has a major influence on glucose metabolism in neonates. During fetal life, glucose production, and thus gluconeogenesis does not occur.[1] Immediately after birth, the glucose supply which is obtained from the mother by rapid flow through the placenta, is abruptly stopped and the neonate has to regulate its own glucose level.

Blood glucose levels fall and rise again due to hormonal regulation, with the nadir at 1-2 hours after birth. Glucose production has been measured during the first day of life in term AGA and SGA infants [2] as well infants of diabetic mothers and preterm infants [3,4] during fasting or infusion of very small amounts of glucose. In addition, Sunehag and others have measured GNG from glycerol in both term healthy newborns, [5-9] but also in infants of diabetic mothers during the first eight hours of life before any feeding [10] and from glycerol in very premature babies during the first 24 h of life during infusion of about 2 mg/kg min of glucose only [11] and shown that even premature infants activate GNG enzymes during the first day of life. Gluconeogenesis and glycogenolysis are a drain on the limited neonatal resources. In preterm neonates, the amount of glucose stored as glycogen is low and glycogenolysis will be limited. Therefore, most preterm neonates receive a parenteral administration of glucose soon after birth. Several studies addressed the glucose turnover in preterm infants at one point of time in the first day of life. [4,7,12] As far as we are aware no data exist about the course of glucose turnover during the first 24 hours of life. It is not known how the neonatal glucose production rate develops during a constant glucose intake the first day of life, nor what the precursors for the endogenous glucose production are.

Since meta-analysis show that antenatal steroids reduce neonatal morbidity in infants born at less than 31 weeks, more and more infants are exposed to antenatal steroids, given to women who are likely to deliver prematurely. [13] Our own observations after the NIH Consensus [14] and the publication of Ryan and Finer [15] are that about 70% of the infants delivered before 32 weeks gestation have had antenatal steroids. Glucocorticoids have an important role in many metabolic pathways which has not been subject to much study in foetuses and neonates to date. In a study we performed before, [4] we found that infants born to mothers who had received steroids antepartum had higher glucose production rates.

The aim of this study was to evaluate glucose turnover and endogenous glucose production during the first 24 hours of life in preterm infants with RDS. Secondly we wanted to estimate the amount of glucose carbon recycling. Finally we wanted to evaluate the effect of antenatal corticosteroids on endogenous glucose production and recycling.

METHODS

The study was approved by the medical ethics committee of the Sophia Children's Hospital Rotterdam.

Patients: As a subgroup from a larger study regarding surfactant metabolism the first 24 hour of life by the incorporation of carbon from the U-13C glucose into surfactant, 27 premature infants (19 male, 8 female) requiring mechanical ventilation and admitted to the neonatal intensive care unit immediately after birth were studied between december 1994 and march 1997. Inclusion criteria were: gestational age $\leq$ 32 weeks, information about prenatal medication, and written parental informed consent. Exclusion criteria were: congenital infection, maternal diabetes and chromosomal abnormality.

Whether antenatal steroids were administered was a decided by the obstetrician. On retrospective evaluation, one group of 11 infants had not received antenatal steroids, one group received an incomplete course of antenatal steroids of just one dose within 24 hours before delivery (6 infants) and one group of 10 patients received a complete treatment of two antenatal steroid doses at least 24 hour before delivery. (Table 1)

Table 1 Patient data

	BW	GA	Cord pH	Apgar 5 min.	VI
Average no steroids	1172 (462)	28.9 (2.3)	7.21 (0.10)	7.6 (1.1)	216 (151)
Average 1 dose steroid	1163 (479)	28.7 (2.8)	7.26 (0.11)	7.8 (1.6)	170 (54)
Average 2 doses steroid	995 (218)	29.0 (2.1)	7.21 (0.12)	7.3 (1.1)	112 (72)
Total Average	1104 (387)	28.6 (2.0)	7.21 (0.11)	7.5 (1.2)	168 (118)

BW = birth weight in gram, GA= Gestational Age in weeks
VI = Ventilatory Index (= Mean Airway Pressure*ventilatory frequency*FiO_2)

All infants suffered from respiratory distress syndrome and were ventilated and treated with surfactant. Every infant was receiving amoxycillin and tobramicin, as is standard practice in premature ventilated infants in our neonatal intensive care unit, pending the results of their initial blood cultures. 8 infants were SGA according to Usher and McLean. [16] No infant needed vasoactive medication. Intravenous glucose was provided as soon as possible after birth, at a rate sufficient to keep the infants glucose levels at or above 2.6 mmol/l.

Glucose studies:

Patients received a constant intravenous infusion of [U-^{13}C]glucose (99% enriched, Campro Scientific, Veenendaal, The Netherlands) for 24 hours at 0.170 mg/kg/min. The start of the study was defined by the start of the infusion of the labeled glucose. The labeled glucose was infused by a high precision pump (M22; Harvard Apparatus Co. Inc., South Natick, MA). Before and during the glucose infusion, 1.0 ml arterial blood was drawn every 6 hours for determination of glucose enrichment. Blood was collected in lithium-heparin containing vacutainers and directly centrifuged to separate plasma and cells. The plasma was stored at –70° C until further processing.

Analytical methods: Plasma was delipidated with chloroform and methanol. The water fraction was passed over a column with anion-exchange resin (AG-1X8 [acetate], 100-200 mesh; BioRad Laboratories, Richmond, CA) and cation-exchange resin (AG-50W X8 [hydrogen], 200-400 mesh; BioRad). The elute containing the glucose was evaporated to dryness at 80o C and derivatized to an aldonitril pentacetate derivative. [17]

Mass Spectrometry analysis : The enrichment of total carbon-13 glucose aldonitril acetates was measured by gaschromatography combustioninterface isotope ratio mass spectrometry (GC-CI-IRMS)(Micromass, Weesp, The Netherlands) with Helium (0.6 ml/min) as carrier gas, as previously described. [18,19]

Briefly, analysis was done by injecting 1 μl with a split ratio of 30:1 at an injection temperature of 280° C on a 25 m x 0.22 mm, 0.11 μm coated HT-5 capillary column (Scientific Glass Engineering, Victoria, Australia), isothermally held at 220° C. The enrichment was expressed in atom percent excess (APE), which represents the increase in the percentage of carbon-13 atoms in total carbon dioxide obtained by combustion of the glucose derivative compounds above baseline enrichment (before infusion).

To determine the M0-M6 isotope cluster enrichment, the same samples were analysed with an SSQ7000 GC/MS instrument (ThermoQuest, San Jose, Ca, USA) in the positive chemical ionisation mode applying methane as the reagent gas. Glucose aldonitril pentacetate was separated on a 20m x 0.18 mm AT-1701 capillary columns, film thickness 0.4 μm (Altech Associates Inc., Deerfield, Ill., USA). Helium served as the carrier gas at a constant flow rate of 0.7 ml/min. The samples were injected splitless at 275°C. The oven temperature started at 80°C (1 min.), was programmed to increase to 280°C with 5°C/min and remained at 280°C for 5 min. The interface temperature was 275°C. Analysis was performed in the selected ion monitoring mode applying the mass ions m/z 328 (MO)-334 (M6). Conditions were adjusted to obtain at least 20 data points over the GC peak and sufficient signal intensities to assure stable area ratio measurements.

Calculations.

Total turnover of glucose was calculated from IRMS data by the following equation:

$Ra_{apparent} = ((IE_{infusion}/IE_{plasma\ apparent})-1) \times I(G)$

Where $Ra_{apparent}$ is the apparent rate of appearance including a contribution of recycled glucose, $IE_{infusion}$ is the isotopic enrichment of the infusate (99%), IE_{plasma} is the total isotopic enrichment of the plasma glucose measured by GC-CI-IRMS, and I(G) is the [U-^{13}C]glucose infusion rate in μmol/kg/min.

True turnover calculation using M6 enrichment was done by the following equation:

$Ra_{true} = ((IE_{infusion}/IE_{plasma\ M6})-1) \times I(G)$, where $IE_{infusion}$ is the M6 enrichment in the infusate, which is 94% in a 99% atom percent enriched tracer. $E_{plasma\ M6}$ is the isotopic enrichment of M6 in the plasma, measured by GCMS.

Glucose production (GPR) was calculated as the difference between the true rate of glucose turnover and the rate of total glucose infused.

Recycling.

Infused labeled glucose carbons can recycle back to glucose as the result of glycolysis of labeled glucose in peripheral tissues. The chance that glucose is formed with 6-labelled carbon atoms, is negligible as the 3-carbon atoms which are produced during glycolysis are diluted with a manifold greater unlabelled pool. Labeled 3-carbon molecules will therefore be combined with unlabelled molecules to produce partially labeled glucose. This partially labeled recycled glucose molecules will contribute labeled carbon, which will be included in and thus overestimating the enrichmentmeasured by the GC-C-IRMS. As a consequence the measurement of glucose Ra will be underestimated by this method. Since the probability of recombination of two uniformly labeled three carbon units to form a uniformly labeled glucose molecule is very low, the enrichment of M+6 glucose will not include any contribution from recycled glucose and will, therefore, provide a true measurement of glucose Ra.

The difference between the total and true glucose turnover therefore reflects the glucose recycling.

The glucose carbon recycling was also estimated by Tayek and Katz from the isotopomer distribution of glucose, measured by GC/MS. [20,21] These authors defined the fraction of recycled glucose carbons in plasma glucose (GCR) as: $GCR = \Sigma_1^3 iM_i / \Sigma_1^6 iM_i$ where $\Sigma_1^3 iM_i$ is the weighted sum of the excess mass isotopomers fraction of glucose molecules containing 1, 2 or 3 ^{13}C atoms, expressed in ^{13}C atoms and $\Sigma_1^6 iM_i$ is the weighted sum of the excess mass isotopomers fraction of glucose molecules containing 1, 2, 3 or 6 ^{13}C atoms.

Excess mass isotopomers are designated by M_i, in which the subscript indicates the number of ^{13}C atoms per molecule. M_0 refers to glucose into which no ^{13}C atoms were incorporated from [U-^{13}C]-glucose, and M_1, M_2, M_3 and M_6 to molecules containing 1, 2, 3 and 6 ^{13}C atoms per molecule glucose respectively. M_4 and M_5 were not present in significant amounts.

Excess mass isotopomer fractions are derived from recorded mass isotopomer distributions of the aldonitril penta-acetate derivative of glucose of M/Z 328 (=M0) to M/Z 334 (=M6) after correction for background abundance, impurities in the infusate and normalization of the corrected spectra according to Lee et al [22,23].

Statistics.

Data are presented as mean ± SD unless stated otherwise. Statistical analysis was done by paired t-tests using the computer software program Excel 5.0 (Microsoft Corporation) and by two-way ANOVA. A p value of less than 0.05 was considered significant.

RESULTS

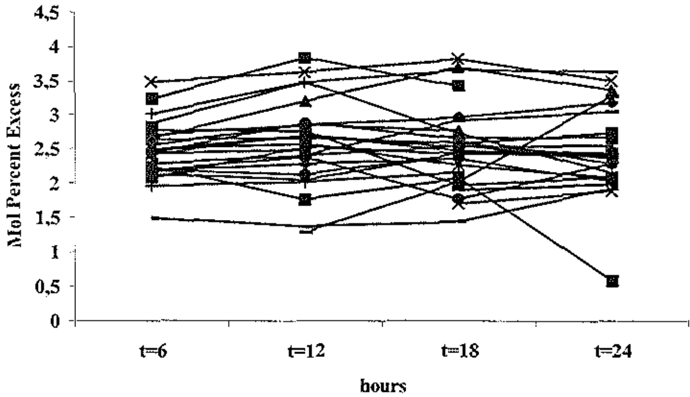
The study started at an average of 5.1 (± 2.7) hr. after birth. Clinical data of the patients are given in table 1. All infants received an intravenous glucose intake of 5.27 ±0.7 mg/kg/min. All plasma glucose levels were kept at or above 2.6 mmol/l during the study. A plateau in isotope enrichment was observed in all patients, both in the total- (Fig 1) and the M+6 enrichment.

The true rate and apparent rate of appearance of glucose for all different time points are given in table 2. In some patients reliable measurements could not be obtained at all timepoints explaining the blank spaces in the table. Since Ra-true and Ra-apparent were constant during 24 hours in each patient, the mean Ra-true and Ra-apparent for the whole 24 hours was calculated for each patient (table 3). Mean Ra-true was significantly higher (8.37 ± 1.5 mg/kg/min) than mean Ra-apparent (6.78 ± 1.4 mg/kg/min) in all patients ($p<0.001$). As Ra-true and glucose intake did not change over the 24 hr period, GPR was also constant. The mean GPR was 2.56 ± 1.5 mm/kg/min.

The glucose recycling calculated from the difference between Ra-true and Ra-apparent was 1.45 ± 0.8 mg/kg/min.

In 24 patients we were able to calculate the glucose carbon recycling according to the method described by Tayek and Katz. In these infants, plasma from all data points was combined in order to have sufficient plasma for analysis. In the remaining patients, insufficient plasma was available for the analysis. The mean glucose carbon recycling calculated by this method was 0.67 ± 0.5 mg/kg/min.

Figure 1 C13 enrichment in Glucose measured by GC-IRMS (Expressed as Mol percent excess)



There was no correlation between birthweight and gestational age with glucose turnover or GPR. The average GPR of the 27 infants was positively correlated with glucose turnover ($p < 0.001$). Also the average recycling was positively correlated with the average GPR ($p = 0.005$).

With respect to antenatal steroids, the infants were divided in three groups.

Glucose turnover in infants who had been exposed to a complete course of antenatal steroids was not significantly different from the group with no steroid. However, the endogenous glucose production was significantly higher in the two dose steroid group versus no steroids ($p=0.05$). (Table 3) There was no difference in recycling between steroid groups (1.25 ± 0.7, 1.81 ± 1.5 and 1.44 ± 0.8 mg/kg/min for no steroids, one dose or two doses respectively).

Glucose turnover in small for gestational age (SGA) infants was not significantly different from appropriate for gestational age (AGA) infants. GPR of SGA-infants was 1.62 mg/kg/min ($\pm$ 1.5) compared to 2.15 ($\pm$ 1.6) in AGA-infants (not significant).

The ventilatory index was not correlated with either GPR or glucose turnover. Although there was a trend for lower ventilatory index in children after antenatal steroids, the difference between the steroid groups was not significant. ($p=0.08$ for 2 doses versus no antenatal steroids)

Table 2

Patient glucose turnover data, true versus apparent for each point of measurement.

Patiënt	RA true 6	RA apparent 6	RA true 12	RA apparent 12	Ra true 18	RA apparent 18	RA true 24	RA apparent 24
1	6,3	5,0	5,7	4,2	5,9	4,8		
2					7,7	6,4	7,4	6,0
3	8,2	6,7	8,1	6,6			7,6	6,2
4	10,2	7,5	9,6	7,7	8,9	6,8	8,7	6,7
5	5,4	4,7	5	4,5	5,2	4,2	5,3	4,6
6	7,6	6,6	7	6,1	7,1	6,5	7,5	6,4
7	8,5	6,8	7,2	6,1	7	6,6	10,2	6,7
8	8,1	6,7		5,7	7,3	6,2	7,3	6,1
9	8,4	6,2	7,6	6,2	7,4	6,3	7,5	6,9
10	7,6	6,6	8,2	6,4	8,7	7,3	8,4	7,9
11	7,5	7,2	6,9	6,8	6,4	6,4	7,4	7,0
12		7,0	7,2		8,8	8,7	8,4	8,3
13	8,1	7,5	8	6,9			8,5	7,2
14	8,9	8,4	7,6	8,1	7,8	7,6		
15		8,4		8,1		6,7		6,8
16	6,7	5,7	5,8	4,7	5,5	4,4	5,7	4,5
17	7,6		6	6,8	5,5	5,6		
18	7,5	6,0	6,4	5,7	6,4	5,5	5,7	5,1
19	7,8	5,9	7,6	5,9	11	8,4	10	7,9
20	6,8	5,4	5,9	4,7	7,1	6,0	8,2	7,7
21	13,7	11,1	12,8	12,1	12,3	11,5	11	8,6
22	8,5	7,3	10	9,4	8,9	7,9		
23	7,4	6,2	8,2			6,3		7,2
24	9,5	7,9	7,9	6,5	8,4	6,7	8,3	6,7
25	9,7	7,6	9,2	7,2	8,8	7,0	10,1	8,1
26		6,2		5,1		4,4		4,8
27						9,7		8,8
Average (SD)	**8,2 (1,7)**	**6,9 (1,3)**	**7,6 (1,7)**	**6,6 (1,7)**	**7,7 (1,8)**	**6,7 (1,7)**	**8,1 (1,7)**	**6,8 (1,2)**

RA true 6 = true turnover at 6 hours, RA app. 6 = apparent turnover at 6 hours, etc

DISCUSSION

The results of our study show that there is no change over time in the period 11-29 hrs after birth in very preterm infants in the glucose enrichment after U-^{13}C glucose infusion, either in the glucose enrichment measured with IRMS, or the M+6 enrichment measured by GCMS. Also we observed a persistent and significant difference between both methods. From these observations the following conclusions can be drawn.

First, despite the tremendous changes in fuel supply and hormonal status which accompanies birth, glucose turnover, endogenous glucose production and glucose recycling are constant

Table 3 Average daily kinetics

Patient	daily RA true	daily RA apparent	glucose intake	daily GPR	GCMS-IRMS	T/K	T/K % of RA true	T/K % of GPR
5	5,23	4,50	5,57	-0,35	0,72	0.37	7	
6	7,30	6,39	5,47	1,83	0,91	0.51	7	28
8	7,57	6,16	5,52	2,05	1,24	0.38	5	19
10	8,23	7,03	4,98	3,25	1,20	0.49	6	15
11	7,05	6,86	5,43	1,62	0,19	0.35	5	22
12	8,13	8,01	5,51	2,62	2,14	0.33	4	13
17	6,37	6,20	5,64	0,73	1,90	0.38	6	52
18	6,50	5,60	4,78	1,72	0,90	0.39	6	23
19	9,10	7,02	6,495	2,61	2,08	1.64	18	63
27		5,11	4,13					
28		9,27	4,14					
no steroid	*7.27 (1.2)*	*6.56 (1.3)*	*5.24 (0.7)*	*1.79 (1.1) #*	*1.25 (0.7)*	*0.54 (0.42)*	*7.11 (4.2)*	*29.38 (18.21)*
4	9,35	7,19	5,26	4,09	2,16	1.03	11	25
14	8,10	8,05	5,57	2,53	0,05	0.41	5	16
15		7,51	5,62					
16	5,93	4,81	3.98	1,95	1,12	0.77	13	39
23	7,80	6,57	5,46	2,34	4,19	0.47	6	28
24	8.53	6.97	5.53	3.00	1,55	0.43	9	14
1 dose steroid	*7.94 (1.3)*	*6.85 (1.1)*	*5.24 (0.6)*	*2.78 (0.8)*	*1.81 (1.5)*	*0.62 (0.27)*	*8.8 (3.35)*	*24.4 (10.06)*
1	5,97	4,66	4,09	1,88	1,30	0.30	5	16
2	7,55	6,18	5,63	1,92	1,37	0.45	6	23
3	7,97	6,50	4,42	3,55	1,46	0.48	6	14
7	8,23	6,55	5,6	2,63	1,68	0.58	7	22
9	7,73	6,39	5,39	2,34	1,33	0.31	4	13
13	8,20	7,20	5,47	2,73	1,00	0.90	11	33
20	7,00	5,93	4,12	2,88	1,07	1.12	16	39
21	12,45	10,83	4,98	7,47	1,62	2.12	17	28
22	9,13	8,21	7,13	2,00	0,92	0.91	10	46
25	9,45	7,46	6,13	3,32	1,99	1.04	11	31
2 doses steroid	*8.35 (1.9)*	*6.91 (1.8)*	*5.06 (0.8)*	*3.30 (1.8) #*	*1.44 (0.3)*	*0.82 (0.6)*	*9.3 (4.5)*	*27 (11)*
Average (SD)	**8.37 (1.5) ***	**6.78 (1.4) ***	**5.18 (0.7)**	**2.56 (1.5)**	**1.45 (0.8)**	**0.67 (0.5)**	**8.4 (4.1)**	**27 (13)**

= p value 0.05 (2 doses steroid compared to no steroid), * = p value <0.001 (Ra true versus Ra total)
In italics the average values per steroid group are given. Glucose intake in mg/kg/min.
GCMS minus IRMS is the calculated recycling by GCMS data minus IRMS data. T/K gives the calculated recycling according to Tayek and Katz and the percentage of the calculated recycling of total glucose turnover and endogenous glucose production repectively.

over time. In view of all changes that take place around birth, this is a remarkable phenomenon. Secondly, since we did not know wether the glucose label would recycle via synthesised amino acid and/or fatty acids during the 24 hours infusion, we observed no recycling of tracer from other compounds than glucose. Our results also indicate that endogenous glucose production and glucose recycling are not completely suppressed at a glucose intake of 5.3 ±0.7 mg/kg/min. Finally, the enzymes involved in gluconeogenesis must be active already 11 hrs after birth, since part of the GPR is due to glucose recycling,

In utero, glucose is the main fuel supplied to the foetus.[8] The hormonal pattern is directed towards the use of transplacental delivered glucose. There are no indications of endogenous glucose production in utero. After birth, glucose remains an important energy source, notably for the brain and erythrocytes. A constant glucose supply after birth therefore is necessary. In very low birthweight infants, this is usually achieved by starting a glucose infusion after birth. At the same time the hormonal profile changes after birth, the insulin level decreases while glucagon increases. Stress hormones like catecholamines also influence glucose turnover. It is shown that also in preterm infants catecholamines increase after birth [24] which might affect glucose turnover. The fact that glucose turnover was constant the first day after birth indicates that there is somehow a balance in glucose consumption and production, and therefore in the hormonal regulation of glucose turnover and production.

The glucose turnover rate as observed in this study is in correspondence with data presented earlier. We did not observe a complete suppression of the endogenous glucose production at a glucose intake of 5.27 mg/kg/min. This is in accordance with the results of Sunehag et al and Herz et al, but in contrast with the study of Cowett et al. [25-27] An explanation for this difference could be the difference in gestational age between the study of Cowett and the other studies. In the study of Cowett they included three groups, term aga infants, term sga and preterm infants. In the preterm infants the GPR was not suppressed but showed an average of 1.4 mg/kg/min, not very different from our results. Also, our infants were suffering from IRDS, and this might be another reason why they did not suppress GPR
Studies regarding the source of the endogenous glucose production on the first day of life in term infants have all been done after fasting, a rather different situation from our study with an ongoing glucose infusion. Total GNG has not been measured during the first day of life in term or preterm infants.[7] Alanine contributes to 10-15% of new glucose carbon [28] and recycled glucose carbon represents around one-third of hepatic glucose production.[6] The contribution of glycerol to glucose production is around 5-20%.[29,30] Essentially no

quantitative gluconeogenic data exist of very low birthweight infants on the first day of life, which is rather important as these infants only have very limited glycogen stores, fewer alternative fuel stores, less developmentally mature enzyme systems and diminished exogenous fuel intakes relative to term infants. The background of providing glucose immediately after birth is not only to prevent hypoglycaemia, but also to reduce the use of endogenous reserves for energy as much as possible. Data on the source of glucose carbon during gluconeogenesis therefore is important.

Sunehag et al studied the gluconeogenesis on day 5 of life in preterm infants while receiving an infusion of glucose, aminoacids and lipids.[9] They used U-^{13}C glucose, $2H_2O$ and 2 ^{13}C-glycerol as tracers and the isotope distribution of glucose and deuterium incorporation in carbon-6 of glucose as indicators for gluconeogenesis. They found that app. 70% of the GPR under these circumstances can be explained by gluconeogenesis, glycerol explained 64% of total gluconeogenesis. The different methods showed comparable results. These results cannot simply be compared to our study, as the conditions in the study of Sunehag were quite different from ours. We studied preterm infants on the first day of life while receiving glucose only. It is conceivable that the contribution of glycerol to the GPR is lower in these circumstances than during TPN. Also, their infants received glucose at about half the rate our infants did, which could explain why they had to make more glucose by themselves via GNG and glycogenolysis.

We used the difference between the apparent glucose turnover and the true glucose turnover as a measure for glucose recycling. One assumption of this method is that both analytical methods give reliable results. Both methods are well validated and robust. Secondly, we used the same derivative, for both methods. Finally, the fact that in all cases the apparent glucose enrichment was higher than the M+6 enrichment makes us confident that the observed results are correct.

Gluconeogenesis has been estimated from glucose carbon recycling in term infants. [6,7] Recently, other methods of estimating gluconeogenesis have emerged. The first is the incorporation of deuterium in glucose during its synthesis from pyruvate. This method was adapted to be used for studies in human infants by Landau et al.[31] Using this method we found the GNG via pyruvate contributed to 20-40% of GPR in 3 days old preterm infants receiving a glucose infusion of 5.9 mg/kg/min.[Kalhan et al, accepted Am J Physiol.] Another

technique is the mass isotope distribution analysis. The glucose production is calculated from the M+1 to M+5 enrichment in glucose after U-^{13}C glucose infusion. This method has its limitations due to the loss of label in the TCA cycle. Another disadvantage of this method is that in order to measure the M 1-5 enrichment reliably, a rather high dose of U-13C glucose tracer has to be infused. A modification and simplification of this method was recently described by Haymond and Sunehag.[32] In our study we infused only a relative low dose U-^{13}C glucose. Therefore we could not measure the M 1-5 enrichment reliably in all samples and therefore used the mean values of each isotope over the 24 hrs. Using the calculations as suggested by Tayek and Katz, we found that glucose recycling was 0.67 ± 0.5 mg/kg/day. Calculating recycling by the difference between Ra-apparent and Ra-true is a method that relies on C-atom measurements, where isotopomer distribution calculations rely on molecule measurements. Although there is some difference between both calculated recycling values, the results indicate that glucose recycling is an active process already during the first day of life. Part of the difference between both results obtained by us can be explained by the relative low dose U-^{13}C glucose used in our study. Further studies, using a higher U-^{13}C-glucose dose, are necessary to validate our method of using the difference between Ra-total and Ra-true as measure for glucose recycling, and to evaluate whether it is possible to calculate the percentage of glucose made available via recycling.
That we observed no trend in enrichment of glucose with either method indicates that within the time frame of the study, glucose carbon was not - to a significant amount - released from other compounds than glucose after being incorporated. When glucose carbon which might have been incorporated in for instance aminoacids and was released from these aminoacids during the period of the study, total enrichment would have increased. In the present study the M+6 enrichment did not increase over the 24 h study period. This finding most likely excludes any significant recycling of tracer via glycogen.

In a previous study we showed evidence that antenatal administered dexamethasone increased glucose turnover in preterm infants.[4] Steroids have been known for a long time to enhance gluconeogenesis at virtually every step in the gluconeogenic process from augmenting muscle proteolysis and the delivery of aminoacids to the liver, to stimulating hepatic aminoacid transport, gluconeogenic enzyme activity, and the hepatic conversion of gluconeogenic precursors to glucose including the induction of foetal gluconeogenesis in the liver of the near-term foetal sheep.[33,35] In this study we could not confirm the earlier observation on antenatal corticosteroids and glucose turnover. The GPR on the other hand was significantly higher in the group receiving two doses of corticosteroids. No effect on recycling was observed. Whether the higher GPR is due to a higher glucose production from the release of aminoacids, or to a higher maturation of enzyme needs further studies.

There was no correlation between the ventilatory index and the GPR or glucose turnover. In other words, we could not find a relation between severity of illness and glucose metabolism in our patients. IRDS is considered to be a state of inflammation with increased levels of cytokines. Whether these increased levels of cytokines are involved in the lack of suppression of GPR is unknown. As we expected, there was a correlation between the number of doses steroids antenatal and the ventilatory index but not significant (p=0.08 for 2 doses versus no antenatal steroids)

Conclusion: in VLBW infants glucose turnover and endogenous glucose production remain constant during the period from 11 to 29 hours after birth. There is no recycling of tracer other than the return from glucose and this glucose recycling is constant over time. As part of the GPR is due to glucose recycling, the enzymes involved in gluconeogenesis must be active already 11 hrs after birth.

Antenatal steroids increase postnatal GPR. There was no difference in glucose kinetics between AGA and SGA infants.

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Chapter 3

Estimation of Gluconeogenesis in Newborn Infants

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ABSTRACT

The rate of glucose turnover (Ra) and gluconeogenesis (GNG) via pyruvate were quantified in seven full-term healthy babies between 24-48 hours after birth, and twelve low birth weight infants on day 3-4 using [$^{13}C_6$]glucose and [$^2H_2$]O. The preterm babies were receiving parenteral alimentation of either glucose or glucose plus amino acid with or without lipids. The contribution of gluconeogenesis to glucos
e production was measured by the appearance of ^{2}H on C-6 of glucose. Glucose Ra in full term babies was 30 ± 1.7 μmole/kg.min (mean ± SD). GNG via pyruvate contributed ~31% to glucose Ra. In preterm babies, the contribution of GNG to endogenous glucose Ra was variable (range 6-60%). The highest contribution was in infants receiving low rates of exogenous glucose infusion. In an additional group of infants of normal and diabetic mothers, lactate turnover and its incorporation into glucose was measured within 4-24 hours of birth using [$^{13}C_3$]lactate tracer. The rate of lactate turnover was 38 μmole/kg.min and lactate C, not corrected for loss of tracer in TCA cycle, contributed ~18% to glucose C. Lactate and glucose kinetics were similar in small for gestational age infants and in normal infants or infants of diabetic mothers.

These data show that gluconeogenesis is evident soon after birth in the newborn infant, and that even after a brief fast (5 h), gluconeogenesis via pyruvate makes a significant contribution to glucose production in healthy full term infants. These data may have important implications for the nutritional support of the healthy and sick newborn infant.

INTRODUCTION

The fetus in utero, under normal unperturbed physiological circumstances, is entirely dependent upon the mother for a continuous supply of glucose, and no significant production of glucose by the fetus has been demonstrated, neither in human nor in other mammalian species (Reviewed in 15). In addition, although significant activity of key enzymes involved in gluconeogenesis has been documented early in gestation in human fetal liver, gluconeogenesis in vivo has not been documented (32,33,11,19,29). Whether cytosolic phosphoenolpyruvate carboxykinase, the key regulatory enzyme involved in gluconeogenesis, is expressed in human fetal liver is not known. In rodents, cytosolic phosphoenolpyruvate carboxykinase is first expressed immediately after birth and is associated with the appearance of gluconeogenesis (15,12). Thus the newborn at birth relies entirely on the mobilization of accumulated hepatic glycogen stores and the initiation of gluconeogenesis for a continuous supply of glucose. Both of these processes, i.e. glycogenolysis and gluconeogenesis, are stimulated by the birth associated surges of catecholamines and pancreatic glucagon (20). That the human newborn can incorporate alanine carbon into glucose as early as six hours of age has been demonstrated by Frazer *et al* using ^{13}C labeled tracer (10). However, the contribution of gluconeogenesis to glucose production in healthy full term newborns has not been quantified. In the present study, using stable isotopic tracers, we have quantified the turnover rate of lactate and its incorporation into glucose in the period immediately after birth. In addition, using the recently developed deuterium labeled water method (27,4,22), we have quantified the contribution of gluconeogenesis via pyruvate to total glucose production in healthy, normal full term babies. During adaptation to extrauterine environment, perturbations in glucose homeostasis are often observed in preterm infants, in those born small for gestational age and to mothers with diabetes (20). Since both hyperglycemia and hypoglycemia are often seen in these babies, we also quantified the rates of glucose turnover and gluconeogenesis in clinically stable infants in these groups. Our data show that gluconeogenesis from lactate and pyruvate is established by 4 - 6 hours after birth. Gluconeogenesis from pyruvate contributes as much as 30% to total glucose production in healthy term babies between five and six hours after a feed.

MATERIALS AND METHODS

Gluconeogenesis was measured in normal, preterm, small for gestational age (SGA) infants and in infants of diabetic mothers using either the deuterium labeling of body water or by quantifying the incorporation of ^{13}C from labeled lactate into glucose.

Deuterium labeled water studies

Gluconeogenesis was quantified in seven full term infants and thirteen preterm infants using the deuterium labeling of body water method (27,4). The study protocol was approved by the Institutional Review Board. Written informed consent was obtained from the mother and, when available, the father, after fully explaining the procedure. The full term infants were appropriate for gestational age, had normal Apgar scores, had no clinical problems and were receiving either formula feeds or maternal breast milk (Table 1). Two infants (#6 and #7) were born to mothers with gestational and insulin dependent diabetes. They had normal plasma glucose concentrations and did not develop any neonatal problems.

Majority of the term infants were studied on the second day after birth in the General Clinical Research Center at MetroHealth Medical Center. Two indwelling cannulae were placed in the superficial vein of each hand to draw blood samples and infuse the isotopic tracer [$^{13}C_6$]glucose (99% ^{13}C; Isotec Inc., Miamisburg, Ohio). The study protocol is displayed in Figure 1.

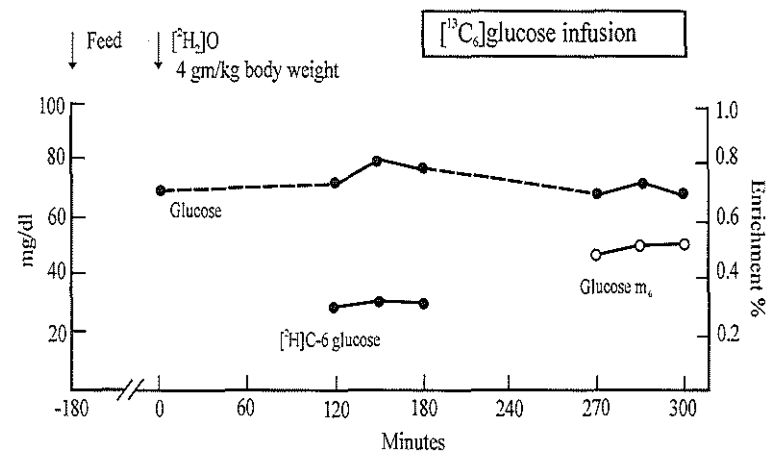

Figure 1
Study design for the quantification of gluconeogenesis using [$^2H_2$]O. Three hours after the last feed, the infants were given [$^2H_2$]O 4.0 gm/kg body weight mixed in sterile water. Plasma samples for the measurement of ^{2}H enrichment in body water and on C-6 of glucose were obtained starting at 120 min following [$^2H_2$]O administration. A prime constant rate infusion of [$^{13}C_6$]glucose was started at 180 min and continued for two hours to quantify the rate of appearance of glucose. The plasma glucose concentration (□) mg/dl, glucose m_6 enrichment and ^{2}H enrichment on C-6 glucose from one study are displayed.

Approximately three hours after the last feed, an oral dose (4 g/kg body weight) of [$^2H_2$]O, (99% ^{2}H; Isotec Inc., Miamisburg, Ohio), mixed with sterile distilled water was administered. Two hours later, three blood samples for the measurement of [^{2}H] enrichment of C-6 of plasma glucose were obtained at twenty minute intervals. This was followed by intravenous administration of a prime constant rate infusion of [$^{13}C_6$]glucose for the next 120 minutes to quantify the rate of appearance (Ra) of glucose. The bolus prime was 0.6 mg/kg body weight and the constant rate infusion was 30 μg/kg.min. Blood samples were obtained for the measurement of glucose m_6 enrichment at 90, 105 and 120 minutes of [$^{13}C_6$]glucose infusion. The infants were comfortable throughout the study period and did not show any evidence of stress. Cardiopulmonary monitoring was performed and plasma glucose concentration measured with each blood sampling. Their plasma glucose concentration remained unchanged throughout the study. The blood samples were centrifuged in cold and plasma stored at -70° C for later analysis. The study was completed at approximately eight hours following the last feed. In one infant (#8, not reported here), the study was discontinued because of inability to obtain venous blood samples.

The preterm infants were studied between 48 and 72 hours after birth using a similar protocol as described above with minor modifications. As anticipated, all preterm infants were initially on ventilatory support. At the time of study, three infants were on ventilatory support and one required continuous positive airway pressure (CPAP). None of the babies were on vasopressors at the time of study. All were receiving antibiotics and three were on caffeine due to apnea or prematurity. All had indwelling vascular cannulae for clinical indications. Within 24 hours after birth, they were assigned to either glucose, glucose plus amino acids (1.2 g/kg.day), or glucose, amino acids (1.2 g/kg.d) plus lipids (1.25 g/kg.d = ~1μmole/kg.min). The rate of glucose infusion was based on the clinical protocol and adjusted by the patient's physician on clinical and laboratory information. However, the rate of glucose infusion was constant for several hours prior to and throughout the tracer infusion study. Plasma samples for deuterium enrichment on C-6 of glucose were obtained 3, 4 and 5 hours after the nasogastric dose of [$^2H_2$]O. [$^{13}C_6$]Glucose infusion was started at 5 hours and blood samples for the ^{13}C enrichment of glucose obtained at 6.5, 6.75 and 7 hours of the study. The dose of [$^2H_2$]O and [$^{13}C_6$]glucose was the same per kg body weight as for term infants. These studies were performed at the Sophia Children's Hospital, Rotterdam, The Netherlands. Study protocol was approved by the Institutional Review Board and written informed consent was obtained from the parents after fully explaining the protocol. The investigators were not responsible for the clinical care of the infants.

Lactate Kinetics

Glucose and lactate kinetics were quantified in ten normal appropriate for gestational age infants, six infants of insulin dependent diabetic mothers (IDDM) and four small for gestational age infants (Table 2).

The mothers with IDDM were managed with multiple insulin doses per day or by continuous subcutaneous insulin infusion in order to maintain normoglycemia throughout the day. Their hemoglobin A_1 concentrations at delivery were within the normal range. All infants were born at term gestation and had no intrapartum or neonatal problems. They had normal physical examinations and had no apparent clinical problems. The tracer isotope infusions were started within 4-5 hours after birth in eight normal AGA infants and three IDDM infants. Other infants were studied between 8 and 24 hours after birth. Tracer infusion in the later group was started 4-5 hours after the last feed and continued for the next 4 hours. [6,6-$^{2}H_2$]Glucose (98 atom % excess ^{2}H) (Merck & Co; Dorvall, Quebec, Canada) and [$^{13}C_3$]lactate (90 atom % ^{13}C) (Merck & Co; Dorvall, Quebec, Canada) were infused as prime constant rate infusion. The rate of [6,6-$^{2}H_2$]glucose infusion was 30 μg/kg.min and the prime was 1.8 mg/kg, while [$^{13}C_3$]lactate was also infused at 30 μg/kg.min following a prime of 1.8 mg/kg. Heparinized blood samples were drawn from an indwelling cannula at 30 minute intervals; plasma was separated and stored at -20°C for later analysis. An aliquot of the whole blood was also precipitated with an equal volume of 10% perchloric acid and neutralized with 10% potassium carbonate for the measurement of blood lactate.

Analytical Methods

Plasma glucose was measured by glucose oxidase method. Blood lactate levels were measured using lactate dehydrogenase. Deuterium enrichment of hydrogens on C-6 of glucose was measured as described previously (4,22). Briefly, C-6 of glucose with its hydrogens, after preparatory isolation and purification, was cleaved by periodate oxidation to formaldehyde which, when condensed with ammonium hydroxide, forms hexamethylenetetramine. The latter was analyzed on a gas chromatography-mass spectrometry system (HP 5970 equipped with an HP 5890 gas chromatograph; Hewlett-Packard Co, Palo Alto, CA). The GC-mass spectrometry conditions were as follows: a non-polar cross-linked methyl siloxane capillary column (HP-1, Hewlett Packard Co, Palo Alto, CA) was used. The column dimensions were: length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm, injection temperature 220°C, oven initial temperature 105°C for 5.6 minutes, final temperature 230°C and ramp rate 45°C/min. The retention time for hexamethylenetetramine was ~4.2 minutes. Electron impact ionization (70 eV) was used, and ions m/z 140 and 141 were monitored using selective ion monitoring technique. Standard solutions of glucose of known enrichment were run along with unknown to calibrate for instrumental variations.

The (m+6) enrichment of glucose (glucose m_6, i.e. all carbons of glucose labeled with ^{13}C) was measured using chemical ionization mass spectrometry as described previously (40,1). The (m+2) enrichment of glucose in [6,6-$^{2}H_2$]glucose was measured using pentacetate derivative as previously described (23). The deuterium enrichment of glucose was corrected for the contribution of ^{13}C from lactate. Plasma lactate enrichment was measured using n-propylamide heptafluorobutyrate derivative (38). The ^{13}C incorporation into glucose was estimated from the enrichment of C-1 of plasma glucose using the enzymatic decarboxylation method (21). ^{2}H enrichment of total body water (plasma sample) was measured using zinc reduction method on an isotope ratio mass spectrometer (Dr. W. Wong; Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX).

Calculations

The rate of appearance of glucose and lactate (Ra) was calculated from the dilution of tracer glucose in plasma, as described (39).

Ra (μmole.kg^{-1}.min^{-1}) = [(Ei/Ep) - 1] x I, where Ei and Ep are the enrichments of isotopic tracer infused and of the substrate in plasma, respectively, and I is the rate of tracer infusion in μmole.kg^{-1}.min^{-1}.

The contribution of gluconeogenesis (GNG) from pyruvate was calculated as follows:

GNG from pyruvate = 100 x 0.5 x [^{2}H] enrichment of glucose C-6/[^{2}H]enrichment in water.

As discussed previously (27,16), it is assumed that methyl hydrogens of pyruvate C-3 that forms C-6 of glucose exchange with hydrogens in body water, so that ^{2}H enrichment of hydrogens bound to C-3 of pyruvate or that of phosphoenolpyruvate become similar to that of water. This exchange reaction in fasting adults has been shown to be over 80% complete. It has not been examined in the neonate.

The enrichment in C-6 is multiplied by 0.5 because of two hydrogens on C-6. Total gluconeogenesis is calculated by multiplying the fractional contribution of GNG with total glucose Ra.

The rate of appearance of lactate was calculated using the tracer dilution equation as above (39). The incorporation of lactate carbon into glucose was estimated by precursor product relationship. Fraction of glucose from lactate = $^{13}C/^{12}C$ ratio of glucose C-1 / $^{13}C/^{12}C$ ratio of plasma lactate. It was assumed that $^{13}C/^{12}C$ ratio of glucose C-1 represents the enrichment of all carbons of glucose. No correction was made for the loss of tracer carbon via exchange in the tricarboxylic acid cycle and therefore the estimates of lactate C incorporation into glucose represent minimal estimates.

All data are reported as mean ± S.D. Group comparisons were made using parametric and non-parametric statistical methods, using Statistix[R] software (Analytical Software, Inc, La Jolla, CA).

RESULTS

Gluconeogenesis via Pyruvate [$^2H_2$]O Studies

Term Infants: As displayed in Table 1, the term infants were appropriate for gestational age and were studied at a mean age of 46 hours. They maintained normal plasma glucose concentration throughout the eight hours of fasting. The mean plasma glucose concentration was 68 mg/dl (3.8 mM).

Table 1 Clinical characteristics of full term infants

Subjects	Gestational age weeks	Birth weight Kg	Age at study hours	Fast hours	Plasma glucose Mg/dl
1	40	3.36	35	3.00	57.3
2	40	3.28	45	3.25	85.0
3	38	2.47	35	3.50	59.6
4	38	3.18	54	3.75	90.1
5	39	3.56	68	4.00	68.1
6*	40	4.22	35	3.75	54.9
7*	39	3.19	48	3.50	61.6
Mean ± SD	**39 ± 0.9**	**3.32 ± 0.52**	**45.75 ± 12.33**	**3.54 ± 0.34**	**68.1 ± 13.9**

*Infants 6 and 7 were born to mothers with gestational and type I diabetes, respectively
The ^{2}H enrichment of plasma water and that of hydrogens on C-6 of glucose are displayed in Table 3.

Table 2 Clinical characteristics of the infants – Lactate kinetics

	Birthweight gm	Gestational Age weeks	Age at study hours	Males/ females
Normals (n=10)	3230 ± 349	39 ± 1	7.8 ± 11.4	6 / 4
SGA (n=4)	2435 ± 105	41 ± 1	19.8 ± 11.0	2 / 2
IDM (n=6)	3365 ± 694	39 ± 1	12.2 ± 8.6	4 / 2

mean ± SD

Table 3 Gluconeogenesis in the full term newborn infant

Subjects	$[^2H]$ C-6 Glucose %	$^2H]$ water %	Glucose m_6 %	Glucose Ra µmole/kg.min	GNG %	GNG µmole/kg.min
1	0.34	0.487	0.51	30.61	34.9	10.67
2	0.37	0.459	0.53	30.33	39.9	12.11
3	0.34	0.504	0.56	27.56	33.7	9.28
4	0.26	0.435	0.47	32.50	29.6	9.61
5	0.20	0.438	0.53	31.61	22.5	7.11
6	0.03	0.076	0.54	29.22	21.9	6.44
7	0.31	0.442	0.56	28.67	34.5	9.89
Mean ± SD				**30.07 ± 1.72**	**31.02 ± 6.7**	**9.30 ± 1.96**

The rate of appearance of glucose (Ra) was quantified using $[^{13}C_6]$glucose tracer dilution.

Infant #6 had received a smaller dose of $[^2H_2]O$ due to spillage. Also displayed are the m_6 enrichments of glucose and the calculated contribution of gluconeogenesis via pyruvate. A steady state isotopic enrichment was evident in all infants. The rate of appearance of glucose as measured by $[^{13}C_6]$glucose tracer dilution ranged between 4.9 and 5.9 mg/kg.min (28-33 µmole/kg.min). Gluconeogenesis from pyruvate contributed between 22% and 40% to the total glucose production, or an average of 1.7 mg/kg.min (9.3 µmole/kg.min). There was no correlation ($r = 0.025$; $p = 0.96$) between glucose Ra and the contribution of gluconeogenesis.

Preterm Babies: The preterm infants were studied between day 3 and 4 after birth. As anticipated in this group, because of clinical considerations, they were receiving a wide range of total calories (64-143 kcal/kg.d). The rate of exogenous glucose infusion (I) ranged between 3 and 10.6 mg/kg.min (16-59 µmole/kg.min) (Table 4).

Their plasma glucose concentration ranged between 4 and 7 mmoles/L. Total Ra of glucose quantified by $[^{13}C_6]$glucose tracer dilution was 6 - 16 mg/kg.min (33-83 µmole/kg.min) and the endogenous rate of glucose production (Re) was 0 - 7 mg/kg.min.

In the infants receiving glucose alone, the contribution of gluconeogenesis via pyruvate ranged between 6% and 60% of endogenous glucose production. The highest contribution (53% and 60%) was observed in the two infants (#4 and #5) who were receiving glucose at 3 mg/kg.min (17 µmole/kg.min), a rate much lower than the endogenous glucose production rate seen in normal term infants.

There was no difference in glucose Ra, glucose Re and the contribution of gluconeogenesis in infants receiving glucose plus amino acids with or without intravenous lipids. There was no correlation ($r = 0.12$) between glucose Re and the contribution of gluconeogenesis.

Table 4 Gluconeogenesis in the preterm babies

	Birth weight	Gest. Age	Glucose			Gluconeogenesis	
			Ra	I	Re		
	Grams	Weeks	(µmoles/kg.min)			% of Ra	% of Re
Glucose							
1	960	26.0	82.29	43.06	39.23	7.1	14.9
2	820	26.0	88.35	41.72	46.62	3.1	5.9
3	1770	30.4	46.32	32.33	13.99	5.0	16.7
4	1545	32.00	48.79	17.22	31.57	34.1	52.8
5	1335	32.70	60.09	16.50	43.59	43.4	59.8
Gluc/aa							
6	1470	29.6	41.39	29.83	11.56	6.4	22.8
7	1350	31.1	47.31	29.44	17.86	12.5	33.1
8	1315	28.0	56.39	58.89	0.00	3.2	
9	886	28.0	42.65	33.17	9.50	3.6	16.1
Gluc/aa/lipids							
10	1265	30.0	56.39	37.72	18.67	6.3	19.0
11	1275	28.4	48.04	29.33	18.71	9.6	24.6
12	1810	30.4	33.53	18.11	15.41	14.3	31.1
13	725	28.1	62.50	40.67	21.83	14.7	42.1

Ra: Rate of appearance of glucose measured by $[^{13}C_6]$glucose tracer dilution.
I: Rate of infusion of glucose.
Re: Endogenous rate of appearance of glucose

Glucose and Lactate Kinetics

The plasma glucose and lactate levels were unchanged throughout the study period and no significant difference was evident between normal, SGA or IDDM infants (Table 5).

The plasma C-peptide levels were slightly higher (p = n.s.) in the infants of diabetic mothers. The rate of appearance of glucose was also similar in the three groups. The measured rates of glucose Ra using [6,6-$^2H_2$]glucose were slightly lower than those estimated using [$^{13}C_6$]glucose tracer. The reason for this difference remains undetermined (17).
The estimated rate of plasma lactate turnover was 38 µmole/kg.min in normal babies. It was slightly lower in the small for gestational age babies and higher in the IDDM infants (p = 0.07 by one-way analysis of variance).

Table 5 Glucose and lactate kinetics in newborn infants

	Glucose mmoles/L	Lactate mmoles/L	C-Peptide ng/ml	Glucose Ra µmoles/kg.min	Lactate-Ra µmoles/kg.min	Glucose from lactate %
Normals (n=10)	2.96 ± 0.51	1.83 ± 0.29	0.87 ± 0.29	20.66 ± 3.28	38.3 ± 11.90	17.9 ± 5.2
SGA (n=4)	3.46 ± 0.43	2.22 ± 0.36	0.64 ± 0.58	17.71 ± 3.34	32.5 ± 5.74	18.0 ± 9.4
IDDM (n=6)	3.42 ± 1.12	2.14 ± 0.43	1.15 ± 0.37	25.74 ± 8.92	48.6 ± 10.40	18.4 ± 4.1

mean ± SD
Lactate Ra: rate of appearance of lactate measured by [$^{13}C_3$]lactate tracer dilution.
Glucose Ra: rate of appearance of glucose quantified by [6,6-$^2H_2$]glucose tracer dilution.

The fractional contribution of gluconeogenesis estimated from the incorporation of lactate carbon into glucose was also similar in the three groups and accounted for 18% of glucose Ra. This (uncorrected) estimation does not take into consideration the loss of tracer carbon via exchange in the tricarboxylic acid cycle intermediates. There was no significant correlation (r = 0.29; p = 0.23) between glucose Ra and lactate Ra, nor between glucose Ra and the fractional contribution of lactate.

DISCUSSION

In the present study, we have documented for the first time that healthy full term neonates establish gluconeogenesis from lactate 4-6 hours after birth, and that gluconeogenesis from pyruvate contributes ~30% of total glucose release at approximately five hours after the last feed. In preterm infants, even in the presence of exogenous glucose and other nutrient administration, gluconeogenesis is a significant contributor to the total glucose turnover. These data are important for the nutritional management of sick newborns, and contribute to our understanding of extrauterine adaptation of the human neonate.

Although gluconeogenesis has been estimated indirectly in the human newborn by quantifying the glucose carbon recycling (8,18) or in the small for gestational age infants by the estimation of incorporation of alanine C into glucose (10), no data exist documenting the contribution of gluconeogenesis or the appearance of gluconeogenesis in the full term healthy newborn infant. In the present study, using deuterium labeling of body water method, we have documented that in the healthy newborn between 35 and 68 hours after birth and 5 hours after the last feed, GNG contributed ~30% to the total glucose Ra. These estimates are of a similar magnitude to those seen in normal healthy adults after an overnight fast (13,37). The deuterium incorporation in the hydrogens of C-6 of glucose quantifies the contribution of pyruvate to glucose and does not include the contribution of glycerol (27,4). In addition, because of a lack of complete equilibrium between deuterium in body water and methyl hydrogens (C-3) of pyruvate, the measurement of ^{2}H enrichment on C-6 of glucose results in underestimation (by ~10%) of the contribution of gluconeogenesis via pyruvate to total glucose Ra. The incorporation of ^{2}H on C-5 of glucose, although more precise for the estimation of total gluconeogenesis, is difficult to use in neonates because of the very large sample size requirements (4,3).

These measurements of GNG via pyruvate are similar to the estimates of glucose carbon recycling made by us in similar neonates using [$^{13}C_6$]glucose (8). In that study, the estimated glucose Ra from tracer dilution in eleven healthy full term infants after an 8-9 hour fast was 28 μmole/kg.min, and glucose C recycling amounted to 35% or 10.4 μmole/kg.min. Since glucose carbon recycling involves return of lactate and pyruvate from the periphery back to the liver and their incorporation into glucose, estimation of glucose C recycling gives an estimation of gluconeogenesis via pyruvate. If the contribution of glycerol, ~4.5 μmole/kg.min (31,35), is also included, then the total gluconeogenesis in a healthy newborn will amount to ~14 μmole/kg.min, or ~46% of total glucose Ra, of 30 μmole/kg.min. This is significant and implies an important role of gluconeogenesis in glucose homeostasis even after a brief fast. The quantitative contribution of various precursors to gluconeogenesis via pyruvate remains undetermined.

The data in preterm babies, although variable, are also significant in that it underscores the quantitative large contribution of gluconeogenesis to glucose production, even when endogenous rate of glucose production was low, e.g. infants infused with glucose plus amino acids with or without lipids. It is of interest that in these babies also, GNG via pyruvate contributed 20-40% to endogenous production of glucose. In babies who were receiving glucose alone parenterally the contribution of GNG was variable. It was highest (~50%) in the two infants (#4 and #5) who were receiving intravenous glucose at the lowest rate. These measurements are of similar magnitude to those reported by Sunehag *et al* and Keshen *et al* in low birth weight babies using different isotopic tracer methods (36,25).

These data suggest that gluconeogenesis should be considered as one of the several substrate cycles like triglyceride fatty acid cycle and protein turnover which are active at all times, and which can be rapidly accelerated at the time of acute demand.

Only one other study has quantified lactate turnover in the human newborn. Cowett and Wolfe (7), using [$^{13}C_3$]lactate tracer dilution technique, reported the rate of lactate turnover to be 77.2 ± 13.0 μmole/kg.min in full term infants between 9 and 42 hours after birth, and 100 ± 19.2 μmole/kg.min in preterm infants. Their estimates are much higher than those reported in the present study. The differences may be related to age of the infants, intercurrent illness or the relationship to the last feed. While the majority of the infants in the present study were investigated during the first few hours after birth (8/10 normal and 4/6 IDDM), and were not receiving any intravenous fluids or antibiotics, such was not the case in the study of Cowett and Wolfe. Our data on lactate turnover are similar to those reported in infants and children aged one to 25 months, which range between 25 and 44 μmole/kg.min (2), and much higher than those reported in normal and diabetic adult subjects using similar tracer methods (9). The calculated contribution of lactate to glucose in our study without accounting for loss of tracer in the tricarboxylic acid cycle was ~18%. Correction for the loss of tracer (correction factor ~1.5) (14,24) would yield lactate's contribution to be ~27% - similar to that observed using [$^2H_2$]O method.

Of interest, the rate of lactate turnover (Ra) was almost two-fold the rate of glucose turnover. This is in contrast to the data in adults, where lactate turnover has been reported to be much lower than the rate of glucose turnover (in lactate equivalents) (9,24,14,5,6,34,26,28). This would suggest either a rapid rate of equilibrium of the tracer between various compartments of lactate (plus pyruvate plus alanine) in babies, or an influx of carbon into the lactate pool from non-glucose sources, e.g. amino acids. Such an hypothesis remains to be examined. The high rate of lactate turnover is also significant since lactate has been suggested to be an important metabolic fuel for the brain in the newborn period (30). Finally, the lack of any significant difference between normal, small for gestational age infants and the infants of diabetic mothers was not surprising. It essentially reflects the clinical practice of rigorous intrapartum regulation of maternal metabolism. Furthermore since a large fraction of lactate C is derived from glucose, a difference in lactate kinetics will not be anticipated in the presence of similar rates of glucose turnover (41).

In summary, the present data show that gluconeogenesis from lactate is apparent soon after birth in the healthy newborn infant, and it contributes ~30% of the total glucose produced. Significant contribution of gluconeogenesis to glucose Ra could be observed in healthy babies within 5 hours of last feed and was also seen in preterm infants while receiving parenteral glucose and other nutrients.

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Chapter 4

Lipogenesis on the first day of life in very low birth weight infants

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ABSTRACT

Indirect calorimetry studies suggest that part of the glucose intake in preterm neonates is converted to lipids while at the same time lipids are oxidised. Lipogenesis in very preterm infants on the first day of life has not directly been measured. We infused ^{13}C glucose to measure lipogenesis from the ^{13}C incorporation in palmitate of plasma triglycerides and phospholipids. A second objective was to determine the influence of antenatal corticosteroids on lipogenesis.

Twenty-eight preterm infants were studied. Mean birth weight 1074 ± 383 gram, mean gestational age 28.4 ± 2.1 week.

Plasma levels of triglyceride and phospholipid palmitate were 7.27 ± 3.8 mg/100 ml and 25.7 ± 8.9 mg/100 ml and did not change with gestational age. The fractional synthetic rate (FSR) of plasma triglyceride palmitate was 16.6 ± 11.2 % per day, of phospholipid palmitate 9.4 ± 6.3 % per day. The FSR of palmitate in plasma phospholipids decreased with increasing gestational age ($p< 0.01$).

Prenatal glucocorticoids had no effect on the FSR of palmitate in plasma triglycerides and plasma phospholipids.

Conclusion: Lipogenesis is present in very low birth weight infants on day one of life. The FSR of plasma phospholipid palmitate decreases with increasing gestational age. Antenatal steroids has no effect on palmitate production.

INTRODUCTION

Glucose is the main source of energy before birth, together with lactate and aminoacids [1]. The transport of fatty acids across the placenta is limited [2]. At the same time the fetus is storing substantial amounts of fatty acids [3], originating predominantly from glucose via lipogenesis [4,5].

After birth lipids become an important source of energy, as fat supplies 50% of the energy in human milk. Therefore, after birth the role of glucose as an energy source reduces. Glucose oxidation studies on the first days of life [6,9] have shown that glucose oxidation is supplying only approximately 50% of the energy expenditure at this age. In preterm infants on day 1 of life, receiving a glucose intake of 6 $mg.kg^{-1}.min^{-1}$., approx. 4 mg was oxidised yielding approx. 20 $kcal.kg^{-1}.d^{-1}$ at an energy consumption of 45 $kcal.kg^{-1}.d^{-1}$. Indirect calorimetry indicated a higher glucose utilisation than glucose oxidation measured by stable isotopes. We hypothesised therefore that glucose was converted to lipids while at the same time lipid was

oxidised [6]. No studies so far have shown lipogenesis in very preterm infants on the first day of life. Developments in stable isotope methodology have made it feasible to measure low levels of ^{13}C enrichment in plasma triglycerides and phospholipids.
The aim of the study was to prove our hypothesis that preterm infants show lipogenesis while receiving a glucose infusion only on the first day of life. Therefore, we infused ^{13}C glucose and measured the ^{13}C incorporation in palmitate of plasma triglycerides and phospholipids in preterm infants on day 1 of life. A second objective was to determine the influence of antenatal corticosteroids on lipogenesis.

METHODS

Patients: Twenty-eight preterm infants (19 male, 9 female) were studied between December 1994 and March 1997. All infants required mechanical ventilation and were admitted to the neonatal intensive care unit of the Sophia Childrens Hospital Rotterdam immediately after birth. Inclusion criteria were: gestational age $\leq$ 32 weeks, and written parental informed consent. Exclusion criteria were: congenital infection, maternal diabetes and chromosomal abnormality. All infants suffered from respiratory distress syndrome and were treated with surfactant. Data on surfactant metabolism in these patients was reported earlier [10]. Every infant was receiving ampicillin and tobramicin, as is standard practice in our unit in ventilated premature infants, pending the results of their initial blood cultures. Intravenous glucose was provided as soon as possible after birth, at a rate sufficient to keep the infants glucose levels at or above 2.6 mmol/l.

Lipogenesis: Patients received a constant intravenous infusion of [U-^{13}C]glucose (Campro Scientific, Veenendaal, The Netherlands) for 24 hour at 0.17 mg/kg/min. The start of the study (5.3 ± 0.5 hours after birth) was defined by the start of the infusion of the labelled glucose. The labelled glucose was infused by a high precision pump (M22; Harvard Apparatus Co. Inc., South Natick, MA). Before and during the glucose infusion, 1.0 ml arterial blood was drawn every 6 hours for isotope enrichment determination of plasma glucose. Blood was collected in lithium-heparin containing vacutainers and directly centrifuged to separate plasma and cells. The plasma was stored at –70° C until further processing. Plasma was delipidated with chloroform and methanol. The water fraction was passed over a column with anion-exchange resin (AG-1X8 [acetate], 100-200 mesh; BioRad Laboratories, Richmond, CA) and cation-exchange resin (AG-50W X8 [hydrogen], 200-400 mesh; BioRad). The eluate containing the glucose was evaporated to dryness at 80° C and derivatized to an aldonitril pentacetate derivative [11].

Plasma lipids were extracted according to Folch et al [12] after the addition of the following internal standards: trinonanoin and triheptadecanoin for triacylglycerols, L-α-phosphatidylcholine-dinonanoyl and L-α-phosphatidylcholine-heptadecanoyl for the phospholipids. Lipid classes were separated by thin-layer chromatography (TLC) developed with heptane:diisopropylether:acetic acid (60:40:3 by volume) and visualised with 1,2-dichlorofluorescein.

Derivatization was performed using 3 molair dry HCl in methanol. The separation and identification of fatty acid methylesters was performed by capillary gas chromatography (Hewlett Packard model 5890II, Amstelveen, The Netherlands) equipped with a fused-silica column (Omegawax 250, 30mx0.25mm internal diameter, 0.25μm film thickness, Supelco, Zwijndrecht, The Netherlands), a flame-ionisation detector (280°C), and a splitless injector. Fatty acids were identified by comparing retention times with known standards (Nu Chek Prep, Elysian, MN).

The ^{13}C-enrichment of plasma glucose and of palmitate in plasma triglycerides and phospholipids was measured by gas chromatography-combustion interface-isotope ratio mass spectrometry (GC-CI-IRMS) (VG Isotech, Midlewich, Cheshire, UK) as previously described [13]. The enrichment was expressed in atom percent excess (APE), which represents the increase in the percentage of carbon-13 atoms in total carbon dioxide from the combusted compounds above natural baseline enrichment (before infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms added during derivatization.

Data are presented as mean ± standard deviation unless otherwise stated.

Fractional synthesis rate (FSR) of palmitate in triglyceride and phospholipid is expressed as the percentage of the plasma palmitate in plasma triglyceride and phospholipid synthesised from glucose per day. It is calculated by dividing the slope of the linear increase of palmitate enrichment by the steady state enrichment value of plasma glucose [14].

RESULTS

Twenty eight infants were studied. Mean birth weight was 1074 ± 383 gram, mean gestational age 28.4 ± 2.1 week (Table 1).

Table 1: Patient characteristics

	All patients	No antenatal steroid	1 dose of steroids	2 doses of steroids
Number of patients	28	11	5	12
Birth weight (g)	1075 (383)	1172 (462)	1161 (535)	949 (179)
Gestational age (wk)	28.4 (2.1)	28.9 (2.3)	28.4 (3.0)	28.0 (1.4)
Ventilatory index	162 (118)	216 (151)	170 (54)	104 (71)
Plasma triglyceride C16 (mg/100 ml)	7.7 (3.5)	6.7 (2.3)	4.22 (3.7)	9.37 (4.7)
Plasma phospholipid C16 (mg/100 ml)	25.7 (8.9)	21.5 (8.3) *	26.8 (5.1)	32.28 (7.9) *
FSR triglyceride	16.6 (11.2)	16.4 (9.6)	17.5 (17.3)	16.6 (11.4)
FSR phospholipid	9.4 (6.30)	7.6 (4.8)	11.5 (9.3)	10.5 (6.7)

Ventilatory Index = Mean Airway Pressure • ventilatory frequency • FiO_2
* = p 0.02 (no steroids versus 2 doses steroids)
FSR Triglyceride = the fractional synthesis rate of palmitate in plasma triglyceride (calculated for 24 patients)
FSR phospholipid = the fractional synthesis rate of palmitate in plasma phospholipids (calculated for 25 patients)

Eleven mothers received no antenatal steroids, 5 received one dose of betamethasone within 24 hours before delivery and 12 mothers completed the antenatal steroid treatment with 2 doses of betamethasone (between 24 hours and 7 days before delivery). The average glucose intake of the infants was 5.24 (± 0.7) mg/kg/min at 6 and 12 hours after start of study and 5.30 (± 0.8) mg/kg/min at 18 and 24 hours after start of study.

The plasma triglyceride and phospholipid palmitate concentrations were 7.7 ± 3.5 mg/100 ml for triglycerides and 25.7 ± 8.9 mg/100 ml for phospholipids and were not related to gestational ages.

The ^{13}C-enrichment of plasma glucose was at steady state in all patients over the period 6 to 24 hours. Mean enrichment values were 2.48 ± 0.43 Mol Percent Excess (MPE), 2.58 ± 0.65 MPE, 2.55 ± 0.59 MPE and 2.48 ± 0.57 MPE at 6, 12, 18 and 24 hours respectively.

^{13}C-enrichment in plasma palmitate of triglycerides and phospholipids increased during the study (Fig.1) as a result of the incorporation of glucose carbon into fatty acids.

In some patients reliable measurements could not be obtained at all timepoints. Therefore, the FSR of triglyceride palmitate was calculated in 24 patients. The FSR of triglyceride palmitate was 16.6 ± 11.2 % per day. The FSR of phospholipid palmitate could be measured in 25 patients and was 9.4 ± 6.3 % per day. The FSR of palmitate in plasma phospholipids decreased with increasing gestational age ($p < 0.01$). (Fig.2).

The plasma triglyceride palmitate concentration was not influenced by antenatal glucocorticoids, but the plasma phospholipid palmitate concentrations was significantly higher in the group with antenatal steroids (Table 1). However, antenatal glucocorticoids had no effect on the FSR of palmitate in plasma triglycerides and plasma phospholipids (Table 1).

DISCUSSION

We show in the study that very low birthweight infants are actively synthesising saturated fat during the first day of life while receiving a low energy intake. In previous studies on the glucose turnover [6-9] part of the disappearance of the glucose carbon was presumed to be explained by lipogenesis. This assumption is supported by the results of the present study.

The biosynthesis of fatty acids is a complex polymerisation with acetyl-CoA as the principal two carbon building block. Low chain fatty acids can either be synthesised from acetyl-CoA itself or by elongation of medium chain fatty acids. Introducing a labelled acetyl-CoA makes it possible to measure lipogenesis.

For several reasons we were not able to calculate total body lipogenesis. First, we could not measure the enrichment of the direct precursor for lipogenesis acetyl-CoA. We used plasma glucose enrichment as the precursor enrichment to calculate fractional synthetic rates of palmitate. Recently, 2 techniques have been used to overcome the problem of the measurement of the enrichment of the direct precursor: in the mass isotopomer distribution analysis (MIDA) the precursor enrichment (acetyl-CoA) is calculated from the labelling pattern of the end product (the fatty acid) [17]. Another method is to use the incorporation of $^{2}H_2O$ into fatty acids [18]. For MIDA studies, significantly higher precursor enrichments than obtained in the current study is required. Secondly, we measured palmitate enrichment in plasma lipids. It is assumed that most of these plasma lipids originate from the liver. The liver releases de novo synthesised lipids mainly in very low density lipoproteins (VLDL). To measure specifically VLDL palmitate would however have required larger amounts of blood samples taken, which is unacceptable in these tiny premature infants.

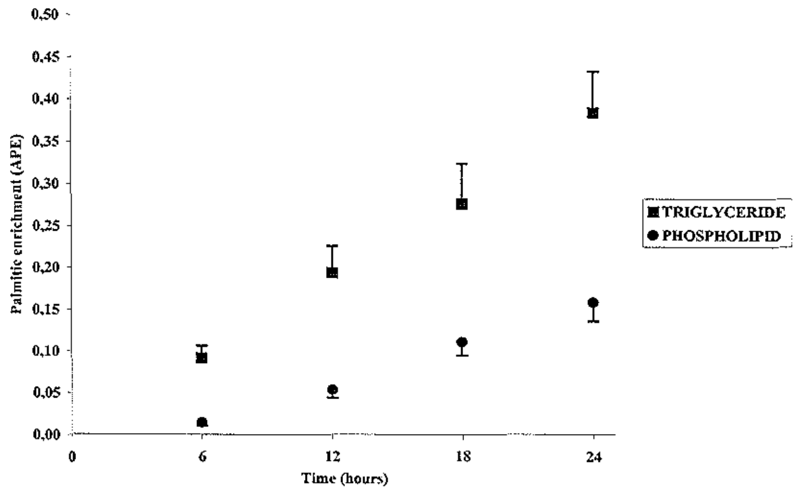

Figure 1
After infusion of [U-^{13}C]glucose (Campro Scientific, Veenendaal, The Netherlands) for 24 hour at 0.17 mg/kg/min , ^{13}C enrichment in plasma palmitate is measured at 6, 12, 18 and 24 hours study-time. Enrichment is expressed as atom percent excess. Values are given as average ± SEM.

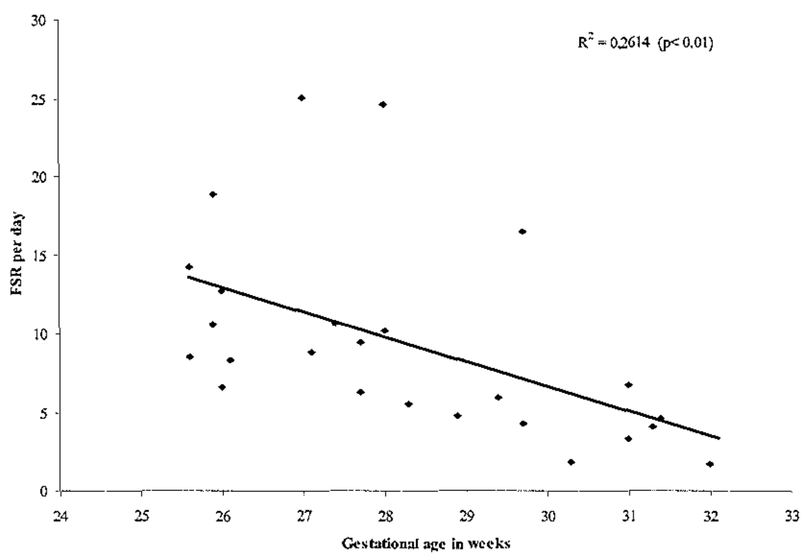

Figure 2
The fractional synthetic rate of plasma phospholipid palmitate is calculated by dividing the slope of the linear increase of palmitate enrichment by the steady state enrichment value of plasma glucose. The FSR is expressed as the percentage of the plasma palmitate in plasma triglyceride and phospholipid synthesised from glucose per day. The FSR of plasma phospholipid palmitate decreases significantly with increasing gestational age ($p< 0.01$).

Thirdly, lipogenesis by fat tissue [19-21] will lead to direct accumulation of fat in the adipose tissue, thereby not increasing plasma palmitate enrichment. The FSR times plasma pool of palmitate in triglycerides or phospholipids will include only a fraction of total palmitate production. Lipid synthesis in pools like brain and adipose tissue are not taken into account in our measurements. Obviously, it is unethical to obtain fat biopsies from these VLBW infants to measure enrichment of fatty acids in adipose tissue.

According to Hellerstein lipogenesis in humans is a rudimentary metabolic process, possibly only of importance in the foetus. From body composition data [22], we know that between 28 and 32 weeks of gestation, 10-12 percent of weight gain consists of lipid, i.e. 1.5 gram per day. Also, central nervous system myelination is one of the major maturing processes during the last trimester of pregnancy, needing large amounts of fatty acids. The mother transfers fatty acids to the fetus, using red blood cell fatty acids and special transport mechanisms in the placenta [2,23]. How much of the daily lipid gain of the fetus is derived from lipid transfer from the mother, and how much is synthesised by the fetus is unknown.

In this study we found that the FSR of palmitate in plasma phospholipids decreased with increasing gestational age. This is not according to our hypothesis, since the foetus is accumulating lipid in the last trimester and central nervous system myelination is especially important. The decreasing FSR with gestational age cannot be explained by a lower plasma pool as phospholipid and triglyceride concentrations did not change with gestational age. In the rat brain the activity of fatty acid synthetase is high during fetal life and decreases slowly after birth, indicating the importance of lipogenesis for fetal brain development [20]. In the rat liver, the enzymatic activity of fatty acid synthetase develops in a similar way up to 3 weeks of age. During weaning however, there is a dramatic increase in the activity of fatty acid synthetase in the liver suggesting that during a low fat intake lipogenesis is present. This is further supported by experiments where the activity of fatty acid synthetase increases during fasting [3,20].

We found no effect of antenatal steroids on the plasma phospholipid palmitate concentration and FSR of phospholipid palmitate. This is in contrast to the finding in a study on surfactant phosphatidylcholine palmitate (PCP) production after corticosteroids [10] where the FSR of PCP increased with 40% per dose of steroid administered. This would suggest that the stimulating effect of corticosteroids on lipogenesis in type-2 pneumocytes is specific for the lung. Several in vitro studies indeed show a stimulating effect of corticosteroids on lipogenetic enzymes in the fetal lung [24]. This is in contrast to findings in fetal and adult rat liver where corticosteroids do not show any consistent effect on lipogenetic enzyme activities or lipogenesis measured using radioactive precursors [19-21,25]. This lack of effect is further supported by adrenalectomy experiments in newborn rats where no effect on liver fatty acid synthetase activity was found [20]. However, in adipose tissue of adult rats corticosteroids led to a marked reduction of the activities of fatty acid synthetase and acetyl-CoA carboxylase. These effects on adipose tissue may be region specific as rat experiments show a different effect of glucocorticoids on lipogenetic pathways in different regions of adipose tissue [19]. Thus, there seems to be an organ specific effect of glucocorticoids on lipogenesis and our human data are concordant with previous rat experiments: glucocorticoids stimulate lipogenesis in the lung but not in the liver.

Plasma phospholipid palmitate concentrations were increased after antenatal glucocorticoids, while plasma triglyceride palmitate concentrations remained constant. As there was no effect of glucocorticoids on palmitic synthesis an increased concentration of phospholipids is likely due to a decreased catabolism. Indeed, glucocorticoids are well known to inhibit the activity of phospholipase A2, an enzyme that degrades phospholipids [26,27].

Conclusion: Lipogenesis on day 1 was measured for the first time in very low birth weight infants while on a low caloric glucose intake. The synthesis of palmitate, reflected by the palmitate enrichment in plasma phospholipids, decreased with gestational age. No effect of antenatal corticosteroids on the FSR of palmitic acid in plasma was observed.

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Chapter 5

Effects of antenatal steroids on leucine turnover in preterm infants, on the first day of life.

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ABSTRACT

Objective. To determine whether antenatal steroids influence leucine turnover and have an effect on splanchnic uptake in preterm infants on day 1 of life.

Methods. Infants with a birth weight of less than 1750g were studied. Within a few hours after birth a primed constant infusion of [1-^{13}C]-leucine was started and continued for 5 hours via the nasogastric tube (i.g.), whereas 5,5,5 D3-leucine was infused i.v. (both tracers priming dose 2 $mg.kg^{-1}$, continuous infusion 2 $mg.kg^{-1}.h^{-1}$). Leucine turnover is calculated using the reciprocal pool method.

Results. Mean birth weight (1271 ± 246 g) and gestational age (30 ± 2.4) weeks did not differ between steroid groups. The plasma KICA enrichment (mol percent excess) from the i.v. tracer was not different between infants who received no antenatal steroids (8.58 ± 1.64), one dose (7.78 ± 0.77) and two or more doses (7.58 ± 1.27). The plasma KICA enrichment from the i.g. tracer were different between the three groups, 7.62 ± 2.35 for 0 doses, 6.0 ± 0.8 for 1 dose and 5.51 ± 1.56 for two or more doses of antenatal steroids; ANOVA, p=0.015. Plasma KICA enrichment from the i.v. tracer was significantly higher than from the i.g. tracer ($p < 0.0001$) in infants who received two or more doses of antenatal steroids. There was no difference in plasma KICA enrichment between the two tracers in infants that had not received antenatal steroids.

Conclusion. Antenatal steroid administration to the fetus shows no effect on total body leucineturnover, however, is associated with an increase in splanchnic leucine uptake, indicating a maturing effect on splanchnic tissue.

INTRODUCTION

Antenatal corticosteroid therapy for fetal maturation reduces mortality, respiratory distress syndrome, and intraventricular haemorrhage in preterm infants. These benefits extend to a broad range of gestational ages (24 to 34 weeks) [1-4]. In animal studies, fetal growth retardation [5] and irreversible damage to neurons [6] has been demonstrated while in humans a reduction in cerebral hemorrage is observed [7-9] . One study described an increased protein breakdown after antenatal corticosteroids[10].

From postnatal administered steroids it is known that protein breakdown increases and nitrogen balance becomes negative [11-13]. The impact of antenatal glucocorticoids on protein metabolism in specific tissues like the gastro intestinal tract has not been established in neonates.

In several studies, a reduction in the incidence of necrotising enterocolitis was observed after antenatal steroids [14]. This is possibly due to decreased intestinal permeability [14]. In rats glucocorticoids influence growth and maturation of fetal gut [15]. Recently a model using stable isotopes was described that measures the uptake of leucine administered to the splanchnic tissues making use of the combined administration of an intravenous and intra gastric tracer [16-18]. A higher uptake of leucine by splanchnic tissue might indicate improved maturation.

The aim of this study was to evaluate the effect of antenatal steroids on total body leucine turnover. Secondly we investigated the effect of antenatal steroids on leucine uptake by the splanchnic tissues as a measure of splanchnic tissue maturation using this double label method.

METHODS

Infants admitted to the neonatal intensive care unit of the Sophia Childrens Hospital Rotterdam with a birth weight < 1750g were included and studied within a few hours after birth. Exclusion criteria were infants of diabetic mothers, major congenital anomalies or proven sepsis. All infants received intravenous glucose. Treatment with antenatal betamethasone was the decision of the attending obstetrician, and not influenced by this study protocol. Informed consent was obtained from at least one of the parents prior to the start of the study.

Experimental Protocol

The stable isotope study was started within a few hours after birth. Baseline blood samples were collected for measurement of natural isotopic "background". All infants had a nasogastric tube for nursing reasons, which was also used for administration of the intragastric tracer. Subjects received primed continuous infusions of [5,5,5-$^{2}H_{3}$]leucine intravenously (i.v.) and [1-^{13}C]leucine intragastrically (i.g.) (both isotopes 99% enriched, Isotec Inc., Miamisburg, Ohio, USA.) These are equivalent tracers of leucine [10-12]. At the start of the tracer infusion, priming doses (2 $mg.kg^{-1}$) of both leucine tracers were given by their respective routes. The leucine tracers were then infused at a constant rate of 2 $mg.kg^{-1}.h^{-1}$ by their respective routes with a double-syringe, screw-driven pump (M22, Harvard Apparatus Co Inc. S. Natick, MA, USA). At 270, 285 and 300 minutes, blood samples were collected for leucine tracer enrichment determination. Blood was spun in a refrigerated centrifuge, and the plasma supernatant was withdrawn.

Plasma Analysis

One hundred μL of plasma was deproteinized by adding 250 μl sulfosalicylic acid (6% (w/v)), after the addition of the internal standards ketocaproic acid and ketovaleric acid. After keeping the samples for 5 min. at 0°C, the samples were centrifuged and 150 μL 1,2-phenylenediamine (2 g%) dissolved in 6 M HCl was added to the supernatant. The tubes were heated at 100°C for 60 minutes and afterwards allowed to cool. After addition of 3 ml dichloromethane-hexane mixture (1:1, v/v) and mixing, the supernatant was evaporated to dryness under nitrogen at 50°C. Derivatization was performed by adding 50 μl N,O-*bis*(trimethylsilyl)trifluoroacetamide after which the vials were immediately sealed under nitrogen and left at room temperature for 30 minutes.

Gas chromatography/mass spectrometry was carried out on a Hewlett Packard HP 5890 (Palo Alto, CA, USA) gas chromatograph coupled to a JEOL JMS-DX303 (Tokyo, Japan) mass spectrometer operating in the electron impact mode. Chromatography was carried out on a 25 m * 0,32 mm capillary column (CP-Sil5-CB, Chrompack BV, Middelburg, The Netherlands). Selective ion monitoring was carried out at m/z 232/233/235 for (m), (m+1) and (m+3) respectively. The plasma α-KICA enrichments were determined using a calibration graph constructed from standard mixtures of known molar ratios of both [$^2H_3$]- and [^{13}C]KICA vs. unlabeled KICA. The coefficient of variation for plasma α-KICA measurements was 2% (n=5). α-KICA enrichments were calculated as mol percent excess above baseline enrichment.

Calculations

Leucine flux was calculated using the reciprocal pool model. Briefly, intracellular leucine is rapidly and reversibly transaminated to its keto analogue; α-ketoisocaproate (α-KICA). The measurement of enrichment of α-KICA following leucine infusion will therefore reflect the intracellular enrichment of leucine [19,20].

Previous studies from this and many others laboratories have used a steady-state, whole body model of amino acid metabolism that developed in part from work by Waterlow and co-workers. This model assumes a common "metabolic" pool, the movement through the metabolic pool is flux. This flux (Q_L) can be calculated according to the following equation:

$Q_L = [(IE_{infusate} / IE_{plasma}) - 1] \times I_L$

where $IE_{infusate}$ is the isotopic enrichment of the infusate, IE_{plasma} is the isotopic enrichment of alpha labelled KICA in plasma and I_L the infusion rate of labelled leucine in micromol. $Kg^{-1}.h^{-1}$.

Because the rate of amino acid intake in this study is zero, flux equals breakdown in steady state.

Statistical Evaluation

Data were analysed with Graph Prism Software (GraphPad Software Inc, San Diego, CA, USA). Data for each isotopic variant were first analysed with linear regression to test for the presence of a significant slope. After determining that isotopic steady state (i.e., absence of slope) existed for the last 30 min of the infusion, mean plateau values were used to calculate substrate flux. Data are presented as mean ± SD. The three groups were compared with the use of one-way ANOVA, with Bonferroni test for pairwise comparison. Differences, within each group, between i.v. and i.g. enrichments were tested by paired t-test. Significance level was set at a p-value <0,05.

The study was approved by the local medical ethical committee.

RESULTS

Thirty-eight infants were studied. Mean birth weight (BW) was 1271 ± 246 g and mean gestational age (GA) 30 ± 2.4 weeks. Twenty-six infants received two or more doses of antenatal corticosteroids before delivery, whereas 8 infants did not receive antenatal corticosteroids. Four patients received only one dose of antenatal steroids. There were no significant differences in BW and GA between groups (Table 1).

A steady state of isotopic enrichment was achieved in the sampled pool by 270 min, as assessed by the lack of a significant slope in all pools by linear regression analysis. The variability was greater for the plasma enrichments of the intragastrically infused tracer, but these enrichments did not have a significant slope.

The plasma KICA enrichments from the i.v. tracer were not different between the infants who received no antenatal steroids (8.58 ± 1.64), one dose (7.78 ± 0.77) and two or more doses (7.58 ± 1.27). The plasma KICA enrichment from the i.g. tracer were different between the three groups, 7.62 ± 2.35 for 0 doses, 6.0 ± 0.8 for 1 dose and 5.51 ± 1.56 for two or more doses of antenatal steroids; ANOVA, p=0.015. Pairwise comparison with Bonferroni correction showed lower plasma KICA enrichment from the i.g. tracer in those infants who received two or more doses of antenatal steroids compared with those who received none (Table 1).

Table 1 Characteristics of Study Infants and Plasma KICA isotopic enrichments

	BW	GA	MPE iv tracer	MPE ig tracer
No steroids (n=8)	1188 ± 313	30 ± 1.9	8.58 ± 1.64	7.62 ± 2.35†
1 dose steroid (n=4)	1259 ± 267	29 ± 2.4	7.78 ± 0.77	6.0 ± 0.8
2 or more doses (n=26)	1299 ± 224	30.6 ± 2.4	7.58 ± 1.27**	5.51 ± 1.56† **

†= 2 doses versus no steroids: $p < 0.05$
** = intravenous versus enteral tracer enrichment : $p < 0.0001$

Plasma KICA enrichment (mol percent excess, MPE) from the i.v. tracer (7.58 ± 1.27 MPE), was significantly higher than from the i.g. tracer (5.51 ± 1.56 MPE) ($p < 0.0001$) in infants who received two or more doses of antenatal steroids. There was no difference in plasma KICA enrichment between the two tracers in infants that had not received antenatal steroids ($p=0.1815$) (Table 1).

Leucine turnover for the intravenous tracer was 162 ± 31 $\mu mol.kg^{-1}.hr^{-1}$ for the no steroid group, 177 ± 19 $\mu mol.kg^{-1}.hr^{-1}$ and 186 ± 33 $\mu mol.kg^{-1}.hr^{-1}$ for the more doses steroid group (no significant differences). The leucine turnover calculated from the intragastrically administered leucine tracer was 195 ± 53 $\mu mol.kg^{-1}.hr^{-1}$, 237 ± 33 $\mu mol.kg^{-1}.hr^{-1}$ and 278 ± 107 $\mu mol.kg^{-1}.hr^{-1}$ for the 0, 1 and more doses steroid groups respectively (ANOVA, $p = 0.09$)

DISCUSSION

In this study, we observed no effect of antenatal steroids on leucine metabolism in preterm infants on day one of life. At the same time however, antenatal steroids seem to stimulate the maturation of the gastro intestinal tract as evidenced by a lower α-KICA concentration in plasma in infants receiving antenatal corticosteroids, compared to controls.

Postnatal steroid treatment is known to cause significant increases in protein breakdown [11-13]. The dose used in postnatal treatment is approximately 8-10 times the basal production. Antenatal steroid administration to the fetus however does not expose the infant to potentially harmful pharmacological levels of betamethasone but rather to levels reached in stress periods [21]. The lower steroid levels obtained after antenatal administration might therefore explain the absence of an effect on protein breakdown. Secondly, it is possible that in different body tissues, steroids express different effects. In rabbits, steroids have a different effect on adipocytes of different fat tissues [22]. No data exist as far as we know on effects of antenatal steroids on different body protein pools. We found a strong association between antenatal steroid treatment and plasma enrichment from intragastrically administered leucine tracer, while no effects on total body leucine turnover measured by the iv tracer are seen. The fact that enrichments for the n.g. tracer are lower indicates that leucine is taken up in the first pass. This might indicate that leucine is used within the splanchnic tissues, most likely for protein synthesis, and not released after deamination into the circulation. We assume that antenatal steroids improve maturation of splanchnic tissue as evidenced by the increase of leucine uptake. This finding can be related to the finding in randomised controlled trials on antenatal steroids where the incidence of necrotising enterocolitis is decreased after antenatal steroids [3,13]. Antenatal steroids appear to decrease gastrointestinal permeability [14]. An increased permeability is associated with mucosal damage and necrotising enterocolitis [23].

The results we reported here with respect to total body leucine turnover are in contrast to a previous study where in a smaller group of infants, an increased protein breakdown was observed after antenatal steroids [10]. The question is whether differences in study populations might explain the differnces, since in that study, 18 patients were studied with a mean gestational age of 29 weeks and a mean birthweight around 1400 gram, which is somewhat larger than in the present study. Another difference is the fact that some of the infants were fed with parenteral amino acid. Protein intake increases the protein turnover which could explain the increase in protein breakdown.

Since the NIH consensus on antenatal steroids [24] it is good practice to administer steroids to a mother who is likely to give birth prematurely. It is therefore not possible anymore to perform a study with randomisation for antenatal steroids versus control with regard to protein metabolism or gut function. Still it is interesting to know what the metabolic effect of antenatal steroids on total body leucine turnover and gut protein turnover is.

Conclusion: Antenatal steroid administration to the fetus shows no effect on total body leucine breakdown, However, it is associated with an increase in splanchnic leucine uptake, indicating a maturing effect on splanchnic tissue.

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Chapter 6

Effect of minimal enteral feeding on splanchnic uptake of leucine in preterm infants

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Submitted

ABSTRACT

We conducted a controlled randomised trial to study the effect of minimal enteral feeding on leucine uptake by splanchnic tissues, as an indicator of maturation of these tissues, in preterm infants in the first week of life.

Patients were randomised to receive solely parenteral nutrition (C), parenteral nutrition and 20 mL breast milk/kg/day (BM) or parenteral nutrition and 20 mL formula/kg/day (F). Within a few hours after birth a primed constant infusion of [1-^{13}C]-leucine was started and continued for 5 hours via the nasogastric (ng) tube, whereas 5,5,5 D3-leucine was infused i.v. (both tracers priming dose 2 mg.kg^{-1}, continuous infusion 2 mg.kg^{-1}.h^{-1}). On day 7 these measurements were repeated. 14 infants were included in group C, 12 in group BM and 12 in group F. There was no difference in energy intake or nitrogen balance at any day. On day 1 plasma enrichment for the ng tracer was lower than for the i.v. tracer for all three groups, for leucine and for α-keto-isocaproic acid (KIC). On day 7 the enrichment for leucine and KIC for the ng tracer was lower than for the i.v. tracer for the groups BM and F (3.65±1.20 ng *versus* 4.64±0.64 i.v. BM, 4.37±1.14 ng *versus* 5.21±0.9 i.v. F). In the control group there was no difference between both tracers. The lower enrichment for the ng tracer compared to the i.v. tracer indicates uptake of leucine by the splanchnic tissues.

We conclude that minimal enteral feeding - even in low volumes of 20 ml.kg^{-1}.day^{-1} -does increase the leucine uptake by the splanchnic tissue.

INTRODUCTION

Advances in perinatal care have lead to a significant increase in survival of preterm infants. There has been an increasing recognition that nutrition plays an important role in the survival and subsequent growth and development of very low birth weight (VLBW) infants. Parenteral feeding is used extensively in clinical care of preterm infants. The timing of initiation of enteral feeding in VLBW infants is still controversial. On one hand anxiety about necrotizing enterocolitis (NEC) has prompted neonatologists to delay enteral feeding in VLBW infants. On the other hand, animal studies have shown a negative effect of replacing enteral by total parenteral nutrition on gut morphology and function (1,2,3,4,5). Minimal enteral feeding has shown to improve gut maturation, as evidenced by improved motility (6,7). The effect of minimal enteral feeding on protein metabolism of splanchnic tissues, an indicator of anabolism of splanchnic tissues, is unknown. Recently a model, using stable isotopes, was described that measures the uptake of leucine administered to the gastrointestinal tract by splanchnic tissues (8,9). We conducted a controlled randomised trial to study the effect of minimal enteral feeding, either as breast feeding or formula, on the leucine uptake of splanchnic tissues in preterm infants in the first week of life. We hypothesized that minimal enteral feeding improves maturation of splanchnic tissue as evidenced by an increased leucine uptake.

METHODS

Patients.

Patients admitted to the neonatal intensive care unit with a birthweight of less than 1600 g were included. Gestational age was calculated from maternal history or, in case of uncertainty, estimated from the Ballard score (10). Patients were defined appropriate for gestational age if the weight was within 2 SD according to the chart of Usher and McLean (11). Exclusion criteria were infants of diabetic mothers, major congenital anomalies or proven sepsis. Infants were scored according to the CRIB scoring system (12).

The feeding policy, at the time of the study, was to delay enteral feeding after birth in infants admitted to the neonatal intensive care unit. Instead, on day 1 parenteral nutrition including aminoacids and lipids was started in infants with a birth weight below 1600 g. For this study infants were divided into three groups. One group receiving no enteral feeding, served as controls (group C). In two other groups minimal enteral feeding was started on day 1. It was aimed to start with 10 ml/kg/day on day 1, increasing to 20 ml/kg/day from day 2 till day 7. One group received formula feeding (Nenatal, Nutricia, Zoetermeer, The Netherlands) (group F), while the other group received milk from their own mother (group BM). Infants were, at random, assigned to minimal enteral nutrition or no enteral nutrition for the first week of life. Infants assigned to minimal enteral nutrition and whose mothers intended to give breast feeding were included in group BM. The study was approved by the local Medical Ethical Committee, written consent was obtained from at least one of the parents.

Experimental design.

Measurement of 24 h nitrogen balance was carried out by collection of urine in 3 hourly periods using adhesive bags on day 1 and 7.

For the isotope studies, on day 1 and day 7 a primed constant intravenous (i.v.) infusion of $NaH^{13}CO_3$ (priming dose 7 $\mu mol.kg^{-1}$, continuous infusion 3.5 $\mu mol.kg^{-1}.h^{-1}$, 98.7% enriched, Isotech, Miamisburg, Ohio, USA) was administered for 3 hours using a Harvard infusion pump (M22, Harvard Apparatus Co Inc S. Natick, MA, USA). After the infusion of ^{13}C labelled sodium bicarbonate a primed constant infusion of labelled leucine was started and continued for 5 hours both i.v. and via the nasogastric (ng) tube. 1-^{13}C leucine (99% enriched, Isotech, Miamisburg, Ohio, USA) was infused via the tube (priming dose 2 $mg.kg^{-1}$, continuous infusion 2 $mg.kg^{-1}.hr^{-1}$), whereas 5,5,5 D3-leucine (99% enriched, Isotech, Miamisburg, Ohio, USA) was infused i.v. (priming dose 2 $mg.kg^{-1}$, continuous infusion 2 $mg.kg^{-1}.h^{-1}$.). On day 7, minimal enteral feeding was stopped 5 hours before the start of the isotope infusion.

Expired air was collected before and during the isotope infusion by passing a sample of air leaving the ventilator through an all-glass spiral condenser, containing 10 ml of a fresh 1 M NaOH solution. After liberating CO_2 by adding phosphoric acid (85%) to the solution, $^{13}CO_2/^{12}CO_2$ isotope ratio was measured on an isotope ratio/mass spectrometer. If the infant did not have an endotracheal tube, expired CO_2 was collected by a mask placed over the mouth and nose.

Four blood samples were drawn, one prior to the start of the leucine infusion and three at the end of leucine infusion with a 10 minute interval. Within 15 minutes, plasma was stored at -70°C until further analysis

Measurement of isotope dilution in plasma.

In this study both leucine and alfa-keto-isocaproic acid (KIC) were measured. Leucine is reversibly transaminated to KIC. KIC is a reflection of intracellular leucine enrichment. One hundred µL of plasma were deproteinized by adding 250 µL sulfosalicilic acid (6%), after the addition of the internal standards ketocaproic acid, ketovaleric acid and norleucine. A rapid cation-exchange chromatographic procedure was used to isolate KIC and the aminoacids by passing the mixture through a small column (2 cm x 7 mm i.d.) of AG 50W-X8 resin, eluting KIC with HCl (0.01 N, 1 mL) and the aminoacids with distilled water (2 mL) followed by ammonia (4 M, 2 mL).

KIC was derivatized by adding freshly prepared o-phenyl-enediamine solution (2% in 6 M HCl, 0.5 mL) at 100°C for 1 h. The solution was extracted with methylene chloride:hexane (1:1, 1.8 mL) and dried under nitrogen at 45°C. The residue was dissolved in methylene chloride, dried under nitrogen and reacted with MTBSTFA (0.05 mL) at room temperature for 30 min.

Aminoacids were derivatized to their t-butyldimethylsilyl derivatives. The effluent was dried under nitrogen at 70°C. Methylene chloride (0.5 mL) was added to the residue. After evaporation of the solvent at room temperature under nitrogen, the residue was dissolved in pyridine (0.02ml) and reacted with MTBSFTA (0.02 mL) at 60°C for 1 h.
Gas chromatography/mass spectrometry was carried out on a Hewlett Packard HP 5890 (Palo Alto, Ca, USA) gas chromatograph coupled to a JEOL JMS-DX303 (Tokyo, Japan) mass spectrometer operating in the electron impact mode. Chromatography was carried out on a 25 m x 0.32 mm capillary column (CP-Sil5-CB, Chrompack BV, Middelburg, The Netherlands). Selective ion monitoring was carried out at m/z 259, 260, 262 for KIC, [^{13}C] KIC and [D_3] KIC respectively; and at m/z 302, 303, 305 for leucine, [^{13}C]-leucine and [D_3]-leucine respectively.

Calculations

Previous studies from this and many other laboratories have used a steady-state, whole body model of amino acid metabolism (13,14). This model assumes a common "metabolic" amino acid pool through which all amino acids move, either to enter from the diet or protein breakdown or to exit for protein synthesis or oxidative catabolism. Plasma leucine fluxes, Q_L, were calculated from plasma enrichment levels for both nasogastric and intravenous tracers, in equation:

$Q_L = I \times ((E_i/E_p)-1)\ \mu mol.kg^{-1}.h^{-1}$

Where I is the rate of leucine tracer infusion ($\mu mol.kg^{-1}.h^{-1}$) and E_i and E_p are isotopic enrichments of the tracer infused and of the tracer in plasma.

Calculations were also performed with the reciprocal pool model by substituting KIC to leucine enrichment.

Assuming a constant pool size, leucine leaving the pool (for protein synthesis (Non Oxidative Leucine Disposal, NOLD) and oxidation (O)) should equal leucine entering the pool (from dietary intake (I_{leu}) and from protein breakdown (Leucine Released from Proteins, LRP). In equation:

$Q_L = I_{leu} + LRP = O + NOLD$

Leucine oxidation was calculated by multiplying the recovery of the label in expiratory air with the flux of leucine, based on plasma [^{13}C] KIC. The recovery of the label in expiratory air was measured according to the following equation, assuming a constant rate of excretion of CO_2 during the study:

$Rec = (Ie_L \times I_B)/(IE_B \times I_L)$

Where Ie_L is the $^{13}CO_2$ enrichment at plateau during the [^{13}C]-leucine infusion, IE_B is the $^{13}CO_2$ enrichment at plateau during the $NaH^{13}CO_3$ infusion, I_L is the infusion rate of [^{13}C]-leucine in $\mu mol^{13}C.kg^{-1}.h^{-1}$ and I_B is the infusion rate of $NaH^{13}CO_3$ in $\mu mol^{13}C.kg^{-1}.h^{-1}$.

Statistical analysis

Mean plateau enrichment values were used to calculate substrate flux values as noted above. Data were analysed with Graph Prism Software (GraphPad Software Inc, San Diego, CA, USA). The three groups were compared with the use of one-way ANOVA, with Bonferroni test for pairwise comparison, and Chi-square test for analysis of contingency tables. Differences, within each group, from birth to day 7 and between i.v. and ng enrichments were tested by paired t-test.

RESULTS

38 Infants were included in the study, 14 in group C, 12 in group BM and 12 in group F. Clinical details of the patients are given in Table 1.

The gestational age of patients receiving breastmilk was lower compared to infants receiving formula, while more infants in the group receiving formula were small for gestational age. Less patients in the control group received antenatal corticosteroids compared to the other two groups. No other differences between groups were observed.

The enteral intake in the two groups with minimal enteral feeding is given in figure 1.

The aim of giving 20 $ml.kg^{-1}.day^{-1}$ was reached in the formula group on day 3 and only on day 4 in the group receiving breastmilk. No difference between intake in the enteral fed groups was observed on days 4, 5, 6 and 7. There was no difference in daily parenteral aminoacid nor energy intake between the groups on any day. Weight gain measured as weight above birthweight on postnatal day 21, and tolerance of enteral feedings, measured as the age at which infants tolerated 120 or 150 $ml.kg^{-1}.day^{-1}$ was not different between groups (Table 2).

Table 1: Clinical characteristics of the infants. Mean ± SD

Characteristics	Total Parenteral Nutrition Group (N=14)	Parenteral Nutrition + Breastmilk Group (N=12)	Parenteral Nutrition + Formula Group (N=12)
Birth weight (g)	1366 ± 240	1252 ± 235	1300 ± 231
Gestational age (wk)	31 ± 2	29 ± 1*	32 ± 2*
SGA (no./total no.)	1/14*	0/12*	6/12*
Male sex – no. (%)	9 (64)	5 (42)	7 (58)
CRIB score	4 ±3	4 ± 5	1 ± 1
On ventilation (no./total no.)	5/14	4/12	1/12
Antenatal steroids (no./total no.)	6/14*	10/12*	10/12*
Caloric intake day 1	24 ± 9	20 ± 9	27 ± 19
Caloric intake day 7	77 ± 15	76 ± 10	71 ± 11
Nitrogen intake day 7 ($mg.kg^{-1}.d^{-1}$)	386 ± 86	385 ± 9	367 ± 31

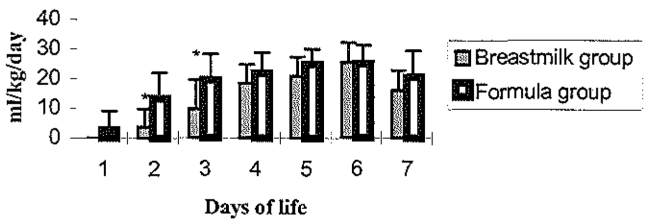
* Statistically different at $p<0.05$.

Table 2: Clinical outcomes.

Outcome	Total Parenteral NutritionGroup (N=14)	Parenteral Nutrition + Breastmilk Group (N=12)	Parenteral Nutrition + Formula Group (N=12)
Weight above birthweight at day 21 (g)	190 ± 132	155 ± 94	221 ± 162
Age infants tolerate 120 ml/kg/d enteral feeding (d)	16 ±4	14 ± 6	13 ± 2
Age infants tolerate 150 ml/kg/d enteral feeding (d)	17 ± 5	18 ± 8	16 ± 2

Mean ± SD

Figure 1. Enteral intake. Mean ± SD

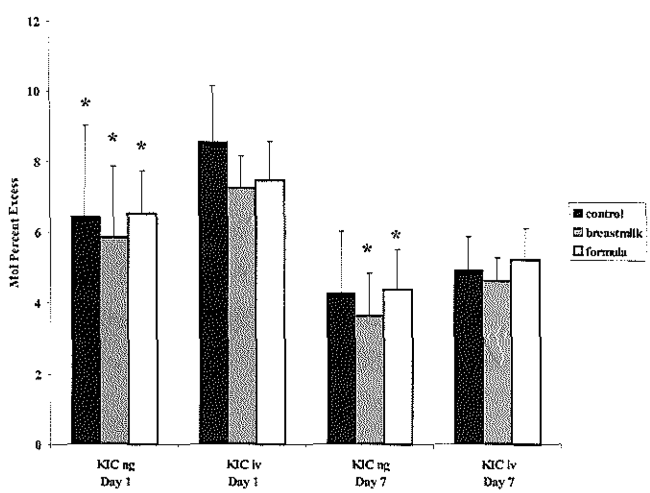


There was no difference in nitrogen intake, nitrogen excretion or nitrogen balance between groups at either day 1 or day 7. Nitrogen balance was negative in all groups at day 1, -70±21, -66±35 and -95±35 $mg.kg^{-1}.d^{-1}$ in groups C, BM and F respectively, to become positive on day 7, 229±73, 206±111 and 247±36 $mg.kg^{-1}.d^{-1}$ in the three groups, respectively.

In all studies a plateau in isotope enrichment was observed after the i.v. as well as the ng infusion. The variability in plasma enrichment was higher for the ng compared to the i.v. tracer. On day 1 no difference in leucine enrichment between ng and i.v. tracer was observed in any of the groups (6.36±2.1 ng *versus* 7.82±1.44 i.v. C, 6.42±2.16 ng *versus* 7.75±1.22 i.v. BM, 6.77±1.34 ng *versus* 7.82±1.63 i.v. F); the KIC enrichment was significantly higher for the i.v. tracer compared to the ng tracer in all three groups (6.45±2.57 ng *versus* 8.55±1.61 i.v. C, 5.87±1.99 ng *versus* 7.26±0.9 i.v. BM, 6.53±1.2 ng *versus* 7.47±1.11 i.v. F) (Fig 2 A and B).

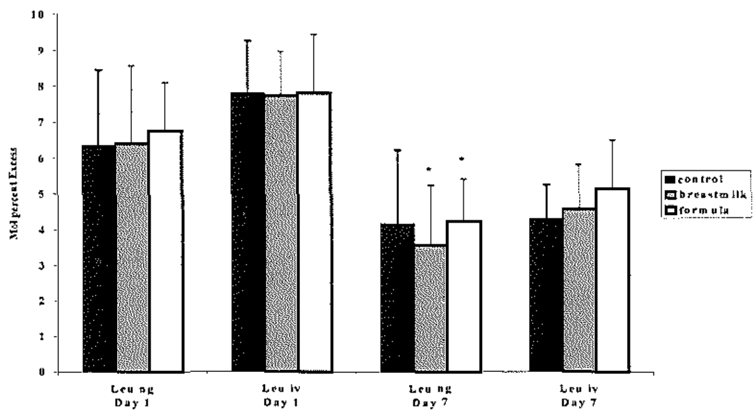
On day 7 leucine enrichment between ng or i.v. tracer was not different in the control group (4.18±2.05 ng *versus* 4.23±0.95 i.v.), while the enrichment was significantly lower for the ng tracer in groups BM and F (3.59±1.67 ng *versus* 4.60±1.24 i.v. BM, 4.25±1.19 ng *versus* 5.16±1.36 i.v. F); KIC enrichment gave the same results, no difference between i.v. and ng tracer in group C (4.29±1.73 ng *versus* 4.93±0.94 i.v.) and a significantly lower enrichment for the ng tracer in group BM and F (3.65±1.20 ng *versus* 4.64±0.64 i.v. BM, 4.37±1.14 ng *versus* 5.21±0.9 i.v. F). In all three groups the leucine enrichment as well as the KIC enrichment was lower for both tracers on day 7 compared to day 1 (Fig 2 A and B). The release of leucine from proteins (a measure for protein breakdown) based on plasma [^{13}C]KIC and [$^{2}H_{3}$]KIC enrichment was not different on day 1 between the 3 groups, either for the ng tracer 262±144, 268±105 and 215±46 μmol.kg^{-1}.h^{-1} for groups C, BM and F, respectively, or the i.v. tracer 158±27, 193±27 and 183±41 μmol .kg^{-1}.h^{-1} in the three groups. The release of leucine from proteins at day 7 was also not different between the three groups measured from the plasma enrichment of either the ng or i.v. tracer. Pooling the data of the three groups together we found a statistically significant higher leucine release from proteins on day 7 (206±40) vs. day 1 (178±35 μmol.kg^{-1}.h^{-1}) ($p=0.0025$). Reliable data on non oxidative leucine disposal, a reflection of the amount of leucine that is being used for protein synthesis, could be obtained in 7 infants in group C, and in 5 infants in each of the other two groups, BM and F. Protein synthesis increased in all groups from day 1 to day 7, 228±141 at birth vs. 357±234 μmol.kg^{-1}.h^{-1} in group C, 228±107 vs. 338 ±190 in group BM, and 183.5±57 vs. 292±121 μmol.kg^{-1}.h^{-1} in group F. No differences were observed between the three groups on day 1 or day 7.

Figure 2A Plasma KIC isotopic enrichments. Mean ± SD



Statistically different at p<0.05 vs. i.v. plasma KIC enrichment

Fig 2 B. Plasma leucine (Leu) enrichments. Mean ± SD



* Statistically different at p<0.05 vs. i.v. plasma leucine enrichment

DISCUSSION

In this study we have shown that in preterm infants five to six days of minimal enteral feeding increases protein uptake by splanchnic tissues, compared to control infants receiving parenteral nutrition only.

The introduction of enteral feeding in preterm infants is rather different between neonatal centres (15,16,17). Some centres start enteral feeding as soon as possible after birth, aiming to have the newborn infant and also the very preterm infant on full enteral feeding preferably within the first week of life. In other centres the introduction of enteral feeding in very low birth weight infants is delayed for periods of 7-14 days as physicians are afraid of increasing the risk of feeding intolerance, thereby inducing aspiration and the risk of necrotising enterocolitis.

Small amounts of feeding, also called trophic feeding, are used to promote the maturation of the gastrointestinal tract. Whether trophic feeding in preterm infants has a positive effect on splanchnic maturation is unknown. We evaluated the maturation of the splanchnic tissues from the uptake of leucine by the gastrointestinal tract. We used a double tracer infusion of leucine, administered simultaneously through the nasogastric and intravenous route as described by Hoerr et al (8). At the same time our studies are hard to compare to those performed so far with this dual tracer method. Hoerr et al (8) compared in a short term cross-over design the difference in splanchnic uptake of leucine between the fed and postabsorptive state in adult man. Matthews et al (18) investigated the fate of enterally delivered leucine in the postabsorptive state in adult man. Beaufrere et al (9) used this method in very low birthweight infants to study the first pass splanchnic extraction of enterally delivered leucine in the fed state. In our study we were interested in the effect of minimal enteral feeding on the splanchnic uptake of leucine. The amount of feeding given is very low and nutritionally of no importance. Moreover, we stopped the minimal enteral feeding in all groups five hours before the tracer study. At the same time parenteral nutrition was continued. It is therefore hard to compare our results with previously published results in either the fed or postabsorptive state. We hypothesized that minimal enteral feeding would have a positive effect on the splanchnic uptake of leucine, resulting in a lower enrichment of leucine and KIC from the ng than the i.v. tracer. On day 1 we observed a lower KIC enrichment for the ng compared to the i.v. tracer, indicating uptake of aminoacids in the splanchnic tissues. For leucine, the difference was in the same direction but not significant. On day 7, in both groups receiving minimal enteral feeding, the enrichment for the ng tracer was lower than the i.v. tracer both for leucine and KIC. In contrast in the control group there was no difference in enrichment, neither for leucine or KIC, between the ng and i.v. tracer. The lower enrichments for the ng tracer indicates that leucine is taken up in the first pass in the groups receiving minimal enteral feeding but not in the control group. The results on day 1 can be explained by the positive effect of amniotic fluid on the maturation of the gastrointestinal (GI) tract. In the fetus it is estimated that nutrients absorbed from amniotic fluid provide 10-15% of fetal requirements for energy and nutrients (19). Our studies on day 1 might reflect the intra-uterine situation.

Antenatal corticosteroids might have an effect on splanchnic uptake of leucine. Less patients in the control group received antenatal corticosteroids compared to the feeding groups. There was no difference between the results of groups in day 1. The differences found on day 7 might have been influenced by antenatal corticosteroids. We compared the results of only those patients who received antenatal steroids in the three groups. The results remained significant different between groups.

In all studies, we observed a KIC-leucine enrichment ratio of around 1 for both tracers. This, together with the lower enrichment for the ng tracer, might indicate that leucine taken up in splanchnic tissues is used within the cell, most likely for protein synthesis, and not released after deamination into the circulation (20). In our study we did not aim to quantitate the protein turnover of splanchnic tissue. Our aim was to evaluate whether minimal enteral feeding does have a positive effect on the maturation of the GI-tract as indicated by increased splanchnic uptake of leucine.

Due to technical problems, no reliable CO_2 collection in extubated patients, we could measure $^{13}CO_2$ excretion only in approximately 50% of the infants studied. On day 1 only 20% of the enterally delivered tracer was excreted as CO_2, and 30% on day 7. This indicates that 70-80% of the enterally delivered leucine is used for protein synthesis.

Different studies have shown a negative effect of withholding enteral feeding on growth and development of the GI tract. The weight of the jejunum and total mucosal thickness decreases significantly after parenteral feeding compared to enterally feeding and protein synthesis decreases in the small intestine (21,22,23).
There is evidence that luminal nutrients stimulate intestinal growth and maintain mucosal integrity in adult and neonatal animals (24,25)

In some centers enteral nutrition is started in order to provide nutrition enterally, while in other centers it is given in nutritionally inconsequential amounts and only to improve the maturation of the GI tract. The questions when feeding in preterm infants should be started, how much should be given, at which intervals, how much the increments should be, and what the optimal composition is, are still unanswered. Many neonatal centers in Europe start enteral feeding on the first or second day after birth. To our knowledge, in the USA several neonatal centers are used to start enteral feeding only at day 5-7. On the other hand there seems to be an increasing trend to start minimal enteral feeding or trophic feeding in the first days of life (15). Minimal enteral feeding is associated with a more normal pattern of gastro-duodenal motility, improved tolerance of enteral feeds, earlier full enteral feeding and later more rapid achievement of full nipple feedings (6). A recent study indicated that minimal enteral feeding is associated with a reduced hospital stay and less reliance on total parenteral nutrition (26). Infants who received minimal enteral feeding had fewer episodes of culture confirmed sepsis (26). This might be explained by the decreased mucosal permeability seen after feeding (27).
In our study, either due to the small sample size or the amount of feeding given, we did not find a difference in clinical parameters (weight gain, time to reach full enteral feeding)

between groups. Infants were on full enteral feeding at the mean age of 16 days, earlier than in most published studies.

Conclusion: Our results indicate that minimal enteral feeding - even in low volumes of 20 $ml.kg^{-1}.day^{-1}$ - improves maturation of splanchnic tissue as evidenced by an increase of leucine uptake. More studies are needed to define the optimal amount and composition of minimal enteral nutrition.

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Chapter 7

Leucine kinetics during simultaneously administered insulin and dexamethasone in preterm infants with severe lung disease.

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ABSTRACT

The objective of this study was to determine whether insulin administration would prevent the well-documented catabolic effect of dexamethasone given to preterm infants with chronic lung disease. We studied leucine metabolism in 11 very-low-birth-weight infants before dexamethasone treatment and on d 2, 4 and 7 thereafter. During the first 4 d of dexamethasone, insulin was administered intravenously at a dose of 0.5 (n=7) or 1.0 (n=5) IU/kg/d. Leucine turnover was not significantly different between d 0 (337 ± 41.3 μmol leucine/kg/h), d 2 (288 ± 27.2), d 4 (302 ± 22.1) and d 7 (321 ± 21.2 μmol leucine/kg/h), and neither was leucine breakdown (272 ±21.9 on d 0, 225 ± 21.5 on d 2, 231 ± 21 on d 4 and 242 ± 17.6 μmol leucine/kg/h on d 7). Weight gain rates were significantly lower during the first week of dexamethasone treatment compared to the week before treatment or the second and third week.

We conclude that during insulin and corticosteroid administration in very-low-birth-weight infants no changes were observed in leucine kinetics in contrast to previous studies. The decrease in weight gain was not reversed.

INTRODUCTION

Due to improvements in neonatal therapies, increasing numbers of very-low-birth-weight infants are surviving. However, lung damage can occur at these low gestational ages because of the infant's underdeveloped lungs combined with its requirement for artificial ventilation at sometimes-high airway pressure. Consequent respiratory complications can result in chronic lung disease (CLD). Fluid restriction and diuretics, traditionally the first line of therapy in such cases, result in lower weight gain rates, which presents a concern because of the fact that growth is essential to overcome the pulmonary problems.

Subsequent to Avery's 1985 publication on the use of dexamethasone to treat respirator-dependent infants with BronchoPulmonary dysplasia [1], corticosteroids have increasingly been used in the management of infants on respirators in an attempt to achieve extubation and lessen the development of CLD. Recent studies indicate a trend toward the use of steroids at earlier postnatal ages in smaller infants [2,3], whose growth is already suboptimal.

The fact that corticosteroids are catabolic has been shown in a number of studies. Not only are urea and plasma amino acid levels increased [4 -10], but protein anabolism is impaired, as shown by direct measurements [8 -13]. We conducted a study which demonstrated that higher leucine catabolic and proteolytic rates occur when high doses of dexamethasone are administered to preterm infants with CLD [10], effects which previously had been observed in adults [8,9]. It has long been known that insulin is anabolic with regard to protein metabolism. This is caused by inhibiting protein breakdown, rather than by stimulating protein synthesis [14 -16] Part of the protein-wasting effect of glucocorticoids is the result of steroid-induced resistance to the anti-proteolytic effect of insulin [17] We hypothesized that insulin treatment during the first few days of treatment with dexamethasone would counteract the well-documented increase in protein degradation. Furthermore, we hypothesized that the rate of weight gain would be positively influenced by the addition of insulin to the dexamethasone treatment of preterm infants with CLD.

Because studies have shown that the effect of dexamethasone is maximal during the first week of treatment [5,10], we administered insulin during the first 4 d only. We evaluated plasma C-peptide levels daily to determine whether the endogenous insulin production was suppressed by the administration of exogenous insulin.

METHODS

This study was approved by the medical ethics committee of the Academic Hospital Rotterdam/Sophia Children's Hospital. Informed consent was obtained from the parents of 12 infants who could not be weaned from a ventilator on which they had been dependent since birth and were selected for dexamethasone treatment by the attending neonatologist. Patients excluded from this study were those infants suspected of having contracted sepsis at the beginning of dexamethasone treatment, as well as any infants who had a metabolic disease or liver failure. Dexamethasone was administered intravenously in two equal doses per day, totaling 0.5 mg/kg/d for the first 3 d, 0.3 mg/kg/d for the second 3 d, then gradually tapering off to alternate days of 0.1 mg/kg/d by d 21, and stopped at d 28 (adapted from 1). During the first 4 d of dexamethasone treatment, short-acting insulin (Actrapid HM, Novo) was continuously administered intravenously (0.5 IU insulin/kg/d in seven patients and 1.0 IU insulin/kg/d in five patients) beginning with the first dose of corticosteroids.

Weight was measured daily on an electronic scale with a resolution of 5 g. Average daily weight gain was calculated from daily weight changes in the week prior to the start of dexamethasone therapy and in the three subsequent weeks, and expressed as g/kg/d.

According to the feeding protocol of the neonatal unit, all patients were parenterally fed during the first 7 d of life (dextrose 10%, amino acids (Primene^R 10%, Clintec Benelux, Brussels, Belgium) and lipids (Intralipid^R 20% Kabi Pharmacia, Stockholm, Sweden)) after which oral feeding was gradually introduced in the second week (Nenatal^R, Nutricia Zoetermeer, the Netherlands).

Leucine turnover studies

Patients were studied prior to the start of dexamethasone treatment and on d 2, 4 and 7 afterward. Leucine turnover studies were conducted by intravenous administration of [1-^{13}C]leucine (99% enriched, Isotec Inc., Miamisburg, Ohio, USA) 2 mg/kg bolus and 2 mg/kg/h maintenance dose for 3 h. The tracer was given with the use of a Harvard infusion pump (model M22;Harvard Apparatus Co. Inc., South Natick, Massachusetts, USA). Blood samples were drawn before the administration of the stable isotope, at 30 min before the end, and at the end of the [1-^{13}C]leucine administration. Blood was stored immediately at 0^0C, centrifuged at 3000 rpm within 15 min, and plasma was then stored at -80^0C. Per study day, three blood samples of 0.5 ml were taken. In total, 6 ml blood was taken per patient during the entire study.

Enrichment of alpha-keto-isocaproic acid , which reflects the intracellular enrichment of leucine [18, 19], was determined as described previously [10]. In brief, plasma is deproteinized with sulfosalicylic acid, and the alpha-ketoacids are reacted with phenylenediamine to form quinoxalinol derivatives. The quinoxalinol derivatives are extracted with a mixture of dichloromethane/hexane, dried and silylated with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoracetamide (MTBSTFA, Pierce; Omnilabo, Breda, The Netherlands) to form the butyldimethylsilylquinoxalinol derivatives. Analyses were carried out by injecting 1 µL with a split ratio of 50:1 on a fused silica capillary column of 25 m x 0.22 mm, coated with 0.11 m HT5 (SGE, Victoria, Australia). Plasma enrichment of [1-^{13}C]-ketoisocaproic acid was analyzed on a Hewlett-Packard 5890 gas chromatograph coupled to a JMS-DX303 mass spectrometer (Jeol LTD, Tokyo, Japan) in electron impact ionization mode with an interface temperature of 280°C and a source temperature of 200°C. Selected ion monitoring was done at mass 259 and 260 for natural and enriched ketoisocaproic acid. Standard deviation was 0.2 mol% (range 0 – 20 mol%) for [1-^{13}C]-ketoisocaproic acid; a concentration effect was not observed for the measured mol% enrichment and the amount injected. Background enrichment at d 4 and 7 was not different from that on d 2, so recycling of infused tracer was negligible.

Leucine turnover rates were calculated as described before, using the reciprocal pool model [10] Leucine turnover was calculated according to the following equation:

$Q_L = [(IE_{infusate} / IE_{plasma}) - 1] \times I_L$

where $IE_{infusate}$ is the isotopic enrichment of the infusate, IE_{plasma} is the isotopic enrichment of alpha [1-^{13}C]KIC in plasma and I_L the infusion rate of [1-^{13}C]leucine in micromol/(kg.h). In a steady state, leucine that leaves the pool should equal leucine that enters the pool. Leucine can enter the pool through the diet (I) and from breakdown of protein (B), and leave the pool via protein synthesis (S) and excretion (E). Breakdown was therefore calculated according to the following equation:

$Q = B + I$

Urine was collected using adhesive bags in order to calculate the nitrogen balance. Nitrogen balance is expressed as grams protein/kg/d, assuming that 6.25 g protein contains 1 g nitrogen.

On each study day, plasma C-peptide levels as an indicator of endogenous insulin production were determined using a C-peptide ^{125}I-RIA kit (Incstar Corporation, Stillwater, Minnesota, USA.)

Statistical analysis

All data are expressed as mean ± SEM unless otherwise stated. Statistical analysis was performed with the use of one-way ANOVA with repeated measurements. Differences between 2 study days were considered to be statistically significant at $p \leq 0.05$ (two sided).

RESULTS

The average birth weight of the participating infants was 841 (range 535 - 1140) g, with a gestational age of 26.7 (range 25.3 - 28.3) wk.. The average postnatal age at start of the corticosteroid therapy was 17 d (range 10 - 33 d). Clinical characteristics of the individual patients are given in table 1. Due to the improvement of the clinical status of the children, the attending neonatologist was able to increase protein and energy intake significantly on d 7 compared to d 0. No statistically significant differences were found between protein or energy intakes on d 0 compared to d 2 or 4 [table 2].

Table 1 Clinical characteristics of the individual preterm infants with CLD receiving dexamethasone therapy

Patient	Insulin dose	Gestational age (wk)	Birth weight (g)	AGA/SGA	Age at time of study (d)	Weight at time of study (g)
1	0.5 IU	28.3	755	sga	16	865
2	0.5 IU	26.9	890	aga	15	955
3	0.5 IU	24.7	735	aga	10	815
4	0.5 IU	28.0	700	sga	20	910
5	0.5 IU	25.3	685	aga	18	675
6	0.5 IU	27.2	1140	aga	13	1210
7	0.5 IU	26.2	810	aga	23	905
8	1 IU	27.2	1125	aga	33	1380
9	1 IU	25.7	1000	aga	14	1225
10	1 IU	27.3	1075	aga	13	1105
11	1 IU	25.3	640	sga	11	710
12	1 IU	27.7	535	sga	21	635
Mean ± SEM		**26.7 (0.3)**	**841 (58)**		**17.3 (1.8)**	**949 (68)**

No significant differences between the 0.5 IU and the 1.0 IU group. AGA = appropriate for gestational age, SGA = small for gestational age

Table 2: Protein and energy intakes

	Protein intake g/kg/d mean (sem)	Energy intake kcal/kg/d mean (sem)
Day 0	2.25 ± 0.3	88.7 ± 8.7
Day 2	2.32 ± 0.3	90.5 ± 6.9
Day 4	2.74 ± 0.2	100.4 ± 6.9
Day 7	3.16 ± 0.3 *	118.4 ± 10.0 *

*Statistically significant difference at $p< 0{,}05$ from day 0

Insulin did not prevent the drop in weight gain during the first week of dexamethasone treatment, as we have observed earlier [10]. The weight gain rates were determined according to intrauterine values [20] from the third week of dexamethasone treatment onward.. Weight gain rates in the infants receiving 0.5 vs. 1.0 insulin were not significantly different. Fig 1

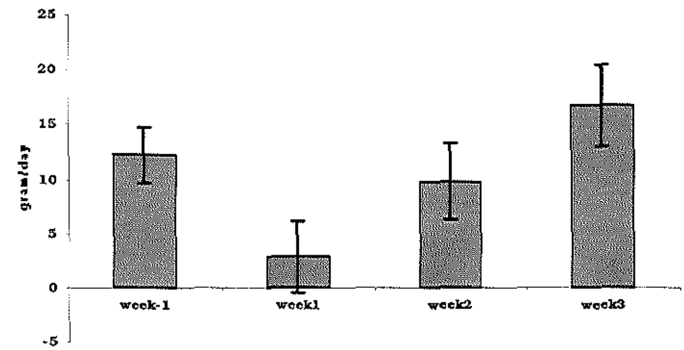

Figure 1: average daily weight gain in g/kg./d (sem) 1 wk before dexamethasone and in the first, second and third week after dexamethasone treatment.

Glucose levels remained between 3.5 – 7 mmol/L during insulin treatment from d 1 – 4 without the need to infuse extra glucose. No difference was found between infants treated with 0.5 or 1.0 IU insulin/(kg.d). After stopping insulin administration on d 4, some patients had transiently elevated blood glucose levels, as are often observed in patients treated with dexamethasone without providing insulin. Insulin administration did not have to be restarted in any patient.

No differences in leucine metabolism were found between the infants treated with 0.5 vs. 1.0 IU insulin/kg.d (data not shown). Since there were no differences in birth weight, gestational age, study age and weight, the results were therefore combined. Whole-body leucine turnover and breakdown rates are shown in Figure 2.

Leucine turnover was not significantly different between d 0 (337 ± 41), d 2 (288 ± 27), d 4 (302 ± 22) and d 7 (321 ± 21 μmol leucine/kg/h). Leucine breakdown also was not different between study days (272 ±22 on d 0, 225 ± 22 on d 2, 231 ± 21 on d 4 and 242 ± 18 μmol leucine/kg/h on d 7).

We were able to obtain a reliable 24-h urine collection from only four infants on d 0 and d 2, due to the small size of our subjects. In those four infants, the net protein balance remained positive and significantly different from zero despite the dexamethasone treatment (1.16 ± 0.55 g/kg/d).

In our previous study [10], we found a negative nitrogen balance during treatment with high dosages of dexamethasone. Despite the infants' severe clinical condition, net protein balance was also positive prior to the start of the dexamethasone (1.94 ±0.97 g/kg/d.)

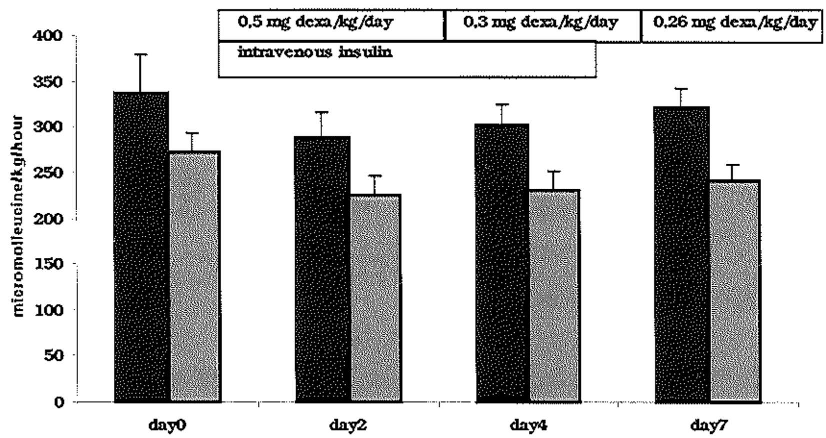

Figure 2 Leucine turnover (black bars) and breakdown (grey bars) on d 0, 2, 4 and 7 after start of dexamethasone therapy. Leucine data are presented in micromol/kg/h mean (sem)

Endogenous insulin production was measured via plasma C-peptide levels. Despite exogenous insulin administration, endogenous insulin production was not suppressed either in infants receiving 0.5 IU insulin/(kg.d) or in those receiving 1.0 IU insulin/(kg.d). C-peptide concentrations increased significantly after cessation of the exogenous insulin administration Fig 3

DISCUSSION

In this study, we found no increase in either leucine turnover or breakdown during a combined infusion of high-dose corticosteroids and insulin. This finding contrasts with that of a study we previously conducted in infants with BronchoPulmonary dysplasia who were treated with dexamethasone without insulin [10], after 4 d of high-dose corticosteroids, the infants displayed a significant increase in protein breakdown.

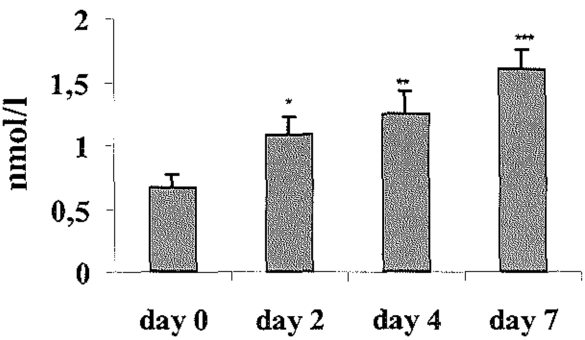

Figure 3: Average plasma C-peptide levels in nanomol/L mean (sem).
* = $p < 0.05$, ** = $p< 0.01$, *** = $p < 0.001$ compared to day 0

We did not include a control group of infants who did not receive insulin for the following reasons. First, multiple studies have shown that glucocorticosteroids increase proteolysis in animal models [12, 13,], adults [6 - 8, 14] and preterm infants [5, 10]. Second, the number of infants who receive dexamethasone treatment for chronic lung disease is small, and this group of infants is very hard to study. The addition of a control group either would have reduced the number of infants receiving insulin treatment or considerably prolonged our study, which encompassed nearly 2.5 y. Changes in treatment protocols over a period of several years, which are inevitable in a rapidly developing subspecialty like neonatology, increase the variability in the results, which are already significant. In table 3, we compare the results of this study with a previous study in which leucine kinetics were studied during the same dexamethasone treatment, but without insulin administration. Although there is a difference in birth weight and post natal age at time of study, the leucine turnover and breakdown on day 0 are in the same range. However, on day 4, a significant increase in protein breakdown was measured in the infants studied by VanGoudoever et al. This was not present in the group with steroids and insulin.

In this study we chose to form two subgroups receiving either 0.5 IU or 1.0 IU insulin/(kg.d). Those dosages were chosen arbitrarily, since no previous studies have been performed with the combination therapy of dexamethasone and insulin. However, in hyperglycemic infants receiving total parenteral nutrition, insulin doses up to 2.8 IU/(kg.d) have been used [21.], and

Table 3. Comparison of the present study data with a previous study in infants with only dexamethasone treatment (ref 10)

	BirthWeight (gram)	Gestational Age (Weeks)	Age at study (days)	Turnover day 0	Turnover day 4	Breakdown day 0	Breakdown day 4
Dexa and insulin	841 (± 202)	26.7 (±1.2)	17.3 (± 6.4)	337 (± 143)	302 (± 73)	272 (± 76)	231 (± 70)
Dexa only	1100 (± 300)	27.5 (±2.0)	24.7 (±12.4)	302 (± 52)	376 (± 85)*	207 (± 54)	272 (± 75)*

Dexa and insulin: data from present study, Dexa only: data from Van Goudoever et al 1994. Effect of dexamethasone on protein metabolism in infants with bronchopulmonary dysplasia. J Pediatr124:112-118.
Turnover and breakdown are leucine in micromol/kg/hr. All data with Standard Deviation. * indicates a statistical significant difference from day-0 values at $p \leq 0.05$.

in a study with four clinically stable, preterm infants, a dosage of 1.2 IU/(kg.d) reduced proteolysis, although protein synthesis decreased to the same extent, resulting in no net protein gain [22.] In order to avoid an increase in glucose intake, we chose to randomly assign the infants to either 0.5 IU or 1.0 IU insulin/(kg.d). The apparent absence of a dose response in leucine kinetics in response to 0.5 IU versus 1.0 IU insulin/kg/day may be due to the number of infants studied.

The fact that insulin reduces proteolysis has been demonstrated in a number of studies [17, 22]. Administration of insulin and glucose partially blocked branched chain alpha-keto acid dehydrogenase activity. Smith found that protein catabolism is exaggerated in diabetic rats compared with normal rats [13]. Since the major effect of dexamethasone treatment is proteolysis, the choice for concomitant insulin treatment was an obvious one.

Our results show that with simultaneously administration of insulin and dexamethasone, there is no increase in protein breakdown. After discontinuation of insulin we did not see an increase in protein breakdown. The reason for this could be that on day 7 the endogenous insulin production has increased more than twofold compared to baseline (Fig 3). Second, the dose of dexamethasone has been decreased by 50% on day 7.

We did not measure leucine oxidation, so we are not able to calculate whether protein synthesis was affected. However, in a small subgroup of infants, we found a positive net protein balance, indicating that the overall effect of insulin in infants treated with

dexamethasone was anabolic. This result stands in contrast to the weight gain rates we observed. Contrary to the leucine results, and similar to our previous study in infants receiving dexamethasone without insulin, weight gain rates decreased during high dosages of dexamethasone and insulin, whereas they normalized at the end of the tapered treatment. The reasons for the discrepancy in leucine results and nitrogen balances vs. weight gain rates are not clear. It may be that leucine is not a very good indicator of whole-body protein metabolism, as leucine is an essential amino acid that is relatively abundant in formula, or that the small number of infants in whom we were able to obtain reliable urinary nitrogen excretion data did not react as a representative subgroup. Another explanation could be that dexamethasone has an effect on body composition and weight gain therefore is not a good parameter for growth in this situation. There was however no effect on daily fluid balances that could explain the reduced weight gain during high dose dexamethasone (data not shown)
Theoretically, exogenous insulin administration could suppress endogenous insulin production. To be sure that endogenous insulin production was not suppressed by exogenous insulin administration, we measured plasma C-peptide levels. Although plasma-measured C-peptide levels are less accurate than computerized compartimental analysis of C-peptide concentration in predicting prehepatic C-peptide production [23], it appeared that even during exogenous insulin administration, the C-peptide levels increased, indicating that endogenous insulin production increased. This means that there are no indications that endogenous insulin production is suppressed by the doses of insulin used in this study.

Because protein breakdown is accelerated by corticosteroids, we believe that blocking such an increase in these infants is preferable to increasing their already high protein synthesis rate.

Other strategies to counteract the steroid-induced increase in protein breakdown are the use of recombinant human growth hormone (rhGH) and/or insulin-like growth factor (IGF). IGF has failed to reverse corticosteroid-induced protein breakdown in piglets, and in a pilot study, growth hormone did not prevent protein breakdown in BronchoPulmonary dysplasia-afflicted infants with dexamethasone [24,25]. In the past few years, studies have been published on adult patients chronically treated with corticosteroids, whose protein balance showed an improvement after providing a therapy combining corticosteroids with rhGH [26-28]. This effect stemmed solely from increased protein synthesis; protein breakdown was not affected. Combination of rhGH with IGF-I appeared to result in a substantially more positive protein balance than either one alone [29]. However, data are conflicting since rhGH did not modify the protein breakdown in rats given dexamethasone [30]. In infants, protein turnover (i.e., protein breakdown and synthesis) occurs at a much higher level than in children and adults [10]. Furthermore, as recent publications on the use of rhGH in critically ill adults have shown a twofold increase in mortality rates [31.], the use of rhGH in critically ill preterm infants should not be advocated in the absence of a thorough examination of the effect of rhGH in such patients. During our study, as the clinical status of most of the patients improved, their nutrient intake changed; total caloric and oral protein intake increased and parenteral protein intake declined. There were, however, no significant differences in either protein or energy intake between d 0 and 4, the period in which insulin was administered.

In conclusion, the results of this study show that during combined insulin and steroid treatment in preterm infants with CLD, leucine kinetics remain unchanged. This was, however, not accompanied by an increase in weight gain, so that the anabolic effect of insulin in preterm infants receiving dexamethasone has yet to be established.

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Chapter 8

Summary and conclusions

SUMMARY

Over the past decades, successes in perinatology and neonatology have resulted in an increasing survival rate, also of the smallest neonates. A major factor in the successful clinical care of premature infants, is the improved understanding of the principle's of metabolism during the neonatal period. Still, many issues regarding neonatal metabolism remain to be investigated. The aim of the studies reported in this thesis is to further elucidate the neonatal glucose-, lipid- and protein metabolism of neonates as part of studies performed in the neonatal unit of the Sophia Childrens Hospital, and to evaluate a possible effect of antenatal steroids on these substrates.

In **chapter one**, an introduction is given regarding the glucose, protein and lipid metabolism in preterm infants.

Some pro's and cons regarding enteral feeding early after birth are discussed with the introduction of small, nutritional inconsequential amounts called minimal enteral feeding as alternative.

Also the literature on antenatal and postnatal corticosteroids in the fetus end preterm infant is briefly reviewed. Antenatal steroids have proven to be the most cost reducing medical treatment thus far, reducing neonatal mortality and morbidity. Side effects on the central nervous system, growth and hormonal homeostasis in animal studies however should prompt us to consider the use of antenatal steroids with care. The long-term side effects of post natal steroids are much more pronounced compared to antenatal steroids as neurological deficits, cerebral palsy and lower psychomotor development is seen.

Early after birth, the caloric intake of a preterm newborn is insufficient to satisfy the energy needs, resulting in a catabolic state. Premature infants are at risk for hypoglycaemia as the glycogen that is stored in the last trimester of the normal pregnancy is very limited while the glucose demands for thermo-regulation and illness are high.

The blood glucose level is the sum of the amount of glucose entering and leaving the blood compartment (= glucose turnover). Glucose enters the blood compartment from intake (intravenous or enteral) and endogenous glucose production (either from glycogenolysis or gluconeogenesis). Glucose leaves the blood compartment for glucose oxidation or glucose storage, either as glycogen or as part of other metabolites, such as glycoproteins. The endogenous glucose production of the term and preterm infant when not given exogenous

glucose is 5 - 6 mg/kg/min. No data exist on glucose turnover over the whole period of the first 24 hours after birth, when major clinical and hormonal changes take place. In **chapter 2** therefore, glucose turnover and endogenous glucose production (GPR) during the first 24 hours of life in preterm infants with Respiratory Distress Syndrome is evaluated.

Glucose metabolism can well be studied using the stable isotope dilution technique. Since the early 1970s, the first studies using stable isotopic tracers in human neonates and children are reported. Stable isotopes differ from radioactive isotopes in that the nucleus of the atom is stable and thus does not release radiation. Stable isotopes are naturally occurring nuclides and therefore have a natural abundance while the use of radioactive tracers would be considered unethical because neonates and children undergo a period of rapid cell division, growth and embryogenesis. Processes that can be influenced by irradiation. Stable isotopes can be used without any harm for studies in newborn infants and pregnant women.

Stable isotopes in biologic samples can be quantified by mass spectrometry. The principle of mass spectrometry is detection of compounds (e.g. glucose) on the basis of their molecular weight, or mass. The investigated compound is introduced in the mass spectrometer after separation in for instance a gas chromatograph (GC-MS). In the mass spectrometer a characteristic mass spectrum is produced. An ion with its corresponding labelled pair (the isotopomer) can be measured due to the difference in molecular mass. The change in abundance from natural abundance is called the enrichment. From this enrichment the kinetics can be calculated. A second method of determining the enrichment of a compound is the use of a so called isotope-ratio-mass-spectrometer (IRMS). The compound of study is hereby burned to CO_2 and water. Through mass spectrometry the ratio in $^{12}CO_2$ and $^{13}CO_2$ is determined. This provides the enrichment of ^{13}C. Glucose turnover can be studied by using ^{13}C-labelled glucose. When glucose is used in which all carbons are ^{13}C, the following measurements can be made. The ratio of glucose molecules with all carbons as ^{13}C compared to glucose molecules with 0-5 ^{13}C –atoms can be measured by GC-MS. The glucose turnover measured in this way is called Ra-true. Since glucose will be reduced in the human body to three carbon molecules by glycolysis, a small amount of the ^{13}C-atoms will recycle back to glucose via gluconeogenesis. The glucose formed in this process will contain 1-5 ^{13}C atoms. With the GC-CI-IRMS method it is not possible to distinguish between uniformly labelled glucose (all C-atoms are ^{13}C) and recycled glucose molecules consisting of a varying number of labelled carbon. The enrichment measured by GC-CI-IRMS therefore gives an apparent rate of glucose turnover (Ra-apparent).

In the study on glucose turnover during the first 24 hours of life, twenty-seven premature infants were studied immediately after birth, of whom 11 infants had not received antenatal steroids, 6 infants received only one dose steroid and 10 patients received two antenatal steroid doses. Patients received a constant intravenous infusion of the stable isotope [U-^{13}C] glucose for 24 hours. The labelled glucose consisted of 6 carbon atoms with atom weight 13 instead of 12. Glucose turnover and endogenous glucose production remained constant during the period from 11 to 29 hours after birth. Mean Ra-true was significantly higher than mean Ra-apparent. The mean GPR was 2.56 ± 1.5 mg/kg/min while the glucose infusion was 5.27 ± 0.7 mg/kg/min. The glucose recycling calculated from the difference between Ra-true and Ra-apparent was 1.45 ± 0.8 mg/kg/min.
The GPR in infants who had been exposed antenatally to 2 doses of steroids was significantly higher compared to infants of mothers who did not receive corticoids before birth.

In conclusion: During the first 24 hours of life, a period with tremendous metabolic and hormonal changes for the neonate, the glucose turnover and endogenous glucose production remain constant indicating that the neonate is relatively well able to maintain glucose homeostasis for a short period of time. No effect of antenatal steroids is observed with regard to glucose turnover. GPR on the other hand is higher after antenatal steroids. Whether the higher GPR is due to a higher glucose production from the release of aminoacids, or to a higher maturation of enzyme needs further studies.

The fetus in utero is entirely dependent upon the mother for a continuous supply of glucose, and no significant production of glucose by the fetus has been demonstrated. Although significant activity of key enzymes involved in gluconeogenesis (GNG) has been documented early in gestation in human fetal liver, fetal gluconeogenesis in vivo has not been documented. Glycogenolysis is the breakdown of glycogen to glucose molecules. Gluconeogenesis is the synthesis of new glucose molecules from tri-carbon molecules. These two processes are stimulated by the birth associated surges of catecholamines and pancreatic glucagon. During gluconeogenesis, an exchange of H^+ of glucogenic intermediates with the H^+ of body water occurs. If the body water pool is labelled with deuterium (^{2}H), the specific hydrogen of glucogenic intermediate will achieve the same magnitude of enrichment as that of body water. The C-6 of glucose originates from the C-3 (methyl group) of pyruvate. Quantification of the deuterium enrichment on C-6 of glucose will therefore give an estimation of the contribution of pyruvate to gluconeogenesis. The contribution of gluconeogenesis to glucose production in healthy full term newborns has not been quantified. In **chapter 3** aim number four is addressed: what is the contribution of gluconeogenesis via pyruvate to total glucose production in healthy, normal full term babies and in premature infants.

The contribution of gluconeogenesis to glucose production was measured by the appearance of ^{2}H on C-6 of glucose in seven full-term healthy babies between 24-48 hours after birth, and twelve low birth weight infants. GNG via pyruvate contributed ~31% to glucose Ra. In preterm babies, the contribution of GNG to endogenous glucose Ra was variable (range 6-60%). The highest contribution was in infants receiving low rates of exogenous glucose infusion.

The data show that gluconeogenesis is evident soon after birth in the newborn infant, and that even after a brief fast (5 h), gluconeogenesis via pyruvate makes a significant contribution to glucose production in healthy full term infants. Hypoglycaemia after birth could well be explained by insufficient GNG because of lack of glucogenic substrate.

After birth lipids become an important source of energy, as fat supplies 50% of the energy in human milk. Therefore, after birth the role of glucose as an energy source reduces. Glucose oxidation studies on the first days of life have shown that glucose oxidation is supplying only approximately 50% of the energy expended at this age. In preterm infants on day 1 of life, receiving a glucose intake of 6 $mg.kg^{-1}.min^{-1}$., approx. 4 mg was oxidised yielding approx. 20 $kcal.kg^{-1}.d^{-1}$ at an energy consumption of 45 $kcal.kg^{-1}.d^{-1}$. Indirect calorimetry indicated a higher glucose utilisation than glucose oxidation measured by stable isotopes. This led us to hypothesise that glucose was converted to lipids while at the same time lipid was oxidised.

Lipogenesis is the biosynthesis of fatty acids, a complex polymerisation with acetyl-CoA as the principal two carbon building block. Lipogenesis however, has not been shown directly in preterm infants until now.

In **chapter 4** the aim was to evaluate if ventilated preterm infants show lipogenesis on the first day of life while receiving a hypocaloric intake. Twenty-eight preterm infants (19 male, 9 female) requiring mechanical ventilation received a constant intravenous infusion of the stable isotope [U-^{13}C] glucose for 24 hours. Glycolysis of uniformly labelled ^{13}C-glucose provides ^{13}C labelled pyruvate, oxaloacetate and citrate that can serve as substrate for lipid synthesis. The enrichment of palmitic acid in plasma triglycerides and palmitic acid in plasma phospholipids was measured by GC-CI-IRMS. The plasma palmitate concentration was not related to gestational ages. ^{13}C-enrichment in plasma palmitate increased during the study indicating that palmitate was synthesised from glucose during the study. The fractional synthetic rate (FSR) of palmitic acid in plasma triglycerides was 16.6 ± 11.2 % per day and the FSR of palmitic acid in plasma phospholipid was 9.4 ± 6.3 % per day. When the FSR of palmitic acid in plasma triglycerides and plasma phospholipids between the steroid groups is compared, there is no significant difference. In conclusion: Lipogenesis was shown for the first time directly in human infants using stable isotopes. Plasma phospholipid palmitate production decreases with increasing gestational age. No effect on palmitate production was observed after antenatal steroids.

Protein is continuously synthesised from, and broken down to amino acids. Several hormones are known to have an effect on protein turnover. Glucocorticoids for instance, cause an increase in protein breakdown. In the past decade, antenatal steroids are increasingly used with the purpose of enhancing lung maturation. The possible negative effect of antenatal steroids on protein turnover of the neonate remains to be elucidated. In **chapter 5**, leucine turnover on the first day of life is measured in 38 infants. In 26 infants, the mother had been treated with two or more doses corticosteroids before giving birth, eight infants had received no antenatal steroids whereas 4 infants had been treated with an incomplete course of only one dose steroids. Protein turnover can be studied using stable isotope labelled amino acid (e.g. ^{13}C-leucine or 5,5,5 D3-leucine). The labelled amino acid can be administered intravenously, giving total body turnover rates. An intra-gastrically administered amino acid enters the circulation after passing the splanchnic tissue. When part of the amino acid is used for splanchnic metabolism, the measured plasma enrichment after intra gastrically administered tracer will be lower. In the present study, a double tracer method was used with an intravenous (5,5,5 D3-leucine) and an intra-gastric administered leucine tracer (^{13}C-leucine). The total body leucine turnover calculated from the intravenous leucine showed no effect of the antenatal steroid treatment. The leucine turnover calculated from the intra gastric tracer showed a significant increase of leucine turnover with the treatment of antenatal steroids suggesting a maturational effect of antenatal steroids on splanchnic protein turnover in the preterm infant. It is reassuring that antenatal steroids have no significant effect on total body protein turnover. The effect on splanchnic protein turnover is considered beneficial. In clinical studies a decreased gastro intestinal permeability after antenatal steroids is associated with a decrease in necrotising enterocolitis.

Parenteral feeding is used extensively in clinical care of preterm infants. The timing of initiation of enteral feeding in VLBW infants is still controversial. Administration of nutritional inconsequential quantities (minimal enteral feeding) has shown to improve gut maturation, as evidenced by improved motility. The effect of minimal enteral feeding on protein metabolism of splanchnic tissues, an indicator of anabolism of splanchnic tissues, is unknown.

In **chapter 6**, aim number five regards the possible effect of minimal enteral feeding together with total parenteral nutrition on splanchnic protein uptake, measured from the leucine metabolism. A primed constant infusion of labelled leucine was administered intravenously (5,5,5 D3-leucine) and enterally (^{13}C leucine). Thirty-eight infants were included in the study of whom 14 in group control group C without enteral feeding, 12 in a breastfed group BM receiving 20 ml/kg/day expressed breastmilk and 12 in a formula fed group F receiving 20 ml/kg/day premature formula. On day one of life, the leucine enrichment was significantly lower for the enteral tracer compared to the i.v.tracer in all three groups. On day 7 leucine enrichment between enteral or i.v. tracer was not different in the control group, while the enrichment was significantly lower for the enteral tracer in groups BM and F indicating leucine uptake by splanchnic tissues. Protein synthesis increased in all groups from day 1 to day 7. The conclusion is that: minimal enteral feeding - even in low volumes as 20 $ml.kg^{-1}.day^{-1}$ - improves maturation of splanchnic tissue as evidenced by an increase of leucine uptake by these tissues.

Corticosteroids have increasingly been used in the management of infants on respirators in an attempt to achieve extubation and lessen the development of CLD.

The fact that corticosteroids increase protein breakdown (e.g. are catabolic) has been shown in a number of studies. According to some studies is protein synthesis (anabolism) impaired. It has long been known that insulin is anabolic with regard to protein metabolism by reducing protein breakdown.

In **chapter 7**, the effect of insulin administered simultaneously with dexamethasone in BPD-infants, on leucine turnover was investigated. The hypothesis was that insulin treatment during the first few days of treatment with dexamethasone would counteract the well-documented increase in protein degradation. 12 premature ventilated infants with signs of BPD were studied. During combined insulin and steroid treatment, leucine kinetics remain unchanged. This indicates that the catabolic effect of steroids on protein was counteracted.

This was, however, not accompanied by an increase in weight gain, so that the positive effect of insulin in preterm infants receiving dexamethasone needs further study.

To conclude: In this thesis, it is shown that glucose turnover and endogenous glucose production is stable in preterm neonates, even during the first 24 hours of life when major metabolic and hormonal changes take place. In the first days after birth, gluconeogenesis is evident and even after a brief fast, gluconeogenesis via pyruvate makes a significant contribution to endogenous glucose production. Therefore, the enzymatic machinery to maintain glucose homeostasis is well available. Hypoglycaemia can still occur, especially in the absence of sufficient glucogenic substrates.

For the first time we showed that lipogenesis is present in preterm infants on the first day of life. In our study it was not possible to measure total body lipogenesis. The phospholipid palmitate production decreases with increasing gestational age. Further studies using the MIDA technique should be performed to further elucidate neonatal lipid metabolism.

With minimal enteral feeding the maturation of splanchnic tissue is improved as evidenced by an increase in leucine uptake by these tissues. This supports the observation in clinical studies that minimal enteral feeding enhances gut motility and feeding tolerance.

As for the effect of steroids on metabolism, endogenous glucose production is increased after antenatal steroids. Whether this means that a neonate is better adapted to risks of hypoglycaemia remains a question. No effect on glucose turnover was seen. With regard to lipid metabolism, also no effect of antenatal steroids was measured on fractional synthetic rate of palmitate. The same is to be said about the effect of antenatal steroids on total body leucine metabolism. On the splanchnic tissue however, antenatal steroids have a positive effect. Splanchnic protein uptake is enhanced. This might explain the beneficial effect of antenatal steroids on the incidence of necrotising enterocolitis seen in other studies.

Antenatal steroids therefore seem to be beneficial also in metabolic point of view. No serious negative side effects were seen in these studies.

Postnatal steroids however should be considered with great care. Reviewing the literature makes one cautious as serious long term side effects as neurological deficits and lower psychomotor development are reported. The short term side effect of steroids on protein metabolism might be counteractable. The enhanced protein breakdown reported before was absent when insulin was administered simultaneously. Further studies to evaluate the clinical relevance of this finding need to be done.

SAMENVATTING

In de afgelopen 20-30 jaar hebben successen in de perinatale en neonatale zorg geresulteerd in een toenemende overleving, zelfs van de hele kleine vroeggeborenen. Een belangrijke factor in deze ontwikkeling is de toegenomen kennis omtrent het neonatale metabolisme. Toch moet er nog veel met wetenschappelijk onderzoek verduidelijkt worden. Het doel van de studies die in dit proefschrift beschreven worden is dan ook het verder verduidelijken van het neonatale glucose-, vet- en eiwit metabolisme en tevens het mogelijke effect van antenatale steroïden op deze stofwisseling te evalueren. Alle studies maken onderdeel uit van studies die op de afdeling neonatologie van het Sophia Kinderziekenhuis worden verricht.

In **hoofdstuk 1** wordt een introductie gegeven met betrekking tot het glucose- vet- en eiwit metabolisme van te vroeggeborenen

Enkele voor en nadelen van enterale voeding kort na de geboorte worden gegeven, met de introductie van minimale hoeveelheden enterale voeding als alternatief.

Er wordt een overzicht gegeven van de literatuur over antenatale en postnatale steroïden in de foetus en te vroeggeborene. Antenatale steroïden hebben bewezen tot nu toe de meest kost effectieve behandeling te zijn door de reductie van neonatale morbiditeit en mortaliteit. Bijwerkingen betrekking hebbende op het centrale zenuwstelsel, de groei en de hormonale status in dierstudies moeten ons echter manen tot voorzichtigheid in het gebruik van antenatale steroïden. De lange termijn bijwerkingen van postnatale steroïden zijn veel uitgesprokener dan van antenatale steroïden en hebben betrekking op neurologische schade en verminderde psychomotore ontwikkeling.

Kort na de geboorte is de hoeveelheid calorieën in de voeding onvoldoende om te voldoen aan de calorische behoefte. Dit resulteert in een katabole status. Te vroeg geborenen hebben een groter risico op het ontwikkelen van lage bloedsuikers omdat de hoeveelheid glycogeen die normaal wordt opgeslagen in het laatste trimester gedeeltelijk ontbreekt terwijl aan de andere kant de glucose behoefte voor temperatuursregulatie en ziekteprocessen juist hoog is.

De bloedsuiker spiegel is de som van de hoeveelheid glucose die de bloedbaan inkomt en verlaat: de glucose turnover. Glucose komt de bloedbaan binnen via intra veneuze en enterale inname, en via endogene glucose productie (van glycogeen afbraak en gluconeogenese) Glucose verlaat de bloedbaan voor verbruik in verbranding of voor glucose opslag, hetzij als glycogeen, hetzij als bouwsteen in andere metabolieten zoals glycoproteinen. De endogene glucose productie (GPR) van à-terme en te vroeg geborenen zonder exogene glucose toediening bedraagt 5 – 6 mg/kg/min. Er bestaan geen gegevens over de ontwikkeling van de glucose turnover gedurende de gehele eerste 24 uur na de geboorte, een periode waarin de pasgeborene grote klinische en hormonale veranderingen ondergaat. In het **tweede hoofdstuk** worden de glucose turnover en endogene glucose productie gedurende de eerste 24 uur van het leven in te vroeg geborenen met respiratoire insufficiëntie geëvalueerd Het glucose metabolisme kan goed met behulp van de stabiele isotopen verdunnings techniek worden bestudeerd. Al sinds de jaren '70 worden studies met door stabiele isotopen gemerkte stoffen bij pasgeborenen en kinderen beschreven. Stabiele isotopen verschillen van radioactieve isotopen omdat de kern van het atoom stabiel is en dus geen straling afgeeft. Stabiele isotopen zijn in de natuur voorkomende kernen en hebben dus ook een natuurlijke verrijking. Het gebruik van radioactieve tracers is onethisch aangezien pasgeborenen en kinderen een periode met embryogenese, snelle celdelingen en groei doormaken. Al deze processen kunnen beïnvloed worden door straling. Stabiele isotopen kunnen daarentegen zonder schade in studies bij zwangeren en pasgeborenen gebruikt worden.

De stabiele isotopen in biologische monsters kunnen gekwantificeerd worden met behulp van massa spectrometrie. Het principe van de massa spectrometrie berust op het onderscheiden van stoffen zoals bijvoorbeeld glucose op basis van het moleculaire gewicht of massa. De te onderzoeken stof wordt in de massa spectrometer gebracht na separatie door bijvoorbeeld een gaschromatograaf. In de massa spectrometer wordt een karakteristiek massa spectrum gemeten. Een ion zonder isotoop en het corresponderende gemerkte ion met isotoop (de isotopomeer) worden onderscheiden op basis van het verschil in hun moleculair gewicht. De toename in het voorkomen van isotopen ten opzichte van de aanwezigheid van isotopen onder natuurlijke omstandigheden heet de verrijking. Vanuit deze verrijking kan de kinetiek worden berekend. Een andere methode voor het bepalen van de verrijking van een bepaalde stof is het gebruik van de zogenaamde "Isotopen-ratio-massa-spectrometer". De te bestuderen stof wordt hierbij verbrand tot water en kooldioxide. Met behulp van de massa spectrometer wordt de ratio tussen kooldioxide met koolstof gewicht 12 ($^{12}CO_2$) en het isotoop met koolstof gewicht 13 ($^{13}CO_2$) bepaald. Dit geeft de verrijking van ^{13}C weer. Glucose turnover kan bestudeerd worden met gebruik van ^{13}C-gemerkt glucose. Als glucose wordt gebruikt waarin alle 6 de koolstof atomen massa 13 hebben kunnen de volgende berekeningen worden gedaan. De verhouding tussen glucose moleculen waarin alle koolstofatomen ^{13}C zijn, met glucose moleculen die 0 tot 5 ^{13}C-atomen bevatten kan worden berekend. De zo te berekenen glucose turnover heet "de ware glucose turnover"of "Ra-true". Omdat glucose in het lichaam wordt afgebroken tot 3-koolstof atoom bevattende moleculen zal een klein gedeelte van de ^{13}C-atomen terugkeren via gluconeogenese. Aangezien de kans verwaarloosbaar klein is dat 2 moleculen met ieder 3-^{13}C atomen weer glucose vormen zal de nieuw gevormde glucose 0 tot 5 C-atomen met massa 13 bevatten. Met de GC-CI-IRMS methode is het niet mogelijk om een onderscheid te maken tussen uniform gemerkt glucose (alle C-atomen ^{13}C) en gerecyclede glucose moleculen met een variabel aantal gemerkte koolstof atomen. De gemeten verrijking met de GC-CI-IRMS geeft daardoor een schijnbare glucose turnover (Ra-apparent).

In de studie naar de glucose turnover gedurende de eerste 24 uur van het leven werden zevenentwintig te vroeg geborenen kort na de geboorte bestudeerd. Elf van de pasgeborenen hadden geen steroïden voor de geboorte gehad, bij 6 pasgeborenen had de moeder slecht 1 dosis steroid gehad en de moeders van 10 pasgeborenen hadden twee doses gekregen. Alle patiënten kregen een continue infusie van het stabiele isotoop [U-^{13}C] glucose gedurende 24 uur. De gemerkte glucose bevatte dus 6 koolstof atomen met atoomgewicht 13 in plaats van 12. De glucose turnover en endogene glucose productie (GPR) bleken constant te blijven in de periode van 11 tot 29 uur na de geboorte. De gemiddelde ware glucose turnover was significant hoger dan de gemiddelde schijnbare glucose turnover. De gemiddelde endogene glucose productie was 2.56 ± 1.5 mg/kg/min tijdens een glucose infusie van 5.27 ± 0.7 mg/kg/min. De hoeveelheid gerecyclede glucose die berekend werd uit het verschil tussen Ra-true and Ra-apparent was 1.45 ± 0.8 mg/kg/min. De GPR in de pasgeborenen wiens moeders behandeld waren met steroïden voor de geboorte was significant hoger dan bij de kinderen wiens moeders geen steroïden hadden gehad.

In conclusie: gedurende de eerste 24 uur van het leven, een periode met enorme metabole en hormonale veranderingen in de pasgeborene, blijven de glucose turnover en de endogene glucose productie constant. Dit indiceert dat de pasgeborene vrij goed in staat is om gedurende een korte tijd de bloedsuiker spiegel constant te houden. Er is geen effect van antenatale steroïden op de glucose turnover. De endogene glucose productie daarentegen is hoger na antenatale steroïden. Of de hogere GPR het gevolg is van een hogere glucose productie uit vrijgekomen aminozuren, of van een verdere rijping van enzymsystemen zal verder onderzocht moeten worden.

In de baarmoeder is de foetus volledig afhankelijk van de moeder voor een continue voorziening van glucose aangezien er geen significante glucose productie door de foetus is aangetoond. Hoewel er al vroeg in de zwangerschap in de foetale lever significante activiteit is aangetoond van sleutelenzymen betrokken bij de gluconeogenese, is foetale gluconeogenese nog nooit aangetoond. Glycogenolyse is de afbraak van glycogeen tot glucose moleculen. Gluconeogenese is de synthese van nieuwe glucose moleculen uit trikoolstof moleculen. Deze twee processen worden gestimuleerd door de bij de geboorte vrijkomende catecholamines en glucagon. Tijdens gluconeogenese vindt er uitwisseling plaats van een waterstof atoom (H^+) tussen de trikoolstof intermediairen en het lichaams water. Indien het lichaams water gemerkt is met deuterium (^2H), zal het specifieke waterstof in de gluconeogenese intermediairen dezelfde mate van verrijking bereiken als in het lichaams water. Koolstof atoom nummer 6 (C-6) van het glucose molecuul komt van origine van C-3 (de methyl groep) van pyruvaat. Het bepalen van de verrijking van deuterium aan de C-6 van glucose levert een maat voor de bijdrage van pyruvaat in de gluconeogenese. De mate waarin gluconeogenese bijdraagt aan endogene glucose productie bij te vroeggeborenen op de eerste levensdagen is nog nooit gekwantificeerd. In **hoofdstuk 3** is de tweede vraagstelling onderzocht, namelijk: wat is de bijdrage van gluconeogenese via pyruvaat aan de totale glucose productie in gezonde à terme pasgeborenen en in te vroeg geborenen. De bijdrage van gluconeogenese aan de totale glucose productie werd gemeten door de verrijking van ^2H op C-6 van glucose in zeven à à-terme pasgeborenen in de periode van 24 tot 48 uur na de geboorte, en in twaalf te vroeg geborenen te meten. De gluconeogenese via pyruvaat droeg voor ongeveer 31% bij aan de totale glucose turnover. In de te vroeg geborenen varieerde deze bijdrage van 6 tot 60%. De hoogste bijdrage werd gemeten in de pasgeborenen die de laagste glucose infusie kregen. Deze data laten zien dat gluconeogenese al kort na de geboorte aanwezig is en dat zelfs na een korte periode van vasten de gluconeogenese een significante bijdrage levert aan de totale glucose turnover in gezonde à-terme pasgeborenen.

Na de geboorte worden vetten een belangrijke energie bron aangezien vetten voor 50% in het energie aanbod van moedermelk voorzien. De rol van glucose als belangrijkste energie bron vermindert dus na de geboorte. Glucose verbranding studies op de eerste levensdag hebben laten zien dat glucose verbranding voor ongeveer 50% voorziet in de energie behoefte op deze leeftijd. In te vroeg geborenen op de eerste levensdag met een glucose inname van 6 mg/kg/dag werd gevonden dat ongeveer 4 mg verbrand werd, hetgeen een bijdrage van ongeveer 20 kcal/kg/dag opleverde, bij een energie consumptie van ongeveer 45 kcal/kg/dag. Indirecte calorimetrie wijst op een hoger glucose verbruik dan glucose verbranding studies met behulp van stabiele isotopen. Dat heeft ons in het verleden geleid tot de hypothese dat glucose omgezet werd naar vetten terwijl tegelijkertijd vetten werden verbrand.

Lipogenese is de biosynthese van vetzuren, een complexe polymerisatie met acetyl-CoA als de belangrijkste twee-koolstof bouwsteen. Lipogenese is echter nog nooit direct gemeten in pasgeborenen.

In **hoofdstuk 4** was het doel te evalueren of beademde te vroeg geborenen op de eerste levensdag lipogenese vertonen terwijl zij een hypocalorische voeding toegediend krijgen. Achtentwintig te vroeg geborenen (19 jongens, 9 meisjes) aan de beademingsmachine kregen een constante intraveneuze infusie met het stabiele isotoop [U-^{13}C] glucose gedurende 24 uur. Glycolyse van dit uniform gemerkte glucose levert ^{13}C gemerkt pyruvaat, oxaloacetaat en citraat dat als substraat kan dienen voor lipogenese. De verrijking in plasma palmitaat in plasma triglyceriden en plasma phospholipiden werd gemeten met behulp van GC-CI-IRMS. De plasma palmitaat concentraties was niet gerelateerd aan de zwangerschapsduur. De ^{13}C-verrijking in plasma palmitaat nam toe tijdens de studie hetgeen aangeeft dat palmitaat werd gesynthetiseerd uit glucose gedurende de studie. De fractionele synthese snelheid (FSR) van palmitaat in plasma triglyceriden was 16.6 ± 11.2 % per dag en de FSR van palmitaat in plasma phospholipiden was 9.4 ± 6.3 % per dag. Met het toenemen van de zwangerschapsduur daalt de FSR van palmitaat in palmitaat in plasma phospholipiden. Als de FSR van palmitaat in plasma triglyceriden en plasma phospholipiden worden vergeleken tussen de verschillende steroid groepen is er geen significant verschil. Concluderend: Lipogenese is voor het eerst direct aangetoond in pasgeborenen met behulp van stabiele isotopen. De plasma phospholipid palmitaat productie neemt af met toenemende zwangerschapsduur. Er is geen effect van antenatale steroïden op de palmitaat productie.

Eiwit wordt continu gesynthetiseerd uit- en afgebroken tot aminozuren. Het is bekend van verschillende hormonen dat zij een effect hebben op de eiwit turnover. Glucocorticoïden veroorzaken bijvoorbeeld een toegenomen eiwit afbraak. In de afgelopen 10 jaar zijn steroïden in toenemende mate toegepast met als doel de longrijping te versnellen. De mogelijke negatieve effecten van antenatale steroïden op de eiwit turnover van pasgeborenen moet nog bestudeerd worden. In **hoofdstuk 5** wordt de leucine turnover in achtendertig pasgeborenen gemeten. De moeders van 26 pasgeborenen waren behandeld met 2 of meer doses corticosteroiden voor de bevalling, 8 pasgeborenen hadden geen antenatale steroïden gehad en 4 kinderen hadden een incomplete kuur van 1 dosis antenataal steroid gehad. De eiwit turnover kan bestudeerd worden met gebruik van, met een stabiel stabiel isotoop gemerkt, aminozuur (bijvoorbeeld ^{13}C-leucine of 5,5,5 D3-leucine). Het gemerkte aminozuur kan intraveneus worden toegediend waardoor de eiwitturnover van het totale lichaam bepaald kan worden. Gastro-intestinaal toegediend aminozuur bereikt de circulatie pas na passage door het splanchnicus gebied. Als een gedeelte van de hoeveelheid aminozuur gebruikt wordt voor het metabolisme van de weefsels in het splanchnicus gebied zal de gemeten plasma verrijking lager zijn. In de beschreven studie werd een zogenaamde "dubbel tracer methode" gebruikt met een intraveneuze (5,5,5 D3-leucine) en een enteraal toegediende tracer (^{13}C-leucine). De totaal lichaams eiwit turnover die berekend werd uit de intraveneuze tracer liet geen effect zien van de antenatale steroïden. De leucine turnover berekend uit de enterale tracer toonde een significante toename van de leucine turnover door de antenatale steroiden, hetgeen een rijping effect op de splanchnische eiwit turnover van antenatale steroïden in de te vroeg geborene suggereert. Het is geruststellend dat antenatale steroïden geen effect hebben op de totale lichaams eiwit turnover. Het effect op de splanchnische eiwit turnover wordt als gunstig beoordeeld. In klinische studies is een verminderde gastro-intestinale permeabiliteit gemeten na antenatale steroiden, iets dat geassocieerd is met een afname in de incidentie van necrotiserende enterocolitis.

Parenterale voeding wordt veel gebruikt in de behandeling van te vroeg geborenen. De timing van het starten met enterale voeding in pasgeborenen met een zeer laag geboortegewicht is nog steeds controversieel. Het toedienen van minimale hoeveelheden voeding (onbelangrijk in perspectief van de totale hoeveelheid calorieën) heeft een verbeterde darmrijping laten zien met een verbeterde darm motiliteit. Het effect van minimale enterale voeding op het eiwit metabolisme van de splanchnische weefsels is onbekend.

In **hoofdstuk 6** wordt de vijfde vraagstelling beantwoord, namelijk het mogelijke effect van minimale enterale voeding (naast parenterale voeding) op de splanchnische eiwit opname, gemeten met behulp van het leucine metabolisme. Na een oplaaddosis werd een constante hoeveelheid leucine intraveneus (5,5,5 D3-leucine) en enteraal (^{13}C-leucine) toegediend. Achtendertig pasgeborenen werden geïncludeerd waarvan 14 in een controle groep C zonder enterale voeding, 12 in een moedermelk gevoede groep BM met 20 ml/kg/dag afgekolfde moedermelk en 12 in een geadapteerde koemelk gevoede groep F die 20 ml/kg/dag prematuren voeding kreeg. Op de eerste levensdag was de leucine verrijking uit de enterale tracer significant lager in vergelijking met de intraveneuze tracer in alle drie de groepen. Op de zevende levensdag was er geen verschil meer in verrijking tussen de enterale en intraveneuze tracer in groep C, terwijl de leucine verrijking uit de enterale tracer in groep BM en F significant lager was dan uit de intraveneuze tracer. Dit is een indicatie voor leucine opname door het splanchnische weefsel. De eiwit synthese nam toe in alle drie de groepen tussen de eerste en zevende levensdag. De conclusie van de studie is dat minimale enterale voeding, zelfs in kleine volumina van 20 ml/kg/dag, de rijping van het splanchnische weefsel bevordert, gebaseerd op de toegenomen leucine opname door deze weefsels.

Corticosteroiden zijn in toenemende mate gebruikt in de behandeling van pasgeborenen aan de beademing in een poging hen te kunnen extuberen en de ontwikkeling van chronische longschade te beperken. Het feit dat corticosteroiden de eiwitafbraak (het katabolisme) verhogen is aangetoond in een aantal studies. Volgens sommige studies is de eiwit synthese (het anabolisme) verminderd. Het is al lang bekend dat insuline een anabool effect heeft op het eiwit metabolisme door het verminderen van de eiwit afbraak. In **hoofdstuk 7** is het effect van insuline, tegelijkertijd toegediend met dexamethason in kinderen met broncho pulmonale dysplasie, op de leucine turnover bestudeerd. De hypothese was dat de insuline behandeling, gedurende de eerste dagen van de behandeling met dexamethason, de bekende toegenomen eiwitafbraak zou tegengaan. Twaalf beademde pasgeborenen met tekenen van broncho pulmonale dysplasie werden bestudeerd. Gedurende de gecombineerde behandeling met insuline en steroïden bleef de leucine kinetiek onveranderd. Dit is een indicatie dat het katabole effect van steroïden was tegengegaan. Het ging echter niet gepaard met een toename in het lichaamsgewicht. De eventuele positieve effecten van insuline tijdens dexamethason behandeling dienen nog verder bestudeerd te worden.

In conclusie: in dit proefschrift is aangetoond dat de glucose turnover en endogene glucose productie stabiel zijn in te vroeg geborenen, zelfs in de eerste 24 uur van het leven als er grote metabole en hormonale veranderingen plaatsvinden. In de eerste dagen na de geboorte is gluconeogenese aantoonbaar, en zelfs na een korte periode van vasten maakt gluconeogenese via pyruvaat een significant deel uit van de endogene glucose productie. De enzymsystemen voor het handhaven van glucose homeostase zijn dus voorhanden. Hypoglycaemie kan nog steeds voorkomen, met name in de afwezigheid van voldoende substraten voor gluconeogenese.

Voor het eerst hebben we aangetoond dat lipogenese aanwezig is in te vroeg geborenen op de eerste levensdag. In onze studie was het niet mogelijk de totale lichaams lipogenese te meten. De phospholipid palmitaat productie neemt af met toenemende zwangerschapsduur. Verdere studies met de MIDA techniek moeten verricht worden om het neonatale vetmetabolisme verder op te helderen.

Met minimale enterale voeding is de rijping van het splanchnische weefsel bevorderd zoals is aangetoond met een toegenomen leucine opname in deze weefsels. Dit ondersteunt de observatie in klinische studies dat minimale enterale voeding de darm motiliteit en voeding tolerantie bevordert.

Met betrekking tot de effecten van steroïden op het metabolisme is aangetoond dat de endogene glucose productie is toegenomen na antenatale steroïden. Of dat ook betekent de pasgeborene minder risoco loopt op het doormaken van een hypoglycaemie blijft een vraag. Er is geen effect van antenatale steroïden op de glucose turnover gemeten. Met betrekking tot het vetmetabolisme kan opgemerkt worden dat er ook geen effecten van antenatale steroïden zijn op de fractionele synthese snelheid van palmitaat en hetzelfde kan gezegd worden van de effecten van antenatale steroïden op het leucine metabolisme. Op de splanchnische weefsels daarentegen hebben antenatale steroïden een positief effect. De splanchnische eiwit opname is toegenomen. Dit kan het gunstige effect van antenatale steroïden op de incidentie van necrotiserende enterocolitis in andere studies verklaren. Antenatale steroïden lijken dus ook gunstig uit metabool oogpunt.

Het gebruik van postnatale steroïden moet echter met grote voorzichtigheid bezien worden. De literatuur beoordelend wordt men voorzichtig. Lange termijn bijwerkingen zoals neurologische schade en verminderde psychomotore ontwikkeling worden gerapporteerd. De korte termijn bijwerkingen van steroïden op het eiwit metabolisme kunnen misschien worden tegengegaan. De voorheen beschreven toename in eiwit afbraak was afwezig indien gelijktijdig insuline werd toegediend. Verder studies om de klinische relevantie van deze bevinding te onderzoeken zijn nodig.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 21 oktober 1959 te Nijmegen. In 1978 behaalde hij het VWO-diploma aan het Canisius College/ Mater Dei te Nijmegen. In datzelfde jaar werd de studie geneeskunde aan de Katholieke Universiteit Nijmegen aangevangen, waar hij in 1984 zijn doctoraalexamen en in 1987 zijn artsexamen behaalde. In juli 1987 werd hij aangesteld als assistent niet in opleiding op de afdeling Urologie van het Radboud Ziekenhuis te Nijmegen. (Opleider Prof. Dr. F.M.J. Debruyne). In oktober 1987 begon hij als assistent kindergeneeskunde niet in opleiding in het Academisch Ziekenhuis te Leiden, waar hij in februari 1988 startte met de opleiding tot kinderarts (opleider Prof. Dr. L.J. Dooren). De niet-academische stage werd gevolgd in het Juliana Kinderziekenhuis te Den-Haag (opleider Dr. H.E. Zoethout). Het laatste jaar van de opleiding werd als fellow doorgebracht op de afdeling neonatologie van het Sophia Kinderziekenhuis te Rotterdam (hoofd Prof. Dr. P.J.J. Sauer). Na zijn registratie als kinderarts januari 1993 bleef hij werkzaam als staflid op de afdeling neonatologie. Het onderzoek dat heeft geleid tot dit proefschrift werd uitgevoerd op de afdeling neonatologie van het Sophia Kinderziekenhuis. Vanaf april 1996 is hij werkzaam als kinderarts in het Ignatius Ziekenhuis te Breda, dat vanaf 1 januari 2001 officieel onderdeel van het Amphia Ziekenhuis uitmaakt.

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