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Energy metabolism in umbilical endothelial cells from preterm and term neonates

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Abstract

Aim: The aim of this study was to investigate the impact of gestational age on energy metabolism in human umbilical vein endothelial cells (HUVECs) of preterm and term neonates.
Methods: Activities of respiratory chain (RC) complexes I–V, citrate synthase (CS), overall mitochondrial fatty acid oxidation (FAO), carnitine palmitoyltransferase 2 (CPT2), glycolytic enzymes as well as energy-rich phosphates in HUVECs from uncomplicated term and preterm pregnancies were measured. Neonatal acylcarnitine profiles were analyzed postpartum.
Results: Activities of RC complexes II + III, IV, V, and CS were higher in HUVECs from immature pregnancies. Overall FAO did not change, whereas CPT2 activity was higher in term neonates. RC complexes II–V and CS correlated inversely to gestational age, as well as CPT2 activity within the term cohort. Phosphofructokinase activity increased with maturation; lactate dehydrogenase and hexokinase as well as energy-rich phosphates remained constant. In blood, long-chain acylcarnitines were higher in term neonates.
Conclusions: Gestational age-dependent differences of energy-providing pathways in HUVECs were shown. Alterations of RC complexes with gestational age may be an adaptive process to cope with metabolic stress during birth; reduced oxidative phosphorylation and high glycolytic activity make HUVECs less susceptible to peripartum hypoxic damage. We hypothesize that HUVECs of premature neonates are metabolically maladapted to birth, which may be responsible for perinatal complications.

Keywords: Energy metabolism; fatty acid oxidation; fetal maturation; fetus; glycolysis; HUVECs; mitochondria; prematurity; respiratory chain.

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Introduction

Alteration of mitochondrial function and biogenesis is often involved in the pathogenesis of human pathologies [31]. Developmental aspects of mitochondrial function during pregnancy are of great importance, since they might be directly related to health problems in preterm neonates and may even have implications for diseases in adulthood [3].

Umbilical cord blood flow is vital for nutritional supply of the fetus and hence has to be tightly regulated. Endothelial cells have an important role in regulating umbilical vascular blood flow. This prompted us to study energy metabolism in human umbilical vein endothelial cells (HUVECs) from mature and premature neonates. Both anaerobic glycolysis and oxidative phosphorylation with fatty acids or glucose as substrates are involved [7, 15]. Long-chain fatty acids are abundantly available in maternal plasma during the last trimester of pregnancy as maternal lipid metabolism switches to a catabolic state [13]. Recently, developmental changes in the expression and activity of fatty acid β-oxidation enzymes at various gestational ages have been demonstrated in the human placenta and fetus [21, 25]. It is assumed that adequate glucose supply and conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase results in inhibition of carnitine palmitoyltransferase 1 (CPT1), thus limiting long-chain fatty acid oxidation (FAO) [24].

We measured activities of mitochondrial respiratory chain (RC) enzymes, FAO enzymes, some glycolytic enzymes, and levels of energy-rich phosphates in HUVECs from preterm and term neonates. Neonatal acylcarnitine profiles were analyzed postpartum.

In previous studies, capacities of oxidative phosphorylation have been shown to increase with gestational age in different cadaveric tissues of human fetuses and neonates [14, 20]. For Ethical reasons, umbilical cord is the only viable tissue available from human neonates.

As HUVECs play an important role in regulating fetal blood supply, metabolic pathways in HUVECs may be regulated differently from other fetal tissues.

Materials and methods

Healthy pregnant women were included in the study. The cohort was divided into preterm (<37 weeks of gestation) and term neonates (≥37 weeks of gestation). The study was approved by our local medical Ethics review board (September 2005). Mothers gave written informed consent prior to enrolment.

HUVECs were harvested from fresh human umbilical cord vessels within 1–5 days of delivery. Umbilical cords were kept dry in 50-mL tubes at 4°C until isolation of HUVECs was started. Isolation and culturing of HUVECs were performed as described in detail elsewhere [16]. Split three to five was used for further experimentation. Purity of the HUVECs cultures was assessed by flow cytometric analysis [16]. Microscopic visual assessment of cultured HUVECs did not show differences between preterm and term cells.

Overall FAO was measured using previously described methods with some modifications: Cultured intact HUVECs were incubated at 37°C with medium containing ^{14}C-labeled and unlabeled palmitic acid. The reaction was terminated by adding perchloric acid followed by the collection of $^{14}CO_2$ and the quantification of ^{14}C acid-soluble products [32, 34].

The activity of carnitine palmitoyltransferase (CPT2) was measured as previously described with some modifications by the incubation of broken HUVECs with labeled and unlabeled palmitoylcarnitine at 37°C. Substrate (^{14}C-palmitoylcarnitine) and product (^{14}C-palmitoyl-CoA) were separated by DAES filter paper, and the product was quantified by radiometry [33].

Cell preparation for spectrophotometric and luminometric assays was done according to procedures described in detail elsewhere [16]. Spectrophotometric determination of enzyme activities was performed at 37°C; luminometric assays were carried out at room temperature.

Activities of complexes I+III were determined by spectrophotometry with slight modifications of the method described by Fischer et al. [10] using rotenone as specific inhibitor at a wavelength of 340 nm.

Activities of complexes II+III were determined by spectrophotometry at 550 nm using antimycin A as inhibitor [29].

Activity of complex IV (cytochrome c oxidase) was assayed by spectrophotometry at a wavelength of 550 nm [35].

Mitochondrial ATP synthase (complex V) activity was measured by spectrophotometry at 340 nm as oligomycin-sensitive ATPase in the direction of ATP hydrolysis as previously described [8, 27].

The citrate synthase (CS) assay was carried out by spectrophotometry at 232 nm [28].

Enzyme activities of lactate dehydrogenase (LDH), hexokinase (HK), and phosphofructokinase (PFK) were measured by spectrophotometry in cell homogenates at 340 nm by following the production or disappearance of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), respectively [5].

An aliquot of cell homogenate supplemented with EDTA was placed in an equal volume of ice-cold dimethylsulfoxide [23]. High-energy phosphates were measured via bioluminescence using a Luminoskan TL Plus luminometer (Fa. Thermo-Labsystems, Helsinki, Finland) with a commercially available luciferin/luciferase assay. ATP was determined based on methods described previously [26]. ADP was measured using pyruvatekinase and AMP with myokinase as coupling enzyme [11].

Protein contents were assayed according to Bensadoun and Weinstein [4].

All enzymatic measurements were done at least in duplicate. Intra-assay and inter-assay variation were typically about 5%–10%.

Blood samples were collected on S&S 2992 filter paper (Schleicher and Schuell, Dassel, Germany) by heel prick or venous puncture. Samples for newborn screening were all collected according to rules and regulations for routine newborn screening in Germany. Sample preparation for tandem mass spectrometry was done as previously described [6]. Infantile acylcarnitine profiles did not show evidence of metabolic diseases.

Tissue culture materials were from PAA (Coelbe, Germany), Sarstedt (Nuembrecht, Germany), Promocell (Heidelberg, Germany), and Nunc (Langenselbold, Germany), and scintillation vials were from PerkinElmer Life Sciences (Rodgau-Juegesheim, Germany). DAES filter paper was obtained from Whatman (Dassel, Germany). Chemicals and enzymes used were purchased from Sigma-Aldrich and Fluka (Deisenhofen, Germany), Merck (Darmstadt, Germany), Dako Cytomation (Hamburg, Germany), Partec (Muenster, Germany), PerkinElmer Life Sciences, La Roche (Mannheim, Germany), Invitrogen (Darmstadt, Germany), and Promega (Mannheim, Germany).

Statistical analyses were performed with SPSS statistical software version 17.0 (SPSS, Chicago, IL, USA). Mean values of parallel measurements were treated as a single measurement for statistical analyses.

In vitro and *in vivo* data from preterm and term neonates were compared by two-factorial analysis of variance [factors are (1) group: preterm vs. term neonates and (2) sex: boys vs. girls]. For presentation, mean values of term neonates were set to 100%. Mean values of preterm neonates were expressed in percent of term mean values ± SD. For correlations, Pearson bivariate correlation coefficients (r) were calculated. P-values <0.05 were considered statistically significant.

Results

Our prospective study included material from 25 preterm (<37 weeks of gestation) and 21 term infants (≥37 weeks of gestation) from uncomplicated pregnancies (Table 1).

In vitro parameters

In HUVECs, activities of RC complexes II+III ($P=0.029$), IV ($P=0.04$), V ($P=0.001$), and mitochondrial marker enzyme CS ($P=0.002$) were higher in preterm neonates, whereas differences of complex I+III were not significant (Figure 1). Ratios of RC complexes to CS were unaffected (data not shown).

Table 1 Demographic data of the cohort.

Group	Gestational age (weeks)	Female (n)	Male (n)	Birth weight (g)	Total (n)
Preterm infants	31.7 ± 4.5 (23.1–36.9)	16	9	1686 ± 804 (485–3080)	25
Term infants	38.9 ± 1.25 (37.1–42.0)	10	11	3389 ± 552 (2330–4460)	21

Values are means ± SDs and ranges (min–max).

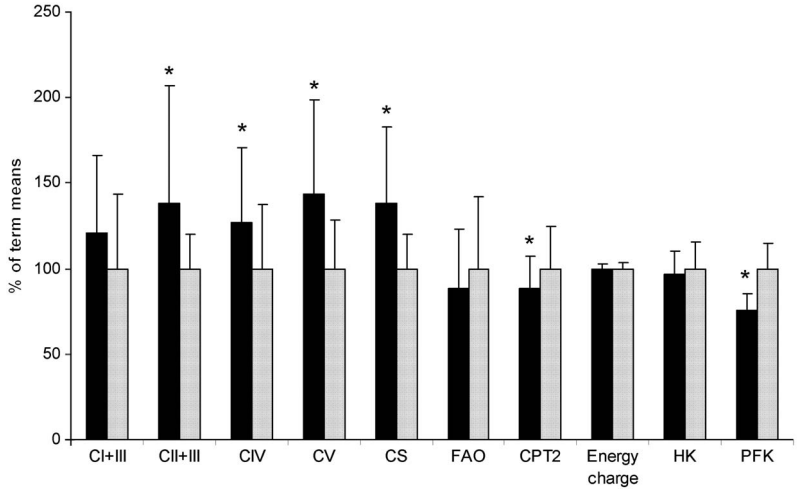

Figure 1 Comparison of relative mitochondrial and glycolytic enzyme activities/energy charges in HUVECs from preterm vs. term neonates. Mean values of term neonates are set to 100% (gray bars). Mean values of preterm neonates (black bars) are expressed in percentage of term means ± SD. *P-values <0.05 were considered statistically significant. RC complexes I + III, *II + III, *IV, and *V: n = 15 term and 24 preterm infants; *CS: n = 15 term and 24 preterm infants; global FAO: n = 14 term and 21 preterm infants; *CPT2: n = 15 term and 13 preterm infants; energy charge: n = 21 term and 25 preterm infants; HK: n = 5 term and 4 preterm infants; *PFK: n = 5 term and 4 preterm infants.

Overall FAO was essentially unchanged between both groups, whereas CPT2 activity was higher in term neonates ($P = 0.04$, Figure 1).

Within the whole cohort (i.e., preterm plus term infants) activities of complexes II + III ($r = -0.32$; $P = 0.045$), IV ($r = -0.44$; $P = 0.005$), V ($r = -0.55$; $P < 0.001$), and CS ($r = -0.65$; $P < 0.001$) decreased with gestational age in HUVECs (Figure 2A–D), whereas activities of overall FAO and CPT2 were not significantly linked to maturation. Regarding correlations of these parameters to gestational age

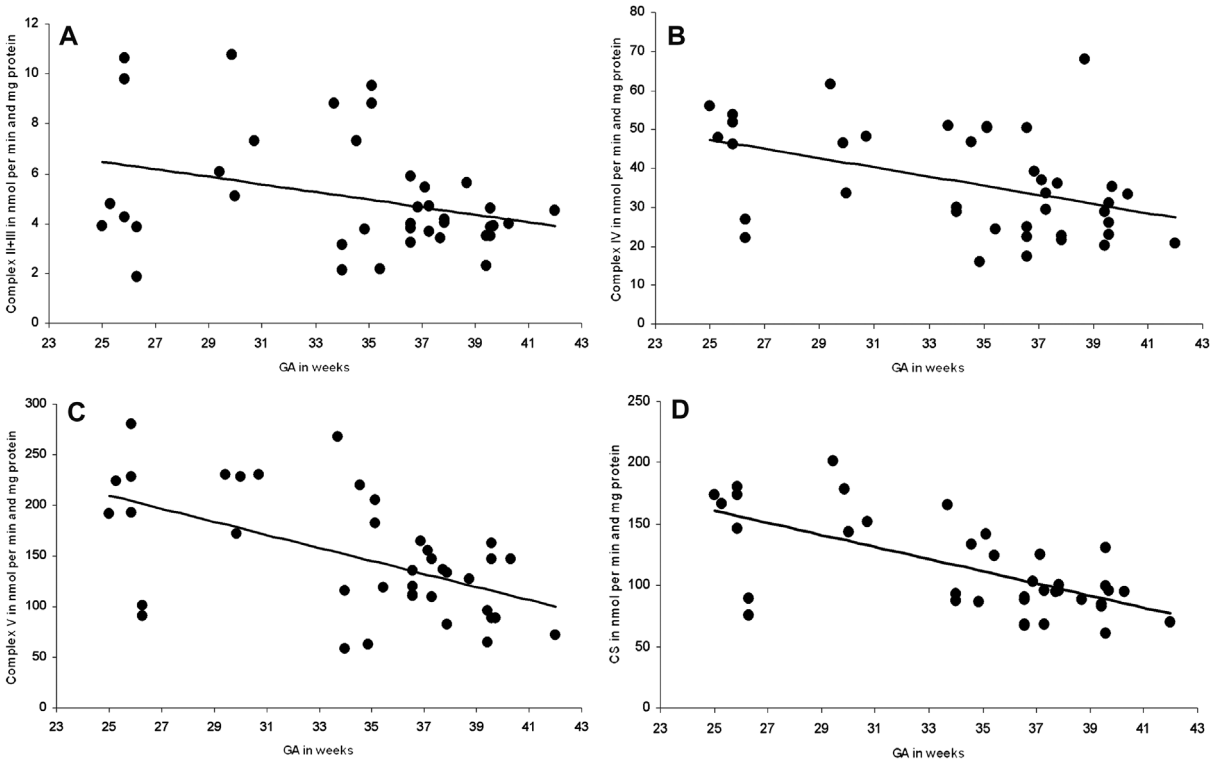

Figure 2 Correlations of RC complexes: (A) II + III ($r = -0.32$, $P = 0.045$), (B) IV ($r = -0.44$, $P = 0.005$), (C) V ($r = -0.55$, $P < 0.001$), and (D) CS ($r = -0.65$, $P < 0.001$) (in nmol/min and mg protein) to gestational age (GA, in weeks) in 39 infants.

within the preterm and term cohort separately, no significant associations could be found except activities of CS in HUVECs of preterm ($r = -0.542$; $P = 0.006$) and CPT2 of term infants ($r = -0.675$; $P = 0.006$).

Activities of glycolytic enzymes in HUVECs were not significantly different between preterm and term neonates except for PFK, which was higher in HUVECs from term neonates ($P = 0.002$, Figure 1). PFK correlated positively with gestational age ($r = 0.781$; $P = 0.013$) within the whole cohort. A limiting factor for interpreting statistical analysis from glycolytic enzymes (in particular, LDH activities) is the small number of measurements; results of LDH measurements are not shown.

Energy charge, defined as (ATP + 1/2 ADP)/(ATP + ADP + AMP), did not change with fetal development (Figure 1).

In vivo parameters

Acylcarnitine profiles from 46 neonates were analyzed. Long-chain acylcarnitines (i.e., the sum of C14– to C18–acylcarnitines) were significantly higher in term neonates ($P = 0.017$, Figure 3). These metabolites correlated positively to gestational age in the total cohort ($r = 0.48$; $P = 0.001$; Figure 4) as well as within the preterm cohort ($r = 0.40$; $P = 0.047$). Short- and medium-chain acylcarnitines as well as free carnitine levels were not linked to maturation (Figure 3).

Results of acylcarnitine profiles were without evidence for inborn errors of metabolic diseases.

Discussion

In our study, we demonstrated developmental changes of mitochondrial as well as glycolytic enzyme activities in cultured HUVECs originating from uncomplicated term as well as preterm pregnancies.

Prenatal development of mitochondrial energy metabolism in HUVECs is apparently opposed to increasing RC enzyme activities in samples taken postmortem from different tissues of human fetuses and neonates [14, 20]. In HUVECs, the bulk of ATP is generated via anaerobic glycolysis, although mitochondria contribute considerably to energy generation. Mitochondria in HUVECs may still have other functions apart from energy generation, such as regulation of cytosolic calcium levels and production of nitric oxide (NO). Mitochondrial RC plays an important role in regulating mitochondrial nitric oxide synthase (mtNOS). NO has been shown to be responsible for the vascular tone of the human umbilical vein [19]. Whereas the main part of NO in HUVECs is derived from endothelial NOS, a small proportion is assumed to be produced via mtNOS. mtNOS is regulated by the potential across the inner mitochondrial membrane, with lower potential leading to a reduction in mtNOS activity [30].

Decreasing complex V activity (which is regulated by mitochondrial membrane potential as well [8]) may indicate a reduction of the membrane potential with increasing gestational age; however, no direct measurements of the mitochondrial membrane potential were performed in this study.

The results of our study could be interpreted as an adaptation to metabolic stress during delivery: with increasing gestational age, a continuous fetal weight-related decrease of umbilical blood flow occurs, which results in a reduction of fetal oxygen transport in the final weeks of gestation [18]. However, total umbilical blood flow increases due to growth of the umbilical vein [18]. Therefore, reduced capacity of oxidative phosphorylation makes HUVECs less susceptible to hypoxia during birth as energy production is shifted to

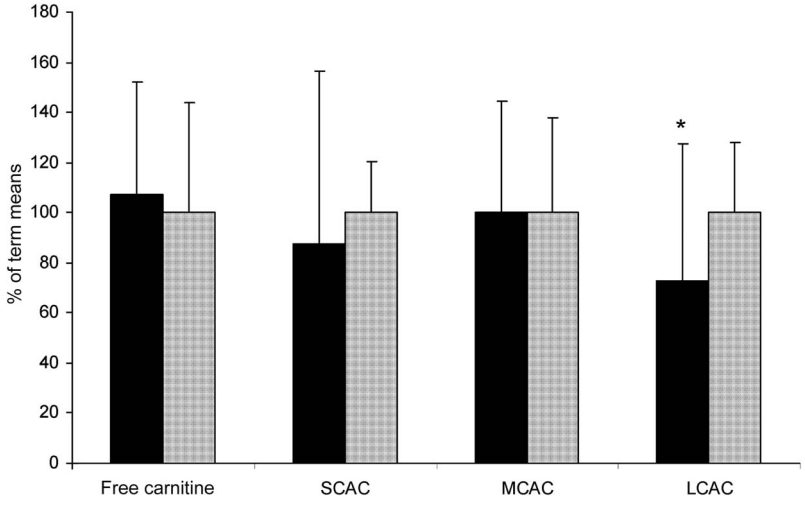

Figure 3 Comparison of relative free carnitine, short-chain (SCAC)-, medium-chain (MCAC), and long-chain (LCAC) acylcarnitine levels in preterm vs. term neonates.

Mean values of term neonates are set to 100% (gray bars). Mean values of preterm neonates (black bars) are expressed in percentage of term means ± SDs. *P-values < 0.05 were considered statistically significant ($n = 21$ term and 25 preterm infants). SCAC = sum of C2-, C3-, C4-, C5-, C5:1-, and C5-OH-carnitines; MCAC = sum of C6-, C8-, C10-, C10:1-, C12-, and C12:1-carnitines; *LCAC = sum of C14-, C14:1-, C14-OH-, C14:2-, C16-, C16-OH-, C16:1-, C16:1-OH-, C18-, C18:1-, C18:1-OH-, C18:2-, and C18:2-OH-carnitines.

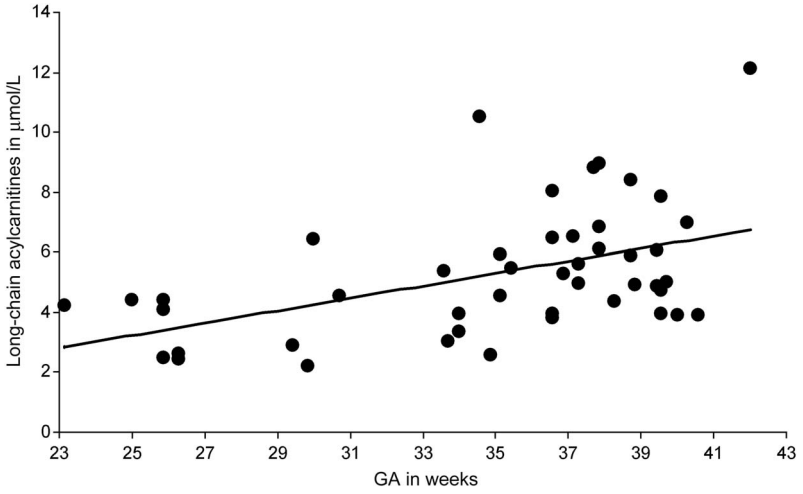

Figure 4 Correlations of long-chain acylcarnitines (µmol/L) to gestational age (GA, in weeks) in 46 infants (r = 0.48, P = 0.001).

anaerobic metabolism. High glycolytic activity may diminish mitochondrial reactive oxygen species and lipid peroxide formation, especially during hypoxia.

Finally, NO is known to be a competitive inhibitor of complex IV [12]. If mtNOS were down-regulated toward the end of pregnancy, mitochondrial NO production is reduced, thus leading to disinhibition of complex IV. This mechanism may protect HUVECs from reperfusion damage during birth by allowing electrons from the RC to be transferred to oxygen.

Following this concept, the umbilical vein of premature neonates is metabolically maladapted to perinatal metabolic stress, which may be responsible for postpartum complications via generation of free electrons and radicals. Electron microscopy of umbilical vein walls from term and preterm neonates revealed that the numbers of necrotizing endothelial cells were higher in preterm than in term neonates [2], which may be related to incomplete adaptation of premature mitochondria in HUVECs as described above.

As FAO did not change during gestation in our study, RC activity is not rate-limiting for FAO in HUVECs, at least *in vitro*. Developmental changes in the expression and activity of FAO enzymes as well as the capacity of carnitine biosynthesis at various gestational ages have been demonstrated in the human placenta and fetus [21, 22, 25]. In HUVECs, active regulation of mitochondrial FAO could be found, suggesting that fatty acids can be a major energy fuel in endothelial cells as well [7].

In our experiments, activities of overall FAO in HUVECs remained substantially unchanged during gestation, whereas CPT2 activity was higher in term infants and decreasing during ongoing gestation within the term cohort. Maturational processes of FAO enzymes are relevant *in vivo* as judged by neonatal acylcarnitine profiles. In line with decreasing CPT2 activities in term neonates, long-chain acylcarnitines increased with maturation *in vivo*.

The importance of intact FAO in the human embryo and fetus is underpinned by several studies in long-chain FAO defects in which a higher frequency of prematurity, intrauterine growth retardation, fetal morbidity, and intrauterine death has been noted [21]. Recently, we demonstrated that pre-eclampsia and HELLP syndrome are associated with impaired mitochondrial function in HUVECs, even in the absence of inborn errors of FAO [16].

In HUVECs, we could show that several mitochondrial enzymes, both mitochondrial and nuclear encoded, were influenced by maturation. Molecular regulation of mitochondrial differentiation processes in HUVECs during the last months of pregnancy may involve an induction of mitochondrial biogenesis, nuclear and mitochondrial gene expression as well as the regulation of both nuclear- and mitochondrial-encoded proteins [1, 9, 20]. However, little is known about the kinetics of mitochondrial assembly during human embryonic and fetal life, especially in HUVECs. In rats with intrauterine growth retardation, altered expression of the peroxisome proliferator-activated receptor gamma coactivator one was shown, which is an important regulator of mitochondrial energy metabolism and which may also play a role in HUVECs and regulation of umbilical vascular tone and blood flow [17].

Up-regulation of glycolytic enzymes during maturation in HUVECs is in line with the hypothesis that endothelial cells generate most of their ATP by anaerobic glycolysis and that increased glycolytic flux could result in the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase, resulting in the inhibition of CPT1 impairing long-chain fatty acid uptake and oxidation [24]. In line with this hypothesis, long-chain acylcarnitines increased with gestational age, whereas PFK, as a regulator of glycolysis, gained function. Accompanied by an impaired carnitine shuttle system, activities of CPT2 declined with maturation in our term cohort.

Our approach (fetal HUVECs in a cell culture model) has some important limitations: reasons for prematurity are supposed to be multifactorial. Preterm neonates, even from apparently uncomplicated pregnancies, are ‘‘pathological

controls'' by definition and the preterm cohort is heterogeneous regarding the etiology of prematurity.

Results of our study demonstrate that energy-providing pathways of oxidative phosphorylation in HUVECs undergo developmental changes. This may be an adaptive process to protect the umbilical vein from damage due to perinatal metabolic stress. Thus, HUVECs and umbilical vein of premature neonates are metabolically maladapted, which may be responsible for peripartum complications and morbidity.

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