Expression of NO synthases and redox enzymes in umbilical arteries from newborns born small, appropriate, and large for gestational age

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BACKGROUND: Modified expression of nitric oxide synthases (NOSs) and an imbalance between the pro-oxidative and the antioxidative system accompany endothelial dysfunction, the first stage of atherosclerosis. Humans born small (SGA) or large (LGA) for gestational age are at higher risk of developing atherosclerosis later in life than humans born appropriate for gestational age (AGA). We hypothesized that indicators of endothelial dysfunction could be detectable at birth. The purpose of this study was to find out whether the expression patterns of NO synthases (endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS)), pro-oxidative enzymes (components of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, NADPH oxidase 1 (NOX1), NOX2, NOX4, p22phox, and p47phox), and antioxidative enzymes (superoxide dismutase 1–3 (SOD1–3), glutathione peroxidase 1 (GPX1), and catalase) in umbilical arteries differ among SGA, LGA, and AGA newborns.

METHODS: Thirty-six umbilical cords were obtained from healthy, normal, full-term SGA, AGA, and LGA newborns. The umbilical arteries were dissected and homogenized. mRNA expression was analyzed with quantitative real-time PCR. Western blotting was performed to determine protein expression.

RESULTS: mRNA and protein expression of NO synthases, pro-oxidative enzymes, and antioxidative enzymes did not differ in the umbilical arteries from newborns of the three groups.

CONCLUSION: Indicators of endothelial dysfunction in terms of differences in enzyme expression in SGA or LGA newborns vs. AGA newborns were not present at birth.

The endothelium is thought to play a pivotal role in the development of atherosclerosis, and endothelial dysfunction seems to be a primary phenomenon of atherosclerosis (1). Humans born small (SGA) and large (LGA) for gestational age are at higher risk of developing atherosclerosis later in life than those born appropriate (AGA) for gestational age (2–5). The endothelial dysfunction is detected early in childhood and persists in these children into adult life (3).

Endothelial dysfunction, as the first stage of atherosclerosis, is due to loss of nitric oxide (NO) function, reduced expression of NO synthases (NOSs), a preponderance of the pro-oxidative system, and a deficit of the antioxidative defense.

Loss of NO function is associated with atherosclerosis (6,7). Three distinct isoforms of NOSs—neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (8)—synthesize NO (9). In human umbilical vessels, all three isoforms are expressed (10,11).

Free radicals, reactive oxygen species (ROS), and reactive nitrogen species are highly reactive molecules (12–14). ROS produced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases promote the development of endothelial dysfunction and atherosclerosis. The neutrophil NADPH oxidase (NOX) contains the following subunits: glycoprotein 91 phagocyte oxidase (gp91phox; NOX2), p22phox, p47phox, and p67phox (15). After the discovery of phagocyte oxidase, further isoforms were discovered: the vascular oxidases NOX1, NOX2, and NOX4. NOX1 is weakly expressed in endothelial cells and is more frequently expressed in smooth muscle cells. NOX4 is the most expressed isoform in endothelial cells. The NADPH oxidases are upregulated in the course of the development of atherosclerosis (15). ROS may react with NO released by eNOS, leading to an uncoupling of eNOS (16). Increase in ROS production can cause oxidative stress, which may result in tissue injury. Excessive production of ROS leads to damage of essential proteins and activation of inflammation (16).

Antioxidants protect the body from damage caused by free radicals (17). The superoxide radical is eliminated by superoxide dismutases (SODs), which catalyze its conversion into hydrogen peroxide plus oxygen. Hydrogen peroxide, in turn, is removed by catalases, which convert it into water and oxygen, and by glutathione peroxidases, which reduce it to water (18). In humans, three distinct types of superoxide dismutases have been described: the cytosolic copper- and zinc-containing Cu/Zn-SOD (SOD1), the mitochondrial manganese-containing MnSOD (SOD2), and the extracellular EC-SOD (SOD3). They all catalyze the same reaction and do so with comparable efficiency (18,19). Cellular glutathione peroxidase (GPX1) is the most abundant intracellular isoform of the GPX antioxidant enzyme family. GPX is a selenocysteine-containing protein that protects against oxidant
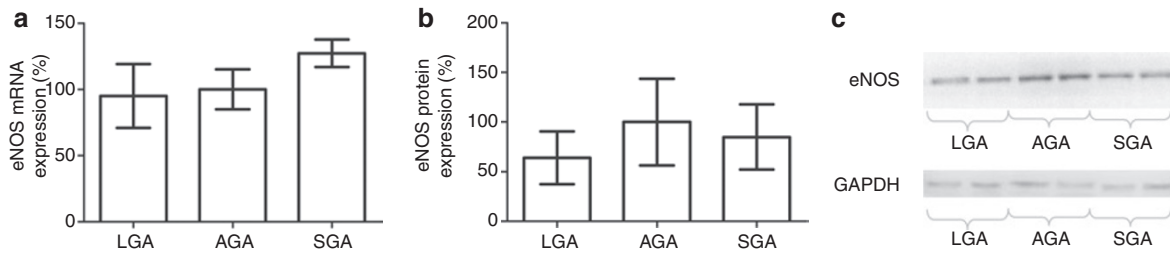
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Figure 1. Expression of eNOS mRNA and eNOS protein. (**a**) Expression of eNOS mRNA in umbilical arteries from newborns born SGA, AGA, and LGA. eNOS was analyzed with qRT-PCR. Columns represent mean ± SEM. Mean values obtained from AGA newborns were used as respective controls. (**b**) Expression of eNOS protein in umbilical arteries from newborns born SGA, AGA, and LGA. eNOS was analyzed with western blot using a monoclonal anti-eNOS antibody. Results of densitometric analyses are shown. Columns represent mean ± SEM. Mean values obtained from AGA newborns were used as respective controls. (**c**) Representative blot of eNOS protein expression in umbilical arteries from newborns born SGA, AGA, and LGA. AGA, appropriate for gestational age; eNOS, endothelial nitric oxide synthases; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LGA, large for gestational age; qRT-PCR, quantitative reverse transcription PCR; SGA, small for gestational age.
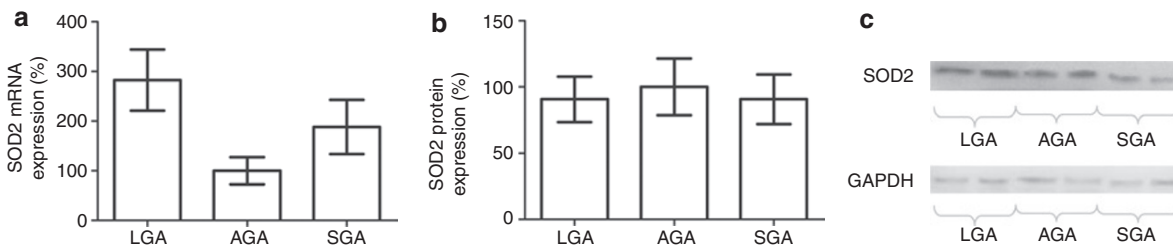
Figure 2. Expression of SOD2 mRNA and SOD2 protein. (**a**) Expression of SOD2 mRNA in umbilical arteries from newborns born SGA, AGA, and LGA. SOD2 was analyzed with qRT-PCR. Columns represent mean ± SEM. Mean values obtained from AGA newborns were used as respective controls. *P* < 0.05. (**b**) Expression of SOD2 protein in umbilical arteries from newborns born SGA, AGA, and LGA. SOD2 was analyzed with western blot using a polyclonal anti-SOD2 antibody. Results of densitometric analyses are shown. Columns represent mean ± SEM. Mean values obtained from newborns were used as respective controls. (**c**) Representative blot of SOD2 protein expression in umbilical arteries from newborns born SGA, AGA, and LGA. AGA, appropriate for gestational age; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LGA, large for gestational age; qRT-PCR, quantitative reverse transcription PCR; SGA, small for gestational age; SOD, superoxide dismutase.

stress by utilizing reduced glutathione (GSH) to reduce hydrogen peroxide (20). Furthermore, catalases are part of the antioxidative defense. The overall reaction catalyzed by catalases is the degradation of two molecules of hydrogen peroxide into water and oxygen (21). A deficit in the antioxidative defense leads to a predominance of ROS and the pro-oxidative system, thereby promoting endothelial dysfunction.

In the pathogenesis of atherosclerosis, endothelial dysfunction is one of the first discernible disorders. An open question is at what earliest time point endothelial dysfunction can be detected. We therefore investigated in this study the expression patterns of NO synthases (eNOS, iNOS, and nNOS), pro-oxidative enzymes (NOX1, NOX2, NOX4, p22phox, and p47phox), and antioxidative enzymes (SOD1, SOD2, SOD3, GPX1, and catalase) in umbilical arteries. We wanted to find out whether they differ between SGA, LGA, and AGA newborns. The aim was to detect indicators of endothelial dysfunction at birth.

RESULTS

Expression of mRNA and protein of the isoforms of NO synthases (eNOS, iNOS, and nNOS), pro-oxidative enzymes (components of NADPH oxidases: NOX1, NOX2, NOX4, p22phox, and p47phox), and antioxidative enzymes (SOD1, SOD3, GPX1, and catalase) were similar in umbilical arteries from newborns born SGA or LGA as compared with newborns

born AGA. Representative examples of the analysis (eNOS and SOD2) are depicted in **Figures 1** and **2**.

There was only a significantly higher mRNA expression of SOD2 in umbilical arteries from LGA newborns as compared with mRNA expression of SOD2 in umbilical arteries from AGA newborns. However, this difference was not confirmed by analysis of the SOD2 protein expression.

In the mRNA expression of nNOS in all groups, the mean of the threshold cycle values was above 35 cycles. Therefore, the amount of RNA was regarded as negligible. In contrast, in western blot analysis of nNOS, we detected protein in all groups. mRNA expression of iNOS could be detected in all groups. However, in the protein analysis by western blot, the amount of protein in all groups turned out to be so low that densitometric analysis was not possible.

DISCUSSION

In 1989, Barker put forth the hypothesis of fetal programming (22). Fetal programming occurs when the normal pattern of fetal development is disrupted by an abnormal stimulus in the *in utero* development (23). In the present study, we did not find differences in mRNA and protein expression of NO synthases and of the pro-oxidative and antioxidative enzymes investigated in umbilical arteries from newborns born SGA and LGA as compared with umbilical arteries from newborns born

AGA. We found no evidence for an effect of fetal programming in terms of factors promoting endothelial dysfunction.

Isoforms of NOS

In the beginning of the development of atherosclerosis, there is an upregulation of eNOS expression, whereas a downregulation of eNOS expression is found in later stages (24). In the present study, we did not find any differences in the expression of eNOS in umbilical arteries from newborns born SGA and LGA as compared with newborns born AGA. The data published by Hracsko *et al.* support the assumption that NO and the NO synthases play important roles in fetomaternal circulation and that intrauterine growth retardation occurs by disturbances in fetomaternal blood flow. In contrast to our experiments, Hracsko *et al.* (25) showed an increase of eNOS mRNA and also of eNOS protein in umbilical arteries of newborns born SGA in comparison with controls.

In the present study, mRNA expression of nNOS could not be demonstrated, whereas in western blot analyses, the expression of nNOS protein could be detected. In our laboratory, the primer used has proven successful in other experiments. Thus, a methodological problem appears to be ruled out. However, the detection of protein without a detection of mRNA cannot be resolved here. nNOS protein may be more stable than nNOS mRNA. Our results are consistent with the findings of Schönfelder *et al.* in this regard (11). Schönfelder *et al.* detected the mRNA expression of nNOS only in the human umbilical vein, not in umbilical arteries. In contrast to our study, they could not find a specific nNOS immunoreactive band in western blot analyses of umbilical arteries (11).

In the present study, the expression of iNOS mRNA could be proven, but no iNOS protein could be detected. Our results are consistent with the findings from Kleinert *et al.* in human cardiomyocytes (26), in which iNOS mRNA, but not protein, was also detectable.

Pro-Oxidative Enzymes: The Family of NOX

To our knowledge, little is known about NOX expression in tissue samples of the human umbilical arteries. Previous studies were based on experiments with human umbilical artery endothelial cells. Among others, Taye *et al.* investigated protein expression of the NOX subunits NOX2 and p47phox in human umbilical artery endothelial cells. They examined the influence of ROS production induced by hyperglycemia in these cells. Their results showed that high glucose concentrations significantly enhanced the protein expression of the NOX subunits (27). In the present study, no difference could be found in NOX expression in tissue samples comprising all layers of human umbilical arteries from SGA and LGA as compared with AGA newborns.

Antioxidative Enzymes: SOD, GPX, and Catalase

We investigated the expression of the antioxidative enzymes SOD1, SOD2, SOD3, GPX1, and catalase in human umbilical arteries from SGA and LGA newborns as compared with AGA newborns. A significant increase in mRNA expression of SOD2 in umbilical arteries was observed in LGA newborns as compared with AGA newborns. This difference was not confirmed by analysis of the SOD2 protein expression. The reasons for this are unclear. Expression of the other investigated antioxidative enzymes did not differ between the three groups. To our knowledge, the expression of antioxidative enzymes has not been investigated in human umbilical arteries. Thus, the present study systematically characterizes the expression of antioxidative enzymes in human umbilical arteries for the first time. Previous data with respect to antioxidative defense of newborns are based on animal models and observations from other tissues, such as lung tissue. Moreover, previous studies mostly compared antioxidative defense between full-term newborns and premature infants. Markers of oxidative stress may be present in higher concentrations in newborns than in adults (28). In general, there is a lower antioxidative activity in the blood of newborns as compared with the blood of adults (29). Our results are partially consistent with the findings of Lee and Chou. These authors investigated the activity of antioxidative enzymes in the venous blood of newborns. Matching our own results, they also could not find any difference in the activity of SOD. Lee and Chou found higher levels of catalase expression in the venous blood of SGA infants than in the venous blood of AGA infants. Results of our study do not point in the same direction (30).

Indicators of Endothelial Dysfunction

One aim of the present study was to detect indicators of endothelial dysfunction at birth. In children aged between 4 and 16 y, Reinehr *et al.* (31) noted that overweight children born SGA have a doubled risk of developing atherosclerosis in the context of a metabolic syndrome as compared with overweight children born AGA. Thus, previous research indicates that endothelial dysfunction may be detectable later in childhood. However, according to our results, factors that favor endothelial dysfunction are not detectable immediately after birth. Other endothelial markers, or rather markers of endothelial dysfunction, and therefore, atherosclerosis that we have not tested for might be altered, e.g., the von Willebrand factor (1), oxidized low-density lipoprotein (32,33), and expression adhesion molecules (selectins, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1) (34). Moreover, another study design, with paired isolation of whole vessel samples and human umbilical artery endothelial cells, could be more beneficial in further studies to detect endothelial specific changes.

Conclusion

In conclusion, our study demonstrates no differences in mRNA expression or protein content of NO synthases, pro-oxidative enzymes (NOX1, NOX2, NOX4, p22phox, and p47phox), and antioxidative enzymes (SOD1, SOD2, SOD3, GPX1, and catalase) in umbilical arteries from newborns born SGA and LGA as compared with umbilical arteries from newborns born AGA. This study does not confirm fetal programming of the expression patterns of the mentioned enzymes. Other intrauterine factors may have an impact on fetal programming (e.g., smoking during pregnancy leading to an increase of homocysteine (35) and placental mitochondrial dysfunction (36)) (37).

Table 1. Characteristics of the newborns from whom the umbilical cords were obtained

	Gestational age (wk)	Birth weight (g)	
	Median (range)	Median (range)	Sex
LGA	38 (37–41)	4,020 (3,720–4,410)	6 Girls, 6 boys
AGA	38 (37–41)	3,290 (2,670–3,920)	5 Girls, 7 boys
SGA	38 (37–41)	2,590 (2,160–3,020)	4 Girls, 8 boys

AGA, appropriate for gestational age; LGA, large for gestational age; SGA, small for gestational age.

Socioeconomic and life-style factors may contribute to the appearance of endothelial dysfunction as the first stage of atherosclerosis, in that they may cause both intrauterine growth restriction and overweight in childhood, and, therefore, the development of a metabolic syndrome (38–40).

METHODS

Vessel Dissection/Sample Preparation
The study was approved by the local ethics committee (Ethik-Komission Landesärztekammer Rheinland-Pfalz). Parental consent was given in each case. Thirty-six human umbilical cords were obtained from healthy, normal full-term deliveries after uneventful pregnancies, born by primary cesarean section. Umbilical cord pH-values were within the normal range (pH: 7.22–7.42) in all cases. The newborns were divided into three groups (with each group containing 12 newborns): AGA (birth weight within the normal range), SGA (birth weight below the 10th percentile), and LGA (birth weight above the 90th percentile). Additional characteristics of the newborns are summarized in **Table 1**. Umbilical arteries were identified by the macroscopic aspects (wall thickness, lumen size, and consistency). After careful preparation on ice, umbilical artery samples were immediately delivered into liquid nitrogen and stored at −80 °C.

Real-Time Reverse Transcription PCR for mRNA Expression Analyses
Total RNA was extracted using the peqGold Total RNA Kit from peqLab Technologies (Erlangen, Germany), following the manufacturer's instructions, and then stored at −80 °C until use. The concentration and purity of total RNA were assessed spectrophotometrically at 260 and 280 nm. Complementary DNA was synthesized from total RNA using the High Capacity cDNA RT Kit from Applied Biosystems (Darmstadt, Germany), according to the manufacturer's instructions. The final reaction volume was 20 μl. The reaction mixture was incubated at 25 °C for 10 min, then at 37 °C for 120 min, and the reverse transcription was terminated by heating the mixture to 85 °C for 5 min. To analyze the expression level of the enzymes of interest by quantitative real-time PCR, SYBR Green was used and the PCR was performed with the iCycler iQ System from Bio-Rad Laboratories (München, Germany). The reaction mixture contained 40 ng complementary DNA diluted in 4 μl diethylpyrocarbonate-treated water, 10 μl SYBR Green Super Mix from Bio-Rad Laboratories, and 0.4 μl gene-specific primers (10 picomol) in a final reaction volume of 20 μl. The housekeeping gene *GAPDH* was used as a control to normalize the values. Threshold cycle values were used for quantitative reverse transcription PCR analysis.

Western Blot for Protein Analyses
For western blotting, samples of the umbilical arteries were homogenized by using the homogenizer Precellys24 from peqLab Laboratories. Proteins of 50 μg were separated by SDS-polyacrylamide gel electrophoresis (15 or 7.5% gels). The proteins were transferred to a PVDF membrane from Millipore (Schwalbach, Germany) by electroblotting. Blots were blocked for 60 min at room temperature in Tris-buffered saline (consisting of 10 mmol/l Tris HCl (pH 7.6) and 154 mmol/l NaCl) containing 5% (wt/vol) nonfat dry milk and 0.05% (wt/vol) Tween 20. Membranes were then incubated overnight at 4 °C with the first antibody

(characteristics summarized in **Supplementary Tables S1** and **S2** online) in Tris-buffered saline containing 5% (wt/vol) bovine serum albumin (for eNOS, p47phox) or 5% (wt/vol) nonfat dry milk (for nNOS, iNOS, p22phox, NOX1, gp91phox, catalase, GPX1, SOD1–3, and GAPDH) and 0.1% (wt/vol) Tween 20. After three washes with Tris-buffered saline containing 0.1% (wt/vol) Tween 20, the blots were incubated for 60 min at room temperature with a rabbit anti-mouse, goat anti-rabbit, or bovine anti-goat peroxidase–conjugated second antibody. After three washing steps with Tris-buffered saline containing 0.1% (wt/vol) Tween 20, bands were developed using Western Lightning chemiluminescence reagent from PerkinElmer (Rodgau, Germany) and visualized using the Imager ChemiDoc XRS from Bio-Rad Laboratories according to the manufacturer's instructions. Optical densities of proteins were normalized to GAPDH protein as a control.

Statistical Analyses
The evaluation of the relative gene expression data from the PCR analysis was carried out by the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (41). The housekeeping gene *GAPDH* was used as a control to normalize the values. Gene expression of the AGA newborns was used as reference.

Protein expression of the target gene was studied by western blot analyses. The bands of target proteins were quantified by densitometry and normalized to the corresponding GAPDH bands. The protein levels were normalized to the AGA control.

Results are expressed as mean ± SEM. ANOVA was used followed by Dunnett's test for multiple comparisons to a single control group. Differences were considered statistically significant at $P < 0.05$.

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