Endothelial Nitric Oxide Synthase Is Up-regulated in the Umbilical Cord in Pregnancies Complicated with Intrauterine Growth Retardation

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Abstract. *Background: NO and NO synthases (NOS) play an important role in the physiology of the fetomaternal blood circulation, although their expression in pathological conditions is unclear. Intrauterine growth retardation (IUGR) is a disorder most probably caused by abnormality of the fetomaternal bloodflow. Materials and Methods: The expression of endothelial NOS (ecNOS) from arteria umbilicalis and the nitrite and peroxynitrite level of umbilical blood were determined. Major consequences of peroxynitrite toxicity are lipid peroxidation and glutathione depletion; these parameters were also measured. Finally, superoxide dismutase (SOD) activity was assayed to evaluate the level of superoxide anions. Results: Elevated expression of ecNOS was found to be coupled with significantly lower SOD activity and glutathione level, and increased lipid peroxidation in IUGR neonates. Conclusion: The increased NO indices could represent a compensatory effort to improve placental bloodflow, but in IUGR neonates it is coupled with inadequate antioxidant defence, resulting in significant oxidative stress.*

Nitric oxide (NO) and NO synthases seem to play an important role in the physiology of the fetomaternal blood circulation, although their expression in pathological conditions is unclear. NO has diverse functions *in vivo*. It is fundamental in signaling *via* the production of cGMP by guanylate cyclase (1). The production of NO is also important for nonspecific immune response (2). NO modulates blood flow, thrombosis and neural activity (3, 4).

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NO also serves many important physiological roles in the regulation of cardiac function including coronary vasodilation, inhibiting platelet and neutrophil adhesion and activation, modulation of cardiac contractile function, and inhibiting cardiac oxygen consumption (5, 6). Most of the cytotoxicity attributed to NO is due to peroxynitrite. It is produced in a diffusion-controlled reaction between NO and the free radical, the superoxide anion (7). Peroxynitrite interacts with lipids, DNA and proteins *via* direct oxidative reactions or indirect, radical-mediated mechanisms. *In vivo*, superoxide is rapidly removed by high concentrations of superoxide dismutases (SOD), so that the level of peroxynitrite under physiological condition is controlled and oxidative damage is minimized by endogenous antioxidant defenses. Strict control is needed since peroxynitrite is a strong oxidant which can react directly with electron-rich groups, such as sulfhydryls (8), causing depletion in the glutathione (GSH) pool.

Another mechanism of peroxynitrite-dependent cytotoxicity relies on its ability to trigger lipid peroxidation (LP) in membranes (9), causing membrane permeability and fluidity changes, with significant biological consequences. The red blood cell (RBC) membrane structure exhibits a special shape and elasticity. Human RBCs are normally biconcave disks, but when exposed to stress, they are known to undergo deformation reversibly. In response to external forces, the RBCs are deformed, but recover their resting shape when the forces are removed (10). If the integrity of the membrane is damaged, RBCs lose this shape and elasticity and may undergo hemolysis, which sets back normal fetal development. Hemoglobin binds not only O_2, but also NO, which plays an important role in blood flow regulation (11). *In vivo*, the measurement of thiobarbituric acid (TBA)-reactive substances, such as malondialdehyde (MDA) which is one of the products of LP, has been used as an index of LP in pathological conditions. The traditional TBA method for the determination

of LP is not sufficiently specific for RBC membranes: the method detects not only the products of membrane LP, but also all the molecules which react with TBA. The concentration of MDA correlates with oxidative lipid damage. TBA reacts not only with MDA, but also with other aldehydes from the intracellular area.

In an abnormality such as intrauterine growth retardation (IUGR), which is most probably a consequence of a disorder of the fetomaternal blood circulation, clarifying the role of NO is reasonable. A fetus or a newborn with IUGR weighs less than 90% of all other fetuses or newborns of the same gestational age. The reported incidence of IUGR ranges between 3 and 10% . This prevalent neonatal disorder is associated with an increased level of morbidity and mortality. The intrauterine environment has long-term effects on subsequent health and survival of neonates. Studies demonstrated a relationship between low birth weight and later development of cardiovascular disease (12), impaired glucose tolerance (13), higher systolic blood pressure and triglyceride levels (14). Moreover, impaired growth in infancy and rapid childhood weight gain exacerbate the effects of impaired prenatal growth.

The aim of this study was to clarify the role of NO and nitrosative stress in the pathophysiology of IUGR. We determined the nitrite and peroxynitrite level of umbilical blood and the expression of endothelial NO synthase (ecNOS) from the umbilical artery. Because major consequences of peroxynitrite toxicity are lipid peroxidation and glutathione depletion, these parameters were also measured and confirmed by hemorheological tests. In addition, the activity of SOD was assayed to estimate the level of superoxide anions. In order to determine the lipid damage to the membranes specifically, we prepared erythrocyte ghosts and applied the TBA method only to the membranes. To confirm the results, we determined the RBC deformability by filtration tests in IUGR and control groups.

Materials and Methods

Sample collection. The blood samples were obtained from the Department of Obstetrics and Gynecology at the Medical University of Szeged, Hungary. The Ethics Committee of the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Szeged approved the study protocol. Twenty-nine full-term neonates of either sex, born between weeks 37 and 40 were selected, 17 of them with normal weight (3,450±550 g) and 12 with symmetrical IUGR (weight 2,000±350 g). In the studied group, there was a multiple birth case: one of the twins was of normal weight (twin A) and the other with IUGR (twin B). The pH of the blood samples was in all cases in the range 7.24-7.39, *i.e.* there was no hypoxic stress on the tissues. Newborns that had a history of difficult delivery and fetal distress, or showed malformations or evidence of genetic disorders were excluded. Hypoglycemia that occurred among the IUGR babies was compensated by a 10% glucose infusion. The nutritional status of the mothers during pregnancy was satisfactory; no case of

malnutrition occurred. The mothers received complete pregnancy care. Smoking mothers and their newborns had a higher concentration of the carbonyl group, lipid peroxides and less total antioxidant capacity. Newborns from these mothers weighed significantly less than others at birth (15). Consequently, the smoking mothers and their newborns were excluded from this study.

Blood was taken from the umbilical artery of the placenta before birth in both groups. Coagulation was inhibited by EDTA. The duration of storage was as short as possible, with a maximum of a week. The blood samples were kept at –20°C until processing. The blood samples were centrifuged at 1000×g for 10 min and the plasma and the buffy coat were removed. The RBC phase was washed twice with 2 volumes of isotonic saline solution at pH 7.0. The RBCs were hemolysed by the addition of distilled water at a ratio of 1:9. Except for SOD activity determinations, the aliquots of the hemolysates were used directly. A Thermo Spectronic Biomate 5 was used for the spectrophotometric measurements.

For studying ecNOS expression, small pieces of the umbilical artery were used. They were cut from the umbilical cord after the blood sample collection, without any storage, and were processed immediately since RNA degradation is very fast.

RNA extraction, reverse transcription and PCR amplification. Approximately 100 mg of umbilical artery was homogenized in TRI reagent (Sigma-Aldrich, Steinheim, Germany) and total RNA was prepared according to the procedure suggested by the manufacturer.

To follow the expression of *ecNOS* gene, semi-quantitative RT-PCR was performed using *β-actin* as an internal control. First-strand cDNA was synthesized using 5 μg total RNA as template. The RNA was denatured at 95°C, mixed with 200 pmol of each dNTP (Fermentas, Vilnius, Lithuania), 200U M-MuLV reverse transcriptase (Fermentas) and 500 pmol random hexamer primers in a final volume of 20 μl and incubated for 10 min at 37°C, followed by 45 min at 42°C.

A total of 1 μl of reverse transcription product was added to 49 μl of PCR reaction mixture containing 250 μM dNTP, 50 pmol primers, 1× PCR buffer/MgCl$_2$ and 5 U Taq polymerase (Fermentas). Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA). The number of amplification cycles during which PCR product formation was limited by the template concentration was determined in pilot experiments: for *β-actin* this was 25 cycles and for ecNOS it was 32 cycles at 95°C for 30 s, 60°C for 40 s and 72°C for 60 s. The amplified products were electrophoresed on 1.8% agarose (Sigma) gel.

For the *β-actin* mRNA used as internal reference, 25 cycles at 95°C for 30 s 60°C for 40 s and 72°C for 60 s were used. The relative level of ecNOS was expressed as a ratio of that to *β-actin* (*ecNOS/β-actin*×100).

The sequences of primers were derived from GenBank entry M24113 for *β-actin* (forward: 5'gcaagagaggtatcctgacc3' and reverse: 5'ccctcgtagatgggcacagt3'), and NM_000603 for ecNOS (forward: 5'gacattgagagcaaagggctg3'and reverse: 5'tgcggcttgtcacctcctgg3') Images of ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analyzed with the GelBase/GelBlot™ Pro Gel Analysis Software (Ultra Violet Products Ltd. Upland, CA).

Nitrite and peroxynitrite assay. Plasma NO concentrations were determined using Griess reagent (Sigma) according to the procedure suggested by the manufacturer.

Peroxynitrite was assayed by diluting aliquots of blood samples into 1.0 M NaOH and measuring the increase in absorbance at 302 nm. As a control, samples were added to 100 mM potassium phosphate (pH 7.4). The decrease in absorbance was measured at neutral pH as peroxynitrite decomposes (16).

SOD activity assay. Before the determination of SOD activity (17), the hemolysates were treated with ethanol:chloroform (2:1) to remove hemoglobin from the samples and were then centrifuged at 3000×g. The supernatants were used for SOD activity determinations *via* inhibition of the epinephrine-adrenochrome transformation. The control sample contained 2.9 ml of 0.05 M carbonate buffer (pH 10.2, warmed to 37˚C) and 0.1 ml of epinephrine (16.5 mg/10 ml 0.1 N HCl). The absorbance was measured at 480 nm for 3 minutes after a one-minute delay. The rate of autoxidation of epinephrine (ΔA at 480 nm/min) was determined. To measure the inhibition of autooxidation by SOD a mixture containing 2.875 ml of the carbonate buffer, 0.025 ml supernatant and 0.1 ml epinephrine was used. Spectrophotometric measurement was carried out at 480 nm. The results were expressed in U/mg protein.

The total quantity of protein was determined with Folin reagent, using bovine serum albumin as standard (18).

GSH measurement. The GSH in the plasma and RBCs was measured using Ellman's reagent (DTNB). Proteins were precipitated with 5% trichloroacetic acid (TCA) in order to omit protein-linked -SH groups from the measurement (19).

LP assay. LP of the plasma and RBCs was determined using the TBA method (20) which reveals the level of total TBA-reactive substances, with slight modifications. Calibration was performed with MDA. A volume of 2.7 ml of TBA reagent solution (a mixture of 0.375% TBA, 0.25 M HCl, 15% TCA) was added to 0.3 ml of the hemolysed blood sample, the components were mixed vigorously and the mixture was heated for 15 min in a boiling water-bath. Subsequently, the samples were cooled in ice-cold water and centrifuged at 3000×g for 10 min; the absorbance of the supernatant at 532 nm was measured spectrophotometrically to calculate concentrations. The LP of the RBC membranes was also measured by preparing erythrocyte ghosts (21).

Deformability tests. For rheological (deformability) tests, 5% hematocrit suspensions were prepared with PBS buffer at pH 7.4. Measurements were performed with an M-100 filtrometer (Mikron, Budapest, Hungary; constructed on the basis of the St. George filtrometer) using a Nucleopore filter (Nucleopore Corp., USA) with a diameter of 5 μm. A computer coupled to the filtrometer calculated the relative filtration speed (F_i), the mean cell transit time (Tc) and the clogging rate (CR) (13).

Statistical analysis. Statistical differences were calculated with one-way analysis of variance (ANOVA) (MedCalc Statistical Software version 9.4.2.0. Broekstraat, Belgium) with a Student–Newman–Keuls *post-hoc* test. Significant difference was accepted at $p<0.05$. The reported values are means±SD.

Results

Expression of ecNOS. RT-PCR was performed on total RNA extracted from the umbilical artery of IUGR and control neonates. The expression of ecNOS in the control samples
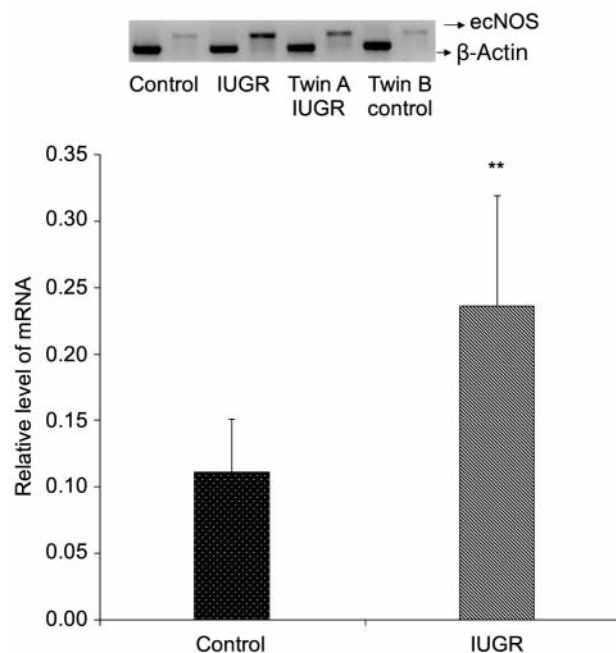

Figure 1. *Detection of ecNOS mRNA from the umbilical artery of IUGR (n=12) and control (n=17) samples. Four representative experiments are presented. The difference was significant at **$p<0.01$.*

was very low (Figure 1). The specific mRNA level varied between undetected (n=7) and around 11% of that of the β-actin transcript (n=10). *ecNOS* mRNA was detectable in all IUGR samples (n=12). The induced expression level measured in neonates with IUGR showed individual variation, with *ecNOS/β-actin* ratio between 0.3-0.6.

Nitrite, peroxynitrite and SOD activity. When both superoxide and NO are present close to each other and in abundance, they will combine spontaneously to form peroxynitrite. Our results show significantly higher serum nitrite and peroxynitrite levels in the IUGR group; moreover, SOD activity was significantly lower in this group which indicates an elevated level of superoxide (Figure 2).

LP assays. Based on the findings that peroxynitrite is a major initiator of membrane damage and rapid oxidation of GSH, we determined the level of LP and GSH in umbilical blood samples of IUGR and control neonates. Not surprisingly, a the level of peroxynitrite was higher in the IUGR group, we also found a significant increase in the level of LP.

Lipid peroxidation of whole red blood cells and purified membrane fractions of erythrocytes were determined (Figure 3). LP in the control group was 0.049 nmol MDA/mg protein, while that in the IUGR group was 0.1064 nmol
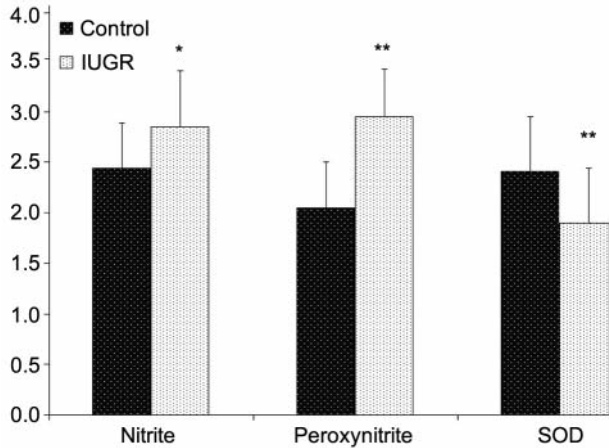
Figure 2. *The level of nitrite (nmol/ml) and peroxynitrite (nmol/mg protein) and the activity of SOD (EU/mg protein) in the IUGR (n=12) and control (n=17) group. Differences were significant at *p<0.05, **p<0.01.*

Figure 3. *The level of LP in RBC and RBC membrane fraction (LP ghost) of IUGR (n=12) and control (n=17) umbilical cord blood samples. Differences were significant at **p<0.01, ***p<0.001.*

MDA/mg protein. The corresponding LP data for the erythrocyte ghost were 7.44 nmol MDA/ml in the control group and 9.97 nmol MDA/ml in the IUGR group.

Deformability test. Deformability tests were applied to RBC suspensions to confirm the results of the LP assay. The relative filtration speed, the cell transit time and the clogging rate were determined by filtrometer. The results of filtration measurements demonstrated the same tendencies (Figure 4). The Fi rate was 0.66 μl/s, in the control group and 0.492 μl/s in the IUGR group; T_c was 9.56 s/cell in the control and 11.69 s/cell in the IUGR neonates, while CR was $5.04×10^{-3}$ pores/μl in the control and $3.14×10^{-3}$ pores/μl in the IUGR newborns. Cells that cross the test membrane relatively quickly have an intact membrane with adequate fluidity, thus our results show that RBC membranes were altered in IUGR neonates.

GSH level. As the level of peroxynitrite was higher in the IUGR group, we found a remarkable diminution in the GSH level (Figure 5). The level of GSH was 6.22 nmol/mg protein and 2.39 nmol/mg protein in the control and IUGR groups respectively.

Discussion

IUGR is a neonatal disorder most probably caused by abnormality of the fetomaternal blood flow. An important finding of our present study is the different level of mRNA expressions of *ecNOS* observed in IUGR and control samples. We detected that *ecNOS* expression in the umbilical artery in IUGR neonates was least 2-fold greater than that of the control group.
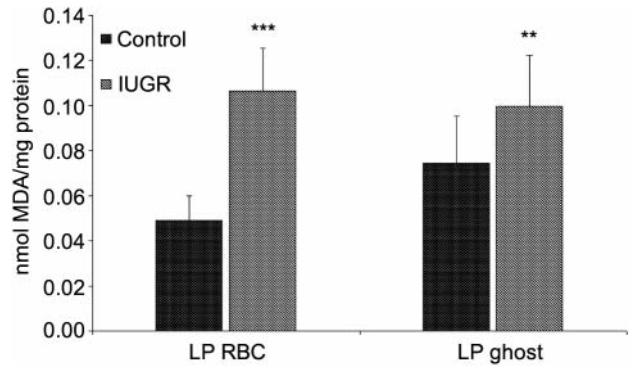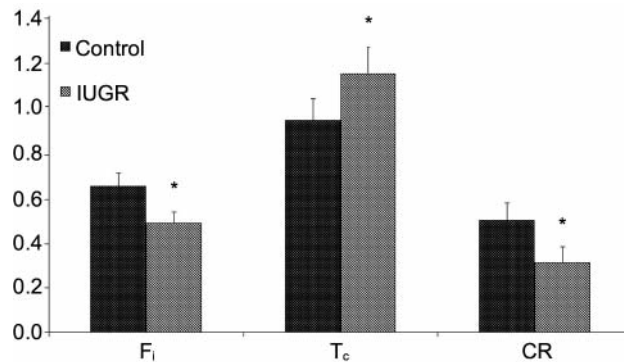

Figure 4. *Rheological parameters Fi (relative filtration speed, μl/s), Tc (cell transit time, s/cell), and CR (clogging rate, pores/μl) of red blood cells of IUGR (n=17) and control neonates (n=12). Differences were significant at *p<0.05.*
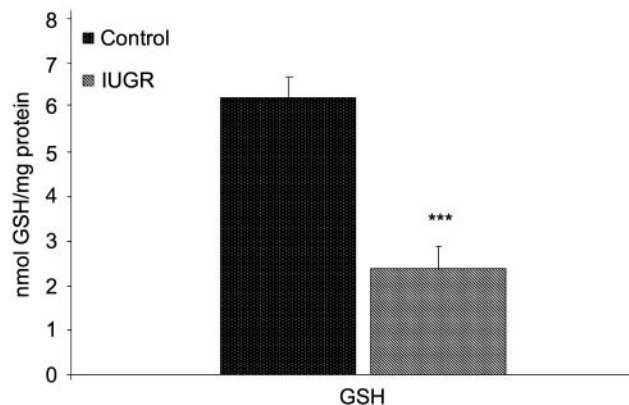

Figure 5. *The level of glutathione (GSH) in IUGR (n=12) and control (n=17) umbilical cord blood samples. The differences were significant at ***p<0.001.*

It is known that NO is generated from the metabolism of arginine by NOS. It diffuses from endothelial cells to underlying smooth muscle, where it activates the enzyme guanylate cyclase and so increases cGMP production leading to vascular relaxation. In the human fetomaternal circulation, NO appears to contribute to the maintenance of low vascular resistance (22).

Our result of elevated ecNOS expression in IUGR concurs the studies demonstrating an increase in ecNOS or NO production in the fetoplacental circulation of IUGR fetuses. Lyall *et al*. (23) determined NO concentrations by measuring indices of NO production in human umbilical venous blood from IUGR and normal. They found in pregnancies complicated by IUGR, umbilical venous plasma had significantly higher nitrite and nitrate levels. Myatt *et al*. (24) used a semiquantitative immunohistochemical technique to show that basal levels of ecNOS were higher in placental tissue in pregnancies complicated by IUGR or by both preeclampsia and IUGR.

In addition, we also determined the level of nitrite, peroxynitrite, LP, GSH and SOD activity to provide more detail regarding this question. We found that in IUGR neonates, elevated expression of ecNOS is coupled with reduced SOD activity. SOD is responsible for superoxide removal. The deficiency of this activity increases superoxide levels. NO reacts very fast with superoxide, forming the highly toxic and stable peroxynitrite. It interacts with biomolecules, especially lipids and GSH, *via* direct oxidative reactions. Our results of GSH depletion and elevated LP confirm these suggestions. GSH is one of the most important antioxidants. It plays essential roles in antioxidant defence, as well as in maintenance of the intracellular redox state.

Lipid peroxides may decrease membrane fluidity, inactivate membrane-bound receptors and enzymes, and increase the membrane permeability. The integrity of erythrocyte membranes is especially important because it is crucial for their transfer function.

We conclude that the increase in NO indices could represent a compensatory effort to improve placental blood flow and decrease the peripherial pressure, but in IUGR neonates, it is coupled with inadequate antioxidant defense, resulting in significant oxidative stress. This oxidative overload then plays important roles in the pathogenesis of the fetal origins of adult disease. Severe oxidative stress can modify the expression of genes in both differentiated and pluripotential cells, which leads to disturbances in cell proliferation and maturation. The fetus also adapts to an inadequate redox status by metabolic changes, redistribution of blood flow and changes in the production of fetal and placental hormones which control growth. Accordingly, an oxidative insult in the intrauterine milieu influences both growth and development of the fetus and the subsequent development of adult diseases.

Adequate fetoplacental circulation is indispensable for the proper development of the fetus and the increased production of NO in IUGR neonates does help to improve this. However, NO is still a problem for these fetuses, as it enhances oxidative stress in the umbilical cord. Nevertheless, our findings indicate a good potential for therapeutic approaches. Adequate antioxidant therapy, after confirmation of these findings through intervention studies, may decrease the indices of oxidative stress arising from increased NO production.

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