Reproductive Toxicology 53 (2015) 10–14

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Major differences in the levels of redox status and antioxidant defence markers in the erythrocytes of pre- and full-term neonates with intrauterine growth restriction

Ágnes Ferencz[a], Hajnalka Orvos[b], Edit Hermesz[a,*]

[a] Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, P.O. Box 533, H-6701 Szeged, Hungary
[b] Department of Obstetrics and Gynaecology, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary

ARTICLE INFO

Article history:
Received 30 September 2014
Received in revised form 16 February 2015
Accepted 17 February 2015
Available online 24 February 2015

Keywords:
Antioxidant defence
IUGR
Oxidative stress
Peroxynitrite
Umbilical cord blood

ABSTRACT

Intrauterine growth restriction (IUGR) is a pleiotropic complication of pregnancy. Prematurity and growth abnormalities are common risk factors for perinatal morbidity and mortality. Free radical damage has been recognized as a common pathogenic mechanism of many neonatal diseases. The aim of the present study was to characterize the possible links between the level of maturity, the birthweight and the antioxidant status of neonates born with IUGR. Our data suggest that the stress markers measured on the cord blood of neonates with IUGR and mature, healthy neonates do not necessarily reflect the extent of oxidative stress. However, significant correlations were found between the maturity of the neonates with IUGR and the oxidative damage. The mature IUGRs exhibited $ONOO^-$ accumulation and increased lipid peroxidation more frequently as compared with the pre-term group. The results suggest that the oxidative injury in IUGR may depend on the level of maturity and the birthweight.

1. Introduction

Pregnancy is a physiological state associated with an enhanced metabolism and an increased demand for oxygen. Premature infants are at particular risk from oxidative stress, as neither the endogenous nor the passively acquired exogenous antioxidant defence system accelerates in maturation until late in the third trimester [1,2]. Intrauterine growth restriction (IUGR) is one of the major complications of pregnancy and accounts for significant neonatal mortality and morbidity [3]. The development of IUGR may be a consequence of various factors, including an abnormal fetomaternal blood circulation, genetic disorders, pregnancy-induced hypertension, pregestational diabetes, a lean umbilical cord, previous intrauterine infections, a poor nutritional state of the mother, frequent cigarette smoking [4] and toxin or drug exposure [5]. However, in the vast majority of the cases the cause remains idiopathic.

IUGR is often complicated by intrauterine hypoxia and may induce the generation of reactive oxygen species (ROS) and foetal oxidative stress, resulting in serious consequences for the foetus, such as low birthweight and prematurity [6]. Neonates with IUGR are more susceptible to ROS-induced oxidative damage because their enzymatic and non-enzymatic antioxidant defence systems and the ability to undergo induction during a hyperoxic challenge are impaired [7].

To eliminate the harmful effects of ROS, cells are equipped with an efficient antioxidant defence system, including enzymes such as superoxide dismutase (SOD), catalase (CAT), hemeoxygenases (HOs), and low-molecular weight antioxidants such as glutathione (GSH) and metallothioneins (MTs) [8,9]. SOD catalyses the reduction of the superoxide anion ($O_2^{\bullet}-$) to hydrogen peroxide (H_2O_2). In a subsequent step, CAT stimulates the degradation of H_2O_2 to molecular oxygen and water [10]. The MTs are small proteins with unusually high cysteine content. This confers their high metal-binding and ROS-reducing properties [11]. The HOs play roles in heme degradation, yielding equimolar quantities of biliverdin, carbon monoxide (CO) with important free radical-scavenging properties and free Fe ions. In mature neonates, a considerable proportion of the plasma total antioxidant capacity originates from bilirubin [12]. HO-2 is a constitutive, and HO-1 an inducible isoform of HO, an antioxidative, anti-inflammatory and cytoprotective enzyme that is induced in response to cellular stress, including oxidative stress [13].

Nitric oxide (NO) may be an important factor for the regulation of blood pressure and oxygen delivery to the foetus [14]. Umbilical cord blood vessels lack innervations, and endothelial cells must

* Corresponding author. Tel.: +36 62 544887; fax: +36 62 544887.
E-mail address: hermesz@bio.u-szeged.hu (E. Hermesz).

http://dx.doi.org/10.1016/j.reprotox.2015.02.008
0890-6238/

Table 1
Clinical parameters of the study groups and the maternal age. Data are expressed as means ± SD. The minimum and the maximum values of the parameters are given in parentheses.

	Full-term neonates with normal weight	Full-term neonates with IUGR	Pre-mature neonates with IUGR
Gestational age at delivery (weeks)	39.2 ± 0.77 (38–40 + 2)	38.74 1.31 (37–40+4)	34.01 ± 1.62 (30–36)
Birth weight (g)	3409 ± 455 (3190–4340)	2354 238.8 (2090–2490)	1518 ± 460.5 (980–2120)
The pH of blood samples	7.25 ± 0.11 (7.04–7.42)	7.24 0.088 (7.1–7.36)	7.20 ± 0.142 (7.05–7.3)
1 min APGAR	8.83 ± 1.37 (6–10)	8.36 1.65 (6–10)	7 ± 1.63 (4–9)
Maternal age (years)	29.9 ± 5.74 (22–42)	28.9 6.55 (20–41)	29.3 ± 7.22 (21–42)

therefore play a major role in the local control of blood flow [15]. NO derived from endothelial nitric oxide synthase (eNOS) is considered the main vasodilator agent in fetoplacental vessels [16]. The simultaneous generation of NO and $O_2^{\bullet-}$ in sufficiently high concentrations in the same compartment favours the production of a toxic reaction product, peroxynitrite anion ($ONOO^-$). $ONOO^-$ and other reactive nitrogen species can affect the cell functions through the oxidation or nitration of various cellular targets [17].

Under stress conditions, genes coding for molecules involved in biological defence and cellular repair are markedly upregulated, and the changes in gene expression can be characteristic, sensitive and measurable endpoints [18]. Members of this antioxidant defence system are useful biomarkers of the oxidant–antioxidant status of neonates with IUGR. The aim of the present study was to characterize the possible links between the level of maturity, the birthweight and the antioxidant status of neonates born with IUGR. We report data on macromolecular damage, the accumulation of powerful oxidants such $ONOO^-$, H_2O_2, the activities of the antioxidant enzymes SOD and CAT, and the expressions of a set of genes coding for members of antioxidant defence system (*sod1*, *sod2*, *cat*, *mt-1*, *mt-2*, *ho-1*, *ho-2* and *enos*) from the aspects of the level of maturity and the birthweight of neonates with IUGR.

2. Materials and methods

2.1. Human subjects

The blood samples were obtained from the Department of Obstetrics and Gynaecology at the University of Szeged, Hungary. The Ethics Committee of the Department of Obstetrics and Gynaecology approved the study protocol (149/2012). 24 mature neonates with normal weight and 28 mature and 28 premature neonates with IUGR of either sex were examined. The neonates were considered premature if they were born before the gestational age of 37 weeks and full-term if the delivery occurred after 37 weeks. Newborns that had a history of difficult delivery and foetal distress, or showed malformations or evidence of genetic disorders were excluded. The nutritional status of the mothers during pregnancy was satisfactory; no case of malnutrition occurred. Smoking mothers and their neonates were also excluded from this study.

Blood was taken from the umbilical artery, before the birth of the placenta. Blood coagulation was inhibited by EDTA. The blood samples were centrifuged at 3000 rpm for 20 min at 4 °C, and the plasma and the buffy coat were removed. The red blood cell (RBC) phase was washed twice with 2 volumes of isotonic saline solution at pH 7.0. The samples were stored at −80 °C until processing (Table 1).

2.2. RNA extraction, reverse transcription and PCR amplification

Approximately, 100 mg of frozen RBC were homogenized in RNA Bee reagent (Tel-Test, Inc.) and total RNAs were prepared according to the procedure suggested by the manufacturer. Total RNA was routinely treated with 100 U RNAse-free DNAseI (Thermo Scientific) to avoid any DNA contamination.

For the quantification of *mt-1* and *mt-2*, *sod1* and *sod2*, *cat*, *enos*, *ho-1* and *ho-2* mRNAs, reverse transcription followed PCR amplifications (RT-PCRs) were performed. First-strand cDNAs were synthesized by using 5 μg total RNAs as templates, 200 pmol of each dNTP (Thermo Scientific), 200 U Maxima H Minus Reverse Transcriptase (Thermo Scientific) and 500 pmol random hexamer primers (Sigma) in a final volume of 20 μL, and incubated for 10 min at 37 °C, followed by 1 h at 52 °C. One microliter reverse transcription product was added to 25 μL DreamTaq Green PCR Master Mix 2x (Thermo Scientific). Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research) using 15 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for the 18S rRNA, used as internal reference and 30 cycles for *mt-1* and *mt-2*, *sod1* and *sod2*, *cat*, *enos*, *ho-1* and *ho-2* mRNAs, respectively. The amplified products were detected on a 2% agarose gel. The relative levels of mRNAs are expressed as ratios (mRNA/18S rRNA).

2.3. Primers

The following primer sets were selected: *sod1*: F: aagatggtgtggccgatgtg and R: ctacagctagcaggataacag; *sod2*: F: caaggctcaggttggggttg and R: gctgggatcattagggtagtatg; *cat*: F: cacagaagatggtaactggg and R: ggcgatgtccatctggaatc; *enos*: F: cactgagcccgtggcagtag and R: ggcaggcagcgccaccgacg; *mt-1*: F: atggaccccaactgctcctg and R: gttcccacatcaggcacagc; *mt-2*: F: atggaccccaactgctcctg and R: cggtcacggtcagggttgtac; *ho-1*: F: gctgctggtggcccacgctt and R: ctctggtccttggtgtcatgg; *ho-2*: F: tggcccacgcatacacccgc and R: ggtctctctggccagtgtgga. For the normalization of *sods*, *cat*, *enos*, *mts* and *hos* mRNAs, the level of carp 18S rRNA was used as internal standard, detected with primer pairs F: gaaacggctaccacatccaagg, and R: ccgctcccaagatccaactacg.

2.4. Densitometry

Images of the ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analyzed with the GelBase/GelBlot™ Pro Gel Analysis Software (UVP).

2.5. Enzyme activity measurement

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. The quantity of protein was determined with Folin reagent, using bovine serum albumin as standard [19]. Biomate 5 Double-Beam UV–vis photometer recording (Thermo Spectronic) was used for SOD measurements and GENESYS 10S UV–vis spectrophotometer (Thermo Scientific) was used for all the other parameters.

SOD (EC 1.15.1.1) activity was determined on the basis of the inhibition of the epinephrine–adrenochrome autoxidation [20]. Spectrophotometric measurement was carried out at 480 nm. The results were expressed in U/mg protein.

CAT (EC 1.11.1.6) activity was determined spectrophotometrically at 240 nm by the method of Beers and Sizer [21] and specific