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ORIGINAL PAPER

Heme oxygenase-1 expression in premature and mature neonates during the first week of life

Zoltán Maróti · Márta Katona · Hajnalka Orvos · Ilona Németh · Ildikó Farkas · Sándor Túri

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Abstract Newborns are exposed to mechanical and oxidative stress during labor and to relative hyperoxia thereafter during the course of adaptation to the extrauterine conditions. Part of the adaptation mechanism is the rapid degradation of fetal hemoglobin and the oxidation of its heme moiety by heme oxygenases (HOs). Heme oxygenase-1 enzyme (HO-1) is the inducible isoform, which is induced by and protective against oxidative stress. We hypothesized that HO-1 may play a role in the physiological adaptation of newborns. We therefore measured the HO-1 mRNA expression with cRT-PCR during the first week after birth in healthy mature and premature newborns. We found that HO-1 was induced until day 2 or 3 after birth, but its level had dropped below the birth HO-1 mRNA level by the end of the first week. HO-1 levels and inducibility were similar in mature newborns and premature newborns. The fact that HO-1 was inducible even in gestation week 26 suggests that HO-1 plays an important role in the early adaptation processes.

Keywords Heme oxygenase-1 · Gene expression · Neonatal adaptation · Oxidative stress · cRT-PCR

Z. Maróti (✉) · M. Katona · I. Németh · I. Farkas · S. Túri
Department of Pediatrics, University of Szeged,
Faculty of Medicine,
Albert Szent-Györgyi Medical and Pharmaceutical Center,
Korányi fasor 14-15,
6725 Szeged, Hungary
e-mail: zmaroti@gmail.com

H. Orvos
Department of Obstetrics and Gynecology, University of Szeged,
Faculty of Medicine,
Albert Szent-Györgyi Medical and Pharmaceutical Center,
Szeged, Hungary

Abbreviations

CDS	coding sequence
cRT-PCR	competitive reverse transcriptase polimerase chain reaction
HGB	hemoglobin
HMOX1	heme oxygenase-1 gene
HO-1	heme oxygenase-1 protein
HO-2	heme oxygenase-2 protein
MCV	mean corpuscular volume
PLT	platelet count
WBC	white blood cell count

Introduction

Birth itself causes mechanical and oxidative stress for newborns. All infants, regardless of gestational age, display evidence of oxidative stress during the first few days after birth; this is especially true for premature infants, who have a much lower antioxidant capacity than that of term babies [11, 15]. The switch to aerial breathing, the increased partial O_2 concentration and the stress caused by birth induce many physiological processes, most notably the neonatal adaptation to the greatly changed conditions.

Part of the adaptation is the rapid degradation of fetal hemoglobin and the oxidation of its heme moiety by heme oxygenases (HOs), which are contributing factors in the development of post parturition hyperbilirubinemia [9]. Although free heme can be cytotoxic directly [1], it can also become toxic by mediating oxidative stress and inflammation [4]. HOs catalyze the rate-limiting step in heme degradation, resulting in the formation of free Fe^{2+}, carbon monoxide and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase [8]. Biliverdin and

Fig. 1 Part of *HMOX1* CDS from exon 3 to 5. HMOX1-T3 and HMOX1-mut were used to create a PCR product from which we transcribed our control RNA, the sequence of which differs from that of wild-type *HMOX*1 mRNA in only a 20-bp deletion. The mRNA and control RNA were reverse-transcribed and consecutively amplified by the same primers HMOX1-F and HMOX1-R

bilirubin are themselves potent antioxidants and play a protective role against oxidative stress [2, 10, 20].

Two functional HO isoforms are known in humans, HO-1 and HO-2. HO-2 is the constitutional form, while HO-1 is the form transcriptionally inducible by a variety of agents, such as heme, oxidants, inflammatory cytokines, UV irradiation, heavy metals and arsenite [5, 7]. HO-1 plays a cytoprotective role in oxidative stress and heme-mediated injury [21]. On the other hand, a transiently enhanced HO-1 mRNA accumulation is a reliable marker of oxidative stress [12, 18, 23].

Although HO-1 induction may be a general and adaptive response to oxidant stress, the inducibility of this enzyme in the early postnatal period remains to be established. We hypothesized that the enzymatic immaturity of HO-1 or its regulation system could play a role in the early transitory adaptation disturbances of premature neonates. Accordingly, we set out to investigate the HO-1 expression in mature and premature neonates during the first week after birth in order to estabilish whether the enzyme is inducible and whether there are any differences between the two groups.

Subject and methods

Patients

A study was carried out to investigate the HO-1 mRNA levels in mature and premature neonates during the first week after birth. The two groups of patients consisted of 21 mature (gestation time 37–40 weeks, birth weight median 3,305 g, quartiles 3,060 g, 3,770 g) and 20 premature neonates (gestation time 26–36 weeks, birth weight median 1,860 g, quartiles 1,450 g, 2,230 g) with transient neonatal adaptation difficulties. We excluded babies with respiratory distress, sepsis, positive bacteriology or any serious complication (intracerebral hemorrhage, necrotizing enterocolitis, pneumonia, pneumothorax, or any congenital heart disease). None of the examined patients were on mechanical ventilation (>24 h) and none of them received a blood transfusion during the investigation period.

The indication of phototeraphy was according to the practice written in *Care of the high-risk neonate* [6]. The patients (mature n=5, premature n=7) requiring phototherapy were on intermittent therapy (425–475 nm light/normal light in 4-h intervals). None of the mature neonates had to be supplied with extra O_2. Ten premature neonates with a transitory adaptational disturbance were on extra O_2 (<24 h). Indication of extra oxygen support was determined by PaO_2 values (the PaO_2 levels were kept in the range 40–60 mmHg in prematures with less than 1,500 g birtweight). The median of FiO_2 values in these patients were 0.4 (quartiles 0.3, 0.6).

Sample collection

Whole blood samples were pipetted in EDTA microtainer tubes (approx. 200 μl per sample) from fresh whole blood taken for other necessary routine analysis. For mRNA analysis, 100 μl was pipetted out immediately into 1 ml of RNA stabilization reagent. A quantitative blood count was performed on an ABX Micros 60 hematological automat.

We obtained blood samples from mature newborns in parallel with the routine serum bilirubin assay: usually 2–3 times from each baby during the first 6 days before they left the obstetric ward. We obtained blood samples from premature newborns in parallel with other routine blood parameter analysis each day following birth.

This study was approved by the Scientific Committee and the Ethical Council of Szeged University.

RNA extraction and competitive reverse transcriptase (cRT)-PCR experiments

The mRNA was extracted from 100 μl of venous blood with the mRNA Isolation Kit for Blood/Bone Marrow

Table 1 Blood count parameters of mature and premature neonates (mean±SD)

	Mature neonates n=21			Premature neonates n=20		
	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
Hgb (g/l)	159.6±19.6	155.4±23.1	156.6±29.8	184.8±16.0*	187.0±18.5*	172.7±18.8*
MCV (fl)	110.7±2.9	110.1±4.7	108.4±4.5	109.1±3.8	108.2±3.6	106.2±4.3
PLT (10^9/l)	187.5±84.1	198.7±79.3	243.1±112.6	233.3±67.4*	248.7±56.7*	280.2±96.6*
WBC (10^9/l)	14.75±4.80	9.91±4.01	10.1±4.30	19.3±2.56**	10.83±1.77	10.10±2.23

Student's t-test: *P<0.001; **P<0.005 healthy premature vs mature neonates

(Roche Diagnostics, Mannheim, Germany). Expression of theheme oxygenase 1 gene (*HMOX1*) was identified using cRT-PCR. The competitor RNA was created by in vitro mutagenesis and transcription with T3 RNA polymerase (Fermentas, Vilnius, Lithuania) according to Waha et al. [22]. The primers to generate the competitor were as follows: HMOX1-T3: 5′-AATTAACCCTCACTAAAGG GAGACGTTTCTGCTCAACATCCAGCTC-3′, and HMOX1-mut: 5′-CCTGGGAGCGGGTGTTGAGTGG GGGGCAGAATCTTGCACTTTG-3′. First-strand cDNA was generated by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) with the specific primer of HMOX1-R. The specific oligonucleotide primers applied for the PCR reaction were designed to span exon boundaries, thereby binding only at the cDNA level (Fig. 1). The PCR amplification was carried out with the following program: initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 20 s, annealing at 61°C for 30 s and extension at 72°C for 20 s, followed by a final extension at 72°C for 10 min. The primers were as follows: HMOX1-F: 5′-CGTT TCTGCTCAACATCCAGCTC-3′, and HMOX1-R: 5′-CCTGGGAGCGGGTGTTGAGTG-3′. The amplified cDNAs were examined on 6% polyacrylamide gel and

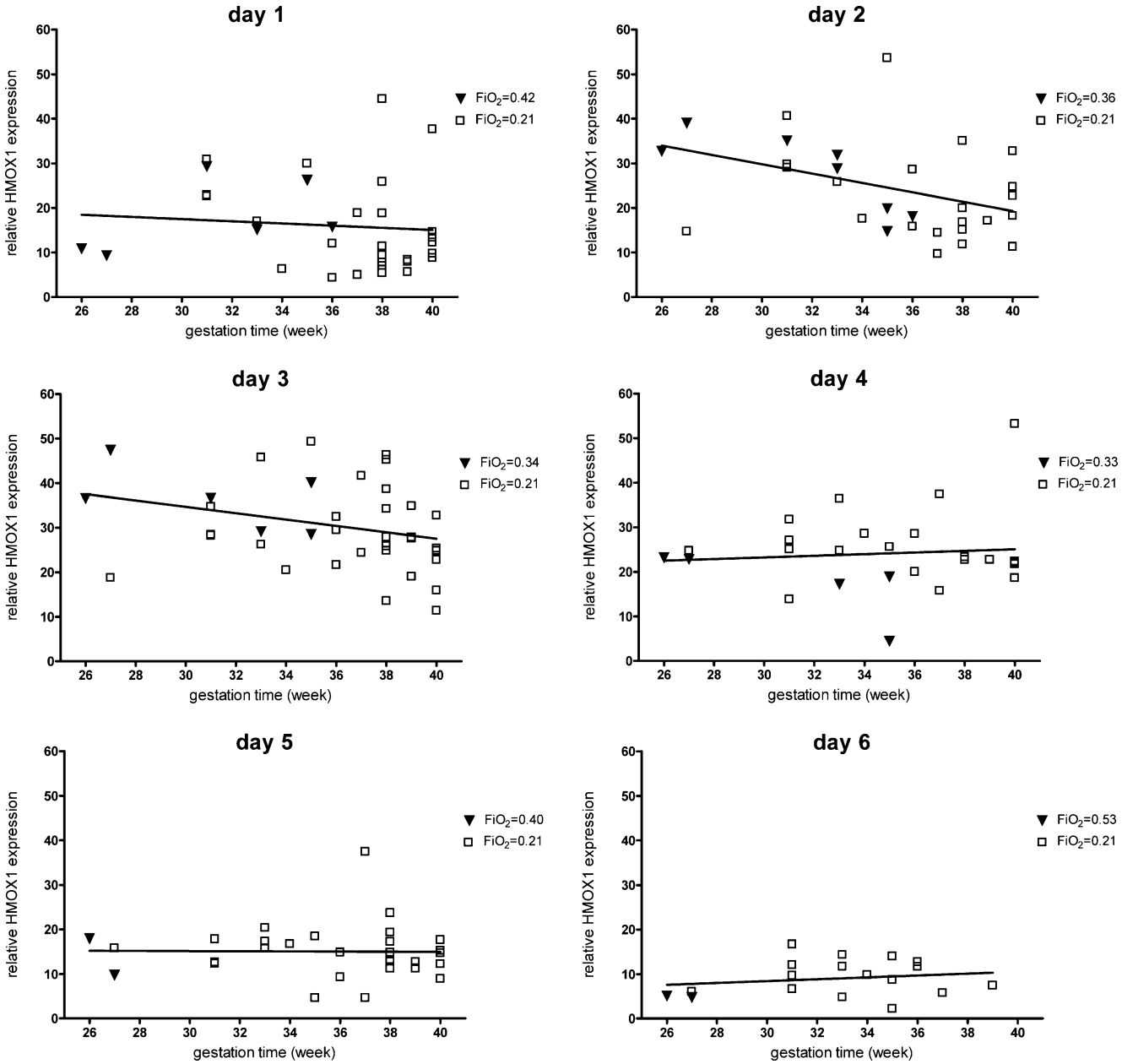

Fig. 2 Relative HO-1 mRNA levels of all examined patients plotted against the gestational age during the first 6 days after birth. The *filled triangles* mark HO-1 expression data of neonates with extra O_2 therapy. The *empty squares* represent babies without extra O_2 therapy. FiO_2 values are shown as medians

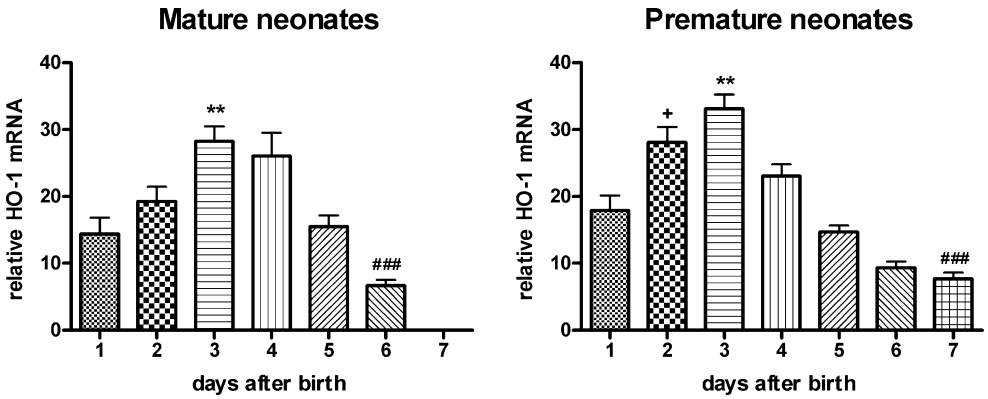

Fig. 3 Relative HO-1 mRNA levels during the first week after birth in healthy mature and premature neonates. Unpaired *t*-test: $^{**}P<0.005$ day 3 vs day 1; $^{\#\#\#}P<0.001$ last day sample vs day 3, $^{+}P<0.05$ 2nd day values of mature vs premature neonates

stained with ethidium bromide. The target HMOX1 band was estimated via the ratio to the competitor by densitometry (AlphaImager™, AlphaEase 5.5). HO-1 mRNA concentrations were expressed with reference to the white blood cell count (WBC); since we did not calculate the copy number of our control RNA, these are relative values (we used the same dilutions of control RNA). Control RNA and mRNA were handled together in the same tubes; their ratio therefore remained the same throughout the whole process.

We established a sensitive cRT-PCR method to measure the mRNA levels of the transcriptionally induced HO-1 from 100 μl of whole blood, which was essential for our experiments because of the limited amount of sample available. We followed the HO-1 expression during the first week after birth in both the mature and the premature newborns.

Statistical analysis

Clinical data on the patients are reported as means ±standard deviations. Statistical analyses included the Kruskal-Wallis test and the Student *t*-test for the analysis of HO-1 expression changes, and the unpaired *t*-test with the Welch correction for analysis of the blood counts of the premature and mature neonates. The level of statistical significance for all tests was taken as $\alpha=0.05$.

Results

The hemoglobin (Hgb), mean corpuscular volume (MCV), WBC, and platelet count (PLT) for the mature and premature newborns were in the normal ranges (Table 1) and their changes during the first week excluded the presence of severe anemia, sepsis or inflammation.

The HO-1 expressions of all examined patients plotted against the gestational age are shown in Fig. 2. The relative HO-1 mRNA levels of the mature and premature neonates plotted against the days passed after birth are shown in Fig. 3. The levels and inducibility of HO-1 in the two groups proved similar although on the second day in premature neonates HO-1 expression was significantly higher than that of mature neonates ($P<0.05$). HO-1 expression was induced on days 2 and 3. Later, the HO-1 mRNA levels decreased and dropped below the day 1 value by the end of the first week. The serum bilirubin levels of the mature and premature neonates are shown in Fig. 4. These levels increased significantly after birth in both groups, and were the highest on day 5 after birth.

Discussion

The degradation of fetal hemoglobin during the adaptation after birth leads to an increased release of free heme, which

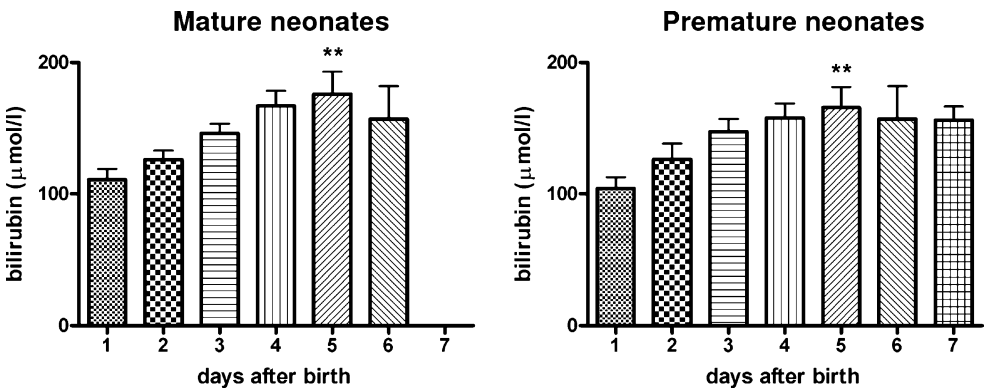

Fig. 4 Bilirubin levels during the first week after birth in healthy mature and premature neonates. Unpaired *t*-test: $^{**}P<0.005$ day 5 vs day 1

is an aggressive oxidative agent. Free heme is a substrate and inductor of its catabolizing enzyme, HO-1, which is also expressed in the lympho-, mono- and granulocytes in blood [13]. HO-1 is transcriptionally induced and we could therefore apply a sensitive cRT-PCR method to measure the increased copy number of HO-1 mRNA. The method is sensitive enough for the quantitative analysis of HO-1 mRNA separated from 100 μl of whole blood.

The one *HMOX1*-deficient patient described in the literature suffered from growth failure, anemia, tissue iron deposition, lymphadenopathy, leukocytosis, and increased sensitivity to oxidant injury. He ultimately succumbed to a premature death [24]. In the animal model of HO-1 deficiency, mice lacking the gene frequently die in utero [14], and those surviving to term display a phenotype similar to the HO-1-deficient boy. It is also suggested that HO-1 activity is required for iron reutilization in mammals [14] for the prevention of abnormal iron accumulation in hepatic and renal cells, which otherwise contributes to oxidative damage, tissue injury and chronic inflammation. Clearly, HO-1 is necessary to the survival of organisms.

It is suggested that even the full-term healthy infants experience oxidative stress, which resolves only with age [3]. In our study, we measured an elevated HO-1 expression level in both mature and premature newborns immediately after birth which could be sign of oxidative stress. As the HO-1 levels increased during the following days (Figs. 2, 3), we can conclude that HO-1 is inducible in this early stage in both premature and mature neonates. The fact that the induction of HO-1 mRNA and its maximum precede the maximal bilirubin levels demonstrates that HO-1 is functional and cleaves free heme to biliverdin, CO and Fe^{2+}.

It has been shown that in species in which hemolysis takes place post parturition, the activity of HO is enhanced in the early newborn period [9]. The present data are the first on HO-1 expression in humans after birth, our data also showed similar induction pattern as observed in animals [17]. We showed that the inducibility of HO-1 does not depend on the gestational age (Fig. 2). Though many enzymes show enzyme immaturity in premature neonates the two groups of patients did not exhibit differences in HO-1 expression pattern. Our study indicates that healthy premature neonates (without any organ manifestation of oxygen radical injury) are prepared to handle the released heme by the induction of the HO-1 system during neonatal adaptation.

Oxygen radical injury is a common pathogenic mechanism in a number of neonatal diseases, including idiopathic respiratory distress syndrome, retinopathy of prematurity, bronchopulmonary dysplasia, subependymal and intraventricular hemorrhage and necrotizing enterocolitis [15, 16]. These disorders display a higher incidence in preterm infants with deficient antioxidant protective systems [19]. Our study suggests that healthy premature neonates can handle the the increased heme released during neonatal adaptation. The question arises of whether the disturbance of HO-1 inducibility plays a role in premature newborns with “oxygen radical disease of prematurity”.

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