Hypoglycemia and nitro-oxidative stress in the neonatal period

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Abstract

Nitro-oxidative stress, i.e. peroxynitrite generation and subsequent nitration and/or oxidation of proteins, lipids and DNA are implicated in neuronal death. In animal models, peroxynitrite generation is increased by hypoglycemia, a condition that occurs frequently in low birth weight newborns. In order to detect systemic nitro-oxidative stress, we developed an assay to measure Plasma NitroAlbumin (PNA) concentration. and investigated its variations according to glycemia during the first days of life. PNA concentrations were measured at days 0, 1 and/or 4 of life in 119 newborns (26 small for gestational age term and 93 preterm infants). We investigated differences in PNA concentrations with regard respect to the occurrence and recurrence of hypoglycemia events (HG: glycemia < 2.5 mmol/l) PNA concentrations at days 0, 1 and 4 were significantly higher in both preterm and SGA term infants who developed at least one HG hypoglycemia than in normoglycemic patients (p = 0.001, 0.001 and 0.04, respectively). PNA concentration at D1 increased with the number of hypoglycemic events and was correlated to the area under curve of glycemia measured 12-24 hours before sampling. We conclude that hypoglycemia during the first 24 hours of life is associated with increased albumin nitration in newborns. This fact suggests that a significant nitrooxidative stress occurs promptly after hypoglycemia and this in turn implying a risk of end-organ damage due to protein nitration, lipid peroxidation and DNA damage. Evidence of nitrooxidative stress in hypoglycemic neonates may open new and exciting perspectives in neuroprotection. Neonatal hypoglycemia is a very challenging issue because of its potential harmful effects on the immature brain. In fact, newborns, especially low birth weight (< 2500 grams) infants, are extremely sensitive to conditions that affect glucose homeostasis during the transition from intra- to extrauterine life (Ward- Platt and Deshpande, 2005). Neonatal glucose metabolism is characterized by

increased glucose utilization, failure of gluconeogenesis, and reduced fat and glycogen stores (Hawdon and Ward-Platt, 1993). Therefore low blood glucose values are frequently observed during the first days of life. Moreover as newborns are unable to oxidize fatty acids into ketone bodies (Stanley et al., 1979), glucose is an essential primary fuel for the brain. Therefore hypoglycemia may be particularly harmful during the early postnatal period.

Neonatal hypoglycemia often occurs during the first hours of life in low birth weight infants. This episode may be unique or recurrent during the first days of life (Gutberlet and Cornblath, 1976). If asymptomatic early transient hypoglycemia is often considered to be benign (American Academy of Pediatrics, 1993) or even physiological (Deshpande and Ward-Platt, 2005), recurrent neonatal hypoglycemia has been associated with developmental delay (Lucas et al., 1988; Duvanel et al., 1999) and specific brain lesions on MRI magnetic resonance imaging (Spar et al., 1994 ; Filan et al., 2006; Yalnizoglu et al., 2007). However the consequences of neonatal hypoglycemia on the developing brain neurological outcome are still a matter of intense debate. FollowDue to methodological weaknesses follow-up studies did not show undisputable evidence that neonatal hypoglycemia, unless severe and prolonged, impairs brain development (Williams, 1997; Cornblatt and Schwartz, 1999; Cowett, 1999). Therefore monitoring, prevention and treatment of neonatal hypoglycemia remain largely empirical (Hay et al., 2009). In factdeed, even the level of glycemia which is safe for the premature newborn is still a matter of debatediscussed (Kalhan and Peter-Wohl, 2000; Cornblath et al., 2000; Inder, 2008; Hay et al., 2008: Rozance and Hay, 2010). On the other hand, fear of hypoglycemia imposes regular monitoring, which means 3-4 hours interval punctures and invasive interventions such as continuous feeding or intravenous perfusion. Careful glucose monitoring exposes newborns to numerous painful procedures which that might in turn affect cerebral development (Fabrizi and Slater, 2012).

An approach to evaluate the potential deleterious effects of hypoglycemia is to search for correlations between blood glucose concentrations and biological markers of local or systemic distress. As the brain is the most sensitive organ to hypoglycemia, the effects of low glucose have mainly been investigated on the central nervous system and in neuronal models. Hypoglycemic neuronal death has been reported to be triggered by different mechanisms (Ferrand-Drake et al., 1999; Suh et al., 2007a) among which nitro-oxidative stress seems to be a key event (Suh et al., 2007b ; Haces et al., 2010). In neuronal cells in culture, glucose deprivation is followed by superoxide ion ($O_2^{.-}$) generation and Nitric Oxide (NO) synthase activation (Liu et al., 2003). Overproduction of $O_2^{.-}$ and nitric oxideNO leads to the generation of peroxynitrite ($ONOO^-$), a highly reactive nitrogen cytotoxic species (Ischiropoulos and Beckman, 2003). Peroxynitrite is responsible for protein nitration, lipid peroxidation and DNA damage, a process referred to as nitro-oxidative stress.

Plasma protein nitration reflects an increased production of $ONOO^-$ and can therefore be used as clinical marker of nitro-oxidative stress (Wayenberg et al., 2009). We developed and clinically validated a sensitive double-sandwich ELISA that allowsing the quantitative determination of Plasma NitroAlbumin (PNA) concentrations in newborns (Wayenberg et al., 2009; Wayenberg et al., 2011). In this articlestudy, we investigated whether neonatal hypoglycemia is associated with albumin nitration in low birth weight infants.

Keywords
hypoglycaemia, nitro-oxidative stress

Abbreviations
AUG : Area Under the Glycemia curve
NO : Nitric Oxide
PNA : Plasma NitroAlbumin

METHODS
Subjects
Study patients were selected from a large database built on the prospective enrolment of 323 infants with various conditions from march 2005 till October 2007 in three maternities in Brussels (Hôpital Français, Hôpital Universitaire Erasme and CHIREC). Inclusion criteria were low birth weight (less than 2500 grams) and no obvious cause of nitro-oxidative stress such as former asphyxia or strong suspicion of perinatal infection. Written consent was obtained from both parents of each infant and the ethics committee of each hospital approved the study was approved by the ethics committee of each hospital.

Blood glucose level determination and monitoring
Glucose levels were checked between the 30th and the 60th minute of life. First glycemia was measured by an automatic multi-analyzer (AVL 990-S Gas Analyser, AVL Medical Instruments). Subsequent glycemias were monitored every 3-6 hours using a strip method (Accu-Chek®, Roche). Hypoglycemia was defined as a blood

glucose concentration < 2.5 mmol/l (45 mg/dl) (Gutberlet and Cornblath, 1976) and was promptly treated either by gavage (milk + 2-5% dextrin maltose) or by intravenous infusion with 10% glucose (60 ml/kg/24 hours) according to the management protocol of each institution and to the clinical situation. In order to evaluate the effects of recurrence, severity and duration of the hypoglycemia, we calculated the Area Under the curve of the Glycemia (AUG) measured during the time elapsed before sampling for nitroalbumin.

Methods for blood sampling and plasma nitroalbumin determination

Blood samples (0.5 - 1 ml) were drawn from umbilical artery and vein (UA and UV) at birth and from a peripheral vein at 1-3 hours of life and at days 1 (D1) and 4 (D4) of life. D1 blood was always collected in the morning of the day following the day of birth. Therefore the time elapsed between birth and D1 blood collection varied from 10 to 34 hours. In order to avoid potential artefactual de novo nitration, blood samples were collected in EDTA containing tubes, put on ice and rapidly centrifuged at 4°C for 10 min. The plasmas were then snap-frozen and stored at -20°C until analysis.

PNA was measured using an ELISA method that which has been described in details previously (Wayenberg et al., 2009). Determinations were performed in triplicate. The detection limit of the assay is < 1 ng/ml and the analytical sensitivity was calculated to be 2 ng/ml.

Statistical Analysis

All PNA values are expressed as median and 25th-75th percentiles. Correlations between continuous variables such as first glycemia and PNA concentrations were tested using Spearman's rank test. Differences in median PNA concentrations between hypoglycemic and normoglycemic infants were assessed by the Kruskall-Wallis ANOVA rank test. Differences in the distribution of discrete variables between the groups were tested using χ^2 analysis. A value of $p < 0.05$ was considered significant. All tests were performed with NCSS® software.

RESULTS

Clinical and biological variables

Based on the criteria defined above, 119 newborns (26 small for gestational age term and 93 preterm infants) were included in the study. Patients were divided into 3 groups according to glucose monitoring (Volpe, 1995): transient hypoglycemia (n = 54), recurrent (> 2) hypoglycemia (n = 8) and normoglycemia (n = 57). 365 blood samples were collected for PNA determinations: 39 from UA umbilical artery, 61 from UV umbilical vein, 77 at H1, 102 at D1, and 86 at D4.

Nitroalbumin during the first hour of life

PNA concentration at H1 was inversely correlated with glycemia at H1 (r = -0.28, p = 0.016). PNA concentration at H1 was significantly higher in hypoglycemic than in normoglycemic infants (medians: 8.7 versus 4.6 ng/ml, respectively, χ^2 = 10.2, p = 0.001). On the contrary, we did not observe any significant variation in umbilical blood PNA concentrations according to patient group. There were no correlations between PNA concentrations at H1 and other variables such as gestational age, gender, oxygen exposure, respiratory course, hemodynamics and biological markers of inflammation.

Plasma nitroalbumin at day 1 of life

The correlation between glycemia and PNA concentration was also observed at D1 (r = -0.26, p = 0.018) and at D4 (r = -0.25, p = 0.024). PNA concentration at D1 was significantly higher in hypoglycemic than in normoglycemic infants (medians: 7.4 versus 4.4 ng/ml, respectively, χ^2 = 10.5, p = 0.001). A finer analysis of D1 samples shows that the PNA increase observed in hypoglycemic infants varies according to the precise

Table 1. Correlations between glycemia at H1 and PNA concentration at D1 according to the time elapsed between birth and sampling for PNA.

	Time elapsed between birth and sampling for PNA.		
	< 20 hours	< 24 hours	> 24 hours
Pearson's Correlation			
Coefficient (r)	- 0.48	- 0.28	- 0.18
p value	0.004	0.04	0.19
ANOVA *			
Median PNA	7.4 vs 3.9 ng/mL	7.2 vs 4.4 ng/mL	6.6 vs 4.5 ng/mL
p value	< 0.001	0.002	0.26

* Hypoglycemic vs normoglycemic infants.

time elapsed between birth and blood collection (table I). PNA concentration increased according to the number of hypoglycemic episodes (fig. 1). Very significant correlations were founded between PNA and AUG's, especially AUG corresponding to a time period of 18 hours-period before sampling for PNA (r = -0.49, p < 0.001)(fig. 2). In contrast, we did not observe any significant variation of PNA concentrations related with respect to other variables such as gestational age, gender, oxygen exposure, respiratory course, hemodynamics and biological markers of inflammation.

Plasma nitroalbumin at day 4 of life

A moderate but significant increase of PNA was still observed at D4 in hypoglycemic infants (5.4 versus 4.3 ng/ml in normoglycemic infants, χ^2 = 4.1, p= 0.043). The increase of PNA is more striking in patients with recurrent hypoglycemia. Like at H1 and D1, we did not observe any significant variation of PNA concentrations at D4 related with respect to the other variables such as gestational age, gender, oxygen exposure, respiratory course, hemodynamics and biological markers of inflammation.

DISCUSSION

We found higher plasma PNA concentrations of nitroalbumin in hypoglycemic compared than in to normoglycemic infants, suggesting that neonatal hypoglycemia is associated with systemic nitro-oxidative stress. Increased albumin nitration is observed during the hypoglycemic event and lasts during the first 4 days of life. PNA increase is correlated to the severity, the number and the duration of hypoglycemic events. As we were not able to demonstrate any confounding factor, the relation

between hypoglycemia and albumin nitration seems to be specific. We have explained previously (Wayenberg et al., 2011) why our results plead in favour ofsupport a causal relationship between hypoglycemia and nitro-oxidative stress (Wayenberg et al., 2011).

Cellular mechanisms and significance of protein nitration

Increased production of reactive oxygen species in hypoglycemia is well documented (McGowan et al., 2006 ; Suh et al., 2008 ; Haces et al., 2010 ; Paramo et al. 2010). $O_2.-$ overproduction may be the result of the activation of NADPH and xanthine oxidases (Paramo et al., 2010) and/ or inhibition of mitochondrial glutathion reductase due to a decreased availability of NAD(P)H (Garofalo et al., 1988). As NO synthase is activated after glucose deprivation (Liu et al., 2003), $O^2.-$ will react with NO to generate $ONOO^-$. In agreement with this, experimental insulin-induced hypoglycemia results in nitration of several proteins in defined areas of the brain (Haces et al., 2010). Albumin nitration suggests that other proteins are nitrated as well. Nitration has been related to modifications of proteins' functional properties, lipids peroxydation and DNA damage, potentially leading to apoptosis. In adults, protein nitration has been reported to be associated with several acute and chronic diseases (Ischiropoulos and Gow, 2005 ; Szabo C et al., 2007). In newborns, nitro-oxidative stress has been implicated in organ damage associated with perinatal asphyxia (Groenendaal et al., 2006; Wayenberg et al., 2009), periventricular leucomalacia (Baud et al., 2004), retinopathy (Beauchamp et al., 2004), severe chronic lung disease (Banks et al., 1998) and prolonged hyperoxia (Sirinyan et al., 2006).
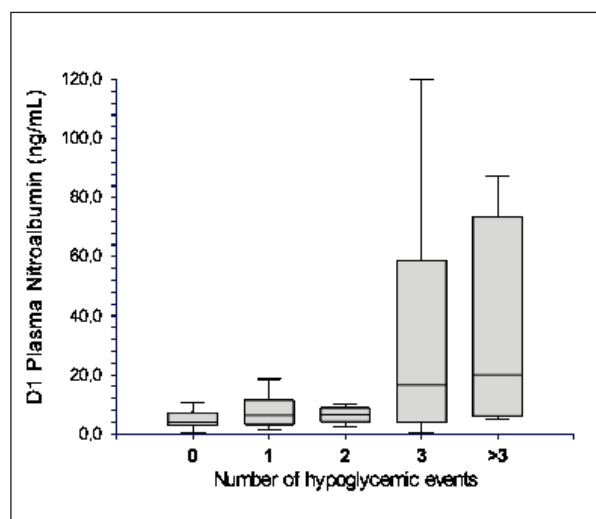

Figure 1. PNA concentration at day 1 according to the number of hypoglycemic events during the first day of life.
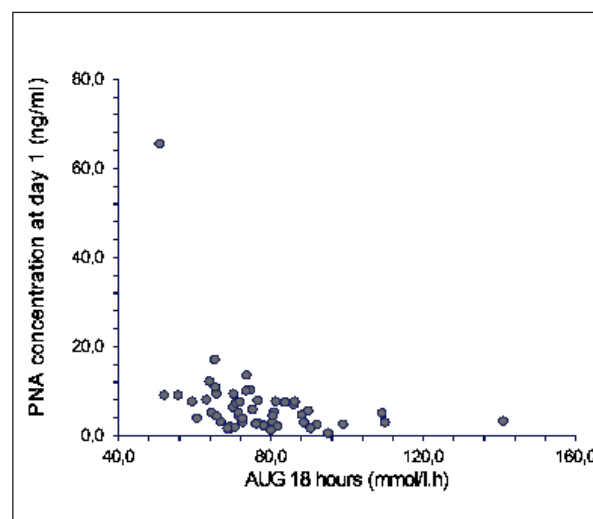

Figure 2. Correlation between PNA concentration at day 1 and AUG 18 hours.

Increased peroxynitrite production is an early and transient event

Our observations also indicate that hypoglycemia-induced albumin nitration occurs very early in postnatal life. Indeed high levels of PNA are found in blood sampled as early as 30 min of life in hypoglycemic infants. Regarding the persistence of albumin nitration, we observed a significant inverse correlation between glycemia at H1 and PNA at D1 only when blood sampling occurred within the first 24 hours of life. The absence of higher PNA concentrations in samples obtained later than 24 hours of life in non-recurrent hypoglycemic infants suggests that ONOO$^-$ generation rapidly falls after restoration of normoglycemia. However, a moderate increase of PNA was still detectable 4 days after the hypoglycemic events. Our results suggest that nitroalbumin is cleared, although the mechanisms involved remain to be defined (Predescu et al., 2002; Leger et al., 2008; Csibi et al., 2010). Whereas protein nitration is reversible, oxidative damage to lipids and DNA by ONOO$^-$ however is not.

Potential confounding factors and limitations of the study

Increased generation of ONOO$^-$ occurs in response to a series of pathological conditions such as infection, hypoxia and ischemia (Szabo et al., 2007). Inflammation is probably a major one as neutrophils are able to generate the nitrating species nitrogen dioxide (Eiserich et al., 1998). In our study, no significant correlation was found between PNA concentrations and leukocytes counts or C-reactive proteinRP. More difficult to assess is the potential effect of exposure to oxygen. Experimental studies on rat pups have shown that hyperoxia leads to nitration in cerebral microvascular cells (Sirinyan et al. 2006) but not in lungs (Cucchiaro et al., 1999) and plasma nitrotyrosine is increased in prematures who develop bronchopulmonary dysplasia after ventilation (Banks et al., 1998). Conversely, Vento et al. (2000) did not find any evidence of increased oxidation in preterms after oxygen exposure at birth. In agreement with this report, nitroalbumin concentrations were not significantly higher in patients resuscitated with 100% oxygen at birth, neither in patients who were hyperoxic during the first hour of life, nor in infants treated by FiO$_2$ > 21% during the first day of life. Thus, among all the variables tested in this study, only hypoglycemia appears to be associated with albumin nitration.

Individual variability in albumin nitration after hypoglycemia

Although there is evidence about a relationship between If the link of hypoglycemia and albumin nitration is indubitable, the effects of hypoglycemia depend on the characteristics of hypoglycemia (severity, recurrence and duration) and on the ability of protective mechanisms such as increase of cerebral blood flow and glucose extraction rate (Volpe, 1995) and the availability of alternative fuels, such as ketones and lactate (Hawdon, 1999). In agreement withsupport to this hypothesis, we found that PNA concentration is correlated with the response to renutrition (oral or intravenous glucose) and lactatemia in preterm infants (Wayenberg et al., 2011).

In conclusion, our Our study indicates shows the occurrence of a systemic nitro-oxidative stress in response to hypoglycemia in low birth weight newborns. Protein nitration has previously been reported in animal models to be a potential link between hypoglycemia and brain damage. The relationship is far from being completely characterized and certainly warrants link is complex, involving numerous factors and needing further research efforts. However, but our findings may contribute to the development of novel targeted strategies for the prevention of brain lesions which may occur in severe and/or recurrent neonatal hypoglycemia.

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