Post hypoxic-ischemic brain injury of the newborn and the role of nitric oxide inhibition

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STELLINGEN BEHORENDE BIJ HET PROEFSCHRIFT

"Post hypoxic-ischemic brain injury of the newborn and the role of nitric oxide inhibition"

1. Bij pasgeborenen die ernstige perinatale asfyxie hebben doorgemaakt ontstaat een substantieel deel van de schade ten tijde van de reperfusie en reoxygenatie.

2. Niet-eiwitgebonden ijzer en stikstofmonoxide spelen een belangrijke rol bij het ontstaan van post-asfyctische cerebrale reperfusieschade.

3. Vroegtijdige inhibitie van de productie van stikstofmonoxide, door toediening van met name een lage dosis N-ω-Nitro-L-Arginine, lijkt de cerebrale reperfusieschade na perinatale asfyxie te kunnen reduceren.

4. Toediening van N-ω-Nitro-L-Arginine lijkt noch een positieve noch een negatieve invloed uit te oefenen op de post-asfyctische linkerventrikelfunctie van de pasgeborene.

5. Inhibitie van de productie van stikstofmonoxide na perinatale asfyxie kan de pulmonale arteriële druk en gaswisseling in de longen van de pasgeborene tijdelijk nadelig beïnvloeden.

6. Voor een optimale preventie van reperfusieschade na perinatale asfyxie is het van belang dat de therapie direct na de geboorte gestart wordt.

7. Bij de behandeling van reperfusieschade na perinatale asfyxie zal uiteindelijk blijken dat een combinatie van verschillende therapieën het meest effectief is.

8. Bij de reanimatie van de pasgeborene dient men terughoudend te zijn met betrekking tot het toedienen van extra zuurstof.

9. Naarmate er meer medische technieken beschikbaar komen wordt het steeds belangrijker dat men zich van tevoren afvraagt of het wel ethisch verantwoord is om een bepaalde behandeling te starten.

10. Pasgeborenen met ernstige perinatale asfyxie dienen na succesvolle reanimatie zo snel mogelijk naar een Neonatale Intensive Care Unit getransporteerd te worden.

11. Primaire preventie van perinatale asfyxie zal dit proefschrift overbodig maken.

12. Iemand die het tijdens zijn leven niet de moeite waard vindt om na te denken over orgaandonatie zou zijn organen niet zonder meer mee moeten kunnen nemen in zijn graf.

13. Het volledig rookvrij maken van treinen zou het daadwerkelijke aantal beschikbare zitplaatsen significant verhogen.

14. Door de steeds hogere eisen die gesteld worden aan een opleidingsplaats kindergeneeskunde houden vrouwelijke assistenten, door het op latere leeftijd krijgen van hun eigen kinderen, de beroepsgroep in stand.

15. Het is belangrijker om je af te vragen hoe je leeft dan of je leeft.

C.A. Dorrepaal
Leiden, 11 juni 1997

The great tragedy of Science: the slaying of a beautiful hypothesis by an ugly fact.
(Thomas Huxley)

To Eric

To my parents and grandparents

CONTENTS

1 PERINATAL HYPOXIA-ISCHEMIA, A REVIEW OF THE LITERATURE

1.1 General background

Despite major improvements in obstetric and perinatal care, with a resultant reduction in perinatal mortality rates over the last decade, the prevalence of perinatal hypoxia-ischemia, also called perinatal asphyxia, has not decreased[1,2]. Estimates suggest that between 3 and 6 out of 1000 full term neonates suffer from hypoxia-ischemia at or before birth, and this incidence figure approaches 60% in small premature neonates[3,4,5,6]. Depending on the gestational age, between 20% and 96% of the neonates suffering from perinatal hypoxia-ischemia die; of the survivors 20% or more will suffer severe brain damage. Extending the figures found by MacDonald et al. to the Netherlands, this would mean that approximately 2000 neonates per year will suffer from perinatal hypoxia-ischemia. About 900 of these neonates will die, and approximately 200 will survive with long term neurologic deficits such as cerebral palsy, mental retardation, learning disabilities and epilepsy. Some of them will be so handicapped that they will be chronically dependent on institutions for mentally and physically disabled. Given these incidence figures it becomes clear that perinatal hypoxia-ischemia is an important health problem with considerable social consequences. Therefore it is not surprising that a lot of research is done to seek effective strategies to prevent or reduce the long-term consequences of perinatal hypoxia-ischemia.

1.2 Definition

Asphyxia [Greek "a stopping of the pulse"] is described as an apparent or actual cessation of life due to interruption of effective gaseous exchange in the lungs[7]. Clinically it refers to an impairment in the exchange of the respiratory gases oxygen and carbon dioxide, leading to a situation of hypoxemia in combination with hypercapnia and eventually ischemia after failure of the heart[1]. When clinicians describe a neonate as "asphyxiated" at birth, they usually mean one ore more of several rather different things[8]: major obstetric complications (placental solution, prolapse of umbilical cord), as well as perinatal markers of fetal distress (meconium passage, low Apgar scores, low arterial umbilical pH, abnormal fetal heart rate pattern), or postnatal neurologic abnormalities, which may be accomplished by multiorgan failure and/or EEG abnormalities. Also in the various studies on perinatal hypoxia-ischemia different definitions of hypoxia-ischemia are used, which makes it difficult to compare them, especially with respect to long term neurodevelopmental outcome. Current evidence suggests that none of the previous mentioned markers alone is able to predict outcome reasonably well. It is the occurrence of a sequence of

indicators of exposure, response, and impact on brain and other functions, that carries predictive weight[8]. Recently, the American Academy of Pediatrics and the American College of Obstetricians Committees on Maternal-Fetal medicine and Fetus and Newborn have defined certain criteria which must be present for the sustainment of perinatal hypoxia-ischemia, and which may lead to long term neurologic deficits[9]. According to these criteria, all of the following must be present: *1.* a profound metabolic or mixed acidity (arterial umbilical cord pH <7.00); *2.* persistence of an Apgar score of 0-3 for longer than 5 min; *3.* clinical neurologic sequelae (e.g. seizures, coma, hypotonia or Hypoxic Ischemic Encephalopathy (HIE)) in the immediate neonatal period; and *4.* evidence of multiorgan failure (e.g. cardiovascular, gastrointestinal, hematological, pulmonary or renal) in the immediate neonatal period. They conclude that a neonate only suffers from perinatal hypoxia-ischemia when all the four characteristics are present, and in that case one must be aware of a possible long term neurologic deficit. Since Apgar scores are quite subjective measurements, and not all of the above mentioned four characteristics of perinatal hypoxia-ischemia are immediately present at birth, we used the following three criteria to ascertain severe perinatal hypoxia-ischemia in our studies: *1.* fetal distress (abnormal heart rate pattern and/or meconium stained amniotic fluid); *2.* arterial cord or first pH of less than 7.00; and *3.* need for immediate neonatal ventilation with mask or endotracheal tube for more than 2 min.

1.3 Etiology

Although there are a number of situations during pregnancy, labor, or delivery in which perinatal hypoxia-ischemia may be anticipated, there is still a percentage of neonates who present with unexpected hypoxia-ischemia upon birth. It has been suggested that in these neonates some unrecognized events during pregnancy may have occurred, leading to subsequent presentation of hypoxia-ischemia at birth[10]. With the recognition from several experimental studies that much of the hypoxic-ischemic injury evolves *after* cessation of the insult, and that it can be interrupted to a considerable extent by several therapeutic approaches (see paragraph 1.5.4 and 1.8), the ultimate possibility of recognition of hypoxia-ischemia both in utero and immediately after birth is desirable for early therapeutic intervention. Presently there are several recognized factors which may place the neonate at high risk for perinatal hypoxia-ischemia[9,11]: *1. impaired placental gas exchange* (in front lying placenta, placental abruption or insufficiency); *2. interruption of the umbilical circulation* (umbilical cord prolapse, knot or compression); *3. risk factors during delivery* (breech or other abnormal presentation, non elective cesarean section, sedative drugs given

shortly before delivery); *4. maternal risk factors* (elderly primigravida (>35), diabetes, previous neonatal death, prolonged rupture of membranes, blood type or group isoimmunization); *5. fetal risk factors* (premature delivery, multiple births, abnormal fetal heart rate pattern, meconium stained amniotic fluid, intrauterine growth retardation, congenital malformation); 6. *inadequate perfusion of maternal side of the placenta* (toxemia of pregnancy, hyper- or hypotension from any cause, abnormal uterine contractions); *7. impaired maternal oxygenation* (cardiopulmonary disease, anemia); and *8. failure to accomplish lung inflation* (surfactant deficiency, neuromuscular disease, severe hernia diafragmatica).

1.4 Long term outcome following perinatal hypoxia-ischemia

It is now generally accepted that severe perinatal hypoxia-ischemia, sufficient to result in an adverse long term outcome with permanent neurological damage, practically always produces signs of neurological dysfunction during the neonatal period[12]. The most useful early predictors correlating with subsequent long term neurological outcome form a constellation of signs commonly called hypoxic-ischemic-encephalopathy (HIE)[13,14]. Generally, the neonatal encephalopathy associated with perinatal hypoxia-ischemia has been described in three stages by Sarnat[14] (see table 1.1). Peliowski and Finer[15] have described significant relationships between each of the three stages of HIE and an adverse long-term outcome, drawing on the results of five different studies. They found that the overall risk of death with all stages of HIE combined was 12.5%, the overall risk of neurologic handicap was 14.3%, and handicap including deaths 25%. Robertson et al.[16] found that the prognostic value of the stage of encephalopathy is greatest when the neurologic examination of the neonate is staged according to the most severe signs, and subsequently the most severe stage of encephalopathy, categorized between 1 hour and 7 days of life, is used to relate to outcome. According to this staging procedure, all the infants in Sarnat stage 1 (mild neonatal encephalopathy), with or without clinical convulsions, had a normal outcome at the age of 3.5 and 8 years[16,17], which indicates that the long term prognosis for infants with a Sarnat stage 1 encephalopathy is good. Neonates with Sarnat stage 2 (moderate neonatal encephalopathy) suffer often from parasagittal injury, with damage at the "watershed" zone of arterial supply between the anterior and middle cerebral arteries. This correlates well with shoulder girdle and proximal upper extremity hypotonia, which is often seen in neonates with Sarnat stage 2[1,17]. About 20% of these infants had an adverse outcome: 5% died and 15% was disabled at the age of 8[17]. Those with prolonged symptoms were the most likely to become disabled.

Symptoms	**Stage 1.** Mild Encephalopathy	**Stage 2.** Moderate Encephalopathy	**Stage 3.** Severe Encephalopathy
Level of consciousness	hyperalert	lethargic or obtunded	stuporous
Neuromuscular control			
- muscle tone	normal	mild hypotonia	flaccid
- posture	mild distal flexion	strong distal flexion	intermittent decerebration
- stretch reflexes	overactive	overactive	decreased or absent
- myoclonus	present	present	absent
Complex reflexes			
- suck reflex	weak	weak or absent	absent
- Moro reflex	strong; low threshold	weak; incomplete high threshold	absent
- oculovestibular reflex	normal	overactive	weak/absent
- tonic neck reflex	slight	strong	absent
Autonomic function	generalized sympathetic	generalized parasympathetic	both systems depressed
- pupils	midriasis	miosis	variable; unequal poor light response
- heart rate	tachycardia	bradycardia	variable
- bronchial and salivary secretions	sparse	profuse	variable
- gastrointestinal motility	normal or decreased	increased, diarrhea	variable
Seizures	none	common; focal or multifocal	uncommon, decerebrated
Electroencephalogram findings	normal (awake)	early: low voltage, delta and theta later: periodic pattern Seizures: focal 1 to 1.5 Hz, spike and wave	early: periodic pattern with isopotential phases later: totally isopotential
Duration	< 24 hours	2 to 14 days	hours to weeks

Table 1.1 Scoring of neonatal encephalopathy according to Sarnat[14]

The nonimpaired survivors of Sarnat stage 2 had a significantly lower intelligence score, and were more likely to be more than one grade level delayed compared to children from a peer group at the age of 8[17]. Sarnat stage 3 (severe neonatal encephalopathy) has the worst prognosis. All children categorized in this stage appeared to have an adverse outcome: 82% died and all survivors (i.e. 18%) were disabled at the age of 8[17]. Widespread cortical and subcortical infarcts in the parasagittal region and white matter have been reported in these children, which may be related to hypoperfusion as well as massive necrosis[1]. Children who have had stage 3 neonatal encephalopathy often suffer from multiple disabilities such as spastic cerebral palsy, mental retardation, cortical blindness, severe hearing loss and convulsive disorders[17]. Such multiple disabled children usually remain dependently handicapped for the rest of their life, and generally have a shortened life expectancy[16,17].

1.5 General pathophysiology of perinatal hypoxia-ischemia

1.5.1 Introduction

For many years the cited mechanisms for neuronal cell death occurring with hypoxia-ischemia were that a deficiency of high energy phosphates resulted in an impaired synthesis of structural components making it impossible for the neuronal cell membrane to maintain electrical stability, resulting in incompetence to maintain cellular integrity with subsequent cell death. It is now clear that this explanation is oversimplified. Several experimental animal models of hypoxic-ischemic injury have demonstrated that, although brain damage starts during the hypoxia-ischemia, it increases during post hypoxic-ischemic recovery[18,19,20,21]. The metabolic perturbations arising in the recovery period after resuscitation contribute substantially to the nature and extent of the neuronal destruction. The term "reperfusion-injury" is often applied to describe the brain damage that evolves after the primary insult per se[18,19,20,21]. However, it is likely that the initial decrease in high energy phosphates and the persistence of a certain extent of energy depletion in the post insult period, are capable of triggering a cascade of additional deleterious events *after* the primary insult which may ultimately lead to neuronal cell death.

1.5.2 Mechanisms of cell death during the actual hypoxic-ischemic insult

Neuronal cell death occurring with hypoxia-ischemia is explained by a sharply decreased production of intracellular high energy phosphate compounds such as phosphocreatinine and adenosine triphosphate (ATP), leading to severe energy failure[19]. During hypoxia-ischemia, only glucose is capable of sustaining energy

metabolism in the brain to a certain extent. All alternative substrates, including keton bodies, lactate and fatty- and amino- acids, require oxygen for their consumption in order to produce energy-equivalents. Under anaerobic conditions however, glucose is able to generate only 2 molecules of ATP per molecule of glucose in contrast to the generation of 36 molecules of ATP per molecule of glucose during oxidative phosphorylation, when oxygen is available[1,19]. To produce the amount of ATP equivalent to that of oxidative phosphorylation, glycolysis would need to increase to a rate 18 times its basal flux. In reality, glycolysis, even when maximally stimulated by total cerebral ischemia, is capable of increasing only 4- to 5- fold, partially owing to the concurrent accumulation of hydrogen ions[19]. A lowered pH due to high levels of anaerobic glycolytic activity has been shown to lead to an impairment of oxidative ATP generation[22]. Although stimulation of glycolysis can supplement oxidative phosphorylation under conditions of partial oxygen debt, it can never completely substitute for mitochondrial oxidative phosphorylation.

1.5.3 Mechanisms of cell death during post hypoxic-ischemic reperfusion

Investigations have been shown that hypoxia-ischemia sets in motion a cascade of biochemical alterations initiated during the course of the insult, which are proceeding well into the recovery period[19]. All of these processes occur at a low level during the actual hypoxic-ischemic insult, but are enhanced during post hypoxic-ischemic reoxygenation[23,24], when oxygen becomes suddenly available. Aggravation of the initial brain damage then occurs due to membrane depolarization, increases in cytosolic calcium and accumulation of extracellular glutamate[18,19,20,21]. The actual hypoxic-ischemic insult will lead to a failure of the ATP-dependent Na^+ K^+-pump followed by inability of the neuronal cell membrane to maintain electrical stability, resulting in membrane depolarization and influxes of Na^+ and Ca^{2+} ions. The Na^+-influx is accompanied by a passive osmotic entry of water and Cl^- ions leading to subsequent cell swelling, which is described as cytotoxic edema. This swelling of the cells may further compromise the microcirculation and, if excessive, lead to cell lysis[20]. The increase in cytosolic calcium occurs as a consequence of at least three mechanisms[18]: *1.* the above mentioned failure of the diverse energy dependent Ca^{2+}-pump mechanisms, normally operating to maintain a low intracellular Ca^{2+}-concentration; *2.* opening of the voltage dependent Ca^{2+}-channels (secondary to membrane depolarization); and *3.* activation of specific glutamate receptors (see paragraph 1.7.3). The increase in extracellular glutamate results from: *a.* excessive glutamate release (secondary to membrane depolarization and to increased intracellular Ca^{2+}); and *b.* failure of energy-dependent glutamate uptake mechanisms in astrocytes and presynaptic nerve endings (see paragraph 1.7.3.4). Other processes at cellular level

leading to secondary damage are apoptosis (programmed cell death) due to deprivation of growth factors and the activity of inflammatory cells[20,21]. Recent studies suggest that the activity of inflammatory cells such as macrophages may cause reperfusion injury by producing the excitatory amino acid glutamate[25], hydrogen peroxide[26], and glial cytotoxins[27].

1.5.3.1 Effects of increased cytosolic calcium

The deleterious effects of an increased cytosolic Ca^{2+}-concentration are numerous[1,18,28,29,30,31]: *1.* degradation of cellular lipids by activation of phospholipases, in particular phospholipase C, which promotes a progressive breakdown in the phospholipid components of the cellular- and subcellular membranes; *2.* degradation of cellular proteins (especially cytoskeletal elements) by activation of proteases; *3.* attack of cellular DNA by activation of nucleases; *4.* crucial indirect mechanisms of destruction mediated by the generation of reactive oxygen species via the formation of nitric oxide, prostaglandins and possibly xanthine oxidase. Finally, high concentrations of intracellular free Ca^{2+} can lead to an uncoupling of the oxidative phosphorylation within the mitochondria: by the utilization of ATP by ATP-dependent Ca^{2+}-transport systems, attempting to correct the cytosolic Ca^{2+}-accumulation, the ATP-reserves are further depleted which perpetuates the process.

1.5.3.2 Effects of the production of reactive oxygen species

The role of reactive oxygen species, including nitric oxide, in post hypoxic-ischemic reperfusion injury has now been well established[20,21,29,30,31,32,33,34,35,36,37,38]. Several studies of hypoxia-ischemia in the newborn lamb and the immature rat have been shown a brain protective effect of treatment with reactive oxygen species scavengers or drugs inhibiting the formation of reactive oxygen species, which suggests that reactive oxygen species may worsen outcome after hypoxic-ischemic injury.

The first source of the production of reactive oxygen species is the mitochondrial electron transport system. Oxygen deprivation prevents the complete passage of electrons in the electron transport system to the terminal enzyme cytochrome *c* oxidase, leading to the generation of reactive oxygen species proximal to this terminal enzyme. These reactive oxygen species can not be further consumed within the mitochondria and "leak" out into the cytoplasm. The following four processes of reactive oxygen species production are directly or indirectly related to cytosolic calcium: *1.* the enzymatic conversion of arachidonic acid (generated by Ca^{2+}-activated phospholipase A_2) to prostaglandins, leukotrienes and tromboxanes by the enzymes cyclo- and lipo- oxygenase [36]; *2.* the auto-oxidation of Ca^{2+}-mediated release of

catecholamines; *3.* the Ca^{2+}-mediated production of nitric oxide, which may lead to the formation of the highly toxic radicals peroxinitrite and hydroxyl radical (see paragraph 1.7.3.8); and *4.* the metabolization of the ATP degradation product hypoxanthine by Ca^{2+}-activated xanthine oxidase to xanthine and uric acid, leading to the formation of the reactive oxygen species superoxide and hydrogen peroxide, which subsequently can react to form the highly toxic hydroxyl radical (see paragraph 1.7.2.1), although this issue is much in debate. Finally, recent data suggest that early reactive cells at the site of the insult or in the cerebral microcirculation, e.g. macrophages, microglia and neutrophils, are potent sources of reactive oxygen species[20,21,25,26]. Extracellular formation of superoxide by neutrophils and macrophages has long been recognized as a bactericidal mechanism. Similar oxidative activity has been observed in microglia. Furthermore, following severe injuries involving additionally a breakdown of the blood-brain barrier, invasion of inflammatory cells such as neutrophils and macrophages occurs, which may lead to subsequent plugging of capillaries (see paragraph 1.6.2.4) and diffusion of inflammatory cells to brain cells. The further role of reactive oxygen species in post hypoxic-ischemic reperfusion injury will be discussed in paragraph 1.7.

1.5.4 Late events after perinatal hypoxia-ischemia

Some 6 to 12 hours after the primary insult, a second and more prolonged (12 to 48 hours) period of edema may occur and the cerebral metabolism may again become disrupted. This phenomenon has been associated with neuronal hyperexcitability, which may be accompanied by clinical seizures and caused by an accumulation of cytotoxins[24,39]. At this time a number of major changes in neuronal and glial expression may become apparent. Microglial cells and astrocytes may become activated and express an inducible form of nitric oxide synthase (see paragraph 1.7.3.2)[40,41]. Recent studies suggest that this activation may play a central role as inhibition of activation of microglial cells and astrocytes before this phase of neuronal hyperexcitability has been shown to have a profound positive effect on the progression of the neuronal loss[42].

1.5.5 Repair processes after perinatal hypoxia-ischemia

As the hyperexcitability phase resolves between about 36 to 72 hours, a marked induction of neurotrophic factors can be observed. These appear to be endogenously protective and may also play a role in a slower repair recovery process. The insulin-like growth factor 1 (IGF-1) is potentially neurotrophic, and early administration of IGF-1 after hypoxic-ischemic injury has been shown to be neuroprotective[43]. Similarly, administration of fibroblast growth factor (FGF)[44] can improve outcome

when given after injury, demonstrating the importance of trophic factors. As apoptosis (programmed cell death) normally occurs in neurons that are cut off from trophic support (e.g. nerve growth factor (NGF)-producing neurons or ciliary neurotrophic factor producing motor endplates), it is tempting to speculate that additional trophic support provided by IGF may overcome the inappropriate activation of the programmed cell death cascade.

1.6 Hemodynamic pathophysiology

1.6.1 General physiology

1.6.1.1 Coupling of cerebral blood flow and metabolism

Several studies have demonstrated a tight coupling of cerebral blood flow with cerebral function and metabolism[45,46,47]. This coupling appears to be mediated by regulation of cerebral blood flow by one or more local vasoactive chemical components. Although nitric oxide (discussed in paragraph 1.7.3) is an important vasodilator, there are conflicting reports concerning the role of nitric oxide in cerebral autoregulation and in coupling of cerebral blood flow with cerebral function and metabolism[48,49,50,51,52]. Important vasoactive species of the brain are: H^+-ions, K^+-ions, adenosine, prostaglandins, perivascular osmolarity and Ca^{2+}. An increase of the perivascular H^+-concentration (i.e., a decrease of the tissue pH due to an increased neuronal metabolic activity or an increased anaerobic glycolysis), will result in arteriolar vasodilatation and thereby increase the blood supply. The vasodilating effect of CO_2 may also be mediated by an increased concentration of perivascular H^+-ions[46]. K^+-ions have been shown vasodilating, as well as vasoconstrictive effects[46,47]. Vasodilatation has been shown to increase linearly with extracellular K^+-levels to 10 mM, whereas levels above 20 mM induce vasoconstriction. Because K^+ is released from nerve cells with electrical activity, but also with membrane depolarization due to oxygen deprivation, this ion may play a role in cerebral blood flow regulation under hypoxic-ischemic conditions. The effect of K^+ on the perinatal brain however, has not yet been studied. Furthermore adenosine, a breakdown product of ATP, and prostaglandins, in particular prostaglandins E and F_2, whose concentrations increase during cerebral ischemia, may lead to vasodilatation[46,47]. On the contrary, agents that inhibit prostaglandin biosynthesis have cerebral vasoconstrictive effects, e.g. indomethacin[53,54,55,56]. An increase in perivascular osmolarity may have a vasodilating effect (whereas a decrease has a vasoconstrictive effect), which may explain the vasodilatation associated with the infusion of hypertonic solutions. Calcium ions may also play a role in the control of cerebral blood flow, e.g. high perivascular

concentrations of Ca^{2+} lead to vasoconstriction and low concentrations to vasodilatation[46].

1.6.1.2 Perinatal autoregulation

Autoregulation of the cerebral blood flow refers to the maintenance of a constant cerebral blood flow despite changing cerebral perfusion pressures[46]. In the fetal and neonatal lamb, as well as in the human neonate, autoregulation appears to be operative over a broad range of perfusion pressures[55,57,58,59]. In most species, including the human neonate, the range of perfusion pressures over which autoregulation is effective extends from a lower limit of approximately 30-40 mm Hg to an upper limit of approximately 70-100 mm Hg. Pryds et al. have been shown that cerebral autoregulation is already fully functional at birth[60]. However, in the fetal and neonatal lamb autoregulation has been shown to be very sensitive to hypoxia[58]. Decreases in arterial pO_2 from 20 to 16 mm Hg in the fetal animal, and from approximately 70 to 30 mm Hg in the newborn animal, completely abolished cerebral autoregulation. Notably, the impairment of autoregulation required only 20 min of exposure to hypoxia, and autoregulation did not recover until 7 hours after restoring normoxia. Moreover, in another study in the newborn lamb it was shown that systemic acidosis, a very common phenomenon with hypoxia-ischemia, was also able to cause a loss in cerebrovascular autoregulation[61]. These findings suggest that perinatal autoregulation may be lost during a hypoxic-ischemic insult such as perinatal hypoxia-ischemia. Lou et al. indeed demonstrated in neonates suffering from perinatal hypoxia-ischemia that the cerebral blood flow was linearly related to the arterial blood pressure and, consequently, varied considerably with spontaneous variations in blood pressure[62].

1.6.2 Pathophysiology

1.6.2.1 Cerebral blood flow during perinatal hypoxia-ischemia

The initial circulatory effect after the onset of perinatal hypoxia will be a prompt redistribution of the cardiac output, leading to an increase of blood flow to the brain, myocardium, and adrenals, at the expense of blood flow to other regions such as the skin, kidneys and gastrointestinal system[63,64]. The effect on the brain circulation is particularly mediated by oxygen chemoreceptors, and is accompanied by reactive hypertension shortly after the onset of hypoxia in order to maintain an effective circulation[65]. The major purpose of these circulatory changes is to supply sufficient oxygen to the most critical organs in face of an impending oxygen dept. As a consequence, there has to be a tremendous increase in cerebral blood flow in order to maintain an effective cerebral oxygen supply. Experimental studies have been shown an increase in cerebral blood flow with perinatal hypoxia-ischemia by up to

100%[65,66]. The mechanisms underlying the initial increase in cerebral blood flow relate in part to cerebral vasodilatation. This may occur secondary to hypoxemia, hypercapnia, or both, presumably due to increased perivascular H^+- concentrations. However, a role may also be played by nitric oxide (see paragraph 1.7.3.8) or by extracellular fluid concentrations of K^+, adenosine and prostaglandins, all of which have been shown to increase markedly in the brain during hypoxia-ischemia[67]. Although the blood flow to the various regions of the brain increases generally in concert with the total increase in cerebral blood flow, distinct regional differences are apparent. In general the increase in blood flow is most marked in brain stem structures and least in cerebral white matter. This effect has been interpreted as an attempt to maintain integrity of vital stem centers.

1.6.2.2 Myocardial function during perinatal hypoxia-ischemia

The common cardiovascular response to acute perinatal hypoxia is bradycardia and hypertension. The bradycardia is induced by chemoreceptor mediated stimulation of the vagal nerve while peripheral vasoconstriction causes hypertension and at the same time stimulates arterial baroreceptors, leading to persistence of the bradycardia[21]. Although the initial cardiovascular response to acute perinatal hypoxia is hypertension, this response is followed by hypotension[68]. The rapidity and severity of this hypotension depends upon the duration and severity of the asphyxial insult. In severe and prolonged hypoxia the blood pressure will eventually fall as a consequence of hypoxia-induced left ventricular myocardial dysfunction, eventually leading to ischemia of the brain. In neonates suffering from severe perinatal hypoxia, left myocardial dysfunction with a reduced cardiac output and even cardiogenic shock has been reported[69,70]. Experimental studies have been shown that this hypotension may be associated with a loss of vascular autoregulation (secondary to hypoxia and systemic acidosis) leading to a severely impaired perfusion of virtually all organs[71]. A study in term fetal sheep subjected to severe hypoxia-ischemia (pH 6.8-7.0) demonstrated a striking pressure dependent cerebral blood flow[71]: marked hyperperfusion with values up to six times normal occurred when mean arterial blood pressure was raised to 60-70 mm Hg, whereas cerebral blood flow was decreased to almost zero in large cortical area's when mean arterial blood pressure was lowered to 30 mm Hg. Also clinical studies in neonates suffering from severe perinatal hypoxia showed left myocardial dysfunction with a pressure dependent cerebral circulation, indicating lack of cerebral autoregulation[69].

1.6.2.3 Regional cerebral blood flow during perinatal hypoxia-ischemia

During severe hypoxia-ischemia the cerebral blood flow is preferentially directed to the brainstem, rather than the cortex, which may in part account for the greater susceptibility of the cerebral cortex for hypoxic-ischemic brain damage. Studies of blood flow to the various regions of the neonatal brain showed that the parasagittal regions of the parietal cortex, especially in the posterior aspects of the cerebral hemispheres are particularly susceptible to hypoxic-ischemic brain damage. This area lies in the border zones between the end fields of the major cerebral arteries, i.e., the anterior, middle and posterior cerebral arteries[72], and is therefore particularly susceptible to a fall in perfusion pressure, such as may occur as a consequence of a decreasing cardiac output and the loss of vascular autoregulation[73]. In term fetal monkeys subjected to prolonged and severe hypoxia-ischemia, impressive deficits in cerebral blood flow up to 80% have indeed been demonstrated, particularly in the parasagittal regions of the cerebral hemispheres and especially posteriorly[73]. A similar parasagittal distribution of cerebral cortical injury was demonstrated in near term fetal sheep subjected to cerebral ischemia[74]. These observations correlate well with the neuropathological and clinical observations made in asphyxiated human neonates[1].

1.6.2.4 Post hypoxic-ischemic cerebral blood flow and oxygen metabolism

Experimental studies in newborn lambs, cats and dogs have been shown that the principal effects on cerebral blood flow and oxygen metabolism during post hypoxic-ischemic reperfusion are an initial cerebral hyperperfusion, followed after 30 to 60 min by a decrease in cerebral blood flow and by up to 30% reduction of cerebral oxygen metabolism[66,75,76]. The early increase in cerebral blood flow is presumably related to the same mechanism operative for the increased cerebral blood flow during hypoxia-ischemia, i.e., a local increase in vasodilator factors, such as H^+-ion, K^+-ion, adenosine, and prostaglandins. Possibly an increased production of nitric oxide (see paragraph 1.7.3) upon reperfusion and reoxygenation may also be, at least in part, responsible for the observed initial cerebral hyperperfusion. Clavier et al.[76] demonstrated that the immediate post hypoxic-ischemic reactive hyperperfusion is mediated by the production of nitric oxide, whereas others showed that inhibition of nitric oxide production could reduce this postischemic cerebral hyperperfusion[77].

The mechanisms for the delayed post hypoxic-ischemic cerebral hypoperfusion and decreased cerebral oxygen metabolism could be related to an excess of vasoconstrictor molecules e.g. tromboxanes and leukotrienes, or reactive oxygen species mediated vascular injury (including nitric oxide mediated injury, see paragraph 1.7.3), or

both[78,79]. Nitric oxide seems to play a dual role: initially it may be related to the observed initial post hypoxic-ischemic cerebral hyperperfusion, due to a vasodilative effect, whereas it subsequently also may be responsible for the delayed post hypoxic-ischemic cerebral hypoperfusion due to reactive oxygen species mediated injury. The critical role of reactive oxygen species was shown by the demonstration that administration of the reactive oxygen species scavenger superoxide dismutase and catalase prior to onset of the hypoxia-ischemia prevented both the delayed cerebral hypoperfusion and the impairment of cerebral oxygen consumption[78].

Reactive oxygen species are known to stimulate leukocyte adhesion within capillaries and venules[80], presumably as a consequence of endothelial damage or leukotriene production. Release of chemoattractants and upregulation of adhesion factors are thought to be general mechanisms that stimulate adhesion, clumping, activation, and emigration of leukocytes during and after ischemic injury. The subsequent plugging of capillaries by these cells might be one of the mechanisms contributing to the delayed post hypoxic-ischemic cerebral hypoperfusion. Earlier suggestions of local edema as a cause for the delayed hypoperfusion turned out to be insignificant by direct measurement of brain edema. The reduction in cerebral oxygen consumption during the period of hypoperfusion also raised the possibility of concomitant mitochondrial injury. Rosenberg et al. showed, by direct measurement of mitochondrial respiratory function, that the decrease in cerebral oxygen consumption likely could be contributed to mitochondrial injury caused by the production of reactive oxygen species[81]. Recent studies suggest a possible role for nitric oxide as one of the reactive oxygen species implicated in the observed delayed cerebral hypoperfusion and the impairment of cerebral oxygen consumption. Beckman has demonstrated that nitric oxide can react with the reactive oxygen species superoxide to form the highly reactive peroxynitrite radical leading to cerebral microvascular damage (see paragraph 1.7.3.8)[79]. Moreover, the observed impairment of cerebral oxygen consumption may be a direct effect of nitric oxide. Brown et al.[82] showed that nitric oxide could inhibit the mitochondrial synaptosomal respiration by competing with oxygen for binding to cytochrome oxidase in vivo.

1.7 Reactive oxygen species

1.7.1 Introduction

Reactive oxygen species are highly reactive compounds with an uneven number of electrons in their outermost orbital. This makes them very unstable as most biologic molecules have their electrons arranged in pairs. Reactive oxygen species donate

(reducing reactive oxygen species) or take electrons (oxidizing reactive oxygen species) from other molecules in an attempt to pair their electrons and generate a more stable molecule. In this way they can react with certain normal cellular compounds such as unsaturated fatty acids of membrane lipids or cellular DNA, leading to irreversible biochemical injury, e.g. membrane injury and damage to DNA and to proteins containing unsaturated or sulfhydryl groups[35]. Reactive oxygen species are very commonly formed during normal metabolism and in fact only cause "oxidative" injury when they exceed the brain's elaborate anti-oxidant defense mechanisms. The term "oxidative stress" was first coined by Sies to account for the imbalance between reactive oxygen species formation and the anti-oxidant defense mechanisms[83]. There are a number of endogenous mechanisms of protection against reactive oxygen species activity, such as the endogenous scavengers cholesterol, ascorbic acid (vitamin C), α-tocopherol (most potent anti-oxidant part of the group of tocopherols of vitamin E) and gluthathione. Gluthathione not only detoxifies reactive oxygen species, but also maintains proteins and anti-oxidants, such as vitamin C en E, in the reduced form, i.e. the reactive oxygen species scavenging form. In addition, the enzymes superoxide dismutase (SOD, which catalyzes the conversion of the superoxide radical to hydrogen peroxide and molecular oxygen) and catalase (which catalyzes the conversion of hydrogen peroxide to molecular oxygen and water) are able to deactivate reactive oxygen species.

Nowadays, there is much experimental evidence that reactive oxygen species play an important role in the so called post hypoxic-ischemic reperfusion injury[35,36,37,37,38]. Several studies of hypoxia-ischemia in the newborn lamb and the immature rat have been shown a brain protective effect of treatment with reactive oxygen species scavengers or drugs which inhibit reactive oxygen species formation, such as allopurinol, oxypurinol, indomethacin, superoxide dismutase, and catalase. Treatment with allopurinol before hypoxic-ischemic injury improved cerebral outcome in the immature rat[37], and post hypoxic-ischemic cerebral survival in newborn sheep was increased after treatment with other reactive oxygen species scavengers and a calcium antagonist[38]. Oxygen derived reactive oxygen species produced during reperfusion seem to be able to overwhelm endogenous scavenger systems and therefore may worsen outcome after hypoxic-ischemic injury.

1.7.2 Non-protein-bound iron

1.7.2.1 Role of non-protein-bound iron in reactive oxygen species formation

Although aerobes need oxygen (O_2) for survival, O_2 concentrations greater than those present in normal air have often been suggested to cause oxidative damage[35]. More

than 90% of the O_2 taken up by the human body is used by mitochondrial cytochrome oxidase. This enzyme, like most others that use O_2, has transition metal ions at its active sites. Transition metals such as iron, but also vanadium, copper and titanium have variable oxidation states: switching between these states allows them to transfer single electrons and thus to facilitate oxidation-reduction reactions[84]. The reduction of O_2 to 2 H_2O by cytochrome oxidase in the mitochondria proceeds in a stepwise fashion, with various partially reduced forms of oxygen normally firmly bound to the metal ions within the enzyme. The rate of leakage of reactive oxygen species under physiological conditions is probably less than 5% of the total electron flow, but it rises with increases in O_2-concentration and mitochondrial damage, such as occurs during post hypoxic-ischemic reperfusion[35]. Moreover, post hypoxic-ischemic reperfusion leads to an increased production of reactive oxygen species, such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), by other sources such as the calcium-induced production of prostaglandins, by activated neutrophils and macrophages, and possibly by xanthine oxidase. Despite the fact that the enzymes SOD and catalase were found to provide neuroprotection in animal models, the reactive oxygen species which are scavenged by these enzymes, i.e. $O_2^{\bullet -}$ and H_2O_2, are weakly reactive oxygen species, and not considered capable of causing significant tissue injury by themselves[38]. However, when they react with transition metal ions, in particular non-protein-bound iron, more reactive oxygen species, such as the highly reactive hydroxyl radical ($OH^{\bullet}$) can be formed by means of the superoxide driven Fenton reaction (reaction 1) or iron catalyzed Haber-Weiss reaction (reaction 2)[35].

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad (1)$$

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{NPBI} OH^{\bullet} + OH^- + O_2 \quad (2)$$

Hydroxyl radicals are able to react at great speed with almost every molecule found in living cells, including DNA (causing strand breakage and chemical alterations of the deoxyribose, purine and pyrimidine bases), membrane lipids and carbohydrates. Moreover they are capable of initiating the process of lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated fatty acid side chain in a membrane lipid[35]. Poly-unsaturated fatty acid side chains (those with two or more carbon double bounds), such as arachidonic acid, are much more sensitive to reactive oxygen species attack than saturated or mono-unsaturated side chains. Abstraction of a hydrogen atom

leaves behind a radical lipid (lipid•) in the membrane. The most likely fate of these lipids• is reaction with O_2 to form peroxyl radicals (lipid-O_2•). In turn, these peroxyl radicals can attack membrane proteins (thus damaging receptors and enzymes), but can also abstract hydrogen atoms from adjacent fatty acid side chains, thereby inducing a chain reaction leading to conversion of many membrane lipids into lipid hydroperoxides (lipid-O_2H)[35]. The existence of lipid hydroperoxides within a membrane severely disrupts its function, altering (usually decreasing) its fluidity, and allowing ions such as Ca^{2+} to leak across the membrane. This occurs in addition to the damage (already) produced by the attack by peroxyl radicals on membrane proteins.

Non-protein-bound iron can contribute to lipid peroxidation in two ways[35]. First it can catalyze the formation of highly toxic reactive oxygen species which are able to initiate the process of lipid peroxidation (e.g. the production of the highly reactive OH•). Second, it is able to stimulate peroxidation by reacting with lipid hydroperoxides, thereby decomposing them into peroxyl radicals (lipid-O_2•) and alkoxyl radicals (lipid-O•) which, in turn, can abstract other hydrogen atoms and so lead to further peroxidation[35]. Under physiological circumstances, the endogenous scavenger α-tocopherol (active part of vitamin E) acts as a membrane bound chain-breaking anti-oxidant[32]. Once lipid peroxidation is initiated, peroxyl radicals react with α-tocopherol instead of an adjacent fatty acid, thus terminating the process. More protection against the formation of new reactive oxygen species can be achieved by elimination of hydrogen peroxide, superoxide, or the catalyst of the reaction, non-protein-bound iron. A recent study has shown that iron scavenging reduces the metabolic decay and delayed hypoperfusion after cerebral ischemia in dogs[75]. An important form of anti-oxidant defense under physiological circumstances is the storage and transport of iron in forms that are unable to catalyze the formation of reactive oxygen species.

1.7.2.2 Storage and transport of iron

Iron has been shown to be a remarkable useful metal in nature[35]. It is required for several important processes, such as oxygen transport (hemoglobin), storage (myoglobin), mitochondrial respiration, proper function of several important enzymes, and antibacterial defense. In mammals it is absorbed from the gut and enters the plasma attached to the protein transferrin. This protein has a very high affinity for non-protein-bound iron at pH 7.4, which means that is it usually firmly bound to transferrin under physiological circumstances. In human plasma, the average iron loading of transferrin is normally 20-30% of its maximum. Hence, there is an excess of iron binding capacity, which implies that the concentration of non-protein-bound iron in

plasma should be effectively nil. This was indeed confirmed in normal adults, having no detectable concentrations of non-protein-bound iron in their plasma[85]. Transferrin enters the cell by endocytosis followed by lowering of the pH of the vacuole containing it, which facilitates the release of iron from the protein. The unloaded transferrin (apotransferrin) is subsequently ejected from the cell and the non-protein-bound iron released from it is used for the synthesis of new intracellular iron proteins[35]. Excess iron is usually stored in the protein ferritin. Iron ions attached to the transport protein transferrin, to the neutrophil-derived protein lactoferrin, to the iron storage proteins ferritin and hemosiderin, to the O_2-binding proteins hemoglobin, and myoglobin, and to other iron proteins, are thought to be incapable of inducing lipid peroxidation or producing hydroxyl radicals by catalyzing the Haber-Weiss reaction, and therefore provide safe iron transport and storage systems at physiological pH[35]. However, lowering of the plasma pH, as occurs during hypoxia-ischemia, enables transferrin to release its iron, thereby inducing the production of reactive oxygen species[22]. These reactive oxygen species in turn, are capable of releasing even more iron by mobilizing it from ferritin[86]. Moreover, attack of iron-bearing proteins by nitric oxide may also lead to the release of non-protein-bound iron (see below). By these mechanisms a cascade of iron release and production of reactive oxygen species can be activated leading to extensive cell damage.

1.7.2.3 Brain and non-protein-bound iron

Although several organs may be the target of oxidative damage, the brain may be especially at risk because of a number of reasons: *1.* the neuronal membranes are very rich in polyunsaturated fatty acids, which are especially sensitive to reactive oxygen species-mediated lipid peroxidation; *2.* several areas of the human brain (e.g. the globus pallidus and substantia nigra) are especially rich in iron[35,87]; *3.* the brain is very poor in catalase activity and has only moderate amounts of superoxide dismutase and glutathione peroxidase[88]; *4.* the cerebrospinal fluid has almost no iron binding capacity, because of a very low concentration of transferrin[89]; *5.* most of the iron will be in its "reactive" ferrous (Fe^{2+}) form because of a high concentration of vitamin C, which is able to reduce the less reactive ferric ion (Fe^{3+}) to the far more reactive ferrous ion, which is unable to bind to transferrin; and *6.* there is a low concentration of the ferrous-ion-oxidizing protein ceruloplasmin in cerebrospinal fluid which is able to convert the reactive ferrous ions back to the less reactive ferric state[89,90].

1.7.2.4 Storage and transport in neonates

As described earlier, the storage and transport of iron normally occurs in (protein-bound) forms unable to catalyze the formation of reactive oxygen species. It is

therefore not surprising that non-protein-bound iron is normally undetectable in adult plasma. However, recent studies showed that apparently healthy neonates often have detectable amounts of non-protein-bound iron in their plasma[91,92]. The appearance of non-protein-bound iron in plasma of neonates seems to be inversely related to gestational age. In a study by Moison et al.[91] none of the adults had detectable amounts of non-protein-bound iron in their plasma, but 6 out of 24 term and 10 out of 21 preterm neonates had detectable concentrations. Almost the same results were found in a study of Evans et al.[92] in which 11 out of 52 term but 3 out of 15 preterm neonates had detectable concentrations non-protein-bound iron in their plasma. This means that neonates may be especially susceptible to oxidative damage, such as may occur following perinatal hypoxia-ischemia. However, the neonate may be even more susceptible for oxidative damage because of a number of other reasons: *1.* the ability of plasma of neonates to inhibit non-protein-bound iron induced lipid peroxidation in vitro is significantly less as compared to the ability of adults' plasma[93]; *2.* neonates may have a relative deficiency of brain superoxide dismutase[94]; *3.* neonates have a relative inability to sequester non-protein-bound iron because of a low concentration of transferrin[93]; and *4.* because of the high concentration of vitamin C and low concentration of ceruloplasmin in the plasma of neonates, the non-protein-bound iron in these neonates is likely to be in the highly reactive ferrous form in stead of the less reactive ferric form[93]. In view of these combined circumstances it may be concluded that neonates are very susceptible to non-protein-bound iron induced oxidative damage.

1.7.3 *Nitric oxide*

1.7.3.1 Introduction

Made of a single atom of nitrogen and a single atom of oxygen, nitric oxide ($NO^{\bullet}$) is one of the 10 smallest molecules of the hundreds of millions found in nature[29,95,96]. Nitric oxide is a gaseous reactive oxygen species with an uneven number of electrons in its outermost orbital, which makes it very unstable as most biologic molecules have their electrons arranged in pairs. Nitric oxide tends to react rapidly with other atoms or molecules that also contain unpaired electrons, such as oxygen, $O_2^{\bullet -}$, and various metals (e.g. iron, copper or manganese), which are usually bound to a protein. Nitric oxide can react with molecular oxygen to form ultimately either of two, largely unreactive anions, nitrite (NO_2^-), and nitrate (NO_3^-). This is typically the path by which nitric oxide is inactivated in cells[29]. Because nitric oxide is so highly reactive with oxygen, cells do not need an enzymatic reaction for its removal. However, the molecule's high reactivity also means that nitric oxide's effects are largely dictated by the amount produced, and by its immediate environment. Nitric oxide is soluble in

water as well as in lipid and, until about ten years ago, was not thought to play any role in the body. The gas had achieved its greatest notoriety as one of the noxious gases appearing in the exhaust from motorcars, as a pollutant that contributes to the formation of smog and acid rain, and as a product that contributes to the destruction of the ozone layer[29,96]. Nowadays nitric oxide has been implicated in a large number of physiologic processes, as well as diseases and disorders. Among the first places in which nitric oxide was found to have a biological role, was in the protective functions of the immune system. It was discovered that nitric oxide was the mediator of tumoricidal and bactericidal effects of stimulated macrophages, natural killer cells, T-lymphocytes and other cells of the immune system[29]. The cells killed by macrophages were found to loose the function of certain iron-bearing enzymes that are involved in cellular respiration. The conclusion was clear: nitric oxide can damage cells by binding to these iron-bearing enzymes, thereby preventing them from working, resulting in cell death[29]. Moreover, attack of iron-bearing proteins may lead to the release of iron from its binding proteins, which may result in massive oxidative injury through the Haber-Weiss reaction (see paragraph 1.7.2.1)[96]. The reaction of nitric oxide with a metal-bearing protein is intriguing because it can have either of two opposite effects. In some cases nitric oxide can activate the protein, for example by triggering the catalytic effect of an enzyme. In other instances however, nitric oxide binds to the metal at the protein active site, thereby preventing it from carrying out its normal function, e.g. inhaled in the lungs, the binding of nitric oxide to heme prevents the binding of oxygen, and therefore its role in respiration[29]. Later on it became apparent that nitric oxide was not only a killer of cells, but also an intracellular messenger in blood vessels, where a continuous production by endothelial cells acts on the underlying smooth muscle cells to maintain the dilatation of blood vessels and nutritious blood flow[29,95,96]. Moreover, nitric oxide turned out to be the same molecule as the well known substance endothelium-derived relaxing factor (EDRF). The beneficial effects of drugs such as glyceril trinitrate in angina pectoris have been known since 1867, but it is only now that we fully realize that they act by releasing nitric oxide in the vascular wall, leading to vasodilatation and relief of chest pain. Other functions of the endothelial derived nitric oxide is the inhibition of the adhesion and aggregation of activated platelets and leukocytes[96]. In the gastrointestinal system, nitric oxide has been shown to be responsible for gastric dilatation and for peristalsis. Moreover, a selective lack of nitric oxide mediated dilatation has been shown in infants with pyloric stenosis[96]. Recently, a new therapeutic window was opened by the finding that inhalation of nitric oxide relieves pulmonary hypertension. Finally nitric oxide has been shown to play a key role in glutamate mediated neuronal transmission and excitotoxicity, a process in

which excessive release of glutamate and nitric oxide leads to overexcitation of neuronal cells with subsequent neuronal cell death (see paragraph 1.7.3.3).

1.7.3.2 nitric oxide biosynthesis and release

Nitric oxide can be produced from a specific nitrogen atom from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS) (see figure 1.1)[29]. The oxygen atom is derived from molecular oxygen gas and the reaction requires nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor and tetrahydrobiopterin as a cofactor. By the removal of the nitrogen atom, the amino acid L-arginine is converted into the byproduct citrulline, which can be recycled back to L-arginine. At least three isoforms of NOS have been identified[97].

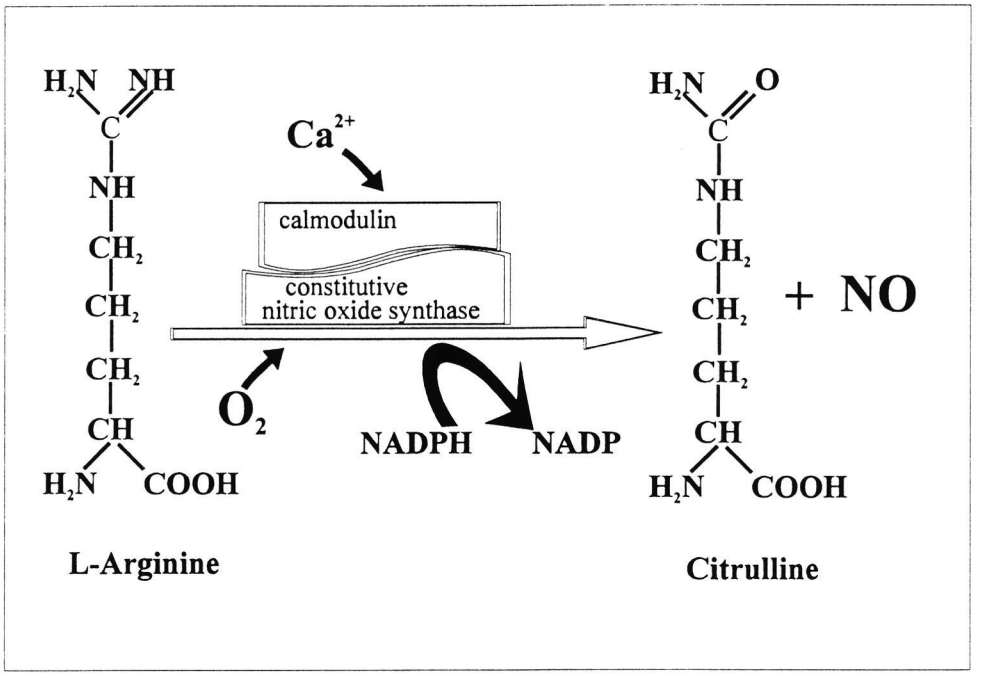

Figure 1.1 Schematic representation of the synthesis of nitric oxide from L-Arginine by the constitutive nitric oxide synthase (NOS) forms. (modified from Lancaster[29])

The forms of NOS found in brain cells (neuronal isoform; nNOS) and in the endothelial wall of blood vessels (endothelial isoform; eNOS) are almost identical. These two forms of NOS are constitutive enzymes, which means that they are present

all the time. When eNOS or nNOS containing cells are given the appropriate stimuli (e.g. acetylcholine, adenosine diphosphate, bradykinin or shear stress [endothelial cell], or glutamate [neuronal cells]), receptor activation leads to an increase in cytosolic calcium, which activates the regulatory protein calmodulin[96].The eNOS or nNOS, in turn, is then activated by the regulatory protein calmodulin resulting in a short bust of nitric oxide. In contrast, the macrophages of the immune system, and a wide variety of other cells, including neutrophils, mast cells, but also endothelial cells and vascular smooth muscle cells, can express an inducible form of NOS (iNOS), which is only produced when the cells receive a special signal (e.g. bacterial toxins, gamma-interferon, interleukin-1)[96,97]. This means that the inducible form is not present all the time: it is only produced several hours after traumatic or hypoxic-ischemic injury[41,98]. Moreover the inducible form of NOS doesn't need to be activated by the regulatory protein calmodulin and also produces much greater amounts of nitric oxide than the constitutive forms. The amount of nitric oxide released per unit of time from fully stimulated macrophages has been shown to be thousand times higher than that released from eNOS in endothelial cells, although the amount of nitric oxide released from eNOS and nNOS enhances during pathological situations (e.g. essential hypertension).

1.7.3.3 Nitric oxide and glutamate mediated neuronal transmission

Neuronal activity, and therefore all functions attributed to the brain, is based on the release of neurotransmitters which act subsequently on a second neuron at the site of a tight coupling between the neurons, called a neuronal synapse[29]. The action can be excitatory (increasing electrical activity), or inhibitory (decreasing electrical activity). Of the excitatory neurotransmitters in the brain, the most significant is the amino acid glutamate. Figure 1.2 shows a schematic representation of the neuronal transmission by the neurotransmitter glutamate[29]. Upon neuronal transmission, glutamate is released from the presynaptic neuron and binds to the postsynaptic N-Methyl-D-Aspartate (NMDA) receptor (see below), leading to Ca^{2+}-influx. Ca^{2+} in turn, binds to the regulatory protein calmodulin, which activates the enzyme nNOS. This enzyme subsequently produces nitric oxide from its precursor L-arginine in a NADPH and oxygen dependent manner. The post-synaptic produced nitric oxide then acts as a retrograde messenger by diffusing back to the presynaptic neuron where it activates the enzyme guanylate cyclase. Guanylate cyclase subsequently catalyzes the reaction that produces the intracellular signaling molecule cyclic guanosine monophosphate (cGMP). In turn cGMP initiates events that ultimately lead to the release of more glutamate, and the circle repeats itself. In the hippocampus, this phenomenon, in which the strength of a synaptic contact is increased as a consequence of frequent use, is

called long term potentation, and has been thought to contribute to the process of learning and the formation of memories[29]. Hypoxia-ischemia leads to a small rise in glutamate and nitric-oxide[24]. During ischemia-reperfusion however, when oxygen becomes suddenly available, excessive amounts of glutamate are released[24]. As a consequence of the repetition of the circle, there may be an excessive production of nitric oxide and glutamate and the neurons may literally excite themselves to death, a process commonly called excitotoxicity.

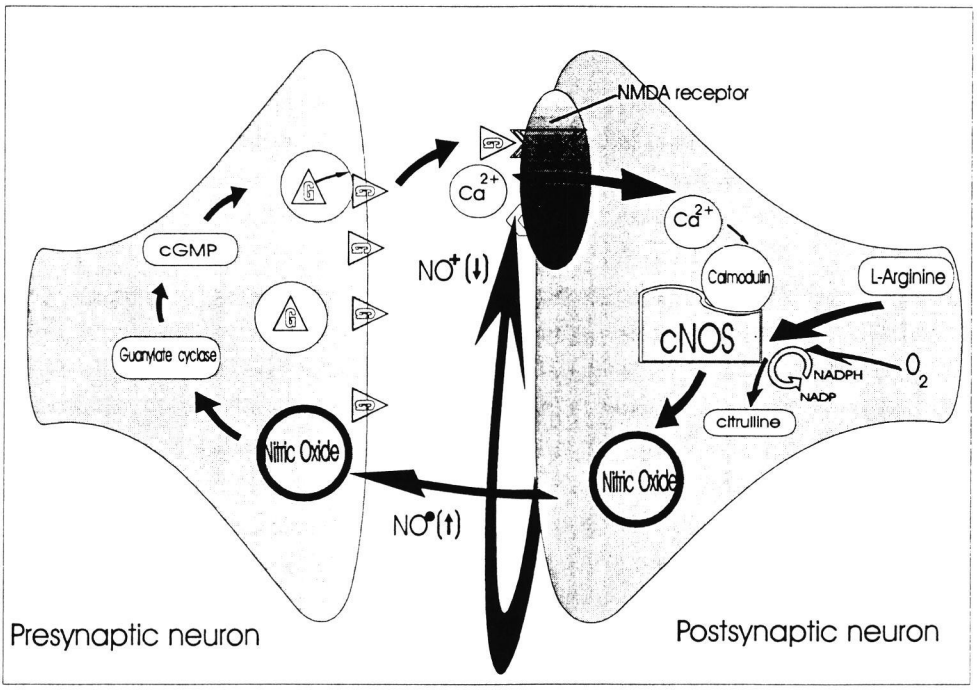

Figure 1.2 Schematic representation of neuronal transmission. Up and downregulation of the neuronal transmission are indicated by respectively (↑) and (↓). = glutamate. (modified from Lancaster[29])

Particular importance for the synthesis of nitric oxide in the mediation of neuronal cell death with glutamate excitotoxicity has been shown by some work, but was not confirmed by all[30,95,96]. The discrepant findings appears to be related to the capacity of derivatives of nitrogen monoxide (NO) to exist in several redox forms. The

chemical state is dependent on the removal or addition of an electron to nitric oxide, a condition that can be influenced by the presence or absence of electron donors such as ascorbic acid or the amino acid cysteine.

The neurotoxic, reactive oxygen species generating form, is nitric oxide ($NO^{\bullet}$) and the apparently neuroprotective form with one electron less is the nitrosium ion (NO^{+}). Work by Lipton and coworkers in cultured neurons has demonstrated that the redox state of nitric oxide is crucial in determining whether neurotoxicity or neuroprotection will occur[99]. Nitric oxide ($NO^{\bullet}$), the toxic form, can be generated by NMDA-receptor activation. Toxicity may occur by the subsequent generation of peroxinitrite ($ONOO^{-}$) (see paragraph 1.7.3.8)[79,100]. However, the nitrosium form of nitrogen monoxide (NO^{+}), which can be generated by sodium nitroprusside, is neuroprotective because it can react with critical thiol(s) on the NMDA-receptor's redox modulatory site to downregulate its channel activity[99], and thereby prevent excessive Ca^{2+}-influx. Up and downregulation of the neuronal transmission are indicated by respectively ($\uparrow$) and ($\downarrow$) in figure 1.2.

1.7.3.4 Hypoxia-ischemia induced glutamate release

Research in animal models during the last decade has shown that the function of certain synapses, especially those that use excitatory amino acids for neurotransmission, is disrupted in the brain following hypoxia-ischemia[101,102]. Moreover, hypoxia-ischemia has been shown to cause a marked increase in extracellular levels of glutamate[101]. One of the first studies establishing this phenomenon has been shown that extracellular glutamate concentrations in vivo increase many-fold with hypoxic ischemic insults in the perinatal animal model. Moreover, glutamate concentrations in the cerebrospinal fluid of asphyxiated neonates are approximately five times larger than concentrations in normal neonates[103]. The elevated levels of extracellular glutamate appear to be caused by a combination of enhanced release by and diminished re-uptake into the synaptic nerve terminals[101,104]. The combination of hypoxia and ischemia has been shown to produce an acute disruption in the ability of synaptosomes to take up glutamate in a sodium-dependent manner[101]. This defect can be modified in vitro by adding serum albumin to the incubation medium, suggesting that the effect may be related to an accumulation of fatty acids within the cellular membranes. Moreover, this experiment demonstrates that the defect in glutamate uptake is not related to neuronal death, but to an acute (and reversible) change in glutamate uptake mechanisms. It is noteworthy that injections of a glutamate agonist, N-methyl-D-Aspartate, also reproduce this acute change in glutamate uptake into presynaptic nerve terminals[105], which means that

overstimulation of the NMDA-type glutamate receptor may initiate a chain of events which cause a reduction in glutamate uptake and is able to trigger a cycle of events which further increase cellular damage.

The reasons for the increase in extracellular glutamate with hypoxic-ischemic insults relate not only to an impaired energy dependent uptake of glutamate by astrocytes and presynaptic nerve endings, but also to excessive release. This excessive release of glutamate is related to at least three factors. The first of these is the persistent membrane depolarization resulting from failure of the Na^+, K^+-(ATP)-dependent pump[106]. Secondly, destruction of the inhibitory neurotransmitter gamma-aminobutyric-acid (GABA) neurons by hypoxia may also contribute to the excessive release of glutamate[107]. A third factor is the rapid blockade of inhibitory synaptic transmission with relative preservation of excitatory synaptic transmission with anoxia in the immature versus the adult animal[108].

1.7.3.5 The role of nitric oxide in glutamate mediated excitotoxicity

It is now well established that overstimulation of glutamate receptors may play an important role in the pathogenesis of neuronal injury from hypoxia-ischemia[109]. The important initial observation was that cultured hippocampal neurons obtained from the fetal rat were resistant to prolonged anoxia before synaptic formation occurred in the cultures, but were very sensitive to the same anoxic insults after synaptogenesis was well developed[110]. Further cell culture experiments indicated that blockade of glutamate receptors markedly increased the tolerance of neurons to severe hypoxia[111]. The particular role of glutamate synapses in hippocampal neuronal death was further supported by the demonstration that hypoxic-ischemic neuronal injury could be prevented in vivo by prior section of glutamatergic afferents[112]. In the brains of animals and humans a good correlation has been found between the distribution of the glutamate receptors and the locations of neuronal injury from hypoxia-ischemia[102]. Moreover, in a neonatal rat model of hypoxia-ischemia, MK-801 (a NMDA-receptor antagonist) has been shown to be neuroprotective even when administered up to 1 hour after the end of a hypoxic-ischemic insult[113]. These observations suggest that the presence of glutamate receptors on neurons may make them more susceptible to hypoxic injury.

There are generally two families of related glutamate receptor subtypes[101]. These receptors have been generally classified into NMDA-type glutamate receptors and non-NMDA-type glutamate receptors, depending on the agonist analogues that preferentially stimulate them. The glutamate receptors have been further divided into

metabotropic and ionotropic (i.e., linked to ion channels) glutamate receptors. The NMDA-type glutamate receptor is an ionotropic glutamate receptor linked to an ion channel for Ca^{2+} and Na^{+} entry. The non-NMDA-type glutamate receptors can be divided in one metabotropic receptor and two ionotropic receptors (e.g. the α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) and Kainate receptor, both linked to an ion channel for Na^{+} entry). All of these receptor subtypes have been implicated in the process of hypoxic-ischemic injury[101]. However, different receptor subpopulations appear to be more susceptible to overstimulation at different ages. The neonatal rodent is particularly sensitive to damage from overstimulation of NMDA receptors, whereas the adult brain is more sensitive to overstimulation of non-NMDA-type receptors[114]. The histological pattern of NMDA-mediated injury in the neonatal animal is very similar to injury from hypoxia-ischemia in the neonate[115]. This enhanced vulnerability to NMDA overstimulation, as well as the efficacy of NMDA-receptor blockade type neuroprotective agents against hypoxia in the neonatal animal, suggest that in particular the NMDA-receptor plays a special role in perinatal hypoxia-ischemia.

The NMDA-receptor channel complex includes receptors for glutamate as well as the simple excitatory amino acid glycine and a receptor operated channel which passes both Ca^{2+} and Na^{+}. It opens in response to glutamate binding, but a relative unique feature of the receptor operated channel complex is, that it is ordinarily blocked by a magnesium ion[18,116]. Blockade of the receptor operated channel complex is voltage dependent and membrane depolarization is needed to remove this block, e.g. when the membrane is partially depolarized, the magnesium ion is not longer able to prevent neuronal Ca^{2+} -influx by glutamate binding. This characteristic means that the receptor channel complex can be opened more easily under conditions in which the tissue membranes are depolarized[117], such as in perinatal hypoxia-ischemia. The depolarized, energy-deficient neuronal tissue might then allow NMDA receptor channel complexes to pass large amounts of calcium *(and produce high amounts of nitric oxide)* even if the extracellular concentration of glutamate is not abnormally elevated.

There are generally two mechanisms of glutamate induced neuronal death[101,102]. One of these is the *rapid cell death* that occurs in the first min after the initial hypoxic-ischemic insult and is initiated by glutamate receptor activation: Na^{+} entry through all three ionotropic receptors, accompanied by passive influx of Cl^{-} down its electrochemical gradient with H_2O following, leading ultimately to cell swelling and lysis. The next mechanism, the so-called *delayed cell death*, occurs over the next hours

following the initial post hypoxic-ischemic "NO-burst". This mechanism is primarily initiated by activation of the NMDA receptor, with influx of Ca^{2+} (as well as Na^{+}) and subsequent production of nitric oxide and glutamate mediated excitotoxicity. It is now well established that the delayed cell death appears to be the crucial form of neuronal death in vivo. Moreover the importance of the NMDA receptor and Ca^{2+} influx is well established by studies of specific blockers of the NMDA receptor channel complex[101,102,113].

1.7.3.6 Ontogeny of nitric oxide synthase expression

The appearance of nitric oxide synthase activity during development seems to depend on the species studied and on the stage of development. In the guinea pig, NOS activity increases from an almost undetectable level at 0.49 of gestation to adult levels before birth and peaking at 140% to 250% of the adult activity in the week after birth. In rats, brain NOS activity does not rise significantly until after birth, reaching adult levels approximately 2 weeks after birth and rising to 150% to 130% of the adult activity at 4 weeks after birth[118]. In both species the appearance of high NOS activity in the brain immediately precedes the period in which maximal synaptogenesis occurs. The predominantly prenatal and postnatal synaptogenesis in the guinea pig and rat respectively is reflected in the general neurologic maturity of these two species at birth. The guinea pig is born essential functional and with its eyes open, whereas the rat is born with limited mobility and with its eyes closed. Studies in fetal and adult sheep demonstrated that the development of the expression of the neuronal isoform of NOS is different in the various regions of the brain, showing a transient, region- and cell type dependent expression[119]. This may indicate a temporally and spatially restricted role for the neuronal isoform of NOS in the maturation of specific neuronal cell populations. The expression of the endothelial isoform of NOS, however, seems to be relatively constant throughout development and parallels the maturation of the cerebrovasculature.

1.7.3.7 Distribution of nitric oxide mediated glutamate excitotoxicity

In animal models immunostaining for nNOS is only observed in 2% of the cerebral cortical, striatal and hippocampal neurons without any apparent colocalization with any known other neurotransmitter[30,95,96]. The highest densities of nNOS are observed in the neurons of the granule cell layer of the cerebellum and in the accessory olfactory bulb[120]. Although only 2% of the cortical neurons seem to contain nNOS, NMDA-receptor mediated excitotoxicity in primary cerebral cortical cultures has been shown to destroy 90% or more of the neurons, whereas the nNOS containing neurons are selectively spared[120]. A way out of this dilemma would be to propose that normally

the nNOS containing neurons elevate cGMP levels in adjacent neurons by means of nitric oxide release without toxicity. However, in the presence of high levels of glutamate, such as following ischemia-reperfusion, the nNOS containing neurons may act like activated macrophages, thereby releasing such large amounts of nitric oxide, that it kills their neighboring nerve cells. Purkinje cells lack nNOS, but have high levels of guanylate cyclase and may therefore be candidate target cells for the neuronal nitric oxide[30,96]. Stimulation by glutamate NMDA receptors results in the triggering of nitric oxide formation, which subsequently diffuses to the neighboring Purkinje cell to activate guanylate cyclase, ultimately leading to cell death. Why nNOS neurons are selectively resistant to glutamate-mediated excitotoxicity is not known. Superoxide dismutase could, in principle, protect these cells against such toxicity. Conceivably nNOS neurons are rich in superoxide dismutase, which would account in part for their resistance[30].

1.7.3.8 Other mechanisms of nitric oxide mediated neurotoxicity

Besides the nitric oxide mediated glutamate neurotoxicity, nitric oxide and its degradation products can cause direct cytotoxicity through: *1.* the formation of iron-nitric oxide complexes with mitochondrial electron transport enzymes; *2.* by the oxidation of protein sulfhydryls; and *3.* by DNA nitration[97]. Moreover, nitric oxide has been shown to be very destructive by means of peroxinitrite mediated vascular damage. It is well known that hypoxia-ischemia may lead to oxidative vascular injury which subsequently causes loss of endothelial barrier function, adhesion of platelets and an abnormal vasoregulation. Recently, Beckman has shown that nitric oxide and $O_2^{\bullet -}$ are important mediators of this post hypoxic-ischemic vascular injury[79]. Figure 1.3 illustrates how nitric oxide and $O_2^{\bullet -}$ may be involved in the vascular injury following ischemia reperfusion. In the first stage, ischemia allows Ca^{2+} to enter into the endothelial cell, due to a failure of the energy dependent ion pumps and opening of the ion channels, thereby stimulating the enzyme eNOS. Although neurons, astrocytes, perivascular nerves and cerebrovascular endothelium have been shown to form some nitric oxide during ischemia[97], the endothelium won't produce large amounts of nitric oxide due to a lack of oxygen. Recent studies have shown a rapid increase of nitric oxide production at the onset of ischemia, possibly as a physiological reaction to increase the cerebral blood flow[121]. During periods of only hypoxia without ischemia up to 1 hour nitric oxide production still remains[122]. However, during periods of ischemia up to 1 hour, the nitric oxide production finally declines due to a lack of oxygen, NADPH and L-Arginine[123]. During reperfusion/reoxygenation however, when oxygen is suddenly available in excess, the already activated enzyme is now able to produce excessive amounts of nitric oxide.

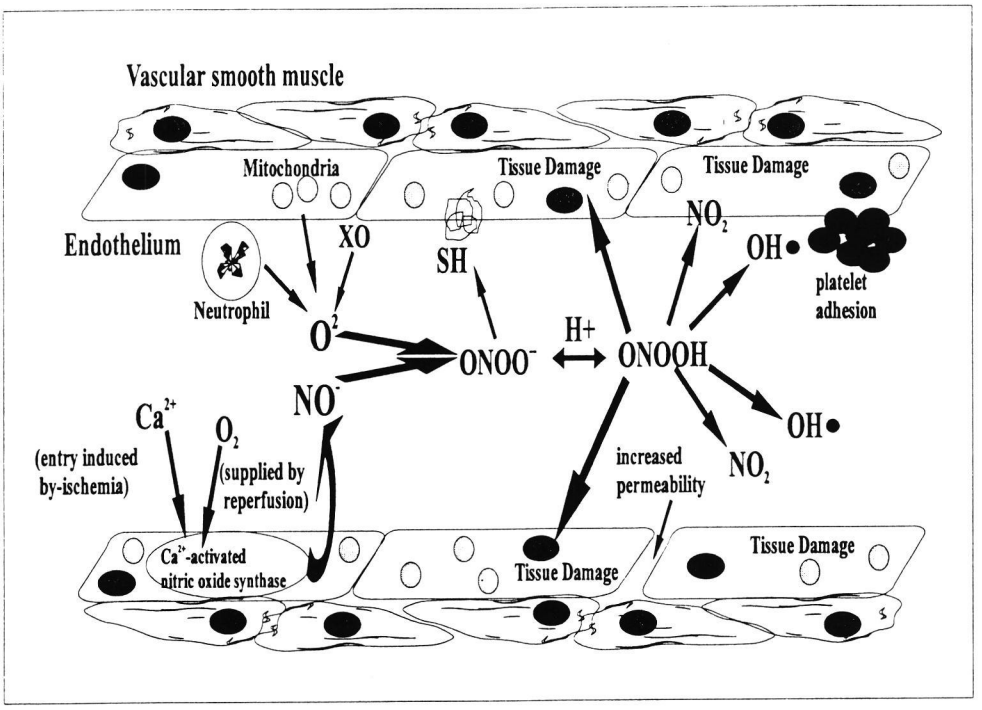

Figure 1.3 Schematic representation of an intracerebral vessel during ischemia reperfusion. (modified from Beckman[79])

Sato et al. have shown an increase in nitric oxide production within 15 min of reperfusion[121]. At the same time $O_2^{\bullet -}$ may be produced by damaged mitochondria, activated neutrophils, and possibly by xanthine oxidase. The produced nitric oxide can then react rapidly with $O_2^{\bullet -}$, both intracellularly and in the vascular lumen to form highly reactive peroxinitrite ($ONOO^-$), which can diffuse for several micrometers before excerting its toxicity. Peroxinitrite may be toxic by at least three mechanisms: *1.* direct reaction with sulfhydryl groups; *2.* reaction with metal ions to form a powerful nitrating agent; and *3.* by decomposing into the highly toxic hydroxyl radical and nitrogen dioxide. Moreover peroxinitrite has been shown to cause lipid peroxidation. The resulting endothelial injury may lead to edema formation, due to the loss of barrier function, adhesion of platelets and neutrophils causing vascular plugging, and abnormal vasoregulation, all of which may exacerbate post hypoxic-ischemic brain injury.

1.8 Therapeutical intervention after perinatal hypoxia-ischemia

Review of the literature made clear to us that there is not only one cause of brain damage after perinatal hypoxia-ischemia. There seems to be a broad range of sequences of events occurring after the primary hypoxic-ischemic insult, which are related to each other. Each of these events may individually influence the course of the post hypoxic-ischemic brain damage and the subsequent neurological outcome. Because these sequences comprise a scale of events, there is in point of fact not one causal therapy. In this paragraph it is tried to simplify the several possible steps of the complex metabolic and biochemical events occurring with post hypoxic-ischemic brain damage in a summarizing figure (figure 1.4). Also speculations are made about potential targets for (pharmaco) therapeutical intervention after perinatal hypoxia-ischemia.

With respect to figure 1.4 there seem to be several potential targets for therapeutical intervention after perinatal hypoxia-ischemia, some of which have already been used in experimental studies.

1. Drugs that inhibit the Ca^{2+}-influx in neurons. Given the strategic role of Ca^{2+} in the regulation of cell metabolism and its potential for neurotoxicity when intracellular concentrations increase to dangerous levels, drugs have been developed that inhibit the Ca^{2+}-influx in neurons. Flunarizine and nimodipine have been shown to reduce the extent of hypoxic-ischemic brain damage in several adult animal models. Although flunarizine was able to improve the ultimate brain damage produced by hypoxia-ischemia in the immature rat, nimodipine was not[124].Thus it remains to be clarified as to whether or not calcium channel blockers are effective in preventing or reducing the extent of perinatal hypoxic-ischemic brain damage. Furthermore, a recent clinical investigation in which the calcium channel blocker nicardipine was used in four severely asphyxiated fullterm neonates[125], reported an accompanying systemic hypotension, indicating that these drugs must be used with caution in the clinical setting to prevent worsening of the brain damage.

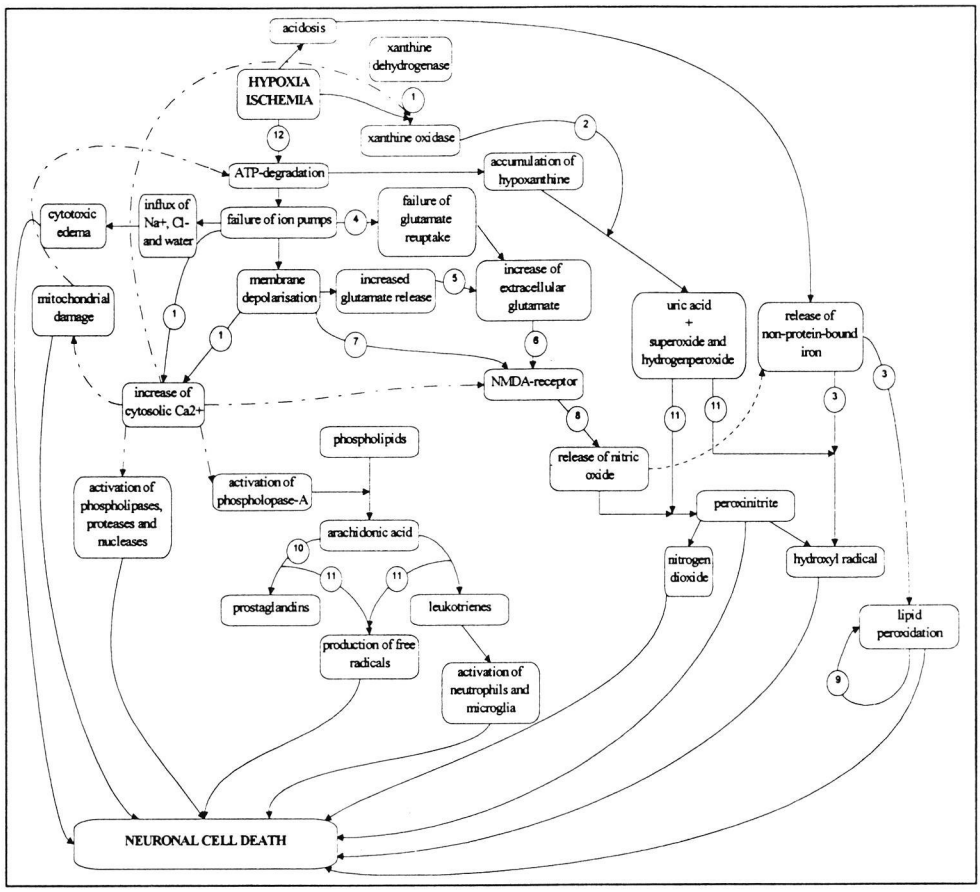

Figure 1.4 Schematic representation of pathophysiology of post hypoxic-ischemic brain damage and potential targets (indicated by numbers 1-12) for (pharmaco) therapeutical intervention.

2. Allopurinol and its active metabolite oxypurinol are inhibitors of the enzyme xanthine oxidase and have been shown neuroprotective properties in perinatal animal models of ischemic brain injury[37]. Although the neuroprotective mechanisms of allopurinol have usually been attributed to its ability to inhibit xanthine oxidase, recent studies have been shown that doses in excess of that required to inhibit xanthine oxidase are needed to produce neuroprotection to cerebral ischemia[126,127]. At these high dosages, allopurinol and oxypurinol could exert other neuroprotective mechanisms besides xanthine oxidase inhibition. For

example, they can both inhibit neutrophil lysosomal enzyme release[128], scavenge hydroxyl radicals[129], chelate transition metals in proportion to their concentration[130,131] and act as an electron transfer agent from ferrous ion to ferric cytochrome c[132].

3. Deferoxamine, the prototype chelating agent, which especially focuses on scavenging the non-protein-bound iron, has been used successfully to diminish oxidative damage in various studies[133,134,135]. However, caution is required, since deferoxamine has been shown to be toxic in premature baboons[136]. Other promising therapies, which focus especially on scavenging the non-protein-bound iron may be the administration of fresh adult plasma (high content of unsaturated transferrin) and/or an exchange transfusion. In neonates, exchange transfusions have been shown to lower iron, ferritin and vitamin C levels and raise the concentration of transferrin, thereby increasing the latent iron-binding capacity[137].

4. The failure of the glutamate re-uptake systems may be a potential target for therapeutical intervention.

5. Inhibition of glutamate release from the nerve terminal (e.g. baclofen). This has however not yet been investigated as a neuroprotective drug[124].

6. Studies searching for antagonists of the NMDA-receptor channel complex generally focus on two distinct sides of regulation: antagonists of the glutamate recognition site of the NMDA-receptor channel complex (e.g. CPP), and antagonists who act at the site of the receptor operated ion channel (e.g. dextromethorphan and MK-801). CPP (3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid), a potent selective competitive antagonist of the glutamate recognition site of the NMDA-receptor channel complex, has been shown to be able to prevent hypoxia induced modification of the NMDA-receptor channel complex in newborn piglets[138]. Recent studies have shown that pre-ischemic administration of dextromethorphan, a common antitussive agent which non-competitively blocks the NMDA-receptor channel complex by acting at the site of the receptor operated ion channel, is able to attenuate post-ischemic cerebral reperfusion injury[139]. Another NMDA-receptor channel complex blocker acting at the site of the receptor operated ion channel is MK-801, which has been shown to be neuroprotective even when administrated up to 1 hour after the end of a hypoxic-ischemic insult[113]. However, MK801 has been shown to cause

neuropsychiatric side effects and must therefore be used very cautiously in a clinical setting[140]. Moreover, since the NMDA-receptor channel complex is expressed especially early in brain development, the long term effects of blockade of the NMDA-receptor channel complex on the neurodevelopment of the brain have to be established before clinical application is possible.

7. Magnesium ions gate the NMDA channel in a voltage dependent manner by producing hyperpolarization. Increasing the extracellular concentration of Mg^{2+} may therefore protect the brain from NMDA-receptor mediated damage. The neuroprotective effect of Mg^{2+} has been evaluated in a neonatal rat model, where a single dose of 2 mmol/kg, 15 min after an NMDA insult, significantly reduced subsequent brain injury. The effect was even greater when higher or multiple doses were used[141]. In a recent clinical study the side effects of two different doses of magnesium sulfate were studied in 15 severely asphyxiated fullterm neonates[142]. A high dose of 400 mg/kg has been shown to have an unacceptable risk of hypotension and a three to six hours lasting respiratory depression, without significant heart rate and EEG changes; the low dose of 250 mg/kg was not associated with hypotension, but one of the eight infants developed a transient respiratory depression. These findings suggest that future clinical studies, elucidated to evaluate the effect of magnesium sulfate on post hypoxic-ischemic brain injury should use a low dose of magnesium sulfate and must be aware of a possible respiratory depression.

8. NOS inhibitors may be used to prevent the excessive production of nitric oxide during post hypoxic-ischemic reperfusion. The existing inhibitors nitro-L-arginine methyl ester (L-NAME), N-ω-nitro-L-arginine (NLA) and monomethyl-L-arginine (L-NMMA) inhibit both the constitutive and the inducible forms of the enzyme, and do not discriminate between the neuronal and endothelial forms[97]. N-ω-nitro-L-arginine is the NOS inhibitor used in this thesis in an experimental setting of hypoxia-ischemia in the newborn lamb (chapters 4 to 7).

9. The α-tocopherol part (most potent anti-oxidant part) of the group of tocopherols of vitamin E is a membrane bound chain breaking anti-oxidant. Once lipid peroxidation is initiated, peroxyl radicals react with α-tocopherol instead of an adjacent fatty acid, thus terminating the process. Its usefulness as a neuroprotective agent, however, is limited by its slow uptake into the brain[124].

10. Indomethacine or other cyclo-oxygenase inhibitors, which may prevent the formation of reactive oxygen species by the cyclo-oxygenase pathway.

11. Endogenous scavengers of reactive oxygen species such as superoxide dismutase, catalase, ascorbic acid, cholesterol and glutathione peroxidase. A recent study has shown that recombinant CuZn superoxide dismutase can be administered safely intratracheally in premature neonates to reduce reactive oxygen species mediated lung injury[143].

12. Hypothermia during an hypoxic-ischemic insult has been shown to reduce post hypoxic-ischemic brain injury[144]. This effect is probably due to a reduction in the brain's energy demands and consequently less ATP depletion. Because it is not possible to induce hypothermia *during* an hypoxic-ischemic insult in the clinical situation, recent studies have investigated the effect of mild hypothermia *after* hypoxia-ischemia and show promising results[145]. The mechanisms involved in the neuroprotective effects of mild hypothermia after hypoxia-ischemia are largely unknown. However it is suggested that mild hypothermia may reduce the release of glutamate, modify regulatory enzymes and protect protein synthesis.

It must be emphasized that thus far, only a few of the above mentioned (pharmaco) therapeutical interventions have been tested in an experimental setting. Because it was not possible to investigate all of the complex metabolic and biochemical events occurring with post hypoxic-ischemic brain damage and its possible (pharmaco) therapeutical interventions, we choose to investigate two specific subjects, i.e. the role of non-protein-bound iron and nitric oxide, in the present thesis. These two issues, which, to our opinion, play a very central role in post hypoxic-ischemic reperfusion injury of the brain, became the research objectives of the present thesis.

1.9 Objectives of the thesis

The research objectives of the present thesis were:

I To investigate post hypoxic-ischemic cerebral hemodynamics and cerebral oxygen metabolism in neonates suffering from perinatal hypoxia-ischemia and relate these parameters to short and long term neurodevelopmental outcome. To elucidate this issue, cerebral hemodynamics and oxygen metabolism of healthy neonates were compared with those of neonates suffering from perinatal hypoxia-ischemia during the first 24 hours of life and furthermore compared with the neurodevelopmental outcome at 1 year of age (discussed in chapter 2).

II It has now well been established that reactive oxygen species are important in post hypoxic-ischemic reperfusion injury. Moreover, non-protein bound iron has been recognized to play a central role in the production of reactive oxygen species. The aim of the study described in chapter 3 was therefore to investigate whether *non-protein-bound iron* was detectable after perinatal hypoxia-ischemia and whether its concentration was associated with the severity of the post hypoxic-ischemic injury and subsequent neurodevelopmental outcome.

Objectives III to VI of the thesis (described in chapters 4 to 8) were set up to investigate whether post hypoxic-ischemic cerebral reperfusion injury might be reduced by immediate post hypoxic-ischemic inhibition of nitric oxide synthesis by N-ω-nitro-L-arginine (NLA). These studies were performed in newborn lambs subjected to severe hypoxia-ischemia. To reduce the total amount of animals in these studies we used each animal for several studies if possible.

III The effect of nitric oxide synthesis inhibition on cerebral perfusion, metabolism, electro cortical brain activity and histological brain damage of the Purkinje cells of the cerebellum is described in chapter 4.

IV In chapter 5 the effect of nitric oxide synthesis inhibition on the production of pro-oxidants (i.e. non-protein-bound iron), anti-oxidative capacity and lipid peroxidation is described.

V Since it is known that inhalation of nitric oxide is a new therapy for the relief of pulmonary hypertension, the aim of the study described in chapter 6 was to investigate whether nitric oxide synthesis inhibition would have adverse effects

on pulmonary vascular resistance, blood gases, ventilator settings and oxygen need in newborn lambs undergoing severe hypoxia-ischemia.

VI Before N-ω-nitro-L-arginine may ever be used for clinical application in neonates with perinatal hypoxia-ischemia, it has first to be elucidated if this drug has positive, or at least no adverse effects on cardiac function. This issue was studied by investigating the effect of immediate post hypoxic-ischemic inhibition of nitric oxide synthesis on myocardial contractility in newborn lambs by means of pressure-volume relations obtained by the conductance catheter method (described in chapter 7).

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2 CHANGES IN CEREBRAL HEMODYNAMICS AND OXYGENATION IN THE FIRST 24 HOURS FOLLOWING BIRTH ASPHYXIA

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2.1 Abstract

Objective: To investigate whether or not postasphyctic cerebral hypoperfusion and decreased cerebral metabolism occur in the perinatally asphyxiated neonate, as has been reported in adults and newborn animals.

Methods: Using near infrared spectroscopy, we monitored changes in oxyhemoglobin (HbO_2), deoxyhemoglobin (HbR), total hemoglobin (HbO_2+HbR, which represents changes in cerebral blood volume [CBV]) and cytochrome oxidase ($Cytaa_3$, which indicates changes in oxidation level of this intracerebral mitochondrial enzyme). Thirty-one neonates (gestational age >34 weeks), divided into three groups, were monitored between 2 and 12 hours or between 12 and 24 hours of life. Group I consisted of healthy neonates: N = 8 (2 to 12 hours) and N = 5 (12 to 24 hours). Patients in group II were moderately asphyxiated neonates but neurologically normal in the first 24 hours of life: N = 6 (2 to 12 hours) and N = 3 (12 to 24 hours). Group III consisted of severely asphyxiated neonates with an abnormal neurologic behavior within 24 hours after birth: N = 5 (2 to 12 hours) and N = 4 (12 to 24 hours).

Results: From 2 to 12 h, CBV in groups I and II were stable. In group III CBV decreased in all neonates. This decrease in CBV was associated with a drop in both HbO_2 and HbR. $Cytaa_3$ was stable in groups I and II, but showed a marked decrease in two of the five neonates of group III. There was a positive relationship between CBV and mean arterial blood pressure in groups II and III. Between 12 and 24 hours all groups showed stable CBV and $Cytaa_3$ patterns. A positive relation existed now between transcutaneous P_{CO2} and CBV in groups II and III.

Conclusions: CBV, HbO_2, HbR and $Cytaa_3$ decreased in the first 12 hours of life in severely asphyxiated neonates who subsequently developed neurological abnormalities. We therefore suggest that post hypoxic-ischemic reperfusion injury of the brain during early neonatal life occurs in neonates with severe birth asphyxia.

2.2 Introduction

Birth asphyxia is an important cause of hypoxic-ischemic brain injury and is associated with an increased mortality and morbidity rate[1,2]. The initial hypoxic-ischemic insult with cerebral oxygen deprivation is of utmost importance in relation to brain cell damage and outcome. Of equal importance, however, may be brain damage caused by post hypoxic-ischemic reperfusion injury[3]. This entity has been extensively reported in adults[4,5] and the (newborn) animal model[6,7,8,9], and consists of a short initial state of reactive cerebral hyperemia followed by a significant decrease in brain blood flow and cerebral oxygen consumption in the postasphyctic period. The post hypoxic-ischemic production of oxygen free radicals and the influx of calcium ions in the brain cell are important in the pathogenesis of this post hypoxic-ischemic reperfusion injury[3,7,10]. Early treatment with calcium antagonists and free radical scavengers has been shown to prevent this hypoperfusion phenomenon and has improved neurologic recovery[7,10,11].

Data of the pattern of postasphyctic brain blood flow and metabolism in the asphyxiated neonate during the first 12 to 24 hours of life are scanty and contradictory, however. Studies in neonates measuring global brain blood flow or cerebral blood flow velocity in this postasphyctic period reported an increased as well as a decreased cerebral perfusion [12,13,14].

To elucidate the issue whether or not postasphyctic hypoperfusion, related to additional brain damage, also occurs in the perinatally asphyxiated human neonate, we compared cerebral hemodynamics and oxygenation of healthy neonates with those of perinatally asphyxiated neonates during the first 24 hours of life using near infrared spectroscopy (NIRS). With NIRS it is possible to monitor changes in oxygenated hemoglobin (HbO_2), deoxygenated hemoglobin (HbR), total hemoglobin (HbO_2+HbR, which represents changes in cerebral blood volume [CBV]) and cytochrome oxidase ($Cytaa_3$, which is supposed to indicate changes in cerebral oxygenation). Recently it has been shown that changes in CBV indicate changes in neonatal brain perfusion[15,16]. The NIRS data were compared further with the neurodevelopmental outcome at 1 year of age.

2.3 Patients and methods

Thirty-five infants with a gestational age of 35 weeks or more, determined by maternal dates or Ballard score[17], consecutively admitted to our neonatal unit were initially

enrolled in the study. Twenty neonates suffered from perinatal asphyxia; 15 healthy neonates, matched for birth weight and gestational age, products of a normal pregnancy and birth with an uncomplicated neonatal course, served as controls. Most of the control neonates were admitted for observation due to suspicion of sepsis (premature rupture of membranes, maternal fever), which eventually was excluded; two neonates had initially low blood glucose levels, which were normal again at the start of the NIRS study. Perinatal asphyxia was defined as fetal distress (abnormal heart rate pattern, meconium stained amniotic fluid, and a cord or first pH of less than 7.10), requiring immediate neonatal ventilation with mask or endotracheal tube for more than 2 min. Admission to our neonatal unit was always within 1 hour after birth. None of the neonates had major congenital anomalies. In four neonates, 2 asphyxiated and 2 control neonates, it was not possible to obtain reliable NIRS recordings for a sufficient period. In two neonates, it was not possible to obtain an acceptable signal, in one neonate an optode was dislocated in the course of the study, and in one neonate parental consent was withdrawn after the start of the study. The final number of neonates studied was 31, 18 neonates with birth asphyxia and 13 healthy neonates. The study was approved by the scientific board of the Department of Pediatrics and the Ethical Committee of the University Hospital of Leiden. Informed parental consent was obtained in all cases.

2.3.1 Assessment of cerebral hemodynamics and oxygenation by NIRS

The head of the neonate is relatively transparent to near-infrared light. Hemoglobin (Hb) and $Cytaa_3$ (the terminal member of the mitochondrial respiratory chain) are natural chromophores and both have an oxygenation-dependent absorption in this wavelength region. By selection of appropriate wavelengths, algorithms have been developed to convert absorption changes into changes in concentration of ΔHbO_2, ΔHbR, $\Delta HbO_2 + \Delta HbR$, and $\Delta Cytaa_3$[18,19]. The NIRS instrument used (Radiometer, Copenhagen, Denmark), consisted of four semiconductor laser diodes with wavelengths of 904, 845, 805 and 775 nm. The lasers were operated sequentially, and pulsed with a repetition rate of 500 Hz for 200 nanoseconds. Because we were dealing with mature neonates, we were not able to use the transillumination mode with the optodes placed symmetrically on either side of the head in the (fronto) parietal region. We therefore placed the transmitting optode on the anterior fontanel in the parasagittal plane, to avoid placement just above the sagittal sinus, with the receiving optode in the usual position. Changes in the optode position will cause changes in pathlength which results in absorption changes not related to changes in cerebral blood or tissue oxygenation. Especially in the present study, with registration times of at least 4 hours, proper fixation of the optodes was critical for reliable registration of ΔHbO_2, ΔHbR

and $\Delta Cytaa_3$. We therefore used a fixation method of the optodes as described by Liem et al.[20]. Briefly, a fixation device of a covered shell, made of hard plastic, was fixed against the skull using collodion as a glue. The fixation was strengthened with pieces of collodion soaked gauzes, placed on the edge of the shell. The optode was then inserted into the shell and further immobilized by a cover screwed on the shell which presses the optode lightly against the skin, thereby minimizing loss of light. Figure 2.1 gives an example of optode-fixation. The energy emitted by each diode was well within the orders of the British Standards Institute safety limits (BS 4803).

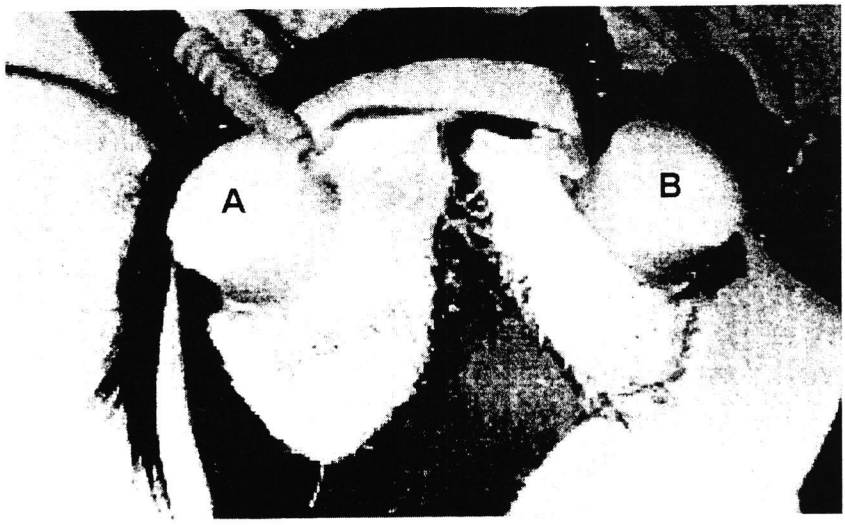

Figure 2.1 Example of the fixation of the optodes on the neonatal skull. The transmitting optode is placed on the anterior fontanel in the parasagittal plane, just beside the midline (A), whereas the receiving optode is positioned in the (fronto) parietal region, just before and slightly above the ear (B).

Assuming a stable hematocrit, changes in total Hb, which reflect the changes in both cerebral arterial and venous systems, will indicate changes in CBV. ΔCBV showed a good relationship with changes in actual cerebral blood flow, determined with the $^{133}Xenon$ clearance method[15,16]. We considered that ΔCBV indicated changes in total brain blood flow if changes in CBV were caused predominantly by changes in HbO_2. $\Delta Cytaa_3$ indicates changes in the oxidation level of the intracerebral mitochondrial

enzyme $Cytaa_3$ and is a relative measure of brain cell oxygenation[19]. The results are thus relative changes from the baseline value and are expressed in millimolar*pathlength (centimeters). Quantification of the NIRS recordings into absolute concentration changes presupposes that the optical path length is known. Much research has been done on the estimation of the exact optical path length[15,21]. However, there is no convincing evidence concerning the accuracy of the mentioned correction factors. This is the reason we used the relative rather than the absolute concentration changes. The interoptode distance in the infants studied ranged from 4.7-7.2 cm.

2.3.2 *Clinical data of the studied infants*

Obstetrical and intrapartum data were collected from hospital records. Neonatal and follow-up data were collected prospectively. Clinical management decisions were made by the attending neonatologist and no attempts were made to influence the clinical care. Samples for blood gas analysis and hematocrit were obtained from an arterial line (umbilical-, radial-, or posterior tibial artery) or from arterialized capillary blood samples, at least once every 2 to 3 hours during the first 12 hours of life and every 6 hours thereafter, more frequently if necessary. When assisted ventilation was necessary time-cycled pressure-limited infant ventilators (Bourns BP 200, Bear Medical Systems Inc., Riverside, CA, or Infantstar, Infrasonics Inc., San Diego, CA) were used. Arterial pressure was determined with an indwelling arterial catheter or with an oscillometric method (Dynamap, Criterion, Tampa, FL).

2.3.3 *Study design, data collection and analysis*

The asphyxiated neonates were divided into two groups based on the presence or absence of subsequent neurological abnormalities in the first 24 hours of life. These abnormalities were considered to be the abnormal results of neurological examination (disturbances in consciousness, hypotonia, hypo- or areflexia including weak or absent suck and Moro reflexes) and convulsions. Transient hyperalertness or hyperreflexia were not considered to be pertinently neurologically abnormal. Thus, the study population consisted of three groups for either time interval (first or second 12 hours of life). Group I consisted of control neonates; group II consisted of those asphxyiated neonates without subsequent neonatal neurologic abnormalities, whereas group III consisted of neonates who had severe birth asphyxia and subsequently developed neurologic abnormalities in the neonatal period. The neonates had to be connected to the NIRS apparatus for between 2 and 6 hours of life (neonates investigated during the first 12 hours of life) or for between 12 and 16 hours of life (neonates investigated between 12 and 24 hours of life). The minimal recording time had to be at least 4

hours. Changes (relative to the value at time point 0) in HbO_2 (ΔHbO_2), HbR (ΔHbR), Hbtot (ΔCBV) and $Cytaa_3$ ($\Delta Cytaa_3$), transcutaneous P_{CO2} and P_{O2} (tcP_{CO2} and tcP_{O2}) and mean arterial blood pressure (MABP) were simultaneously determined every 8 seconds and stored in a personal computer for off-line analysis. For each patient studied the 8 second-to-8-second values recorded every 20 min were averaged. These 20-minute intervals were necessary to reduce the number of data to a more acceptable amount.

2.3.4 Assessment of neurodevelopmental outcome

Follow-up at the age of one year was evaluated with the Van Wiechen neurodevelopmental assessment test, which is used in Dutch child health care for children from 4 weeks to 5 years of age. It is based on milestones and warning symptoms defined by Touwen, such as asymmetry, dystonia, persistence of primitive reflexes, and hearing or visual disturbances, and on five to eight items covering the five fields of development as described by Gesell and Amatruda[22,23]. (The items were chosen in such a way that at least 90% of a normal population will achieve them by the age at examination.)

2.3.5 Statistical analysis

Differences between groups regarding clinical data were assessed by one-way analysis of variance followed by the Student-Newman-Keuls test when a significant difference was found. To investigate whether or not intraindividual differences between lowest and highest hemoglobin values existed within the three groups, the Student's *t*- test for paired observations was used. To investigate within groups which factors were related with hemodynamic changes (ΔCBV), changes in blood oxygenation (ΔHbO_2, ΔHbR) or changes in cellular oxygenation ($\Delta Cytaa_3$), we used a multiple linear regression model. We selected those variables which are supposed to be involved in brain perfusion: MABP, tcP_{CO2} and tcP_{O2}. The regression equation was:

$$Y = a_0 + a_{MABP} \cdot MABP + a_{tcPCO2}\, tcP_{CO2} + a_{tcPO2} \cdot tcP_{O2}$$

where ΔCBV (1), ΔHbO_2 (2), ΔHbR (3) or $\Delta Cytaa_3$ (4) were the dependent variables (Y_1, Y_2, Y_3 or Y_4) and a_0 (a_{01}, a_{02}, a_{03}or a_{04}) their means over all the runs. MABP, tcP_{CO2} and tcP_{O2} were independent variables. These independent variables were either introduced or removed from the equations, based on their significance level. A *p*-value of less than 0.05 was considered statistically significant.

2.4 Results

Of the 31 neonates, 19 were studied in the first 12 hours of life (group I, 8; group II, 6; group III, 5) and 12 were studied between 12 and 24 hours of life (group I, 5; group II, 3; group III, 4). Table 2.1 shows the relevant clinical data of the different groups in both study periods.

	Periods of study					
	2-12 Hours			**12-24 Hours**		
	Group I (N=8)	Group II (N=6)	Group III (N=5)	Group I (N=5)	Group II (N=3)	Group III (N=4)
Birth weight (g)	2798 ± 484	3433 ± 724	3160 ± 666	3222 ± 884	3200 ± 750	3154 ± 222
Gestational age (wk)	37.8 ± 1.2	38.5 ± 1.6	38.8 ± 1.9	37.5 ± 1.7	38.6 ± 2.3	38.8 ± 2.6
Cord / first pH	7.36 ± 0.05	7.01 ± 0.06*	6.68 ± 0.17*	7.31 ± 0.09	7.07 ± 0.02#	6.93 ± 0.06#
Registration time (h)	6.3 ± 2.2	6.2 ± 2.4	6.8 ± 2.2	5.5 ± 1.1	4.4 ± 0.5	4.5 ± 0.7

* $P < 0.05$ vs. group I, # $P < 0.05$ vs. group I

Table 2.1 Important clinical data (means ± 1SD) in the various groups during the two periods of study

Cord or first pH were significantly lower in moderately and severely asphyxiated neonates as compared with the control neonates. Five-minute Apgar scores (median (range)) were 10 (7 to 10), 5 (3 to 9) and 3 (1 to 7) for control, moderately asphyxiated, and severely asphyxiated neonates, respectively. All nine severely asphyxiated neonates needed assisted ventilation beyond the resuscitation period (always with minimal ventilator settings), five had convulsions requiring anticonvulsive therapy during their stay at the neonatal intensive care unit (2 neonates received anticonvulsive therapy during NIRS), and one neonate needed dopamine medication during NIRS to prevent hypotension. No differences were found between groups regarding MABP, blood gases or pH (not shown) at the start of the NIRS. Moreover, pH, tcP_{CO2}, tcP_{O2} and MABP were stable and well within normal limits in almost all cases during the first 24 hours of life. Only in two neonates with severe birth asphyxia, which were on mechanical ventilation, the P_{CO2} values were initially to low (<35 mm Hg), but soon rose to normal values after adjustment of the ventilator settings. No difference was

found in any group between individual lowest and highest hemoglobin value. All control neonates, all but one moderately asphyxiated neonate (1 neonate was slightly hypertonic and hyperreflexic), and 4 of the 6 surviving neonates of the severely asphyxiated group had normal results of neurologic examinations at discharge. Three of the nine severely asphyxiated neonates, all three investigated during the first 12 hours of life, died after supportive treatment was withdrawn because of their deteriorating neurological condition (all were comatous, two neonates having virtually flat electroencephalograms).

2.4.1 Cerebral hemodynamics and oxygenation in the first 12 hours of life

Figure 2.2 A shows the ΔCBV in the three groups as a function of postnatal age. The neonates of group I showed an increase of ΔCBV over time in most cases. In group II, ΔCBV showed an initial drop followed by an increase over time in all but one neonate. All the neonates of group III, however, showed a decrease in ΔCBV. Although there was little difference with control and moderately asphyxiated neonates (groups I and II) until 6 hours of life, the decrease in group III became evident from about 6 hours onwards (Figures 2.2 A and 2.4 A). At 8.5 hours after birth, all control and moderately asphyxiated neonates who were still on registration (five and four respectively), showed higher ΔCBV values as compared with the baseline at the start of the recording, whereas the opposite was true for the severely asphyxiated neonates (three on registration at 8.5 hours of age); they all showed a substantial decrease in ΔCBV as compared with baseline. The two neonates with the largest decrease had virtually no electroencephalographic activity (figure 2.2 A). The decrease in ΔCBV in the neonates of group III was associated predominantly with a decrease in ΔHbO_2 and, to a much lesser extent, with a decrease in ΔHbR (ΔHbO_2 and ΔHbR not shown).

Figure 2.3A shows the $\Delta Cytaa_3$ in the three groups as a function of postnatal age. $\Delta Cytaa_3$ showed a rather stable pattern in groups I and II. In the severely asphyxiated neonates (group III), however, 4 of 5 neonates showed a decrease in $Cytaa_3$ with increasing postnatal age as compared to baseline; in two neonates this decrease was marked (figure 2.3A). One neonate showed an increase in $Cytaa_3$. This was the neonate with the most marked decrease in CBV. Figure 2.4, A and B, shows the patterns of the mean values (standard deviation not shown) of ΔCBV and $\Delta Cytaa_3$ of the various groups.

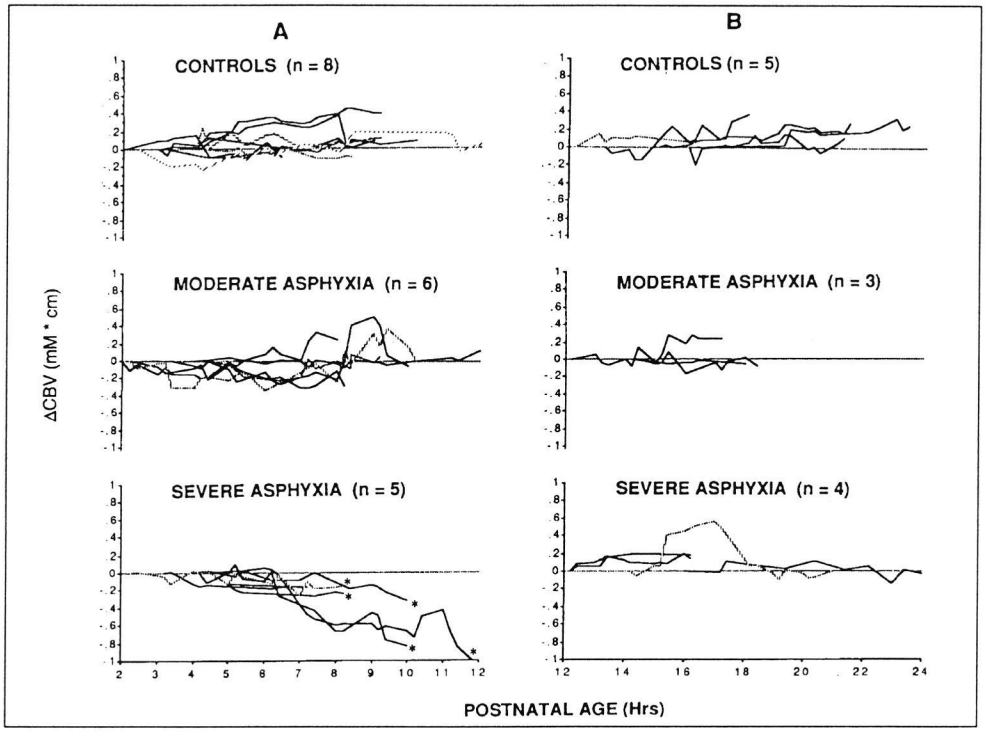

Figure 2.2 Individual changes in cerebral blood volume (ΔCBV) as a function of postnatal age in the three study groups during the first 12 hours of life (A) and from 12 to 24 hours of life (B). Patterns of ΔCBV of infants with an adverse neurologic outcome are indicated by asterix.

Multiple linear regression revealed a positive relation between MABP and ΔCBV in groups II and III (coefficients 0.01 and 0.008 mM*cm / mm Hg, respectively) and a positive relation between ΔHbO_2 and tcP_{O2} in the same groups (coefficients 0.003 and 0.002 mM*cm / mm Hg, respectively). In groups II and III, there was also a positive relation between tcP_{O2} and $\Delta Cytaa_3$ (coefficients 0.001 and 0.001 mM*cm / mm Hg, respectively), whereas MABP showed an inverse relation with $\Delta Cytaa_3$ (coefficients -0.001 and -0.003 mM*cm / mm Hg, respectively). No relations were found between tcP_{CO2}, tcP_{O2}, or MABP and ΔCBV, ΔHbO_2, ΔHbR or $\Delta Cytaa_3$ in the control neonates (group I). In this group, however, blood gases and MABP showed only little spontaneous variation, which may mask a possible relationship with the NIRS variables.

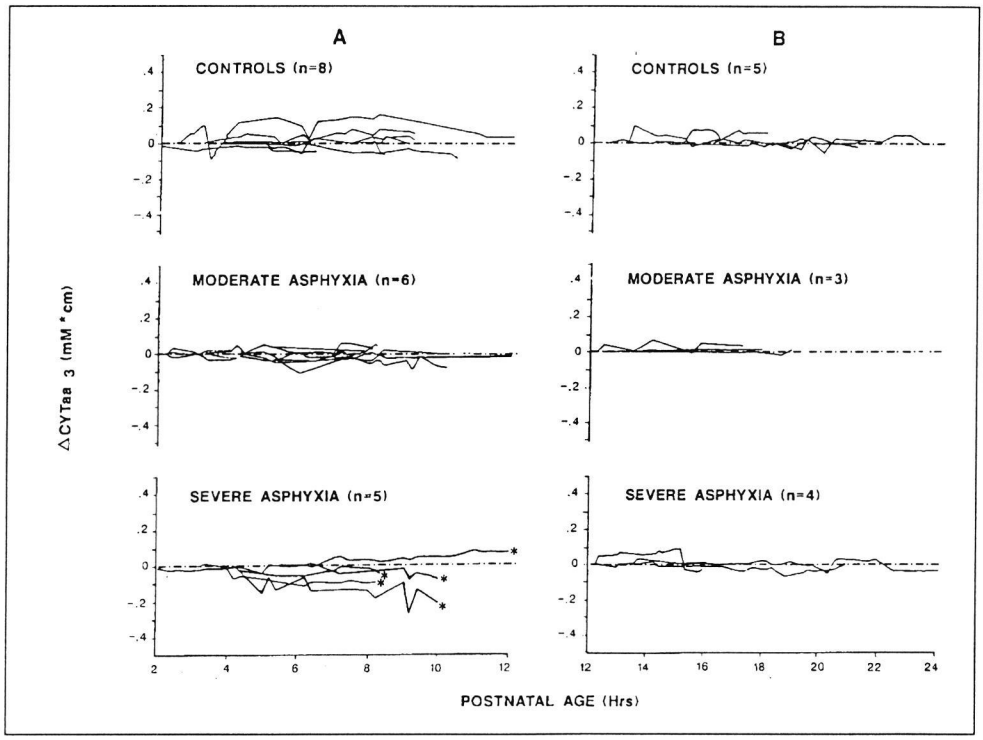

Figure 2.3 Individual changes in cerebral cytochrome oxidase ($\Delta Cytaa_3$) patterns as a function of postnatal age in the three study groups during the first 12 hours of life (A) and from 12 to 24 hours of life (B). Patterns of $\Delta Cytaa_3$ of infants with adverse neurologic outcome are indicated by asterisks.

2.4.2 Cerebral hemodynamics and oxygenation from 12 to 24 hours of life

Figure 2.2 B shows the pattern of ΔCBV of the three groups as a function of postnatal age. The control neonates showed the same pattern as reported in the first 12 hours of life, a slight increase in ΔCBV over time. Although the number of neonates was small, especially in groups II and III, the data showed a more heterogenic pattern of ΔCBV with increases as well as decreases in ΔCBV, but no major changes from the baseline values. So, individual ΔCBV-patterns seemed to be rather stable in this period (figure 2.2 B). ΔHbO_2 and ΔHbR were stable too and showed a similar pattern as ΔCBV. Also $\Delta Cytaa_3$ showed a rather stable pattern in all three groups (figure 2.3 B).

Multiple linear regression revealed a positive relation between tcP_{CO2} and ΔCBV in groups II and III (0.01 and 0.08 mM*cm / mm Hg, respectively). No relation was

found now between MABP and ΔCBV in these groups. Moreover, no relationships were detected between ΔHbO_2 , ΔHbR or $\Delta Cytaa_3$ on the one hand and tcP_{CO2}, tcP_{O2} or MABP on the other. As in the first 12 hours of life, MABP, blood gases and pH of the control neonates (group I) showed little variation in this period.

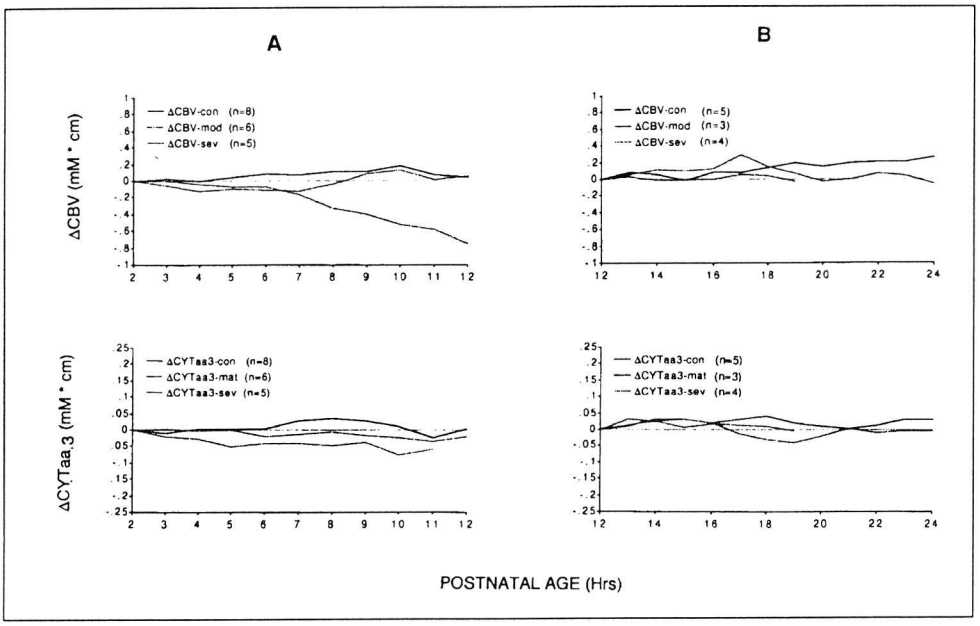

Figure 2.4 Mean values of changes in cerebral blood volume (ΔCBV) and changes in cerebral cytochrome oxidase ($\Delta Cytaa_3$) as a function of postnatal age in the 3 study groups during the first 12 hours of life (A) and from 12 to 24 hours of life (B). con, control; mod, moderate; sev, severe.

2.4.3 Neurodevelopmental outcome at one year of age

Only 8 of the 13 infants of the control group, 7 of 9 infants of the moderately asphyxiated group and all 6 surviving infants of the severely asphyxiated group were available for the 1-year follow-up study. All infants from the control and moderate asphyxia groups were neurologically normal at one year of age (including the one who had slightly abnormal results of the neurological examination at discharge). The two patients with severe asphyxia who had abnormal neurologic results at discharge showed a delayed neurodevelopmental course. It appeared that in those patients with an adverse outcome (death [N = 3] or delayed development at 1 year of age [N = 2])

ΔCBV decreased (figure 2.2, Individual ΔCBV patterns of these 5 infants are indicated with asterix) as compared with their base line values, which was contrary to the patterns of ΔCBV of the infants with a normal 1-year outcome (stable or increasing ΔCBV). The pattern of $\Delta Cytaa_3$ in patients with an adverse outcome as compared with those with a favorable outcome showed, although less clearly, a decrease (figure 2.3, [indicated by asterisk]) of 5 patients. The small number of patients in the 12 to 24 hours of life period prevented any conclusion about the predictive value of ΔCBV or $\Delta Cytaa_3$ in relation to neurodevelopmental outcome.

2.5 Discussion

NIRS can be used to monitor changes in HbO_2 and HbR in the brain and changes in the oxygenation level of $Cytaa_3$. Changes of HbO_2 + HbR in the brain indicate changes in CBV, assuming a stable blood hemoglobin. Earlier studies in preterm and full term neonates showed a relation between carbon dioxide-induced changes in brain blood flow and ΔCBV[15,24]. In the present study, the asphyxiated patients were at risk for hypoxic-ischemic cerebral damage with brain edema and myocardiopathy. Therefore, changes in intracranial pressure and cerebral venous pressure, both of which are important determinants of cerebral perfusion pressure, may be involved as determinants of brain blood flow. However, when a decrease in cerebral venous drainage should have played an important role in the changes of CBV, one should expect increases in HbR. Changes in CBV, however, were predominantly associated with changes in HbO_2. The changes in HbR were much smaller and always in the same direction as the HbO_2 changes. Although in the encephalopathic brain oxygen extraction might be so low that the venous blood is highly saturated and may confound these arguments, we suggest that generally the changes in CBV indeed indicate changes in brain blood flow.

$\Delta Cytaa_3$ is supposed to indicate changes in the oxidation-reduction level of the intracerebral enzyme cytochrome oxidase, the terminal member of the mitochondrial respiratory chain. $\Delta Cytaa_3$ can therefore be used as a relative measure of cellular oxygenation. The enzyme is not maximally oxidized under base line conditions and an increased oxygenation should further oxidize $Cytaa_3$, whereas a decrease in oxygenation should result in a greater concentration of the reduced form of this enzyme[19,25,26]. However, there is some concern that $\Delta Cytaa_3$ does not properly reflect changes in brain cell oxygenation. Use of the wrong algorithms for calculation of changes in $Cytaa_3$ and the low energy requirement of the brain cell in the (preterm) neonate may mask fluctuations in the oxidation-reduction level of cytochrome oxidase

and affect the reliability of $\Delta Cytaa_3$ as a marker of actual changes in oxidation of the enzyme cytochrome $Cytaa_3$[15,27].

Our data showed that severely asphyxiated neonates are often prone to a decrease in cerebral CBV and HbO_2 during the first 12 hours of life, suggesting a decrease in cerebral perfusion and oxygenation. Because the optical path length of the infrared light is not known, quantification of changes in CBV is not possible. To speculate about the magnitude of changes in CBV, we extrapolated our data to those of a study of Pryds et al., which compared changes in CBV, measured with a similar NIRS-device, with changes in brain blood flow (^{133}xenon clearance method)[15]. This suggested that a change of 0.1 mM*cm pathlength in our neonates was equivalent with 8 $mlL^{-1} \cdot 100g^{-1} \cdot min^{-1}$ change in actual cerebral blood flow. Assuming that the cerebral blood flow in healthy neonates is about 60 $mlL^{-1} \cdot 100g^{-1} \cdot min^{-1}$, it should mean that the two neonates without electroencephalographic activity had hardly any cerebral perfusion left at the end of the NIRS-registration[14,28].

The decrease of $Cytaa_3$ in most of the severely asphyxiated neonates during the first 12 hours of life supports the suggestion that oxygenation of brain tissue might be compromised. This is supported further by the positive relation between tcP_{O2} and $\Delta Cytaa_3$ in both groups of asphyxiated neonates.

Because we only monitored relative changes of HbO_2, HbR, CBV and $Cytaa_3$, we are unable to confirm that these changes represent true hypoperfusion or normalization of brain blood flow after postasphyctic cerebral hyperemia. We suggest, however, hypoperfusion, because results of recent studies in (newborn) animals (lambs, piglets, dogs) indicate that the cerebral hyperemia after experimentally induced asphyxia is of short duration and reverses in hypoperfusion of the newborn brain within 30 to 60 min after the end of an hypoxic-ischemic insult[6,7,8,9]. Because we started monitoring CBV at least 2 hours after birth we assumed that this initial period of hyperperfusion did not confound our study results. A clinical study of Shankaran et al. showed a significantly lower brain blood flow, up to the fourth day of life, in full term neonates with evidence for hypoxic-ischemic encephalopathy[14]. The present data do not support the results of an earlier study we performed in severely asphyxiated neonates with a comparable gestational age, in which we found an increased cerebral blood flow velocity from birth until the fourth day of life as compared with healthy controls, suggesting an increased global brain blood flow[13]. In fact, Doppler determined cerebral blood flow velocity reflects changes in blood flow (velocity) in one of the major cerebral arteries. In the present study, however, cerebral blood volume and cerebral oxygenation have

been assessed, especially those of the cerebral cortex and adjacent subcortical white matter in the parietal region of the neonatal brain. Cerebral blood flow velocity may therefore not reflect changes of perfusion of this brain area, which is particularly prone to hypoxia and ischemia and is the site of the so called parasagittal cerebral injury[29]. It is possible that early postasphyctic blood supply, especially in this area, is reduced at microvascular level in the severely asphyxiated neonates, whereas blood flow in the major cerebral arteries may be maintained or even be increased by focal vasoparalysis of cerebral resistance vessels induced by the actual asphyctic insult[30,31]. This causes loss of autoregulatory ability of these vessels and "luxury perfusion" in some regions of the brain in the postasphyctic period[32,33].

The steady decrease of CBV and HbO_2 in the severely asphyxiated neonates in the first 12 hours of life, suggesting a decrease in brain blood flow in the investigated area of the brain, may have several reasons. The most important one could be delayed neuronal cell death with subsequent decrease in brain blood flow, which is supported by the decrease of $Cytaa_3$ in 4 of the 5 neonates of this group. The one neonate however, with the most pronounced decrease in CBV, showed an increase in $Cytaa_3$. We excluded system drift as a cause for this increase, and the only explanation for this phenomenon may be a failure of electron transport within the brain cell due to mitochondrial disruption. This will cause a highly oxidized $Cytaa_3$[34]. To some extent the passive relation between MABP, which was always in the normal range, and ΔCBV in the severely asphyxiated neonates, as far as this relation indicates a blood pressure-passive brain perfusion, may have contributed to the decrease of CBV in these infants. However, the correlation coefficient between changes in MABP and CBV (0.008 mM*pathlength in centimeter change in ΔCBV per mm Hg change in MABP) cannot explain the frequently huge decreases of ΔCBV (up to 1 mM*cm).

The stable CBV in all groups from 12 hours of life onwards suggests no further decrease in cerebral perfusion in the severely asphyxiated neonates and a stable brain blood flow in the control and moderately asphyxiated neonates.

The positive relationship between tcP_{CO2} and ΔCBV in groups II and III from 12 to 24 hours of life suggests an intact P_{CO2}-mediated vasoreactivity, contrary to the situation during the first 12 hours of life. Although, in this period no relationship was found between tcP_{CO2} and ΔCBV, the lack of variability of tcP_{CO2}, especially in neonates of group III, might have masked this relationship.

The relation between a decreasing CBV during the first 12 hours of life and an adverse outcome suggests a relation between cerebral hypoperfusion and brain tissue damage, but the small study population prohibits any conclusion concerning this relation.

We conclude that CBV, HbO_2, HbR, and to a lesser extent $Cytaa_3$ decreased in the first 12 hours of life in severely asphyxiated neonates who subsequently developed neurological abnormalities. We therefore suggest that postasphyctic hypoperfusion of the brain with a decreased oxygen consumption occurs during early neonatal life in neonates with severe birth asphyxia.

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3 NON-PROTEIN-BOUND IRON IN POST ASPHYXIAL REPERFUSION INJURY OF THE NEWBORN

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3.1 Abstract

Objective: To investigate if the availability of non-protein-bound iron after birth asphyxia is related to the severity of the postasphyxial injury and neurodevelopmental outcome.

Methods: Non-protein-bound iron (bleomycin assay) and thiobarbituric-acid-reactive species (TBARS), an index of oxidative lipid damage, were measured in plasma of 50 neonates (GA>34wk) between 0-8 h, 8-16 h and 16-24 h after birth. Three groups were compared: healthy neonates (n=20), moderately asphyxiated neonates (n=15), who were neurologically normal during the first 24 h after birth and severely asphyxiated neonates (n=15), who developed abnormal neurological signs in the first 24 h after birth.

Results: In the severely asphyxiated neonates, liver enzymes, creatinine, urea and uric acid concentrations were significantly elevated. Eleven severely asphyxiated neonates were brain damaged, 9 of them died during the neonatal period. Non-protein-bound iron was detectable in 30% of the control, 60% of the moderately asphyxiated, and 80% of the severely asphyxiated neonates. During the whole study period non-protein-bound iron concentration was significantly elevated in severely asphyxiated neonates as compared to controls. Three of the 4 severely asphyxiated neonates who had a normal outcome at 1 year of age, had no detectable non-protein-bound iron during the study period. Stepwise logistic regression analysis with neurodevelopmental outcome at one year of age (normal versus adverse/death) as dependent variable and all the measured parameters for organ damage as independent variables revealed that the non-protein-bound iron concentration at 0-8 hrs after birth was the most significant variable and at the same time the only variable which entered the model, in relation to neurodevelopmental outcome ($p<0.001$). TBARS tended to be higher in severely asphyxiated neonates, suggesting oxidative lipid damage.

Conclusion: Non-protein-bound iron may play an important role in oxidative damage mediated post-asphyxial brain injury and subsequent neurodevelopmental outcome.

3.2 Introduction

Despite advances in perinatal and obstetric care, perinatal asphyxia is still the most important cause of brain injury in the newborn[1,2]. Although cerebral injury may occur during the actual hypoxic-ischemic insult, recent studies suggest that a substantial proportion of the injury can be attributed to the formation of excess reactive oxygen species upon reoxygenation and reperfusion[3,4]. The preceding hypoxia-ischemia induces the brain to respond on reperfusion with an increased production of reactive oxygen species, such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2)[3,4,5,6]. Large amounts of $O_2^{\bullet -}$ and H_2O_2 are generated upon reoxygenation by mitochondria, calcium-induced production of prostaglandins, activated neutrophils and macrophages, and by circulating xanthine oxidase[7,8]. Although relatively poorly reactive themselves, $O_2^{\bullet -}$ and H_2O_2 can be converted into the highly reactive hydroxyl radical ($OH^{\bullet}$) by transition metal ions, in particular non-protein-bound iron, leading to cellular damage (e.g. protein and lipid-peroxidation)[9]. In normal adults this process is prevented by sequestrating iron into "safe" forms; for example by transferrin-binding, which makes non-protein bound iron undetectable in normal plasma[10]. Recent studies from our group and others however, showed that up to 25% of apparently healthy neonates had detectable non-protein-bound iron in their plasma[11,12]. This may imply that neonates are especially susceptible to oxidative damage as can occur during ischemia-reperfusion. The aim of this study was therefore to investigate if non-protein-bound iron was detectable after birth asphyxia and whether its concentration was associated with the severity of the postasphyxial injury and subsequent neurodevelopmental outcome.

3.3 Methods

The study population consisted of 50 neonates with a gestational age of 35 weeks or more, who consecutively were admitted to our neonatal unit for birth asphyxia or for observation (see also below). Thirty patients suffered from birth asphyxia, defined as fetal distress (abnormal heart rate pattern, meconium-stained amniotic fluid and a cord or first capillary pH of less than 7.1), requiring immediate assisted ventilation for more than 2 min. These neonates were divided into a moderately asphyxiated group (n=15), without neurological abnormalities during the first 24 hours after birth and a severely asphyxiated group (n=15), who subsequently developed neurological abnormalities during the first 24 hours after birth (e.g. disturbances in consciousness, hypotonia, hypo- or areflexia including weak or absent suck or Moro reflexes) and/or convulsions.

Transient hyperalertness or hyperreflexia were not considered to be neurological abnormalities. Twenty healthy neonates served as a control group. Assignment to the moderately or severely asphyxiated group was done within the first 24 hours of life and *before* the results of the various blood samples (*i.e.* non-protein-bound iron, TBARS, liver and renal function tests, see also below) were available. Most of these control neonates were admitted for observation for possible infection (premature rupture of membranes, maternal fever), which was eventually excluded, or for observation for hypoglycemia because of maternal diabetes. None of them became hypoglycemic during the study period. All neonates were admitted to our neonatal unit within two hours after birth. None of them had congenital malformations. The study was approved by the scientific board of the department of Pediatrics and the Ethical Committee of the University Hospital of Leiden. Informed parental consent was obtained in all cases.

3.3.1 Determination of non-protein-bound iron and lipid peroxidation

Blood was collected into heparinized glass tubes and immediately centrifuged (750 g, 10 min); the plasma was stored under argon at -70° C until analysis. Plasma samples which showed pink discoloration (hemolysis), were excluded from the study. Non-protein-bound iron in plasma was measured by the bleomycin assay[13]. Using this assay, the absence of non-protein-bound iron, *i.e.* the presence of iron binding capacity, can be measured as well as the presence of non-protein-bound iron, *i.e.* the lack of iron binding capacity. If non-protein-bound iron is present, the lower detection limit is 0.6 μM. The glass tubes used to collect the blood did not contain detectable amounts of iron. The intra and inter assay coefficients of variation of the bleomycin assay are 6.6% and 7.4% respectively. Lipid peroxidation was detected by measuring the concentration of thiobarbituric-acid-reactive species (TBARS) according to the method of Asakawa and Matsushita[14]. Since bilirubin is known to react with TBA, reagent interference was checked by adding bilirubin in high concentrations to the reaction medium[15]. To eliminate the effect of bilirubin, a correction factor was introduced following the method of Thurnham et al.: measured TBARS (μM) - 0.034 bilirubin (μM) = corrected TBARS (μM)[15]. All TBARS values in this study are expressed as corrected TBARS. Preliminary studies showed no effect of storage on non-protein-bound iron or TBARS concentration.

3.3.2 Study design

When blood was withdrawn from the patients for clinical purposes, a small additional sample was taken to determine non-protein-bound iron and TBARS. These samples were collected during the following time-periods: 0-8, 8-16 and 16-24 hours after birth. Mean time (±1SD) of blood sample collection during the subsequent time periods was

4.0 ± 1.2, 11.5 ± 2.5 and 21.8 ± 2.0 hrs for control neonates; 4.4 ± 2.2, 11.7 ± 1.5 and 20.2 ± 2.1 hrs for moderately asphyxiated neonates; and 4.4 ± 1.0, 11.6 ± 1.7 and 18.8 ± 2.0 hrs for severely asphyxiated neonates respectively. Additional blood samples were taken between 24-36 hours after birth to determine liver enzymes: serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and lactic acid dehydrogenase (LDH), renal function (creatinine, urea) and uric acid levels. Neurologic examinations were performed by the attending neonatologist. When the neurologic observations were abnormal, the newborn was also examined by a pediatric neurologist. Brain tissue damage and abnormal electrocortical brain activity were assessed by cranial 2-D ultrasound, computer-tomography, and/or electroencephalogram registrations. Follow-up at the age of one year was evaluated by the Van Wiechen neurodevelopmental assessment test, which is used in Dutch child health care for children from 4 weeks to 5 years of age. It is based on milestones and warning symptoms, such as asymmetry, dystonia, persistence of primitive reflexes, and hearing or visual disturbances as defined by Touwen, and on five to eight items covering the five fields of development as described by Gesell and Amatruda (The items were chosen in such a way that at least 90% of a healthy population will achieve them by the age at examination)[16,17].

3.3.3 Statistical analysis

Differences between the perinatal and laboratory data of the three groups were assessed by one way factorial analysis of variance. When a significant difference was found, ANOVA was followed by the Scheffe's procedure for comparison between the groups. Because of a skewed distribution of the non-protein-bound iron and TBARS data, non-parametric statistics were used to analyze these data. Only those neonates with observations during at least two time periods were included in this statistical analysis (see also result section). Differences between the three groups within one time period were assessed by the Kruskal-Wallis test, followed by the Mann-Whitney-U-test, to compare between each two groups, when a significant difference was found. Differences between the three time periods within one group were analyzed by the Friedman-test, followed by the Wilcoxon-signed ranks-test to compare between each two time periods, when a significant difference was found.

To investigate if there was an association between the long-term outcome of the neonates in this study and one or more of the parameters for organ damage, a stepwise forward logistic regression (p-value to enter = 0.05; p-value to remove = 0.10) was performed with outcome at one year of age (good [normal] or adverse [abnormal,

death]) as dependent variable and cord/first pH, SGOT, SGPT, LDH, creatinine, urea, uric acid and non-protein-bound iron and TBARS at [0-8hrs], [8-16hrs] and [16-24hrs] as independent variables. The criteria for adding or removing of variables were based on the likelihood criteria.

Results in text and figures are expressed as mean (±1SD) or as median with ranges where appropriate. A *p*-value less than 0.05 was considered statistically significant.

3.4 Results

3.4.1 Patients characteristics and laboratory data

Patients characteristics are shown in table 3.1

	CONT	MA	SA
Birth weight (g)	3158 ± 768	3357 ± 479	3413 ± 622
GA (wk)	37.5 ± 2.6	39.8 ± 1.8	39.8 ± 2.2
Cord / 1st pH	7.27 ± 0.09	7.02 ± 0.08*	6.87 ± 0.12*†
Median Apgar at 5' (range)	9 (7 to 10)	7 (6 to 10)*	4 (1 to 8)*†

*p<0.05 vs. CONT, † p<0.05 vs. MA

Table 3.1 Patient characteristics of the three study groups (means ± 1SD). CONT=control group [n=20]; MA=moderately asphyxiated group [n=15]; SA=severely asphyxiated group [n=15].

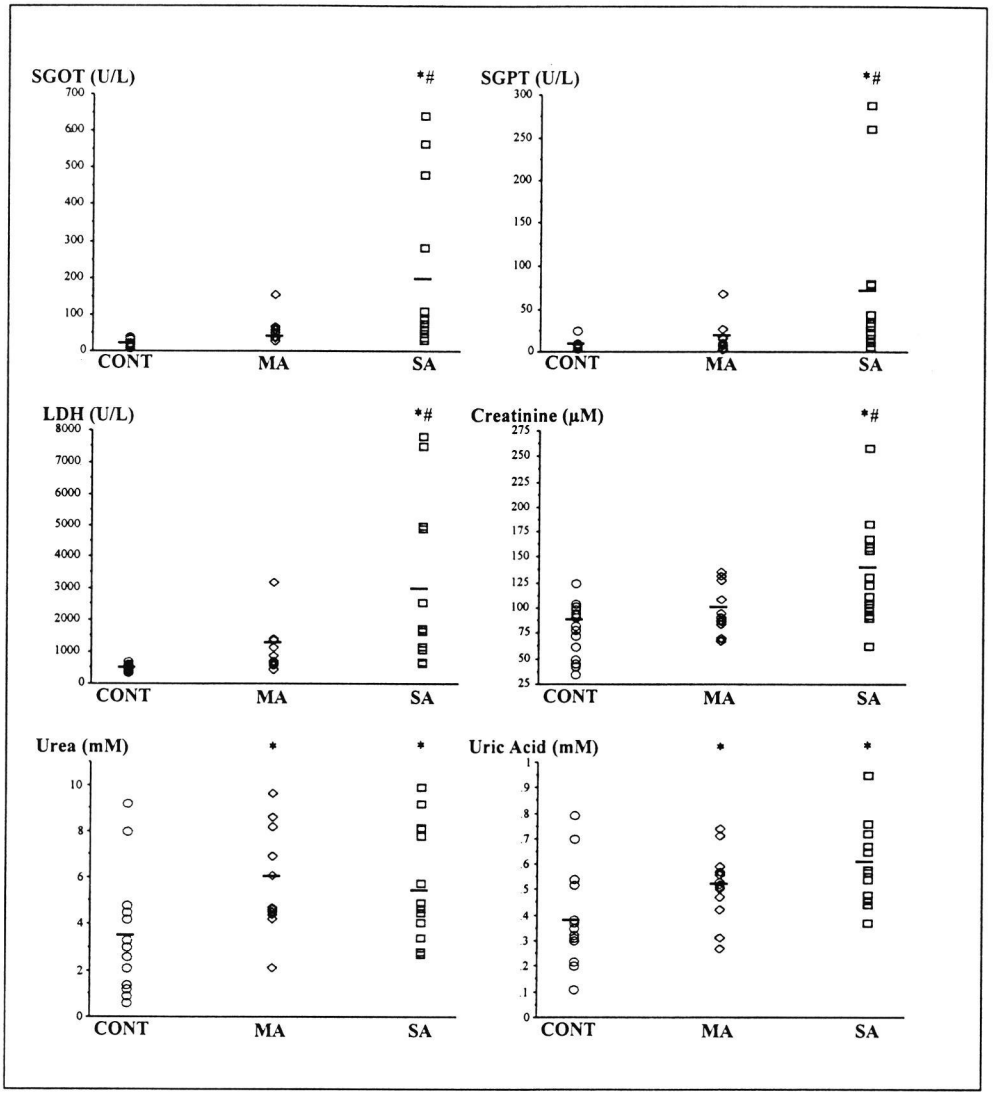
There were no significant differences between the 3 groups regarding birth weight or gestational age, although the neonates of the control group tended to have a lower gestational age. Presumptive causes of the asphyxiated neonates were: abruptio placentae [3], meconium aspiration [6], strangulation of the umbilical cord around the neck [3], insufficient progression of delivery, resulting in cesarean section or vacuum extraction [16], unknown [2]. Cord or first capillary pH (within 10 min of birth) and five minute Apgar scores of the severely asphyxiated neonates were significantly lower than those of the moderately asphyxiated neonates, whose values were significantly lower than those of the control neonates. Individual and mean laboratory data of all the neonates are shown in figure 3.1. The SGOT, SGPT, LDH and creatinine concentrations of the severely asphyxiated neonates were significantly higher than those of the moderately asphyxiated and control neonates. The urea and uric acid

concentrations of the moderately and severely asphyxiated neonates were significantly higher than those of the control neonates.

3.4.2 Non-protein-bound iron

Figure 3.2 and table 3.2 show the individual concentrations of non-protein-bound iron (medians indicated by the little dash) in the 3 groups during the subsequent postnatal time periods. Non-protein-bound iron was detected in 6/20 (30%) of the control, 9/15 (60%) of the moderately asphyxiated and 12/15 (80%) of the severely asphyxiated neonates in at least one of the 3 time periods. The 9 control neonates without detectable non-protein-bound iron in their plasma, were not different for any patient characteristic as compared with the remaining 6 control neonates with detectable non-protein-bound iron. Moreover, no reason was found in these 6 patients for the occurrence for non-protein-bound iron.

In this clinical study it was not always possible to obtain a blood sample from each patient during each of the time periods (*e.g.* rejection of blood sample because of hemolysis, death [severely asphyxiated neonates] or discharge). Fourteen control neonates (n=14, n=12 and n=13 at [0-8 hrs], [8-16 hrs] and [16-24 hrs] respectively), 13 moderately asphyxiated neonates (n=10, n=9 and n=11 at [0-8 hrs], [8-16 hrs] and [16-24 hrs] respectively), and 13 severely asphyxiated neonates (n=12, n=12 and n=11 at [0-8 hrs], [8-16 hrs] and [16-24 hrs] respectively) had at least non-protein-bound iron determinations during two time periods. Patient characteristics and laboratory data of these neonates were not different from the original groups. For statistical analysis of the data shown in figure 3.2 only those neonates with observations during at least two time periods were included. In the severely asphyxiated neonates, the plasma concentration of non-protein-bound iron was significantly elevated during the whole study period as compared to the control neonates, and from [0-8 hrs] as compared to the moderately asphyxiated neonates. No significant changes of non-protein-bound iron concentration were seen between the three time periods within one group.



* p<0.05 vs. CONT, # p<0.05 vs. MA

Figure 3.1 Individual and mean (-) plasma concentrations of SGOT, SGPT, LDH, creatinine, urea and uric acid in the three study groups. CONT [O]=control group (n=18); MA [◊]=moderately asphyxiated group (n=13); SA [□]=severely asphyxiated group (n=13).

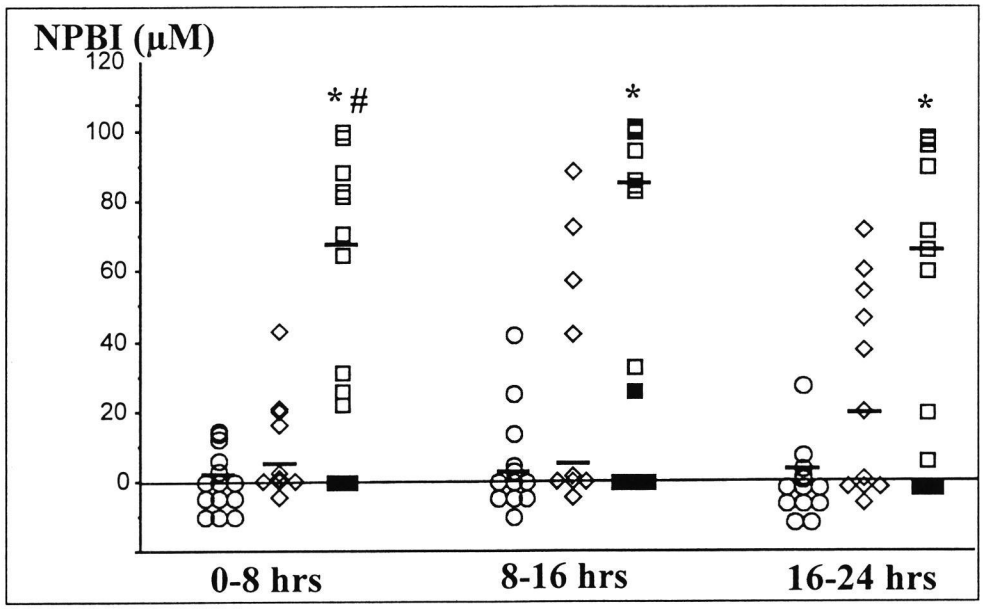


Figure 3.2 Individual and median (-) plasma concentrations of non-protein-bound iron (NPBI) in the three study groups during the various postnatal ages. [O]=control group (n=14, n=12 and n=13 at [0-8 hrs], [8-16 hrs] and [16-24 hrs]); [◊]=moderately asphyxiated group (n=10, n=9 and n=11 at [0-8 hrs], [8-16 hrs] and [16-24 hrs]); [□]=severely asphyxiated group (n=12, n=12 and n=11 at [0-8 hrs], [8-16 hrs] and [16-24 hrs]). [■] indicates the individual plasma concentrations of NPBI of the 4 severely asphyxiated neonates with a normal outcome at 1 year of age.

	NPBI (μM) CONTROLS			NPBI (μM) MODERATELY ASPHYXIATED			NPBI (μM) SEVERELY ASPHYXIATED		
	0-8hrs (n=14)	**8-16hrs (n=12)**	**16-24hrs (n=13)**	**0-8hrs (n=10)**	**8-16hrs (n=9)**	**16-24hrs (n=11)**	**0-8hrs (n=12)**	**8-16hrs (n=12)**	**16-24hrs (n=11)**
1	3.0	42.0	28.7	19.6	72.6	21.1	31.1	94.4	68.2
2	13.3	25.3	2.0	1.7	88.6	56.0	65.2	84.7	100.0
3	11.8	4.6	9.3	16.2	0		100.0	86.2	99.4
4	5.8	3.3	5.2	0	0		71.3	99.6	97.6
5	0	0	0	43.0		2.2	89.0	100.0	73.7
6	14.8	13.8		2.3		61.5	81.7	82.9	91.6
7	0	0		20.5		0	0(+)	0(+)	0(+)
8	0		0	0		0	22.3	32.5	
9	0		0	0		0	0(+)	0(+)	
10	0		0		1.2	39.1	26.9		7.3
11	0		0		41.8	48.4		98.9	61.9
12	0	0			57.3	73.6		26.2(+)	21.5(+)
13		0	0		0	0		0(+)	0(+)
14		0	0	0			82.9		
15	0				0		97.6		
16	0								
17		0							
18		0							
19			3.5						
20			0						
median	0	0	0	2.0	1.2	21.1	68.3	83.8	68.2
(range)	(0-14.8)	(0-42.0)	(0-28.7)	(0-43.0)	(0-88.6)	(0-73.6)	(0-100)	(0-100)	(0-100)
mean	3.5	7.4	3.7	10.3	29.1	27.4	55.7*†	58.7*	56*
(SD)	(5.6)	(13.4)	(8.0)	(14.4)	(36.3)	(28.9)	(37.3)	(43.0)	(41.4)

* $p<0.05$ vs. controls, †$p<0.05$ vs. moderately asphyxiated (0-8hrs), (+) severely asphyxiated neonates with a normal outcome at 1 year of age

Table 3.2 Individual plasma concentrations of non-protein-bound iron (NPBI) in the control, moderately asphyxiated and severely asphyxiated neonates during the various postnatal ages. The median values with the ranges and the mean (± 1SD) values are indicated at the bottom of the table.

3.4.3 TBARS

The median (range) plasma concentration of TBARS for the 3 groups were as follows: 6.79 (4.43-15.01), 6.36 (4.74-11.30) and 6.35 (4.72-17.29) μM at [0-8 hrs]; 7.81 (5.53-10.9), 9.87 (3.60-13.89) and 9.50 (4.27-27.54) μM at [8-16 hrs]; and 7.99 (6.21-11.3), 9.22 (6.01-12.32) and 9.43 (6.32-16.58) μM at [16-24 hrs] for control, moderately asphyxiated and severely asphyxiated neonates respectively. TBARS tended to be higher in the asphyxiated neonates as compared to the control neonates at [8-16 hrs] and [16-24 hrs], but this difference never reached significance. No significant changes of TBARS concentration were seen between the three time periods within one group. Mean values (±1SD) for maximal total bilirubin plasma concentrations during the first 24 hours of life were 94.8 ± 43.8, 61.8 ± 14.9 and 62.5 ± 41.4 μM for control, moderately asphyxiated and severely asphyxiated neonates respectively and did not differ between groups.

3.4.4 Short-term outcome

Fourteen of the 15 severely asphyxiated neonates needed assisted ventilation beyond the resuscitation period, 9 neonates had convulsions requiring anticonvulsive therapy, one neonate received an erythrocyte transfusion because of a low hemoglobin and two needed dopamine medication to treat hypotension during the study period. Nine of the 15 severely asphyxiated neonates developed major neurological abnormalities. All died during the early neonatal period after supportive treatment was withdrawn because of their deteriorating neurological condition (e.g. coma, extensive cerebral damage), determined by computer tomography and/or a virtually flat electroencephalogram. Among them were those two severely asphyxiated neonates, which were excluded from the statistical analysis, because of only one blood sample (both died in the first 8 hours after birth and had rather high non-protein-bound iron concentrations: 82.9 and 97.6 μM). At discharge two of the 6 surviving severely asphyxiated neonates had abnormal neurological examinations (hypo- or hypertonia and little spontaneous movement). All the control and moderately asphyxiated neonates were neurologically normal at discharge.

3.4.5 Long-term outcome

All the control and moderately asphyxiated neonates were neurologically normal at 1 year of age. Of the 6 surviving severely asphyxiated neonates, two showed a delayed neurodevelopmental outcome at one year of age. The other 4 survivors were neurologically normal at 1 year of age. Three of these neonates had no detectable non-protein-bound iron during the study period. Stepwise logistic regression analysis with

outcome at one year of age (good [normal] or adverse [abnormal, death]) as dependent variable and cord/first pH, SGOT, SGPT, LDH, creatinine, urea, uric acid and non-protein-bound iron and TBARS at [0-8hrs], [8-16 hrs] and [16-24 hrs] as independent variables, revealed that the non-protein-bound iron concentration at [0-8 hrs] was the most significant variable ($p<0.001$) and at the same time the only variable which entered the model. An increase of the non-protein-bound iron plasma concentrations at [0-8 hrs] was associated with an increased risk for an adverse outcome at one year of age (Odds ratio = 1.12 / μM non-protein-bound iron; 95 % confidence interval: 1.00-1.26). Figure 3.3 shows the individual non protein-bound iron concentrations (medians indicated by the little dash) at [0-8 hrs] after birth in the neonates with a good (n=26) and an adverse outcome (n=10).

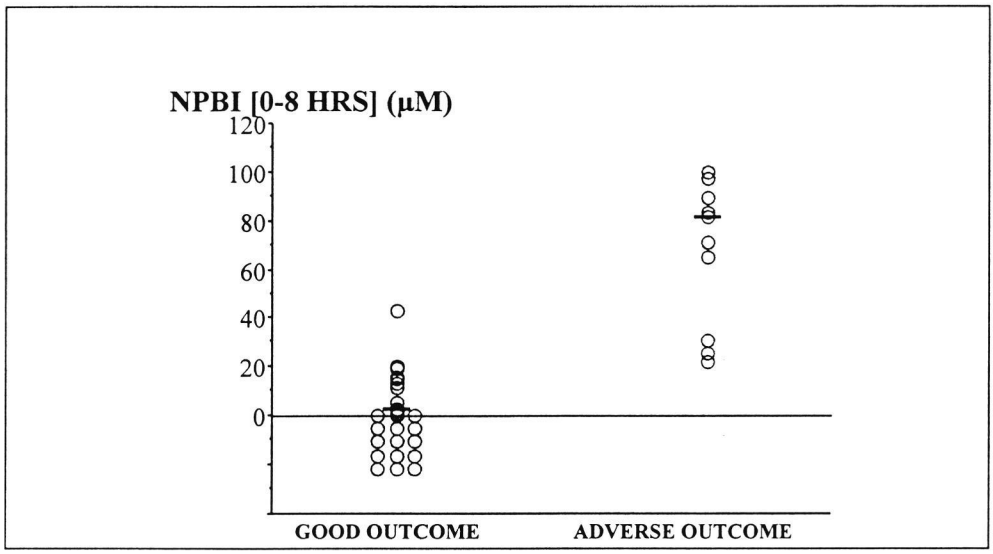


Figure 3.3 Individual and median (-) plasma concentrations of non-protein-bound iron (NPBI) at [0-8 hrs] after birth in the neonates with a normal (n=26) and an adverse outcome or death at one year of age (n=10).

3.5 Discussion

To our knowledge, this is the first clinical study which has investigated the relation between severity of birth asphyxia, plasma concentration of non-protein-bound iron and TBARS, and subsequent neurological outcome. There appeared to be an

association between elevated plasma concentrations of non-protein-bound iron at 0-8 hrs after birth and an adverse outcome after severe birth asphyxia. Moreover, the severely asphyxiated neonates with *no* detectable non-protein-bound iron in their plasma all had a *normal* neurodevelopmental outcome at one year of age. These findings may imply that non-protein-bound iron plays an important role in the pathogenesis of post-asphyxial reperfusion/reoxygenation injury. Interestingly, the association between non-protein-bound iron in plasma and adverse outcome appeared to be lost after 8 hours of age, despite sometimes high concentrations of non-protein-bound iron (figure 3.2). Possibly the reoxygenation of the brain upon early reperfusion is an important co-factor to generate reactive oxygen species such as $O_2^{\bullet -}$ and hydrogen oxide, leading to formation of the highly toxic $OH^{\bullet}$ radical in the presence of non-protein-bound iron and to brain cell damage.

The ability of plasma of neonates to inhibit non-protein-bound iron induced lipid peroxidation in vitro is significantly less as compared to the ability of adult plasma[18]. Moreover, healthy neonates often have detectable non-protein-bound iron in their plasma and this seems to be inversely related to their gestational age. In a study from Moison et al. none of the adults had detectable non-protein-bound iron in their plasma, but 6/24 term and 10/21 preterm neonates had detectable concentrations, which makes neonates more susceptible to non-protein-bound iron induced oxidative damage[11]. The values for non-protein-bound iron in some of the control neonates of the present study were higher than those previously reported by us[11]. This may be related to their somewhat lower gestational age (11 out of the 20 control neonates had gestational ages between 35.0 and 37.0 weeks), since preterm neonates do have higher non-protein-bound iron values than term neonates. With respect to the 3 outlying values of non-protein-bound iron in plasma in the control group (2 values in the 8-16 hrs-period [25.3 and 42.0 μM]; 1 value in the 16-24 hrs-period [28.7 μM], see also figure 3.2), we must admit that we did not measure plasma hemoglobin and have to consider the possibility that the key source of these non-protein-bound iron concentrations could be associated with heme or hemoglobin, despite our attempts to clinically exclude this by excluding plasma samples which showed pink discoloration. However, the role of heme or hemoglobin in detecting non-protein-bound iron is not likely to be of great importance in our study. In a previous study we showed that despite similar hemoglobin concentrations, very different levels of non-protein-bound iron were detected[11]. Furthermore, it has been demonstrated that addition of up to 3 mg hemoglobin/ml plasma (*i.e.* 46.5 μmol hemoglobin/l plasma) did not influence the measurement of non-protein-bound iron[19]. This amount of hemoglobin results in a discoloration of

plasma that can easily be identified. Since we excluded samples showing pink discoloration, the amounts of hemoglobin in the remaining samples must have been lower than 46.5 μmol.

The very high non-protein-bound iron levels in the asphyxiated neonates could be related to injury-induced iron release into the plasma. In the normal situation, iron is required for several important processes: oxygen transport (hemoglobin), mitochondrial respiration, proper function of several important enzymes and antibacterial defense and is usually firmly bound to transferrin in plasma or intracellularly to ferritin. These forms of iron are not capable to induce free radical production by catalyzing the Haber-Weiss reaction and therefore provide safe iron transport and storage systems[5]. However, lowering of the plasma pH, as occurs during ischemia, enables transferrin to liberate its iron, thereby inducing free radical production[20]. These free radicals are capable of releasing even more iron by mobilizing it from ferritin[21]. By these mechanisms a cascade of iron release and free radical production can be activated and lead to extensive cell damage. The injured cells may release their intracellular iron into the surrounding environment, thereby further increasing the plasma concentration of non-protein-bound iron. Non-protein-bound iron levels as high as 21.5 μmol/l were reported in an adult treated for leukemia despite the fact that plasma transferrin was only 50% saturated prior to therapy[22]. The authors suggested that chemotherapy induced destruction of leukemic cells released sufficient iron to saturate the transferrin and produce high non-protein-bound iron levels. Iron released after anoxic-ischemic damage may produce even higher non-protein-bound iron concentrations: the latent iron binding capacity of plasma is very limited in the neonate and much less of the released iron can be bound[23]. We therefore suggest that the increased plasma concentration of non-protein-bound iron in our asphyxiated neonates reflects the increased liberation of non-protein-bound iron from iron-binding proteins and/or damaged cells within the organ systems.

The involvement of non-protein-bound iron in free radical mediated damage is well established. Halat et al. showed the essential role of non-protein-bound iron in the promotion of post-ischemic lipid peroxidation[24]. Others found that local cerebral injection of iron salts increased lipid peroxidation, whereas lipid peroxidation was inhibited by the iron chelator deferoxamine[25,26]. We have recently demonstrated in newborn lambs, subjected to severe hypoxic-ischemic reperfusion injury, that there was a very rapid increase in non-protein-bound iron concentration within 15 min after the completion of the hypoxic-ischemic insult. Highest values were reached at 60 min after completion of the hypoxia-ischemia[27].

These processes can occur in all the important organ systems, which may contribute to the elevated liver enzymes and abnormal renal function tests in especially the severely asphyxiated neonates[6]. The brain however, may be especially at risk for free radical mediated injury because neuronal membranes are very rich in polyunsaturated fatty acids (sensitive to free radical attack) and several areas of the human brain are especially rich in iron[5,28]. Moreover, the iron binding capacity of cerebrospinal fluid is low (low concentration of transferrin) and most of the iron will be in its active ferrous form because of a high concentration of vitamin C and a low concentration of ceruloplasmin in cerebrospinal fluid[29,30].

It can be questioned whether the increased plasma concentration of non-protein-bound iron in our severely asphyxiated neonates is merely a marker of an increased leakage from injured cells, or if it is also actively inducing peroxidative damage itself (e.g. endothelial damage). Lesnefsky et al. found that intracellular, but not extracellular iron-administration augmented myocardial reperfusion injury[31]. Recent studies however, showed that chelating agents which were confined to the intravascular space improved outcome following head injury or cardiac arrest[32,33]. Another important question is whether the iron in this study will be in the active ferrous or less reactive ferric form. Because of the high concentration of vitamin C and low concentration of ceruloplasmin in the neonate, the non-protein-bound iron in these neonates is likely to be in the highly active ferrous form[18,34,35]. We therefore suggest that the increased plasma concentration of non-protein-bound iron in this study is more than only a marker indicating multi organ damage and may have played an important role in the extent of the post-asphyxial reperfusion injury and the generally poor neurological outcome of the severely asphyxiated neonates. In this respect studies concerning the prevention of free radical mediated reperfusion injury, which especially focus on scavenging the non-protein-bound iron, showed that deferoxamine, the prototype chelating agent, has been used successfully to diminish oxidative damage in various studies[32,33,36]. However, caution is required since deferoxamine has shown to be toxic to premature baboons[37]. Other promising therapies may be the administration of fresh adult plasma (high content of unsaturated transferrin) and/or an exchange transfusion. In newborns, exchange transfusions have shown to lower iron, ferritin and vitamin C levels and raise the concentration of transferrin, thereby increasing the latent iron-binding capacity[38]. However, further clinical studies are indicated to evaluate the effect of these therapies and to search for new agents, which can prevent free radical production, scavenge non-protein-bound iron *and* can be used safely in neonates.

Although the plasma concentration of TBARS in the severely asphyxiated neonates tended to be higher, suggesting an increased lipid peroxidation, these differences did not reach significance. However, it is important to realize that TBARS, although they are the most frequently quoted evidence for the involvement of free radicals in human disease, do have their limitations. They only measure lipid peroxidation, while proteins and DNA are more often the targets of oxidative damage than are lipids. Moreover lipid peroxidation often occurs late in the oxidative injury process[39]. Therefore we suggest that oxidative damage may play a role in the observed association between elevated plasma concentrations of non-protein-bound iron and an adverse outcome after severe birth asphyxia.

In summary, the results of this study suggest a relation between elevated plasma concentrations of non-protein-bound iron and an adverse outcome at one year of age after severe asphyxia. Although non-protein-bound iron may play an important role in the pathogenesis of postasphyxial reperfusion injury and subsequent neurological outcome, further study is warranted to evaluate the exact mechanisms of this relationship.

3.6 Acknowledgments

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4 EFFECT OF POST HYPOXIC-ISCHEMIC INHIBITION OF NITRIC OXIDE SYNTHESIS ON CEREBRAL BLOOD FLOW, METABOLISM AND ELECTROCORTICAL BRAIN ACTIVITY IN NEWBORN LAMBS

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(submitted for publication)

4.1 Abstract

Post hypoxic-ischemic (HI) brain injury is characterized by a short initial cerebral hyperperfusion, followed by cerebral hypoperfusion, decreased cerebral metabolic rate of oxygen ($CMRO_2$) and decreased electrocortical brain activity (ECBA). Since an excessive production of nitric oxide upon reperfusion/reoxygenation may play an important role in post-HI brain injury, we investigated whether immediate post-HI blockade of nitric oxide synthesis by N-ω-Nitro-L-Arginine (NLA) may reduce this injury. In 18 newborn lambs, subjected to severe HI, changes from pre-HI values were measured for carotid blood flow (Qcar [ml/min]) as a measure of changes in brain blood flow, (relative) $CMRO_2$, and ECBA at 15, 60, 120 and 180 min after HI. Upon completion of HI, 6 lambs received a placebo (CONT-group), 6 a low dose NLA (10 mg/kg/i.v.; NLA-10 group), and 6 a high dose NLA (40 mg/kg/i.v.; NLA-40 group). Histological damage of cerebellar Purkinje cells was assessed after termination of the experiment. Only the CONT-group showed a distinct initial post-HI cerebral hyperperfusion. From 60 min post-HI onward Q_{car} was decreased to about 75% of pre-HI Q_{car} in all 3 groups, although none of these changes in Q_{car} reached statistical significance. Despite the decreased Qcar in all 3 groups, only CONT-group showed a significantly decreased $CMRO_2$. ECBA and its bandwidth decreased in all groups, but only recovered in the NLA-10 group at 180 min post-HI. Brain to body mass ratio (%) and percentage necrotic Purkinje cells were respectively 15.3 ± 0.8 / 56.0 ± 10.0 (CONT-group), 12.5 ± 1.2 / 36.3 ± 8.6 (NLA-10 group) and 11.3 ± 1.0* / 34.5 ± 13.9 (NLA-40 group) (*$p<0.05$ vs. CONT-group). In conclusion, preservation of $CMRO_2$ in both NLA-groups, but a recovery of ECBA and its bandwidth only in the NLA-10 group, suggests that NLA, and especially a low dose NLA, may reduce post hypoxic-ischemic brain injury.

4.2 Introduction

Previous studies in newborn animals, including newborn lambs, who developed severe brain damage after subjection to hypoxia-ischemia (HI), showed abnormalities of cerebral hemodynamics, metabolism and electrocortical brain activity in the immediate post-HI period[1,2,3,4,5]. These abnormalities were characterized by a short initial cerebral hyperperfusion, followed by cerebral hypoperfusion, a drop in cerebral metabolic rate of oxygen ($CMRO_2$) and a decreased electrocortical brain activity during the first hours after the HI-insult. Although cerebral injury may occur during the actual hypoxic-ischemic insult, recent studies suggest that a substantial proportion of the injury can be attributed to the formation of reactive oxygen species, in particular nitric oxide, upon reoxygenation and reperfusion[6,7,8,9,10,11]. Nitric oxide can cause neuronal damage by inducing glutamate excitotoxicity, but also by damaging mitochondria, proteins and DNA[6,9,10,11]. Moreover, in the cerebral microcirculation it can react with superoxide ($O_2^{\bullet -}$) to form highly reactive peroxynitrite ($NO\bullet + O_2^{\bullet -} \rightarrow ONOO^-$), which in turn can decay to form the extremely toxic hydroxyl radical and nitrogen dioxide. This will result in endothelial damage, with loss of its barrier function, abnormal vasoregulation, and adhesion of platelets and white blood cells, leading to plugging of the cerebral microcirculation[7,8].

The aim of the present study was therefore to investigate the effect of immediate post HI-inhibition of nitric oxide synthesis by N-ω-Nitro-L-Arginine (NLA), a nitric oxide synthase inhibitor which easily crosses the blood-brain barrier[12], on post-HI cerebral perfusion, oxygen metabolism, electrocortical brain activity and histological damage of cerebellar Purkinje cells. Because it has been suggested in earlier studies that low rather than high dosages of NLA showed a maximal effect on reduction of brain cell damage[13], the treatment group was divided into a low dose (10 mg/kg NLA) and a high dose (40 mg/kg NLA) subgroup. An earlier study from Fineman et al. reported that the latter dose was necessary to produce near maximal inhibition of endothelium-dependent vasodilation in the newborn lamb[14].

We hypothesized that a low rather than a high dose NLA will reduce nitric oxide-induced cerebral tissue injury, since a low dose will not completely block endothelium-dependent vasodilation and therefore will be able to maintain sufficient perfusion to the critical regions of the brain.

4.3 Materials and Methods

4.3.1 Animal preparation

Surgical and experimental procedures used were reviewed and approved by the Animal Research Committee of the Leiden University Hospital and the scientific board of the Department of Pediatrics. Eighteen newborn lambs, ages ranging from 2 to 11 days (median: 7 days), weighing 4.6 ± 1.4 kg (means ± SD) were studied. General anesthesia was induced with a bolus of ketamine hydrochloride (3 mg/kg i.v.) and supplemented by xylazine (1 mg/kg/3hrs i.m.) and local subcutaneous injection of lidocaine 1% before each skin incision. During the study the wounds were sprayed with 1% lidocaine at regular intervals. After intubation the lambs were paralyzed with pancuronium bromide (0.2 mg/kg i.v.) and ventilated with oxygen and air, using a continuous flow, pressure-controlled ventilator (Bourns BP 200, Bear Medical Systems Inc., Riverside, CA). Ventilation was adjusted to keep arterial P_{O_2} and P_{CO_2} within the normal range throughout the study. An intravenous infusion of 5% glucose in NaCl 0.9% was continued throughout the study at about 15 ml/kg/h. $NaHCO_3$ was supplemented if the arterial pH was lower than 7.30 and the base deficit more than 5 mmol/l.

Self sealing sheaths, 5F or 6F, were introduced into both left and right femoral arteries and veins. Into the left femoral artery a 5F micromanometer catheter (Millar Instruments, Houston, TX) was advanced into the descending thoracic aorta for continuous measurement of the mean aortic blood pressure (MABP). The right femoral artery was used for sampling of arterial blood gases and pH. Both veins were used for blood withdrawal and infusion of drugs. In the right external jugular vein a 4F catheter was advanced retrogradely into the internal maxillary vein, to sample blood from the cerebral sinuses for measuring of the cerebral venous oxygen saturation. Blood from the cerebral sinuses is drained eventually by the internal maxillary veins, which unite with the external maxillary veins to form the external jugular veins[15]. Appropriately sized ultrasonic flow transducers (Transonic Systems Inc., Ithaca, NY) were applied to fit around the carotid artery for continuous measurement of the carotid blood flow (Qcar) by the transit-time technique. Changes in electrocortical brain activity (ECBA) were monitored using a filtered and selectively amplified one channel cerebral function monitor (Lectromed, Oxford Instruments, Oxford, UK), described by Prior[16]. The cerebral function monitor has a special filter which sharply attenuates frequencies below 2 and above 15 Hz, giving an amplitude-integrated recording which contains the main EEG frequencies, but with little disturbances from artefacts. The EEG signal was obtained from a pair of silver-chloride disk electrodes, placed with electrode cream at the P3 and P4 position of the 10-20 International System, i.e. in the left and right

parietal region[17]. The ECBA was recorded on a semilogarithmic scale (0-100μV). The paper speed was 25 mm/min. Simultaneously with the amplitude curve, an impedance curve records the reliability of the signal by a reference electrode positioned anterior to the scalp, and shows artefacts from movement, experimental procedures, or loose electrodes.

4.3.2 Histology

After completion of the experimental protocol (see below), the brain was removed and placed immediately in 4% formaldehyde for at least one week for subsequent histological investigation of the Purkinje cells of the cerebellum. Purkinje cells with ischemic cell changes, characterized by acidophilic (red) and/or shrunken cytoplasm, were assessed as pycnotic, whereas all others were considered viable[18]. Each section was examined by light microscopy and scored for the total proportion of necrotic and viable Purkinje cells by counting 3 regions of 100 cells each. This was done by an investigator who was blinded to the study.

4.3.3 Physiological measurements

Changes in brain blood flow were assessed by changes in Qcar [ml/min]. Previous studies have shown a close linear relationship between Qcar and the actual brain blood flow as determined by radioactive microspheres, also during hypoxia and/or hypotension[19,20]. The (relative) cerebral metabolic rate of oxygen was calculated with Qcar as a measure of the actual brain blood flow: (arterial Co_2 - cerebral venous Co_2) × Qcar; the results are expressed as ml O_2/min, where Co_2 is the blood oxygen content[21]. Co_2 is calculated as: grams Hb/dl * 1.36 ml O_2/g Hb * % saturation of Hb with O_2. The mean voltage of the ECBA (μV) was determined over a period of 2 min at each time point, and its bandwidth ($ECBA_{bw}$; μV), which reflects the variations in minimum and maximum amplitude, was calculated as described by Viniker at al.[22]. The MABP, Qcar and ECBA-signal were measured continuously, digitized with a sample frequency of 200 Hz, and stored on a personal computer. Arterial blood gases and pH were measured using a Corning 178 pH/blood gas analyzer (Corning, Halstead, UK). Cerebral venous blood lactate concentrations were determined spectrophotometrically.

4.3.4 Experimental procedure

After completion of the surgical preparation, the lambs were allowed to achieve hemodynamic stability and wash out their ketamine, to minimize a possible effect of ketamine on the brain[23]. The period between ketamine medication and the start of the

experiment was always at least 3 hours. After this stabilization period, blood samples for the various determinations as discussed above were taken, heart rate, MABP, Qcar and ECBA registered, and used as pre-HI values. Severe HI was then induced by ventilating the lamb with 6-8% O_2 supplemented with a mixture of 10% CO_2 in N_2 for 30 min, followed by a 5 min period of hypotension (MABP < 35 mmHg), achieved by careful withdrawal of blood (50 to 150 ml). Upon resuscitation (e.g. after completion of HI), 6 lambs received an intravenous infusion with a placebo (30 ml of a solution of 0.1N HCl in NaCl 0.9%; CONT-group), 6 lambs received a low dose NLA (10 mg/kg/i.v. in 30 ml of 0.1N HCl in NaCl 0.9%; NLA-10 group), and 6 lambs received a high dose NLA (40 mg/kg/i.v. in 30 ml of 0.1N HCl in NaCl 0.9%; NLA-40 group). Resuscitation was principally performed in a way similar to that routinely used in our neonatal unit: extra ambient oxygen, which was progressively decreased depending on the color of the tongue and the arterial blood, and on the blood gas determined 2 min after the start of the resuscitation. Cardiac arrest and hypotension were treated with adrenaline (0.01%) and/or dopamine when appropriate. The blood withdrawn to achieve hypotension was reinfused immediately after completion of the HI period.

At 15, 60, 120 and 180 min after completion of the HI, MABP was registered, blood samples were taken and % changes relative to pre-HI values were measured for Q_{car} [ΔQ_{car}], mean voltage and bandwidth of the ECBA [ΔECBA and $\Delta ECBA_{bw}$] and $CMRO_2$ [$\Delta CMRO_2$]. After termination of the experiment the lamb was sacrificed with nembutal (150 mg/kg). The brain was removed and weighed before fixation. Brain to body mass ratio (%) (used as an indicator for cerebral edema) was assessed by dividing the brain mass by the total body mass, which was determined before the start of the experiment.

4.3.5 Statistical analysis

Differences between the age, total body mass, brain mass, brain to body mass ratio (%) and percentage of necrotic Purkinje cells of the three groups were assessed by one way factorial analysis of variance (ANOVA). When a significant difference was found, ANOVA was followed by the Scheffe's procedure for comparison between the groups. To evaluate whether there were significant changes of MABP, ΔQcar, ΔECBA, $\Delta ECBA_{bw}$, $\Delta CMRO_2$, arterial pH, blood gases and cerebral venous lactate concentration within each group between the several time points and whether these changes were significantly different for the NLA-treated lambs as compared to the CONT-group, a repeated measurements analysis of variance was used with 3 main factors, 2 fixed (time and dose) and 1 random factor (animal, nested within group). To evaluate whether the hypoxic-ischemic insult had an effect on arterial pH and blood

gases a paired *t*-test was performed on the pre-HI and 2 min post-HI values. A *p*-value <0.05 was considered statistically significant.

4.4 Results

Five CONT, 4 NLA-10 and 5 NLA-40 lambs needed adrenaline and/or dopamine during the early resuscitation period because of cardiac arrest or persistent hypotension after completion of the HI-insult. There was no difference among the groups with respect to the amount of bicarbonate infused to treat the severe metabolic acidosis in the immediate post-HI period. Hb values were not different between the three groups or between time points. Table 4.1 summarizes the mean values (± SEM) of MABP, arterial pH, blood gases and cerebral venous lactate concentration during the respective time points. One hour post-HI the MABP was significantly increased in the NLA-40 group as compared to the CONT-group. The pH was significantly decreased in all three groups during the whole post-HI period. The pCO_2 initially increased in all 3 groups post HI, although this was only significant for the NLA-10 group at 2 and 15 min post-HI and in the NLA-40 group at 2 and 60 min post-HI. There were no differences in the pH and blood gases between the three groups within the various time points except a higher pCO_2 in the NLA-10 group 15 min post-HI. The cerebral venous lactate concentration was significantly increased in all 3 groups during the whole study period. Three hours post-HI the cerebral venous lactate concentration was significantly lower in the NLA-10 and NLA-40 groups as compared to the CONT-group.

4.4.1 MABP, Q_{car}, and ECBA during the actual HI period

During the period of hypoxia all the animals showed a small increase in MABP. Qcar showed a tremendous increase in all groups (pre-HI mean Qcar (± SEM): 62 ± 6 ml/min; highest Qcar during HI: 132 ± 7 ml/min, $p<0.05$). ECBA and $ECBA_{bw}$ remained almost stable, although a slight decrease was seen during hypoxia, until severe hypotension was reached. During the 5 min hypotensive period MABP and Qcar decreased to very low values in all groups (CONT group: 25 ± 4 mm Hg/14 ± 3 ml/min; NLA-10 group: 27 ± 1 mm Hg/13 ± 3 ml/min; NLA-40 group: 26 ± 3 mm Hg/12 ± 4 ml/min). ECBA decreased to virtually zero in all lambs. Figure 4.1 shows a representative example of the pattern of ECBA and $ECBA_{bw}$ during the HI-period, including the period of severe hypotension.

	pre-HI	2 min post-HI	15 min post-HI	60 min post-HI	120 min post-HI	180 min post-HI
MABP (mm Hg)						
CONT	81 ± 5	70 ± 40	81 ± 6	79 ± 12	78 ± 9	76 ± 8
NLA-10	79 ± 8	69 ± 4	84 ± 6	76 ± 7	85 ± 6	87 ± 4
NLA-40	71 ± 4	67 ± 5	80 ± 7	89 ± 6*	76 ± 14	83 ± 6
pH						
CONT	7.38 ± 0.03	6.99 ± 0.04†	7.02 ± 0.02†	7.19 ± 0.08†	7.23 ± 0.07†	7.22 ± 0.05†
NLA-10	7.38 ± 0.03	6.91 ± 0.04†	6.94 ± 0.05†	7.23 ± 0.03†	7.19 ± 0.05†	7.27 ± 0.06†
NLA-40	7.36 ± 0.04	6.93 ± 0.05†	7.05 ± 0.04†	7.10 ± 0.06†	7.15 ± 0.07†	7.23 ± 0.05†
PCO_2 (kPa)						
CONT	4.7 ± 0.5	7.8 ± 1.3	5.3 ±1.0	4.3 ± 0.6	4.4 ± 0.3	4.8 ± 0.4
NLA-10	5.0 ± 0.5	10.4 ± 1.7†	7.9 ± 0.9*†	5.6 ± 0.6	5.1 ± 0.5	4.9 ± 0.4
NLA-40	4.5 ± 0.7	10.7 ± 1.6†	5.6 ± 0.6	5.8 ± 0.8†	4.9 ± 0.3	5.2 ± 0.2
PO_2 (kPa)						
CONT	16.3 ± 1.9	20.0 ± 3.7	22.5 ± 3.9	15.2 ± 2.3	17.5 ± 1.1	15.4 ± 0.4
NLA-10	14.2 ± 1.1	19.2 ± 2.6	16.0 ± 2.9	11.8 ± 0.5	16.1 ± 1.6	16.7 ± 1.1
NLA-40	13.2 ± 1.5	17.2 ± 2.5	21.8 ± 7.3	12.3 ± 1.7	15.6 ± 1.7	13.5 ± 2.5
Lactate (mmol/l)						
CONT	4.2 ± 0.6		10.3 ± 1.5†	12.4 ± 1.5†	13.5 ± 1.4†	16.7 ± 1.5†
NLA-10	3.0 ± 0.6		10.4 ± 0.8†	9.9 ± 1.4†	10.0 ± 1.4†	7.1 ± 0.7*†
NLA-40	2.5 ± 0.6		7.4 ± 1.5†	7.9 ± 1.14†	10.7 ± 2.0†	10.9 ± 1.1*†

*$p < 0.05$ vs. CONT-group, †$p < 0.05$ vs. pre-HI

Table 4.1 Mean values (± SEM) of mean aortic blood pressure (MABP), pH, PCO_2, PO_2 and cerebral venous lactate concentration in the control (CONT), low-dose N-ω-Nitro-L-arginine (NLA-10) and high-dose N-ω-Nitro-L-arginine (NLA-40) group during the respective time periods after completion of hypoxia-ischemia (HI).

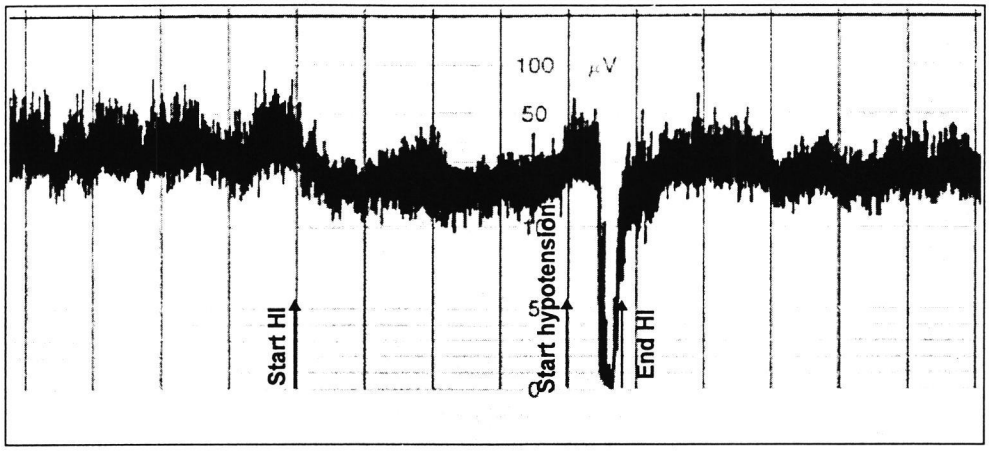
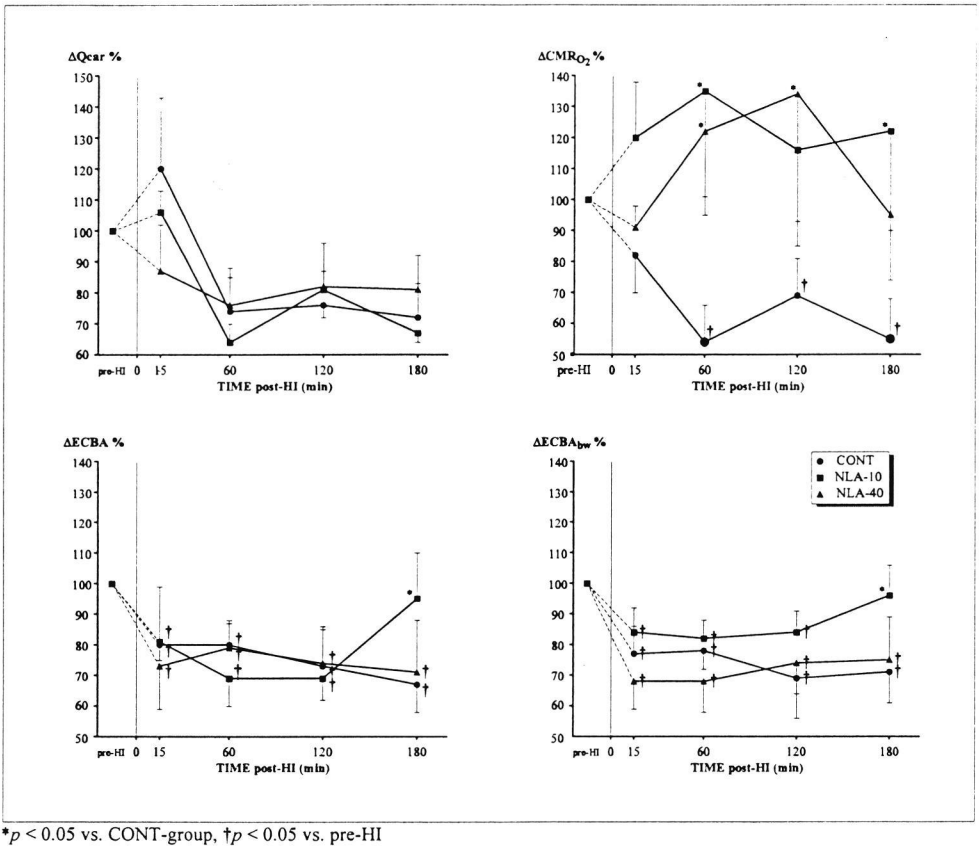


Figure 4.1 Representative example of a registration of the electrocortical brain activity using the cerebral function monitor during the actual hypoxic-ischemic (HI) period, including the period of severe hypotension (see arrows) in one of the experiments.

4.4.2 Q_{car}, $CMRO_2$, ECBA and $ECBA_{bw}$ during the post-HI period

Figure 4.2 shows the mean changes (%) in Q_{car}, $CMRO_2$, ECBA and $ECBA_{bw}$ (ΔQ_{car}, $\Delta CMRO_2$, ΔECBA, $\Delta ECBA_{bw}$) relative to the respective pre-HI values as a function of time after HI in the three different groups. In contrast to the observed cerebral hyperperfusion during the HI-insult in all 3 groups, only the CONT group showed a distinct initial post-HI cerebral hyperperfusion. From 60 min post-HI onward Q_{car} was decreased to about 75% to 80% of pre-HI Q_{car} in all 3 groups, although this reached no statistical significance. In contrast to the NLA-10 and NLA-40 group, $CMRO_2$ was significantly decreased in the CONT-group during the whole study period. Both ECBA and $ECBA_{bw}$ initially decreased significantly in all 3 groups, but recovered to its pre-HI value only in the NLA-10 group at 180 min post-HI. At that time the ECBA and $ECBA_{bw}$ were significantly higher in the NLA-10 group as compared to the CONT-group.



*$p < 0.05$ vs. CONT-group, †$p < 0.05$ vs. pre-HI

Figure 4.2 Percent (%) changes (mean ± SEM) relative to pre hypoxic-ischemic (HI) values for carotid artery blood flow [ΔQ_{car}], cerebral metabolic rate of oxygen [$\Delta CMRO_2$] and mean voltage and bandwidth of the electrocortical brain activity [ΔECBA and $\Delta ECBA_{bw}$].

4.4.3 Pathological and histological data

Table 4.2 shows the mean (± SEM) values of body and brain mass, brain to body mass ratio (%) and the percentage of necrotic Purkinje cells in cerebellar tissue of the three groups.

	CONT-group	NLA-10 group	NLA-40 group
Body mass (kg)	4.3 ± 0.7	4.6 ± 0.5	4.8 ± 0.5
Brain mass (g)	64.5 ± 10.7	55.3 ± 1.5	52.2 ± 3.2
Brain to body mass ratio (%)	15.3 ± 0.8	12.5 ± 1.2	11.3 ± 1.0*
Necrotic Purkinje cells (%)	55.5 ± 10.0	36.3 ± 8.6	34.5 ± 13.9

*$p < 0.05$ vs. CONT-group

Table 4.2 Mean values ± SEM of brain and body mass and histological data of the control (CONT), low-dose N-ω-Nitro-L-arginine (NLA-10) and high-dose N-ω-Nitro-L-arginine (NLA-40) group.

There was no significant difference in body mass between the three groups. Brain mass tended to be higher in the CONT group as compared to NLA-10 and NLA-40 groups, although this reached no statistical significance. Brain to body mass ratio was lower in NLA-10 and NLA-40 groups as compared to the CONT-group, although this difference was only significant for the NLA-40 group. The percentage of necrotic Purkinje cells in NLA-10 and NLA-40 groups was 20% lower as compared to those in the CONT-group. However, this difference was not statistically significant.

4.5 Discussion

The results of the present study indicate that post-HI inhibition of nitric oxide synthesis by especially a low dose NLA prevented a drop in post-HI $CMRO_2$ and resulted in a recovery of the ECBA and its bandwidth. These findings are consistent with the hypothesis that an excessive synthesis of nitric oxide plays an important role in the genesis of these abnormalities, which are characteristic for post-HI brain injury. We therefore suggest that inhibition with a low, rather than a high dose NLA may reduce post-HI brain injury.

The involvement of nitric oxide in post hypoxic-ischemic brain injury is well established. Beckman reported that post-HI cerebral reperfusion can cause a sharp increase of endothelial derived nitric oxide which subsequently can react with the reactive oxygen species superoxide to form the highly reactive compound peroxynitrite[7]. This molecule exerts its toxicity either by direct reaction with sulfhydryl groups, or by decomposing into the highly toxic hydroxyl radical and

nitrogen dioxide, leading to damage of the endothelial layer of the microvessels of the cerebral circulation. Moreover, it has been suggested that nitric oxide and hydroxyl radical can easily cross the blood-brain barrier, thereby also exerting their destructive activity on the brain tissue itself[7]. Another proposed mechanism of nitric oxide-toxicity is via the production of the excitatory neurotransmitter glutamate. There is considerable evidence now that the N-Methyl-D-Aspartate (NMDA) receptor mediated formation of excess neuronal derived nitric oxide results in glutamate mediated neurotoxicity, which is also observed in hypoxic-ischemic brain injury[9,10,11]. Moreover, administration of nitric oxide synthase inhibitors and NMDA receptor antagonists have been reported to exhibit neuroprotective effects in several studies[6,24,25].

Recent studies have shown a rapid increase of nitric oxide production at the onset of ischemia, possibly as a physiological reaction to increase cerebral blood flow[26]. In this study we also found an increase in Q_{car} at the onset of the HI. During periods of only hypoxia without ischemia up to 1 hour nitric oxide production still remains[27], whereas, during ischemia up to 1 hour, nitric oxide production finally declines due to a lack of oxygen, NADPH and L-Arginine[28]. During reperfusion/reoxygenation however, when oxygen is suddenly available in excess, excessive amounts of nitric oxide may be produced. Sato et al. indeed have shown an increase in nitric oxide production within 15 min of reperfusion[26]. Clavier et al. further demonstrated that immediate postischemic cerebral hyperperfusion is mediated by this nitric oxide prduction[29]. Moreover, Greenberg et al. showed that nitric oxide synthase inhibition could reduce postischemic cerebral hyperperfusion[30]. This is in agreement with the findings of the present study in which the post-HI increase in cerebral perfusion was far less pronounced in the NLA-10 group and even absent in the NLA-40 group, suggesting that NLA reduces post-HI cerebral hyperperfusion in a dose dependent manner.

From 1 hour post-HI onwards, Qcar was about 75% of the pre-HI value in all 3 groups. Despite this relative hypoperfusion, $CMRO_2$ was preserved in both NLA-groups but depressed in the control group. The most plausible explanation for this depressed $CMRO_2$ in the control group may be an impaired mitochondrial function, as a consequence of nitric oxide and glutamate induced mitochondrial damage. On the other hand it may also reflect a direct effect of nitric oxide. Brown et al. showed that nitric oxide could inhibit mitochondrial synaptosomal respiration by competing with oxygen at cytochrome oxidase in vivo[31].

Three hours post-HI the lactate production was significantly lower in the groups treated with NLA as compared to the control group. As the lactate metabolism is dependent on oxygen availability, this may indicate a disturbed oxygen metabolism in the control group, possibly because of mitochondrial damage, which has been associated with a poor prognosis following birth asphyxia[32,33].

The semilogarithmic scale of the cerebral function monitor makes it especially sensitive to changes in cerebral activity within the lowest amplitudes, a feature of a compromised brain function with low voltages and inactive tracings[16,17]. Earlier studies in newborn lambs subjected to hypoxia, hypocarbia and hemorrhagic hypotension showed that the ECBA was only affected under severe hypotensive conditions (<30 mmHg)[34]. Although some decrease was seen during the actual period of hypoxia, the ECBA and its bandwidth remained quite stable during HI until the last 5 min, when severe hypotension was reached. Then the ECBA and its bandwidth showed a distinct decrease. Upon resuscitation ECBA and its bandwidth initially recovered partially, but remained depressed in all 3 groups, which may indicate a serious impairment of neuronal metabolism. However, in the lambs receiving a low dose NLA, the ECBA and its bandwidth recovered to pre-HI values 180 min post-HI. To the extent that the speed and the completeness of the recovery of the ECBA after an ischemic episode is closely related to the extent of neuronal recovery, these findings suggest a more favorable neuronal prognosis for the NLA-10 treated lambs[35]. In contrast, the prolonged suppression of the ECBA and its bandwidth in the CONT and NLA-40 group may represent a prelude for a degree of sustained brain damage in these lambs[16].

The brain to body mass ratio was higher in the CONT, as compared to the NLA-treated lambs, which may indicate less cerebral edema in the NLA-treated lambs. Mujsce et al. demonstrated that cerebral edema can be detected as early as 30 min after hypoxic-ischemic injury[36]. Moreover, Nagafuji et al. showed that NLA treatment could mitigate post-ischemic brain edema in rats[25]. We therefore suppose that post-HI treatment with NLA reduces neuronal cell damage, and by that mechanism also reduces cerebral edema. This reasoning is supported by the fact that, although not statistically significant, the percentage of necrotic Purkinje cells was about 20% lower in both groups of NLA-treated lambs.

Of all the tissues so far examined, the brain contains the highest activity of constitutive, Ca-dependent nitric oxide synthase[37]. Immunostaining for brain nitric

oxide synthase has revealed the highest densities in neurons of the cerebellar granular cell layer and the accessory olfactory bulb[38]. We chose to investigate the cerebellar Purkinje cells, because they lack nitric oxide synthase, but have high levels of guanylate cyclase, and are therefore the most vulnerable cells with respect to nitric-oxide mediated neurotoxicity[10,38]. In the presence of high levels of glutamate following ischemia-reperfusion/reoxygenation, the nitric oxide synthase containing cells may act like activated macrophages: by releasing large amounts of nitric oxide they can kill their neighboring cells. In the present study the period between the HI-insult and the sacrificing was only four hours. The period in which neuronal necrosis develops may take several hours to days. Although we indeed found no significant decrease in the percentage of necrotic Purkinje cells, there was a clear trend of less necrosis in both groups of NLA-treated lambs (56% in CONT vs. 36% and 34% in NLA-10 and NLA-40 groups respectively).

Although the above mentioned and discussed results suggest a beneficial effect of especially a low dose NLA, adult and neonatal studies show conflicting results, reporting either increases or reductions in infarction volume after a stroke and subsequent administration of nitric oxide synthesis inhibitors[6,39,40]. It has been suggested that although nitric oxide overproduction plays an important role in reactive oxygen species mediated neuronal injury, endothelial derived nitric oxide may protect against ischemia by increasing regional cerebral blood flow[41]. It is important to stress here that there is an essential difference between *local* and *global* ischemia, the latter occurring during neonatal hypoxia-ischemia. After local (partial) occlusion of a cerebral artery, nitric oxide synthesis may cause vasodilation and thereby "rescue" brain cells in the penumbra surrounding the infarct lesion, indicating a neuroprotective effect. Moreover, absence of reperfusion/reoxygenation of the infarct lesion itself may prevent the production of highly toxic reactive oxygen species. On the other hand, during global ischemia, it is important to reduce the nitric oxide burst in order to prevent excessive reactive oxygen species and glutamate production, which means that nitric oxide synthesis inhibition after global ischemia may be neuroprotective.

It is interesting that the results of the present study suggest that low, rather than high dose nitric oxide synthesis inhibition shows a neuroprotective effect. In this regard it is important to stress that, besides its potential neurotoxic properties, nitric oxide has also some beneficial roles. Nitric oxide has been suggested to act as a mediator of neuronal activity and cerebral blood flow and may have a role in learning and memory[38]. The role of nitric oxide in the brain therefore seems controversial. On the one hand, in physiologic (low) concentrations it has beneficial effects, whereas an excessive nitric

oxide synthesis can have very destructive properties. Therefore it may be important to decrease the excessive amount of nitric oxide produced upon hypoxic-ischemic reperfusion in order to inhibit its neurotoxic properties, but on the other hand not to block the nitric oxide synthesis completely to guarantee an essential cerebral blood supply. A recent study of Marks et al. in near term fetal lambs subjected to cerebral ischemia showed indeed that high dose nitric oxide synthase inhibition increased cerebral injury in stead of reducing it[42]. In the present study we found that the initial cerebral hyperperfusion seen in the control group was diminished in the NLA-10 group and even absent in the NLA-40 group, which may indicate a second, or prolonged period of ischemia in the NLA-40 group. We speculate that there is a balance between the amount of "hyperperfusion" needed to "rescue" dying cells and on the other hand the prevention of "hyperperfusion" in order to prevent the formation of reactive oxygen species. We therefore suppose that partial nitric oxide synthesis inhibition can prevent excessive production of reactive oxygen species and at the same time is able to maintain sufficient perfusion to critical regions of the brain.

In conclusion, the findings of the present study show that post-HI inhibition of nitric oxide synthesis by especially a low dose NLA prevents a drop in $CMRO_2$ and results in a recovery of the ECBA and its bandwidth. We therefore suggest that treatment with especially a low dose NLA may reduce post-HI brain injury, although further (chronic) studies are warranted to investigate the long-term effects of NLA-treatment.

4.6 References

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5 OXIDATIVE STRESS DURING POST HYPOXIC-ISCHEMIC REPERFUSION IN THE NEWBORN LAMB: THE EFFECT OF NITRIC OXIDE SYNTHESIS INHIBITION

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5.1 Abstract

Post hypoxic-ischemic (HI) reperfusion induces endothelium and neurons to produce excessive amounts of nitric oxide and superoxide, leading to peroxynitrite formation, release of protein-bound metal ions (i.e. iron) and cytotoxic oxidants. We produced severe HI in 18 newborn lambs and serially determined plasma pro-oxidants (non-protein-bound iron), lipid peroxidation (malondialdehyde) and anti-oxidative capacity (ratio of ascorbic acid/dehydroascorbic acid: AA/DHA-ratio, α-tocopherol, sulfhydryl groups, allantoin/uric acid-ratio and vitamin A) in blood effluent from the brain before and at 15, 60, 120 and 180 min after HI. The lambs were divided in 3 groups: 6 received a placebo (CONT), 6 received low dose (10 mg/kg/iv) N-ω-nitro-L-arginine (10-NLA) to block nitric oxide production and 6 received high dose NLA (40 mg/kg/iv; 40-NLA) immediately after completion of HI.

Non-protein-bound iron increased in all groups after HI but was significantly lower in both NLA-groups at 180 min post HI ($p<0.05$), the AA/DHA-ratio showed a consistent decrease in CONT (at 60 min post-HI, $p<0.05$), but remained stable in NLA-lambs. α-Tocopherol decreased steadily in the CONT, but not in the NLA-lambs (180 post HI: 1.9±0.9 vs. 4.2±0.7 μM [NLA-40], $p<0.05$). Malondialdehyde was significantly higher in CONT-lambs 120 min post HI as compared to NLA-groups (0.61±017 vs. 0.44±0.05 μM [NLA-40], $p<0.05$). Vit A and sulfhydryl groups did not differ among groups. We conclude that post-HI inhibition of nitric oxide synthesis diminishes non-protein-bound iron increment and preserves anti-oxidative capacity.

5.2 Introduction

Hypoxia and ischemia are major factors in the pathogenesis of brain injury following birth asphyxia[1,2]. More recently it has been recognized that the reperfusion and reoxygenation phase in the immediate post hypoxic-ischemic period also contributes to the mechanism of this so-called reperfusion damage: hypoxia-ischemia "primes" the perinatal brain to respond with an excess production of reactive oxygen species such as superoxide and hydrogen peroxide on reperfusion and reoxygenation[3,4,5,6]. There is increasing evidence that superoxide and calcium-induced production of nitric oxide in neuronal and endothelial cells, both produced in excess when oxygen is readmitted to the brain during the reperfusion phase, can react to form highly cytotoxic oxidants[6,8]. Moreover, this excessive production of nitric oxide and hydrogen peroxide can release metal ions (*i.e.* iron) from its binding proteins (*i.e.* ferritin)[9,10]. This non-protein-bound iron is a powerful pro-oxidant and converts hydrogen peroxide into the very toxic hydroxyl radical[11]. Recent in vitro and in vivo studies suggest that inhibition of nitric oxide production during the immediate post hypoxic-ischemic period prevents free radical mediated reperfusion injury to brain tissue[8,12,13,14].

We investigated in newborn lambs, which underwent a severe hypoxic-ischemic insult, whether inhibition of nitric oxide production with an analog of L-arginine (the precursor of nitric oxide), N-ω-nitro-L-arginine (NLA), in the immediate post hypoxic-ischemic period, decreased (pro) oxidant activity. Because it has previously been suggested that a low rather than a high dose NLA resulted in less brain damage[15,16,17], the treatment group was divided into a low dose (10 mg/kg NLA) and a high dose (40 mg/kg NLA) subgroup: an earlier study from Fineman et al. reported that up to 300 mg NLA was necessary to attenuate endothelium-dependent vasodilation in the newborn lamb[18].

We hypothesized that (low dose and or high dose) NLA would diminish production of pro-oxidants and consumption of anti-oxidants, and thus limit lipid peroxidation.

5.3 Materials and methods

5.3.1 Animal preparation

Surgical and experimental procedures employed were reviewed and approved by the Committee on Animal Experiments of the University of Leiden and the Scientific Board of the Department of Pediatrics.

Eighteen newborn lambs with weights ranging from 3.5 to 5.3 kg (median 4.7 kg) and ages ranging from 2 to 11 days (median 7 days) were studied. General anesthesia was induced with ketamine hydrochloride (3 mg/kg i.v.) and supplemented by xylazine (1 mg/kg i.m.). In addition, local anesthesia was accomplished with 1% lidocaine hydrochloride before each skin incision. During the actual study, the wounds were sprayed with 1% lidocaine at regular intervals. After intubation, the lambs were ventilated with oxygen and air using a pressure-regulated ventilator which was adjusted to maintain arterial pO_2 and pCO_2 in the normal range. Pancuronium (0.2 mg/kg i.v.) was administered for muscle relaxation. The animals were nursed on a heating pad to maintain normal body temperature. An intravenous infusion of 5% glucose in NaCl 0.9% was maintained throughout the study at about 15 ml/kg/h. $NaHCO_3$ was administered if the arterial pH was lower than 7.30 with a base excess of >5 mmol/l.

Into the right and left femoral arteries and veins 5F or 6F self-sealing sheaths were placed using a percutaneous technique. Into the left femoral artery a 5F micromanometer catheter (Millar Instruments, Houston, TX) was advanced into the descending thoracic aorta for continuous measurement of instantaneous mean aortic pressure. The right femoral artery was used for sampling of arterial gases and pH. Both femoral venous catheters were used for blood withdrawal and reperfusion, and for infusion of drugs. Via the right jugular vein a 4F catheter was advanced retrogradely towards the head and positioned in the internal maxillary vein, into which the cerebral sinuses drain[19]. Blood from this vessel was used to sample venous blood effluent from the brain to determine the redox status. After the carotid arteries in the neck were exposed, appropriately sized transonic flow transducers (Transonic Systems Inc., Ithaca, NY) were applied to fit around the vessels to measure carotid artery blood flow (ml/min) for assessment of changes in actual brain blood flow[20]. This was done to be sure that there was indeed cerebral ischemia during the hypoxic-ischemic insult (see also *experimental protocol*).

5.3.2 Physiologic measurements

Arterial blood gases and pH were measured using a Corning 178 pH/Blood Gas Analyzer (Corning, Halstead, UK). Blood gases, pH and hemoglobin were determined at regular intervals and adjusted if necessary. Instantaneous aortic pressure and ECG were continuously displayed on a memory oscilloscope (Gould OS 4100, Hainault, UK), and digitized with a sample frequency of 200 Hz and stored on hard disk using a personal computer.

5.3.3 Measurement of pro- and anti- oxidant capacity and lipid peroxidation

Blood was collected into heparinized glass tubes and immediately centrifuged (750 g, 10 min); the plasma was stored under argon at -70° C until analysis. Plasma samples which showed pink discoloration (hemolysis) were excluded from the study. Non-protein-bound iron, a pro-oxidant, was measured using the bleomycin assay[21]. Using this assay, the absence of non-protein-bound iron, *i.e.* the presence of iron binding capacity, can be measured as well as the presence of non-protein-bound iron, *i.e.* the lack of iron binding capacity due to saturation or dysfunction of transferrin[22]. If non-protein-bound iron is present, the lower detection limit is 0.6 μM. The glass tubes used to collect the blood did not contain detectable amounts of iron. The intra- and inter-assay coefficients of variation of the bleomycin assay were 6.6% and 7.4% respectively. High performance liquid chromatography techniques were used to determine the following anti-oxidants: reduced and oxidized ascorbic acid (ascorbic acid and dehydroascorbic acid respectively), uric acid and its oxidation product allantoin and α-tocopherol and retinol[23,24,25]. Plasma sulfhydryl content was determined spectrophotometrically[25]. The lipid peroxidation product malon-dialdehyde was measured using high performance liquid chromatography[26].

5.3.4 Experimental protocol

After completion of the surgical preparation, the lambs were allowed to achieve hemodynamic stability and wash out their ketamine, to exclude an effect of ketamine on the brain[27]. The period between ketamin medication and the start of the experiment was always at least 3 hours. The skin incisions were sprayed with 1% lidocaine at regular intervals. After having reached steady state (mean aortic blood pressure [MABP], heart rate), blood samples were taken from the jugular vein catheter to determine the various indicators of the redox status, from the aorta to determine the arterial blood gases and pH, and from the femoral vein to determine the hemoglobin, before and at 15, 60, 120 and 180 min after the hypoxic-ischemic (HI) insult. An additional arterial blood sample was taken at 2 min after completion of the HI-insult to determine pH and blood gases to assess the magnitude of HI-induced metabolic acidosis. The HI insult was established by ventilating the lamb with 6-8% oxygen and 10% carbon dioxide (supplemented with nitrogen) for 30 min, followed by 5 min of hypotension (mean aortic pressure (MABP): <35 mm Hg) achieved by careful withdrawal of blood. Upon completion of the HI-insult, 6 lambs received an intravenous infusion with a placebo (30 ml of 0.1 N HCl in NaCl 0.9%; CONT group), 6 lambs received a low dose NLA (10 mg/kg/iv, dissolved in 30 ml of 0.1 N HCl in NaCl 0.9%; NLA-10 group), and 4 lambs received a high dose NLA (40 mg/kg/iv,

dissolved in 30 ml of 0.1 N HCl in NaCl 0.9%; NLA-40 group). Resuscitation was performed in principle in a way similar to that used routinely in our neonatal intensive unit: extra ambient oxygen which was progressively decreased depending on the color of the tongue and the arterial blood, and blood gases determined 2 min after completion of the HI-insult. Cardiac arrest and hypotension were treated with adrenaline (1:10000) and/or dopamine when appropriate. The blood withdrawn to achieve hypotension, was reinfused immediately after completion of the HI-period. Metabolic acidosis was corrected with $NaHCO_3$ (see above).

5.3.5 Statistical analysis

Data are summarized as means ± 1SD or ± SEM. Differences between pre-HI values and values of MABP and blood gases immediately after completion of the HI (2 min post-HI) were compared with a paired *t*-test. Differences between values within the groups during the different time points (pre-HI, 15, 60, 120 and 180 min) were assessed by ANOVA for repeated measurements, and differences between values of the three groups at each time point were assessed by one way factorial ANOVA. When a significant difference was found, ANOVA was followed by the Scheffe's procedure. A p-value of < 0.05 was considered statistically significant.

5.4 Results

5.4.1 Physiologic measurements

There were no differences between the 3 study groups with respect to animal weight or postnatal age. MABP and carotid artery blood flow decreased to extremely low values at the end of the 5 min-hypotensive period. Lowest MABP and carotid artery blood flow values (means ± SEM) during this 5 min period did not differ between groups (CONT-group: 28 ± 2 mm Hg/12 ± 3 ml. min^{-1} [pre HI: 70 ± 8 ml. min^{-1}]; low dose-NLA-group: 27 ± 1 mm Hg/13 ± 3 ml. min^{-1} [pre-HI: 66 ± 4 ml. min^{-1}]; and high dose NLA-group: 24 ± 2 mm Hg/11 ± 2 ml.min^{-1} [pre-HI: 62 ± 7 ml. min^{-1}]). Table 5.1 summarizes the mean values (± 1SD) of MABP, arterial pH, PCO_2, PO_2 and base excess during the respective time points, including 2 min post-HI. Although MABP and PO_2 did not differ between groups during the study, MABP in the NLA-40 lambs increased, as expected, to 89 ± 15 mm Hg 60 min post-HI *vs.* 71 ± 11 mm Hg pre-HI ($p<0.05$). PCO_2 tended to be higher 2 min post-HI, but this was only significant for both NLA-groups. Arterial pH and base excess were significantly lower in all 3 groups at 2 min post-HI, as compared to pre-HI values. Although arterial pH and base excess remained (mostly significantly) lower during the remainder of the post-HI period there was a partial recovery of these parameters in all groups. There were no relevant

differences among groups for hemodynamic parameters or pH and blood gas values, except the rather low PCO_2-values in the NLA-10 group at 180 min post-HI. There was no difference among the groups with respect to the amount of bicarbonate infused to treat the severe metabolic acidosis in the immediate post-HI period. Hemoglobin was not different within or among groups during the various time points.

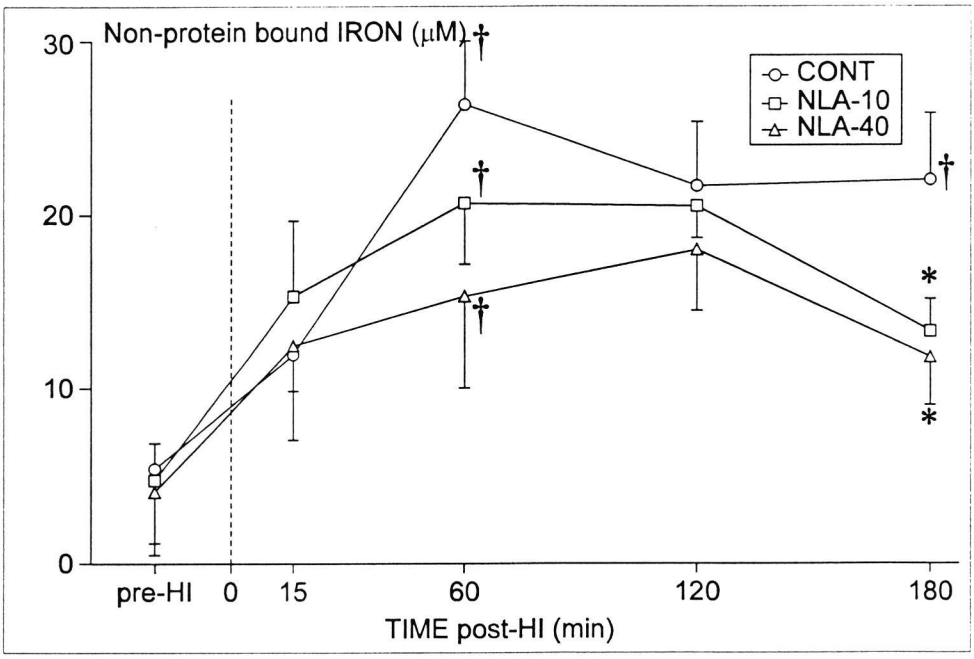
	pre-HI	2 min post-HI	15 min post-HI	60 min post-HI	120 min post-HI	180 min post-HI
MABP (mm Hg)						
- CONT	81 ± 13	70 ± 34	81 ± 16	79 ± 28	78 ± 20	76 ± 17
- NLA-10	79 ± 20	69 ± 25	84 ± 16	76 ± 17	85 ± 14	87 ± 8
- NLA-40	71 ± 11	67 ± 20	80 ± 16	89 ± 15*	76 ± 14	83 ± 11
pH						
- CONT	7.38 ± 0.07	6.99 ± 0.09*	7.02 ± 0.19*	7.19 ± 0.20	7.23 ± 0.16	7.22 ± 0.12
- NLA-10	7.38 ± 0.06	6.90 ± 0.11*	6.95 ± 0.12*	7.23 ± 0.07	7.19 ± 0.11	7.27 ± 0.11
- NLA-40	7.36 ± 0.10	6.93 ± 0.08*	7.05 ± 0.09*	7.10 ± 0.13*	7.16 ± 0.14	7.23 ± 0.12
PCO_2 (kPa)						
- CONT	4.7 ± 1.3	7.8 ± 7.3	5.3 ± 2.1	4.3 ± 1.4	4.4 ± 1.1	4.8 ± 0.9
- NLA-10	5 0 ± 1.3	10.4 ± 3.0*	7.9 ± 2.4	5.6 ± 1.4	5.1 ± 1.0	4.9 ± 1.2
- NLA-40	4.5 ± 1.6	10.7 ± 2.5*	5.6 ± 1.4	5.8 ± 1.9	4.8 ± 3.9	5.2 ± 1.9
PO_2 (kPa)						
- CONT	16.3 ± 4.8	20.0 ± 7.3	22.5 ± 9.6	15.2 ± 5.6	17.4 ± 2.5	15.4 ± 0.9
- NLA-10	14.2 ± 2.6	19.2 ± 7.5	16.0 ± 2.7	11.8 ± 1.2	16.2 ± 2.6	16.8 ± 2.4
- NLA-40	13.2 ± 3.7	17.2 ± 9.8	21.8 ± 7.2	12.3 ± 3.2	15.5 ± 4.8	13.5 ± 3.6
BE						
- CONT	-3.9 ± 3.8	-19.7 ± 4.6*	-12.1 ± 1.3*	-9.6 ± 4.2*	-12.6 ± 5.7	-11.4 ± 2.0*
- NLA-10	-2.2 ± 2.5	-18.7 ± 2.7*	-15.9 ± 8.1*	-12.6 ± 3.5*	-8.8 ± 2.9*	-8.5 ± 4.0
- NLA-40	-2.8 ± 3.4	-18.9 ± 2.1*	-16.2 ± 2.5*	-12.7 ± 3.7*	-9.3 ± 2.0*	-5.7 ± 2.5

* $p<0.05$ versus pre-HI values. HI=hypoxia-ischemia

Table 5.1 Mean values ± 1SD of mean aortic blood pressure (MABP), pH, blood gases, and base excess (BE) of the control (CONT), low-dose N-ω-nitro-L-arginine (NLA-10) and high-dose N-ω-nitro-L-arginine (NLA-40) groups during the respective conditions, including 2 min after hypoxic-ischemic (HI) insult.

5.4.2 Pro-oxidative activity

The patterns of non-protein-bound iron concentrations of the 3 groups (mean values ± SEM) are shown in figure 5.1.



* p<0.05 vs. CONT; † p<0.05 vs. pre HI

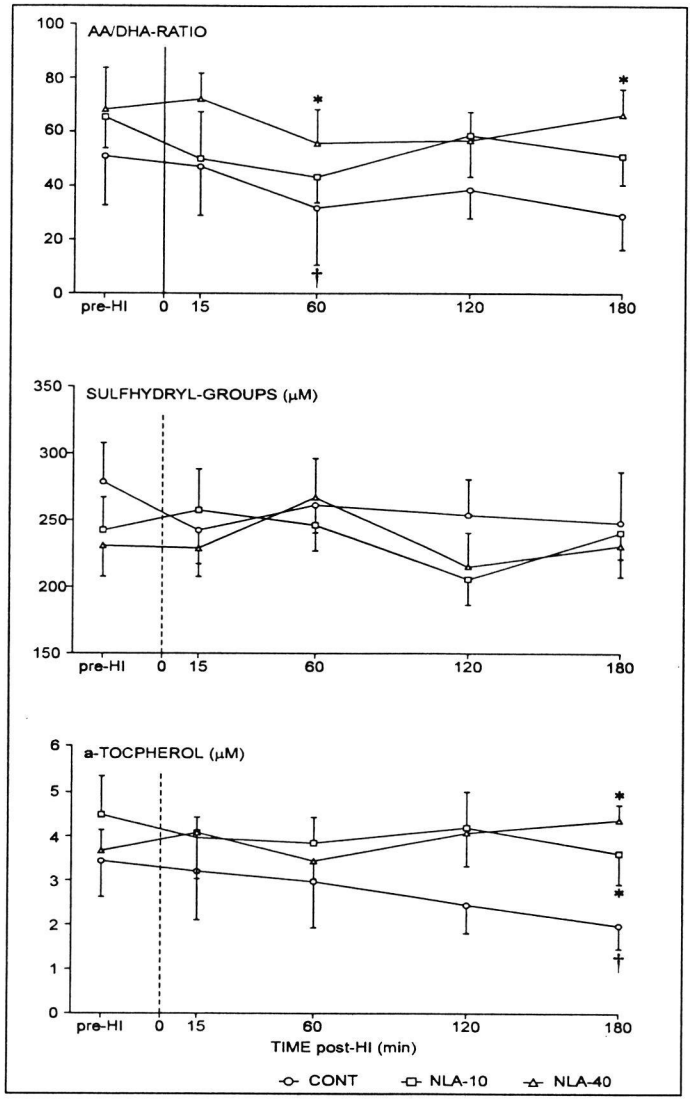
Figure 5.1 Mean values (± SEM) of plasma concentrations of non-protein-bound iron of the control (CONT), low-dose N-ω-nitro-L-arginine (NLA-10) and high-dose N-ω-nitro-L-arginine (NLA-40) groups at the various time points. HI = hypoxia-ischemia.

Although extremely low, non-protein-bound iron was detectable during the pre-HI condition in 5, 4 and 5 animals of the CONT, NLA-10 and NLA-40 groups respectively. Non-protein-bound iron increased significantly in all 3 groups post-HI with highest values at 60 and 120 min post-HI, but remained significantly higher in the CONT-group as compared to pre-HI values and the NLA-10 and NLA-40 groups at 180 min post-HI. The two NLA groups showed no significant difference at 180 min post-HI as compared to the pre-HI values.

5.4.3 Anti-oxidative capacity

Figure 5.2 summarizes the patterns of the mean values (± SEM) of the ascorbic/dehydroascorbic acid-ratio (AA/DHA-ratio), sulfhydryl-groups and α-tocopherol during the study periods. Contrary to the pattern of both NLA-groups, the patterns of AA/DHA-ratio and α-tocopherol in the post-HI period of the CONT-group indicate consumption of these anti-oxidants: ascorbic acid decreased and dehydroascorbic acid increased significantly in the CONT-group post-HI as compared to the pre-HI values, indicated by a consistent decrease of the AA/DHA-ratio during the post-HI period (pre-HI: 5.1 ± 0.9; 60 min post-HI: 2.2 ± 0.9 [$p<0.05$ vs. pre-HI], and 2.9 ± 0.7 180 min post-HI). This was not the case for the NLA-groups, in which the ascorbic acid, dehydroascorbic acid, and AA/DHA-ratio remained stable during the post-HI period, and were not significantly different from the pre-HI values. In the CONT-group α-tocopherol showed a significant and consistent decrease during the post-HI period as compared to pre-HI-values (pre-HI: 3.4 ± 0.5 μM; 120 min post-HI: 2.3 ± 1.2 μM ($p=0.05$), and 180 min post-HI: 1.9 ± 0.3 μM, $p<0.05$). In the NLA-groups, however, α-tocopherol values remained stable. Sulfhydryl-groups showed no significant changes or differences within groups during the various time points or among groups respectively, although the CONT-group showed a transient (non-significant) drop from pre-HI (278 ± 23 μM) to 15 min post-HI (239 ± 21 μM).

Uric acid, allantoin and its ratio (allantoin/uric acid), and retinol (not shown) remained stable in all groups during the study period. Respective mean values (± SEM) of the allantoin/uric acid-ratio ranged from 2.4 ± 0.4 to 3.2 ± 0.2 in the CONT-group; from 3.5 ± 1.0 to 6.2 ± 0.5 in the NLA-10-group; and from 2.7 ± 0.4 to 5.1 ± 1.2 in the NLA-40-group. Respective ranges of retinol were: CONT-group: 0.72 ± 0.19 to 0.85 ± 0.20 μM; NLA-10-group: 0.55 ± 0.05 to 0.79 ± 0.17; NLA-40-group: 0.83 ± 0.11 to 1.11 ± 0.18.

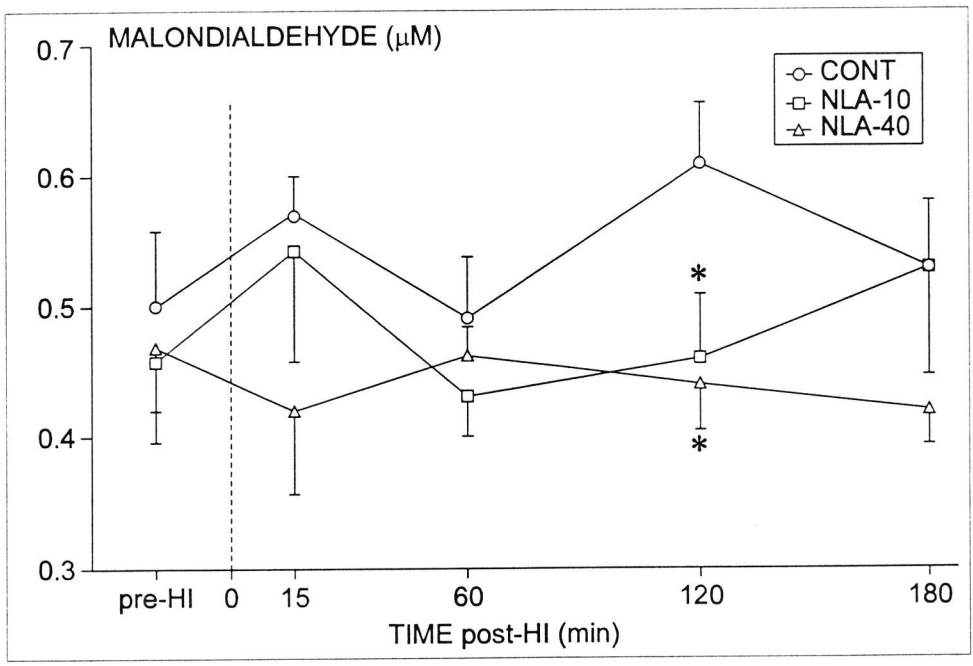


* p<0.05 vs. CONT; † p<0.05 vs. pre HI

Figure 5.2. Mean values (± SEM) of the plasma AA/DHA-ratio, and the plasma concentrations of α-tocopherol and sulfhydryl-groups of the control (CONT), low-dose N-ω-nitro-L-arginine (NLA-10) and high-dose N-ω-nitro-L-arginine (NLA-40) groups at the various time points. HI = hypoxia-ischemia.

5.4.4 Lipid peroxidation

Figure 5.3 summarizes the pattern of malondialdehyde of the 3 groups (mean values ± SEM).



* p<0.05 vs. CONT

Figure 5.3. Mean values (± SEM) of plasma concentration of malondialdehyde in the control (CONT), low-dose N-ω-nitro-L-arginine (NLA-10) and high-dose N-ω-nitro-L-arginine (NLA-40) groups at the various time points. HI = hypoxia-ischemia.

Malondialdehyde did not differ significantly during the post-HI period as compared to pre-HI values in any group. However, values tended to increase in the CONT-group but tended to be lower in both NLA-groups, in particular in the NLA-40-group. At 120 min post-HI, malondialdehyde was significantly lower in both NLA-groups (NLA-10: 0.46 ± 0.03 μM, $p<0.05$; NLA-40: 0.44 ± 0.01 μM, $p<0.01$) as compared to the CONT-group (0.61 ± 0.04 μM).

5.5 Discussion

Ventilation with 6-8% of oxygen and subsequent withdrawal of blood caused severe metabolic acidosis (as indicated by the grossly abnormal blood gases determined immediately after completion of HI), severe hypotension and hypoperfusion of the brain, making it very likely that hypoxia and ischemia of the brain took place during the actual HI-period. The present study indicates that post-HI reperfusion and reoxygenation in our newborn lambs induced an increased pro-oxidative activity by liberation of iron. This was most obvious in the CONT-group, where non-protein-bound iron showed the highest values. These values in the CONT-group remained significantly higher as compared to pre-HI values and the NLA-groups, which showed a decrease to pre-HI values from 120 min post-HI onward. In the CONT-group, there was also a reduction of the anti-oxidative capacity. In particular, we found a consistent increased oxidation of ascorbic acid, which is a sensitive indicator of oxidative stress, and a steady decrease in the anti-oxidant α-tocopherol in the CONT-group during the post-HI period[28]. Inhibition of nitric oxide production with a competitive analog of L-arginine, the precursor of nitric oxide, NLA, mitigated these adverse effects on pro-oxidative activity and the consumption of the anti-oxidants ascorbic acid and α-tocopherol of the newborn lamb. This attenuating effect of NLA was not dose dependent, as indicated by the similar results obtained in low and high dose NLA-groups. Although malondialdehyde did not significantly change in the post-HI period as compared to pre-HI values in any group, it was higher in the CONT-group as compared to the NLA-groups (120 min post-HI, $p<0.05$). However, it is important to realize that malondialdehyde is a rather unstable marker of lipid peroxidation in the in vivo situation. Moreover, it only measures lipid peroxidation, while proteins and DNA are more often the targets of oxidative damage than are lipids. It is also important to realize that lipid peroxidation often occurs late in the oxidative injury process[26]

In 1991 Beckman highlighted the fact that post hypoxic-ischemic reperfusion and reoxygenation induced the production of large amounts of superoxide, hydrogen peroxide and nitric oxide in neonatal brain tissue and in the cerebral microcirculation[6]. Although these substances are relatively poorly reactive oxygen species themselves, superoxide and nitric oxide are able to form peroxynitrite, which can decompose to form the powerful and cytotoxic oxidants hydroxyl and nitrogen dioxide. These oxidants are highly diffusable and can easily cross the blood-brain barrier to exert their destructive action on brain tissue itself[6,28]. Peroxynitrite is able to initiate lipid peroxidation and react directly with sulfhydryl groups at physiologic pH values[29]. Moreover, superoxide and hydrogen peroxide can be converted into the hydroxyl radical by transition metal ions, in particular non-protein-bound iron, by the

so-called superoxide-driven Fenton reaction[4]. Siesjo et al.[30] showed that lowering of the plasma pH, as occurs during and after ischemia, enables transferrin to liberate its iron, thereby inducing reactive oxygen species production, whereas a recent study from our group showed that 12 out of 15 severely birth asphyxiated term neonates had substantial concentrations of non-protein-bound iron in their plasma during the first 24 hours of life[31]. In addition, nitric oxide also reacts with transition metals (*e.g. iron*) releasing them from their binding proteins[32]. It is conceivable that the above mentioned cascade may have contributed to the excessive production of non-protein-bound iron which further increased lipid peroxidation. This suggestion is strongly supported by our finding that inhibition of nitric oxide synthesis with NLA had an attenuating effect on non-protein-bound iron levels measured in blood effluent from the brain and preserved anti-oxidative capacity by preventing oxidation of ascorbic acid and consumption of α-tocopherol. In this respect it is important to stress the findings of a recent in vitro study of Van der Vliet et al.[28]. They reported that peroxynitrite formation in body fluids is likely to cause anti-oxidant depletion and oxidative damage, but that peroxynitrite leads in particular to rapid peroxidation of ascorbic acid and to a lesser extent to a decrease in α-tocopherol. This is in line with the results of the present study. The mechanism of the reaction of ascorbic acid with peroxynitrite is not clear yet, but it has been suggested that it reacts with hydroxyl and/or nitrogen dioxide, the decomposition products of peroxynitrite, rather than with peroxynitrite itself[28]. Although α-tocopherol was significantly decreased at 180 min post-HI, it has been reported that this anti-oxidant may be recycled by ascorbic acid, which prevents further decreases[33,34]. The remarkably steady sulfhydryl levels (although a rather impressive, albeit insignificant, decrease of sulfhydryl levels was found in the CONT-group at 15 min post-HI as compared to pre-HI levels) may be explained by the addition of quite large amounts of bicarbonate in the immediate post-HI period to treat metabolic acidosis and to restore arterial pH: bicarbonate protects sulfhydryl from oxidation and will also scavenge peroxyl radicals[28,35].

Our finding that in most of the newborn lambs low concentrations of non-protein-bound iron were already available during the baseline condition was remarkable because this is usually not the case in healthy adult animals and adult humans[36,37]. It confirms the results of recent studies in apparently healthy human (preterm) neonates, in which substantial levels of non-protein-bound iron are measured during the early neonatal period[37]. Although not very likely, we cannot exclude the possibility that the experimental instrumentation caused an oxidative stress leading to liberation of protein-bound iron.

Finally a short comment should be made on the 2 different NLA-regimes: one group received low dose NLA (10 mg/kg), the other group received high dose NLA (40 mg/kg). We aimed to investigate, whether or not partial rather than total nitric oxide synthesis inhibition had a more favorable effect on the redox status of blood effluent from the brain after severe hypoxia and ischemia. As already suggested by earlier studies we speculated that our low dose NLA regimen could prevent excessive production of reactive oxygen species and at the same time allowed a sufficient blood supply to critical regions of the brain, whereas the high dose regimen prevented adequate brain perfusion by total inhibition of nitric oxide production with consequent constriction of the cerebral vascular bed, eventually leading to more production of reactive oxygen species[15,16,17]. In a previous report we commented on the pattern of electrical cortical brain activity of the studied animals during the post-HI period. Only in the low dose NLA group electrical cortical brain activity recovered to pre-HI values, whereas it remained significantly lower as compared to pre-HI values in the CONT group and the high dose NLA group[38]. In the present study, however, we did not find consistent differences between the low and high dose NLA-groups with respect to the redox status of blood effluent from the brain.

In summary, after a hypoxic and ischemic insult in newborn lambs substantial levels of non-protein-bound iron were measured. Moreover, anti-oxidative capacity was decreased in this post-HI period as indicated by a decrease in AA/DHA-ratio, indicating oxidation of ascorbic acid, and in α-tocopherol levels. The abnormal production of non-protein-bound iron was reduced and the decrease in AA/DHA-ratio and α-tocopherol levels prevented in the NLA-treated lambs. This suggests that inhibition of nitric oxide may have a beneficial effect on the excessive formation of reactive oxygen species upon reperfusion and reoxygenation after hypoxia and ischemia. The present study, however, did not support earlier findings suggesting that partial rather than complete inhibition of nitric oxide production was superior, since we did not find a difference in results with respect to the redox status between high dose and low dose NLA treatment.

5.6 Acknowledgments

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6 NITRIC OXIDE SYNTHESIS INHIBITION AFTER HYPOXIA-ISCHEMIA INDUCES PULMONARY HYPERTENSION AND INCREASES OXYGEN NEED

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(submitted for publication)

6.1 Abstract

Inhibition of nitric oxide production may reduce post hypoxic-ischemic (HI) neonatal brain damage, but may also induce pulmonary hypertension by inhibiting endogenous nitric oxide production in the pulmonary vascular bed. The aim of this study was to evaluate the effect of nitric oxide inhibition on pulmonary artery pressure and oxygen need after hypoxia-ischemia.

Severe HI was produced in 18 newborn lambs. After completion of HI the lambs were divided into 3 groups of 6 animals receiving either placebo (CONT), low dose N-ω-nitro-L-arginine (NLA) (10 mg/kg iv, NLA-10) or high dose NLA (40 mg/kg iv, NLA-40) to inhibit nitric oxide synthesis.

Pulmonary artery pressure (P_{ap}), aortic pressure (P_{ao}), blood gases, inspiratory oxygen concentration (FIO_2) and ventilator settings were recorded before and at 15, 60, 120 and 180 min after HI. Mean P_{ap} rose initially significantly as compared to baseline in all groups at 15 min post HI, decreased to normal in CONT but not in treated animals: 180 min post HI mean P_{ap} was significantly higher in both treated groups as compared to the controls (32 and 34 vs. 25 mmHg, $p<0.05$). Moreover, in both NLA-treated groups the PaO_2/FIO_2-ratio was significantly decreased at 15, 60, 120 and 180 min post-HI, in contrast to the PaO_2/FIO_2-ratio in CONT.

Nitric oxide synthase inhibition after HI causes a prolonged increase in pulmonary artery pressure leading to a higher oxygen need.

6.2 Introduction

Nitric oxide, a free radical gas, is involved in many physiological processes[1]. Nitric oxide plays an important role in modulating pulmonary vasomotor tone: it causes potent and selective vasodilatation of pulmonary vessels in both experimental and clinical settings[2,3]. Therefore nitric oxide inhalation is increasingly used in the treatment of neonatal persistent pulmonary hypertension[3].

Nitric oxide also plays an important role as a messenger molecule in the brain: it acts as a neurotransmitter by regulating glutamate production[1,4]. However, recently is has been recognized that upon reperfusion and reoxygenation, as occurs after birth asphyxia, excessive amounts of nitric oxide are produced[5,6]. This subsequently leads to neuronal damage by nitric oxide mediated excessive production of glutamate and reactive oxygen species, which are, at least partly, responsible for the post hypoxic-ischemic reperfusion damage of brain tissue[5,7]. Although results of studies with respect to focal ischemia are conflicting, inhibition of nitric oxide production in the immediate post hypoxic-ischemic period after birth asphyxia has been shown to reduce brain damage in neonatal rats and newborn lambs[6,8,10].

However, earlier studies have demonstrated a dose-dependent pulmonary vasoconstriction by inhibition of nitric oxide production[11,12]. Protection of brain tissue against hypoxic-ischemic damage by nitric oxide production inhibition may concomitantly cause pulmonary hypertension requiring more aggressive artificial ventilation, extra oxygen need and subsequent lung injury. This study was designed to evaluate the effect of post-HI nitric oxide synthesis inhibition on pulmonary artery pressure, ventilation requirements and oxygen need.

6.3 Materials and methods

6.3.1 Animal preparation

Surgical and experimental procedures employed were approved by the Committee on Animal experiments of the University of Leiden and by the scientific board of the Department of Pediatrics. Eighteen newborn lambs, weighing 4.4 ± 1.6 (means ± SD) kg and ages from 4 to 10 days were studied. General anesthesia was induced with ketamin hydrochloride (3mg/kg i.v.), and supplemented by xylazine (1mg/kg/3hrs i.m.). Local anesthesia, in addition, was accomplished with 1% lidocaine hydrochloride injected locally. During the study the wounds were sprayed with 1%

lidocaine at regular intervals. After intubation, the lambs were ventilated with air and oxygen using a continuous flow pressure-controlled ventilator (Bourns BP 200, Bear Medical Systems Inc., Riverside, CA) which was adjusted to maintain arterial PaO_2 and $PaCO_2$ within physiologic limits. Upon ventilation, pancuronium (0,2 mg/kg i.v.) was administered for muscle paralysis. The animals were nursed on a heating pad to maintain normal body temperature. An intravenous solution of 5% glucose in NaCl 0.9% was infused (15 ml/kg/h) continuously during the experiment.

6.3.2 Instrumentation

In the right femoral artery and vein 5F or 6F self-sealing sheaths were introduced for respectively arterial blood sampling, blood withdrawal and reperfusion, and for drug administration. Via the left femoral artery a 5F micromanometer catheter (Millar Instruments, Houston, TX) was advanced into the thoracic aorta for continuous measurement of aortic pressure (P_{ao}). A 5F Berman catheter (American Edwards Laboratories, Irvine, CA) was introduced via the left femoral vein into the pulmonary artery for pulmonary artery pressure (P_{ap}) measurement. After the carotid arteries in the neck were exposed, appropriately sized transonic flow transducers (Transonic Systems Inc., Ithaca, NY) were applied to fit around the vessels to measure carotid artery blood flow (ml/min) for assessment of changes in actual brain blood flow. This was done to be sure that there was indeed cerebral ischemia during the hypoxic-ischemic insult.

6.3.3 Physiologic measurements

P_{ao} and P_{ap} were monitored continuously using Statham P23Db stain-gauge transducers and Beckman R 612 (Beckman Instruments Inc., Palo Alto, CA) or Gould 2800s (Gould Inc., Cleveland, OH) polygraph. Arterial blood gases and pH were measured with a Corning 178 pH/blood gas analyzer (Corning, Halstead, UK).

6.3.4 Experimental procedure

After completion of the surgical preparation, 3 hours were allowed for recovery from instrumentation. Birth asphyxia was simulated by ventilation with a mixture of 6-8% oxygen and 10% carbondioxide in nitrogen for 30 min, followed by a 5 min period of hypotension (mean P_{ao}<35 mmHg), achieved by a careful withdrawal of blood. Resuscitation was performed in principle in a way similar to that used routinely in our neonatal unit: extra oxygen which was progressively decreased depending on the color of the tongue and the arterial blood, and blood gases determined 2 and 15 min after the start of the resuscitation. Cardiac arrest and hypotension were treated with adrenaline (0.01%) and/or dopamine as appropriate. The blood withdrawn to achieve hypotension

was returned immediately after completion of the HI-period. Metabolic acidosis was corrected with $NaHCO_3$. Upon resuscitation 6 lambs received an intravenous infusion with a placebo (30 ml of a solution of 0.1N HCl in NaCl 0.9%; CONT-group), 6 lambs received a low dose NLA (10mg/kg/i.v. in 30 ml of 0.1N HCl in NaCl 0.9%; NLA-10 group), and 6 lambs received a high dose NLA (40 mg/kg/i.v. in 30 ml of 0.1N HCl in NaCl 0.9%; NLA-40 group). P_{ao}, P_{ap} and FIO_2 were recorded continuously; blood gas samples were taken pre-HI, and at 2, 15, 60, 120 and 180 min post-HI.

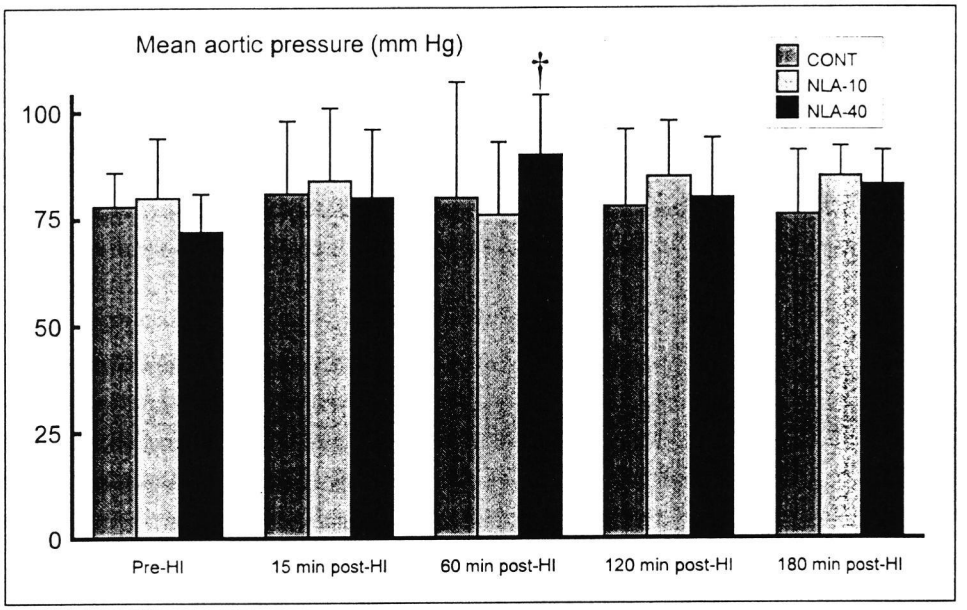
6.3.5 Statistical analysis

All data are summarized as means ± 1SD. Differences between mean values within the groups during different time points were assessed using analysis of variance (ANOVA) for repeated measurements; differences between mean values of the three groups within each time period were assessed by one way factorial ANOVA. When significant differences were found, ANOVA was followed by the Student-Newman-Keuls test. A p-value <0.05 was considered statistically significant.

6.4 Results

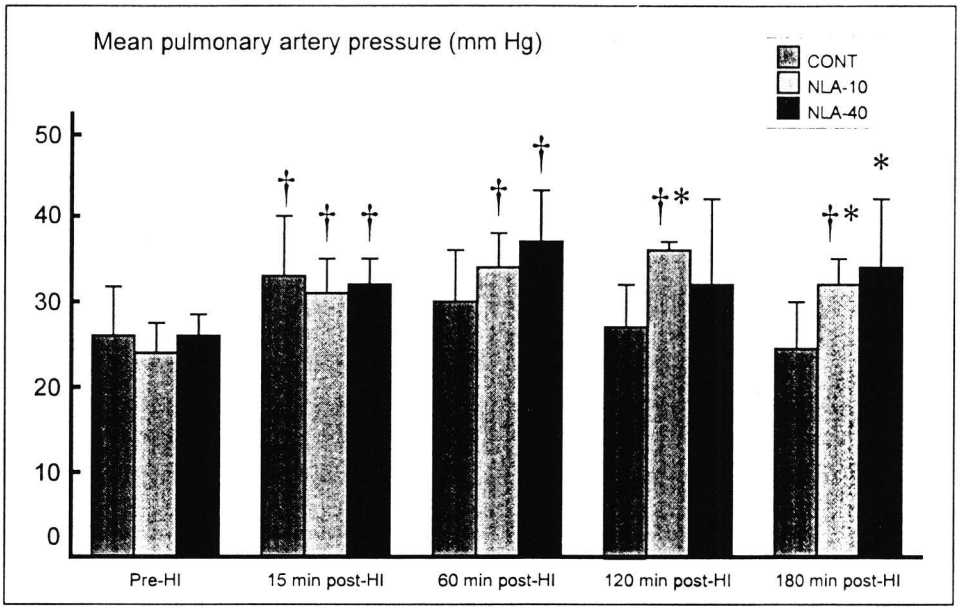
6.4.1 Hemodynamic parameters

Figures 6.1 and 6.2 summarize the pattern of mean P_{ao} and P_{ap} during the study period. Although significant changes of the mean P_{ao}-values between the 3 groups were not found, mean P_{ao} tended to be higher in especially the NLA-40 group. In this group mean P_{ao} was significantly higher as compared to pre-HI values at 60 min post-HI. The hypoxic-ischemic insult induced an increase in mean P_{ap} in all 3 groups (figure 6.2). This increase was only transient in the CONT lambs: at 180 min post-HI mean P_{ap} had dropped below pre-HI values in this group. However, in the NLA-10 treated animals mean P_{ap} was significantly higher as compared to the control animals at 120 min post-HI and at 180 min post-HI mean P_{ap} was significantly higher in both treated groups as compared to the CONT-group ($p<0.05$). Carotid artery blood flow decreased to very low values at the end of the 5 min-hypotensive period. Lowest carotid artery blood flow values (means ± 1SD) during this 5 min period did not differ between groups (CONT-group: 12 ± 3 ml/min [pre-HI 70 ± 8 ml/min]; NLA-10 group: 13 ± 3 ml/min [pre-HI 66 ± 4 ml/min]; NLA-40 group 11 ± 2 ml/min [pre-HI 62 ± 7 ml/min]).



† p<0.05 vs. pre-HI

Figure 6.1 Mean values (± 1SD) of mean aortic pressure (P_{ao}; mmHg) of the three groups at the various time points. HI=hypoxia ischemia, CONT=control, NLA-10=N-ω-nitro-L-arginine 10 mg/kg, NLA-40=N-ω-nitro-L-arginine 40 mg/kg.



* p<0.05 vs. CONT; † p<0.05 vs. pre-HI

Figure 6.2 Mean values (± 1SD) of mean pulmonary artery pressure (P_{ap}; mmHg) of the three groups at the various time points. HI=hypoxia ischemia, CONT=control, NLA-10=N-ω-nitro-L-arginine 10 mg/kg, NLA-40=N-ω-nitro-L-arginine 40 mg/kg.

6.4.2 Blood gases, pH and FIO_2

Table 6.1 summarizes the blood gases and pH of the 3 groups.

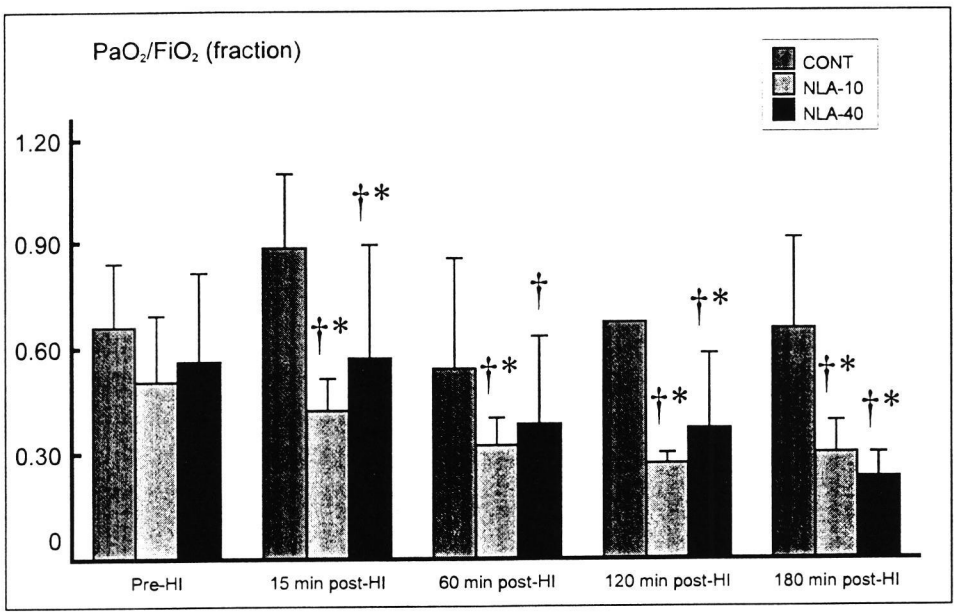
Time (min)	CONT	NLA-10	NLA-40
pH			
pre HI	7.37 ± 0.07	7.38 ± 0.06	7.35 ± 0.09
post HI 15	7.04 ± 0.10†	6.98 ± 0.12†	7.05 ± 0.09†
post HI 60	7.19 ± 0.20†	7.21 ± 0.07†	7.10 ± 0.14†
post HI 120	7.24 ± 0.15	7.23 ± 0.14†	7.18 ± 0.14†
post HI 180	7.25 ± 0.22	7.26 ± 0.09	7.21 ± 0.12†
$PaCO_2$ (kPa)			
pre HI	4.5 ± 1.2	5.0 ± 1.2	4.9 ± 1.5
post HI 15	5.8 ± 2.3	7.2 ± 2.4	5.9 ± 1.4
post HI 60	4.5 ± 1.4	5.9 ± 1.4	6.0 ± 1.9
post HI 120	4.1 ± 0.9	5.7 ± 1.0	5.7 ± 3.5
post HI 180	4.2 ± 1.0	5.2 ± 0.9	5.6 ± 1.6*
PaO_2 (kPa)			
pre HI	15.9 ± 4.4	14.6 ± 3.6	13.1 ± 3.6
post HI 15	18.9 ± 4.7	14.3 ± 2.7	15.8 ± 11.3
post HI 60	14.9 ± 5.6	11.7 ± 1.2	11.8 ± 3.2
post HI 120	16.7 ± 2.2	13.2 ± 2.3†	16.1 ± 11
post HI 180	15.9 ± 0.8	14.0 ± 2.6	11.4 ± 2.6*

* $p<0.05$ vs. CONT; † $p<0.05$ vs. pre-HI

Table 6.1 Mean values (± 1SD) of pH, $PaCO_2$ and PaO_2 of the three groups at the various time points. HI=hypoxia ischemia, CONT=control, NLA-10=N-ω-nitro-L-arginine 10 mg/kg, NLA-40=N-ω-nitro-L-arginine 40 mg/kg.

pH was lower in all groups post-HI as compared to pre- HI values, which reached significance for post-HI 15 and 60 min (CONT), post-HI 15, 60 and 120 min. (NLA-10) and for all post-HI time points for NLA-40. $PaCO_2$ did not differ post-HI or between groups, except for the NLA-40- group at 180 min post-HI as compared to the CONT-group. PaO_2 tended to be higher in respectively the control group as compared to both NLA-groups during the entire post-HI phase. At 180 min post-HI PaO_2 was significantly lower in the NLA-40 group as compared to the CONT-group. Moreover, oxygen need (FIO_2) was significantly higher in the NLA-40 group at 120 and 180 min

post-HI as compared to the CONT-group (120 min post-HI: 0.47 vs. 0.26; 180 min post-HI: 0.45 vs. 0.24). Albeit not significantly different, FIO_2 also tended to be higher in the NLA-10 group as compared to the control group. PaO_2/FIO_2-ratio was significantly decreased in the NLA-groups as compared to the CONT-group as well as to pre-HI values (figure 6.3).



* p<0.05 vs. CONT; † p<0.05 vs. pre-HI

Figure 6.3 Mean values (± 1SD) of the fraction of inspired oxygen (FIO_2) of the three groups at the various time points. HI=hypoxia ischemia, CONT=control, NLA-10=N-ω-nitro-L-arginine 10 mg/kg, NLA-40=N-ω-nitro-L-arginine 40 mg/kg.

6.4.3 Ventilator settings

Table 6.2 summarizes ventilator settings (ventilator rate, positive inspiratory pressure (PIP), positive end-expiratory pressure (PEEP)) of the 3 groups.

Time (min)	CONT	NLA-10	NLA-40
PIP (cm H_2O)			
pre HI	18 ± 6	20 ± 6	18 ± 4
post HI 15	20 ± 6	22 ± 4	21 ± 6
post HI 60	24 ± 3†	25 ± 3	21 ± 5
post HI 120	24 ± 5†	24 ± 3	25 ± 4†
post HI 180	22 ± 5	25 ± 8	26 ± 3†
PEEP (cm H_2O)			
pre HI	3 ± 2	3 ± 1	4 ± 1
post HI 15	4 ± 2	4 ± 1	5 ± 2
post HI 60	4 ± 1	4 ± 1	4 ± 2
post HI 120	5 ± 1	4 ± 2	5 ± 3
post HI 180	4 ± 1	5 ± 1	5 ± 2

† $p<0.05$ vs. pre HI

Table 6.2 Mean values (± 1SD) of positive inspiratory pressure (PIP), positive end-expiratory pressure (PEEP) of the three groups at the various time points. HI=hypoxia ischemia, CONT=control, NLA-10=N-ω-nitro-L-arginine 10 mg/kg, NLA-40=N-ω-nitro-L-arginine 40 mg/kg.

Ventilator settings were not significantly different within groups at various time points nor between groups, although PIP tended to be higher in the NLA-40 group as compared to control animals.

6.5 Discussion

It has been increasingly recognized that an important factor of hypoxic-ischemic brain damage at birth in the neonate occurs during the early reperfusion and reoxygenation phase[13]. Upon reperfusion and reoxygenation large amounts of nitric oxide are released[5,17]. This gives rise to excessive production of the excitatory neurotransmitter glutamate by stimulating cyclic GMP production in the presynaptic dendrites of neurons and glia cells[5,6]. This neurotransmitter has been thought to play a key role in

the reperfusion injury of the developing brain. Therefore, it has been suggested that reduction of nitric oxide production by inhibition of nitric oxide synthesis by N-ω-nitro-L-arginine may reduce post-asphyxial reperfusion injury of the brain[9,10]. However, as nitric oxide plays a major role in the regulation of pulmonary vascular tone as well, inhibition of nitric oxide production could concomitantly cause pulmonary vasoconstriction. Previously, a dose-dependent increase in pulmonary artery pressure has been shown in non-asphyxiated animals[11]. The present study shows indeed that inhibition of nitric oxide synthesis by NLA after HI in asphyxiated newborn lambs also causes prolonged increase in pulmonary artery pressure leading to a higher oxygen need. Although the present study showed no significant differences with respect to pulmonary arterial pressure between low (NLA-10) and high (NLA-40) dose groups, there was a clear tendency for higher mean P_{ap}-values post-HI in the NLA-40 group as compared to the NLA-10 group. Inhibition of nitric oxide production did not result in a significant increase in systemic arterial pressure as compared to the control group, despite the use of high doses of NLA, although we must admit that aortic pressures tended to be higher in the NLA groups. Earlier studies, however, were performed without a hypoxic-ischemic insult. Possibly, the hypoxia-ischemia causes damage to either cardiac myocytes resulting in a decreased myocardial contractility as has been shown in earlier clinical studies, and/or endothelial cell injury leading to capillary leak and relative intravascular hypovolemia[15]. Cellular injury due to HI may render cells less susceptible to nitric oxide synthesis inhibition. Moreover, the use of adrenaline and dopamine in the resuscitation-phase and in the post-HI period might have masked differences in aortic blood pressure between the groups. Despite the observed definite increase in pulmonary artery pressure in both NLA-groups, ventilatory settings were not different in these groups and oxygenation and elimination of carbondioxide did not significantly differ from the animals in the control group. In addition, although oxygen need was significantly higher in the NLA treated animals, FIO_2 never exceeded 0.4. This suggests, but does not prove, that nitric oxide production inhibition did not cause important ventilation and oxygenation problems. Moreover, to treat pulmonary hypertension in NLA-treated post asphyxiated neonates one can consider to ventilate NLA-treated individuals with nitric oxide as recently suggested. A recent study of our group has investigated the systemic adverse effects of inhaled nitric oxide, such as free radical production, changes in systemic and cerebral hemodynamics, and electrocortical brain activity in newborn lambs. This study indicated that nitric oxide inhalation did not lead to any systemic side-effects[16,17].

In conclusion, inhibition of nitric oxide production by NLA in artificially ventilated newborn lambs after hypoxia-ischemia leads to an prolonged increase in pulmonary artery pressure and a moderately higher oxygen need. Reduction of post-asphyxial brain injury by inhibition of nitric oxide synthesis may be complicated by moderate pulmonary hypertension.

6.6 References

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7 THE INFLUENCE OF NITRIC OXIDE SYNTHESIS INHIBITION ON THE POST HYPOXIC-ISCHEMIC NEWBORN LAMB HEART

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7.1 Abstract

Since an excessive production of nitric oxide upon reperfusion and reoxygenation may play an important role in post hypoxic-ischemic (HI) neuronal cell damage, inhibition of nitric oxide synthesis has been proposed to reduce post-HI brain injury in the newborn. However, the reports concerning the cardiac side-effects of post-HI nitric oxide synthesis inhibition are conflicting. On the one hand may an impairment of nitric oxide-induced relaxation of the coronary vessels influence cardiac function negatively, whereas on the other hand post-HI nitric oxide synthesis inhibition may also reduce reactive oxygen species mediated stress to the myocardium. We therefore investigated the effect of immediate post-HI inhibition of nitric oxide synthesis by N-ω-Nitro-L-Arginine (NLA) on cardiac function and reactive oxygen species production in 14 newborn lambs. Five received a low dose NLA (10 mg/kg iv; NLA-10), 4 a high dose NLA (40 mg/kg iv; NLA-40) and 5 a placebo (CONT). Pump and myocardial performance of the left ventricle (LV) were assessed by determination of cardiac output (CO), stroke work (SW), and left ventricular contractility (expressed by the slope (E_{es}) and volume intercept (V_{10}) of the end-systolic pressure-volume relationship, obtained using the conductance catheter). The redox status (determined in plasma obtained from the right atrium) was determined by measurement of pro-oxidative activity (non-protein-bound iron: NPBI), anti-oxidative capacity (ratio of reduced/oxidized ascorbic acid: AA/DHA-ratio, α-tocopherol and sulfhydryl groups) and lipid peroxidation activity (malondialdehyde: MDA). Measurements were performed before and at 15, 60 and 120 min after completion of an HI-insult. There was a significant decrease of CO in all 3 groups and SW in both NLA-treated groups at 60 min post-HI ($p<0.05$), suggesting a subnormal LV-function at this time point. The indices for LV-myocardial contractility showed a transient decrease, although this only reached significance in NLA-10 at 120 min post-HI (increased V_{10}; $p<0.05$) and in CONT at 60 min post-HI (decreased E_{es}; $p<0.05$). Reactive oxygen species production was also highest at 60 min post-HI (significantly increased NPBI concentration and decrease of sulfhydryl groups in CONT lambs ($p<0.05$)), which may indicate a causative relationship between changes in pump and myocardial performance and reactive oxygen species mediated stress. The above mentioned results suggest neither a positive nor a negative effect of nitric oxide synthesis inhibition on post-HI LV-function. However, there was a positive effect of nitric oxide synthesis inhibition on the formation of the pro-oxidant NPBI and the degradation of sulfhydryl groups, suggesting less reactive oxygen species mediated stress in NLA-treated animals.

7.2 Introduction

Nitric oxide (NO•) plays an important role as a messenger molecule in various functions, including blood pressure regulation, tissue perfusion and neurotransmission[1,2,3]. However, there is increasing evidence that during pathological conditions, such as hypoxia-ischemia (HI), excess formation of nitric oxide upon reoxygenation and reperfusion may contribute significantly to glutamate-induced and direct neuronal cell damage[4,5,6]. Moreover, post-HI reperfusion induces the endothelium of the cerebrovascular bed to produce excessive amounts of nitric oxide and superoxide, leading to peroxynitrite formation and other cytotoxic oxidants. This results in endothelial damage, with ensuing loss of its barrier function, abnormal vasoregulation, and adhesion of platelets and white blood cells, leading to plugging of the cerebral microcirculation[7,8]. Competitive inhibition of nitric oxide synthesis with nitro-substituted analogues of L-arginine have therefore been proposed to reduce post-HI reperfusion injury of the neonatal brain[9,10]. However, before clinical application of these analogues is possible, it is important to know the possible side effects of nitric oxide synthesis inhibition on cardiac function. Recent studies report a reduction in cardiac output (CO) after nitric oxide synthesis inhibition, which is often already compromised in the early neonatal period after severe birth asphyxia[11,12,13,14]. On the other hand, a recent study of Matheis et al. in juvenile pigs reported a protective effect of nitric oxide synthesis inhibition on myocardial reoxygenation injury after cardiopulmonary bypass: they postulated that prevention of nitric oxide induced lipid peroxidation prevented damage to myocardial tissue[15]. The aim of the present study was therefore to investigate the effect of immediate post-HI inhibition of nitric oxide synthesis by N-ω-Nitro-L-Arginine (NLA) on myocardial performance, left ventricular myocardial contractility, and on the formation of reactive oxygen species. Because it has been suggested in earlier studies that low rather than high dosages of NLA showed a maximal effect on reduction of brain cell damage[16], the treatment group was divided into a low dose (10 mg/kg NLA) and a high dose (40 mg/kg NLA) group.

7.3 Methods

7.3.1 Animal preparation

Surgical and experimental procedures used were reviewed and approved by the Animal Research Committee of the Leiden University Hospital and the Scientific Board of the Department of Pediatrics. Fourteen newborn lambs, with ages ranging from 6 to 11 days (median: 8 days) and weights ranging from 3.3 to 6.9 kg (median: 4.7 kg) were

studied. General anesthesia was induced with a bolus of ketamine hydrochloride (3 mg/kg i.v.) and supplemented by xylazine (1 mg/kg/3 hrs i.m.) and local subcutaneous injection of lidocaine 1% before each skin incision. During the study the wounds were sprayed with 1% lidocaine at regular intervals. After intubation the lambs were paralyzed with pancuronium bromide (0.2 mg/kg i.v.) and ventilated with oxygen and air, using a continuous flow, pressure-controlled ventilator (Bourns BP 200, Bear Medical Systems Inc., Riverside, CA). Ventilation was adjusted to maintain arterial P_{O_2} and P_{CO_2} in the normal range throughout the study. An intravenous infusion of 5% glucose in NaCl 0.9% was continued throughout the study at about 15 ml/kg/h. $NaHCO_3$ was supplemented if the arterial pH was lower than 7.30 and the base deficit more than 5 mmol/l. Arterial blood gases and pH were measured using a Corning 178 pH/blood gas analyzer (Corning, Halstead, UK). Blood lactate concentrations were determined spectrophotometrically in plasma obtained from the right atrium.

7.3.2 Assessment of myocardial and pump performance

Left ventricular (LV) contractility and pump performance were quantified by the end-systolic pressure-volume relationship (ESPVR) obtained by the conductance catheter[17]. The ESPVR is represented by the slope (Ees) of a straight line connecting the upper left-hand corners of the pressure-volume loops obtained when loading conditions are changed by inflow occlusion (induced by inferior vena cava (IVC) occlusion) and by the volume intercept of the relation at a fixed pressure of 10 kPa (V_{10})[18,19,20]. We used this value to quantify its position along the volume-axis as explained previously[21]. Both an increase in Ees and a leftward shift of the ESPVR (decrease of V_{10}) have been shown to reflect an increase in LV contractility[19,22]. From each loading intervention we also calculated the left ventricular dP/dt_{max} - end-diastolic volume relationship. We used the slope of the dP/dt_{max}-EDV relationship (dP/dt_{max}-EDV) as an additional index of myocardial performance in subsequent data analysis, because of its alleged superior sensitivity to changes in inotropic state[23]. The method to measure LV- pressure and volume by means of the conductance catheter in dogs and also in newborn lambs have been described earlier[17,18,21,24,25].

Briefly, via percutaneous approaches 6 or 7F self-sealing sheaths were placed in both femoral arteries and veins and in the right external jugular vein. All catheters were positioned under fluoroscopic guidance. A 5F balloon catheter (Fogarty, Gould Statham model SP 5005) was advanced via a femoral vein in the IVC-right atrial junction to decrease preload during acquisition of LV pressure and volume data. Via the other femoral vein a 5F catheter was advanced into the right atrium for blood

sampling (determination of redox status (see below) and lactate concentrations). Into the left femoral- and carotid artery 5F micromanometer catheters (Millar Instruments, Houston, TX) were advanced into respectively the descending thoracic aorta and left ventricle for continuous blood pressure measurements (P_{ao} and P_{lv}). An eight-electrode pig-tail conductance catheter (size 6F, custom made by Webster Labs, Baldwin Park, CA) was advanced via the other femoral artery to the apex of the left ventricle.

LV-conductance was measured and converted to LV-volume using a Sigma-5 DF signal conditioner-processor (Cardiodynamics, Zoetermeer, the Netherlands). A 5F Berman angiographic catheter was advanced via the right external jugular vein into the main pulmonary artery for injection of hypertonic saline (1 ml) to determine parallel conductance, necessary to obtain absolute volumes[17]. Because of its possible dependence on volume, calibration for parallel conductance was repeated after each intervention which changed LV volume[20,26]. In addition, blood samples were taken to determine blood conductivity (using the Sigma-5 signal conditioner-processor) and repeated at least every half hour or after infusion of fluids that might alter its value. The right femoral artery was used for sampling of arterial blood gases and pH. Both femoral veins were used for blood withdrawal, determination of Hb, and infusion of drugs.

General hemodynamic measurements were analyzed from the first 10 steady-state beats of each run, obtained during periods of suspended respiration at end-expiration. Mean P_{ao}, stroke work (SW), stroke volume (SV) and cardiac output (CO) were either analyzed or calculated in a beat-to-beat fashion, using a special-application software program, Conduct-PC (Cardiodynamics), which calculated dP/dt_{max}, SV (end-diastolic volume minus end-systolic volume), SW [as the area enclosed by the pressure (P)-volume (V) loop, formula 1], and heart rate (HR) from the pressure and volume data. CO was calculated using formula 2. The program was also used to plot dP/dt_{max} as a function of end diastolic volume and to calculate its slope and volume intercept. Finally, the data were averaged over 10 beats.

$$SW = \int PdV \qquad (1)$$

$$CO = HR * SV \qquad (2)$$

7.3.3 Measurement of redox status

Blood was collected into heparinized glass tubes and immediately centrifuged (750 g, 10 min); the plasma was stored under argon at -70° C until analysis. Plasma samples which showed pink discoloration (hemolysis), were excluded from the study. Non-protein-bound iron in plasma was measured by the bleomycin assay[27]. Using this assay, the absence of non-protein-bound iron, *i.e.* the presence of iron binding capacity, can be measured as well as the presence of non-protein-bound iron, *i.e.* the lack of iron binding capacity. If non-protein-bound iron is present, the lower detection limit is 0.6 μM. The glass tubes used to collect the blood did not contain detectable amounts of iron. The intra and inter assay coefficients of variation of the bleomycin assay are 6.6% and 7.4% respectively. High performance liquid chromatography techniques were used to determine the following anti-oxidants: reduced and oxidized ascorbic acid (AA and DHA respectively) and α-tocopherol[28,29]. Plasma sulfhydryl (SH) content was determined spectrophotometrically[29]. The lipid peroxidation product malondialdehyde (MDA) was measured using high performance liquid chromatography[30].

7.3.4 Experimental procedure

After completion of the surgical preparation, the lambs were allowed to achieve hemodynamic stability. After this stabilization period, blood samples were taken (to determine blood gases, pH, lactate concentrations and the redox status) and LV pressure-volume loops and general hemodynamic measurements were obtained and used as pre-HI values. The IVC occlusions were performed by slowly inflating the Fogarty balloon with 1.5 ml of NaCl solution over about 10 seconds. IVC occlusions were performed during periods of suspended respiration at end-expiration (maximally 20 s), to exclude the influence of respiration and variation in lung volume on LV-volume and/or -parallel conductance. Severe HI was then induced by ventilating the lamb with 6-8% oxygen supplemented with a mixture of 10% CO_2 in N_2 for 30 min, followed by a 5 min period of hypotension (MABP < 35 mmHg), achieved by careful withdrawal of blood (50 to 150 ml). Upon resuscitation, after completion of the HI period, 6 lambs received an intravenous infusion with a placebo (30 ml of 0.1 N HCl in NaCl 0.9%; CONT group), 5 lambs received low dose NLA (10 mg/kg/iv, dissolved in 30 ml of 0.1 N HCl in NaCl 0.9%; NLA-10 group), and 4 lambs received a high dose NLA (40 mg/kg/iv, dissolved in 30 ml of 0.1 N HCl in NaCl 0.9%; NLA-40 group) in 30 min. Resuscitation was performed according to the routine protocol used in our neonatal unit: administration of extra oxygen, which was progressively weaned depending on the color of the tongue and of the arterial blood, and on the blood gas

determined 2 min after the start of resuscitation. Cardiac arrest and hypotension were treated with adrenaline (0.01%) and/or dopamine when appropriate. The blood withdrawn to achieve hypotension was reinfused immediately after completion of the HI period.

At 15, 60 and 120 min after completion of the HI, all hemodynamic measurements and blood sampling were repeated. Pressure-volume loops were continuously displayed on two memory oscilloscopes (Gould OS 4100; Hainault, UK). The following signals were digitized with 12-bit accuracy on a personal computer at a sample frequency of 200 Hz: ECG, P_{ao}, P_{lv} and LV-volume computed on-line from the conductance catheter. Data were stored on a hard disk for subsequent analysis. All pressure values and derived variables are given in units of kPa (13 kPa = 100 mm Hg) or mm Hg (P_{ao}).

7.3.5 Statistical analysis

Data are summarized as mean ± 1 SD. Differences between the perinatal data, hemodynamic parameters and blood determinations of the three groups within each time period were assessed by one way factorial analysis of variance (ANOVA). ANOVA for repeated measurements was used to evaluate if there were significant changes between the several time periods within each group. When a significant difference was found, ANOVA was followed by the Scheffe's procedure for comparison between the groups. A p-value of < 0.05 was considered statistically significant.

7.4 Results

7.4.1 Physiologic data

Animal weight and postnatal age did not differ between groups. Three CONT, 2 NLA-10 and 2 NLA-40 lambs had to be resuscitated at the end of the HI-period including adrenaline infusion intracardially, and subsequently received dopamine during the first 25 to 35 min to prevent hypotension. All lambs were supplemented with $NaHCO_3$ in the early post-HI period in an effort to regain a normal base excess. There was no difference among the groups with respect to the total amount of dopamine and bicarbonate infused in the immediate post-HI period.

Table 7.1 shows blood gases, pH and lactate concentrations of the 3 groups at the subsequent time points during the study period. The pH was significantly lower at 2 and 15 min post-HI in all groups ($p<0.05$). Although the pH showed a tendency to

recover, it never reached pre-HI values. PCO_2-values were significantly higher at 2 min post-HI in all groups ($p<0.05$), but recovered to pre-HI values afterwards. PO_2-values had a tendency to be slightly higher at 2 and 15 min post-HI as compared to the other conditions, but this was not significant. The plasma lactate concentrations increased significantly post-HI in all groups to abnormally high concentrations. The mean lactate concentrations post-HI of the CONT-group were consistently higher than those of both NLA-groups, although this was only significant at 120 min post-HI.

	pre-HI	2 min post-HI	15 min post-HI	60 min post-HI	120 min post-HI
pH					
CONT	7.38 ± 0.09	6.99 ± 0.06*	7.09 ± 0.17*	7.16 ± 0.19	7.20 ± 0.12
NLA-10	7.36 ± 0.03	6.92 ± 0.10*	6.96 ± 0.12*	7.23 ± 0.07	7.20 ± 0.14
NLA-40	7.35 ± 0.13	6.93 ± 0.12*	7.04 ± 0.11*	7.07 ± 0.16	7.16 ± 0.17
PCO_2					
CONT	4.6 ± 1.3	7.9 ± 2.9*	6.1 ± 2.2	4.5 ± 1.3	4.4 ± 1.1
NLA-10	5.4 ± 1.1	9.2 ± 3.0*	7.5 ± 2.2	5.7 ± 1.5	5.6 ± 1.4
NLA-40	4.9 ± 1.8	9.7 ± 2.5*	6.5 ± 0.9*	6.4 ± 2.2	6.8 ± 4.5
PO_2					
CONT	15.3 ± 5.0	19.1 ± 7.5	19.2 ± 4.5	14.6 ± 5.6	13.0 ± 13.3
NLA-10	13.4 ± 2.2	17.4 ± 7.5	11.5 ± 2.7	11.8 ± 1.5	10.5 ± 0.9
NLA-40	14.3 ± 4.1	18.2 ± 9.5	18.8 ± 11.8	12.2 ± 2.8	17.4 ± 12.2
Lactate					
CONT	3.9 ± 1.6		11.1 ± 3.1*	13.0 ± 2.3*	16.5 ± 0.8*
NLA-10	2.8 ± 1.7		10.1 ± 2.5*	9.8 ± 4.4*	10.4 ± 3.2*†
NLA-40	2.0 ± 0.5		7.8 ± 4.3*	8.4 ± 12.9*	10.7 ± 1.9 *†

*$p<0.05$ versus pre-HI, †$p<0.05$ versus CONT

Table 7.1 Values (mean ± 1SD) of pH, blood gases (kPa), and right atrial plasma lactate concentration (mmol/l) in the control (CONT), NLA-10 and NLA-40 group during the various time points during the study period. HI = hypoxia-ischemia.

Table 7.2 shows mean HR, and P_{ao}. No significant changes in mean HR and P_{ao} were found during the study period in the CONT and NLA-10 groups. In the NLA-40 group mean P_{ao} was significantly higher at 60 min post-HI. However, this difference was just significant.

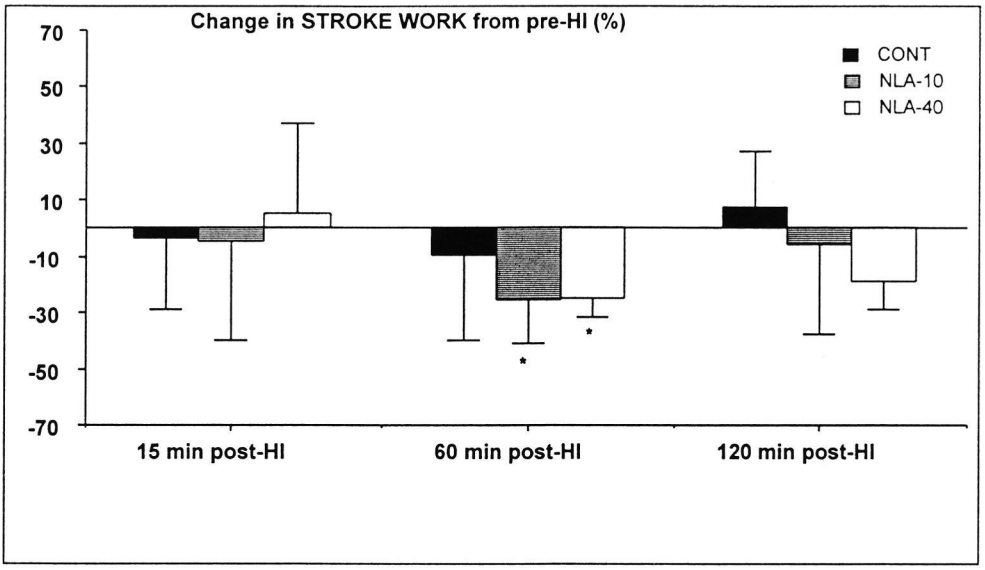
	pre-HI	15 min post-HI	60 min post-HI	120 min post-HI
HR				
CONT	173± 47	192 ± 41	185 ± 66	199 ± 43
NLA-10	157 ± 33	161 ± 30	186 ± 40	179 ± 42
NLA-40	151 ± 34	154 ± 33	132 ± 41	147 ± 13
mean P_{ao}				
CONT	82 ± 12	78 ± 16	82 ± 27	82 ± 20
NLA-10	81 ± 22	86 ± 17	81 ± 17	94 ± 15
NLA-40	72 ± 13	74 ± 17	87 ± 10*	77 ± 16

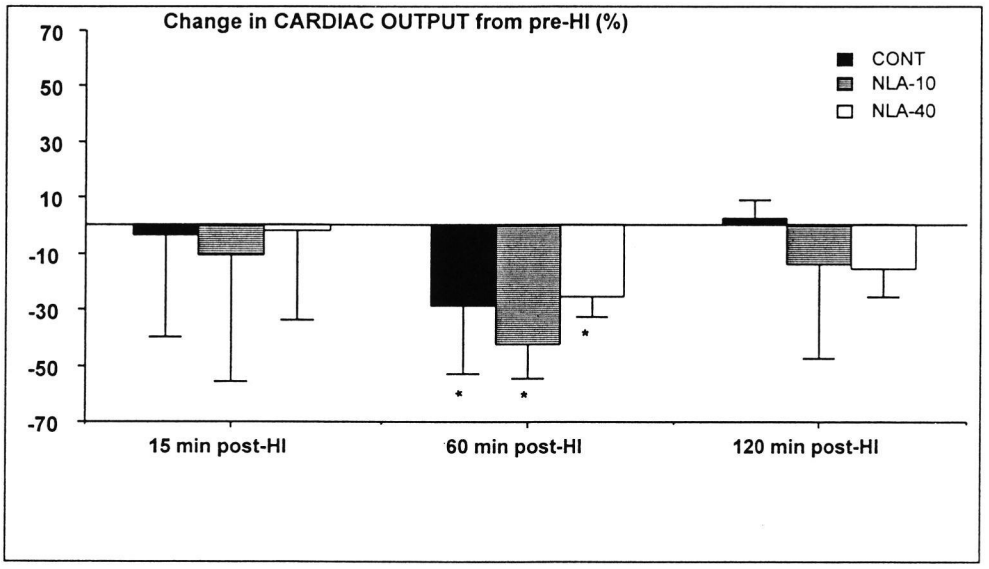
*$p<0.05$ versus pre-HI

Table 7.2 Values (mean ± 1SD) of heart rate (HR) and mean aortic pressure (Pao: mm Hg) in the control (CONT), NLA-10 and NLA-40 group during the various time points during the study period. HI = hypoxia-ischemia.

7.4.2 Assessment of pump and myocardial performance

Figure 7.1 shows the post-HI changes in SW and CO as a percentage from pre-HI values. Both SW (NLA-10 and 40 groups) and CO (all groups) were significantly decreased at 60 min post-HI. Afterwards these hemodynamic parameters almost recovered to pre-HI values.





*$p<0.05$ versus pre-HI, †$p<0.05$ versus CONT

Figure 7.1 Percent (%) changes (mean ± 1SD) relative to pre-hypoxic-ischemic (HI) values for stroke work and cardiac output in the control (CONT), NLA-10 and NLA-40 group during the various post-HI time points.

Table 7.3 shows the mean values (± 1 SD) of Ees, V_{10} and dP/dt_{max}-EDV indicating the pattern of LV-contractility during the study period. Although mostly no significant changes were detected, there was a clear tendency for a transient decrease in myocardial contractility post-HI in the CONT-group as indicated by decreases in Ees and dP/dt_{max}-EDV and increases in V_{10} respectively. In both NLA-treated groups there was a clear tendency for a transient increase in V_{10} (NLA-10 group $p < 0,05$ at 120 min post-HI). This finding of a decrease in contractile performance was accompanied by decreases in Ees and dP/dt_{max}-EDV, although neither of these reached statistical significance.

	pre-HI	15 min post-HI	60 min post-HI	120 min post-HI
Ees				
CONT	7.7 ± 3.7	6.7 ± 4.2	5.1 ± 2.3*	7.0 ± 4.0
NLA-10	5.8 ± 1.9	6.9 ± 4.1	5.9 ± 2.3	4.9 ± 0.9
NLA-40	4.6 ± 1.1	4.3 ± 1.3	5.4 ± 2.4	6.1 ± 2.8
V_{10}				
CONT	2.1 ± 0.4	2.6 ± 1.6	2.9 ± 2.2	2.7 ± 1.6
NLA-10	2.5 ± 1.7	2.4 ± 1.9	3.4 ± 2.7	4.2 ± 3.9*
NLA-40	3.4 ± 2.1	3.9 ± 2.1	3.8 ± 1.6	3.8 ± 1.7
dP/dt_{max}-EDV				
CONT	266 ± 99	177 ± 67	239 ± 202	174 ± 82
NLA-10	132± 100	125 ± 71	122 ± 58	92 ± 27
NLA-40	207 ± 142	103 ± 41	117 ± 60	212 ± 92

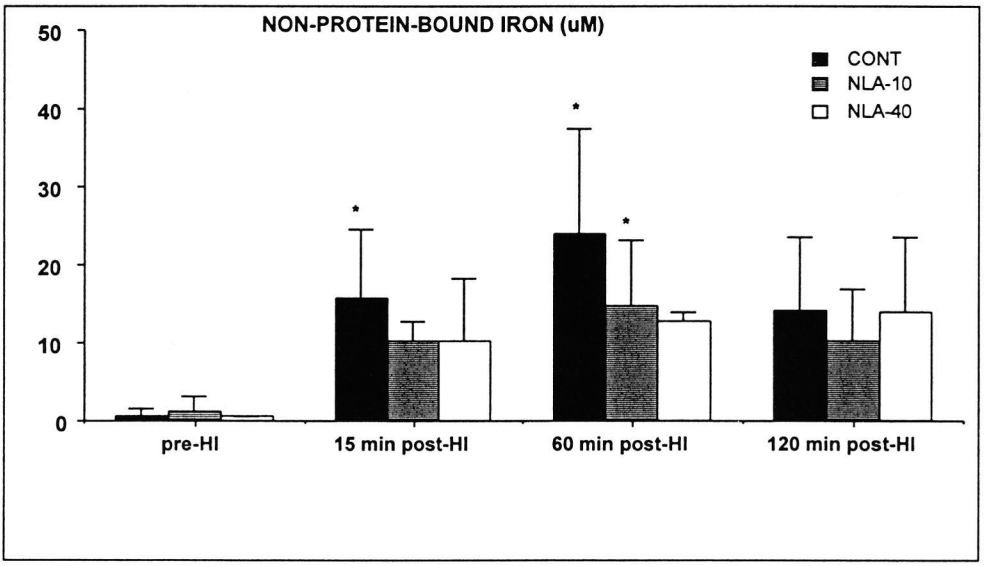
*$p<0.05$ versus pre-HI

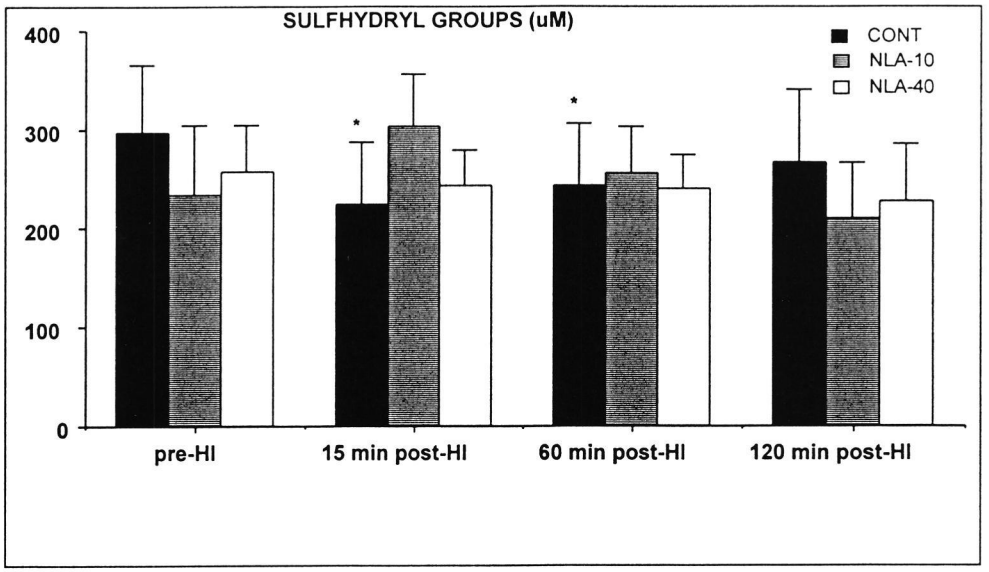
Table 7.3 Values (mean ± 1SD) of Ees (kPa/ml), V_{10} (ml) and dP/dt_{max}-EDV (kPa/s/ml) in the control (CONT), NLA-10 and NLA-40 group during the various time points during the study period. HI = hypoxia-ischemia.

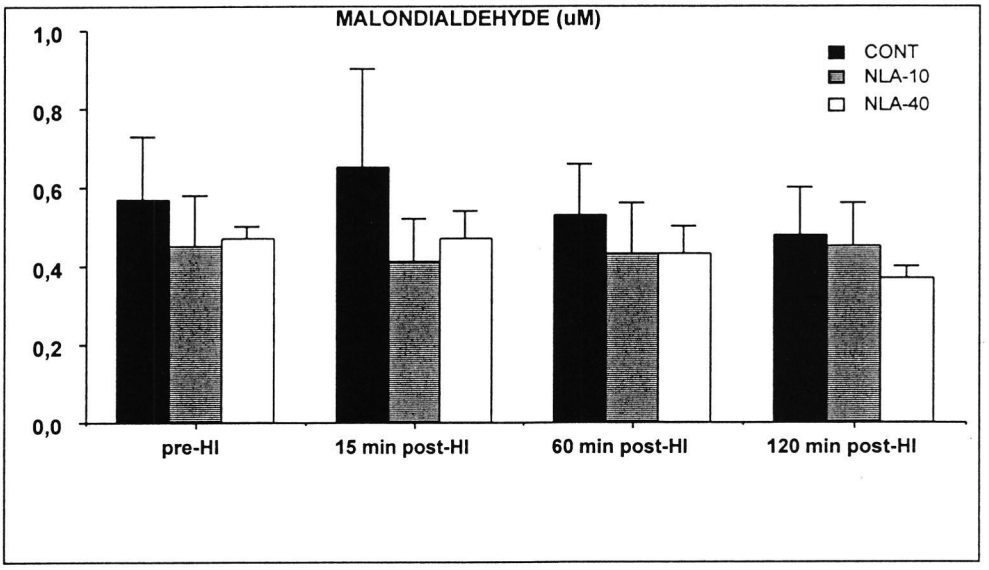
7.4.3 Redox status

Figure 7.2 shows the actual values of NPBI, sulfhydryl groups and MDA during the study period. Pre-HI, NPBI was not detectable or very low in all lambs. Post-HI CONT group showed a sharp and significant increase at 15 and 60 min post-HI ($p<0.05$ and $p<0.001$ versus pre-HI respectively). Although there was a significant increase of

NPBI in the NLA-10 group at 60 min post-HI ($p<0.001$), the rise in NPBI in the NLA-treated groups was more modest as compared to the control group. Sulfhydryl groups were only significantly lower in CONT-group at 15 and 60 min post-HI ($p<0.01$ and $p<0.05$ respectively). Sulfhydryl was stable in both NLA-treated groups. MDA did not change in any group during the study period, although it was higher in control lambs at 15 min post-HI as compared to the NLA-treated animals. This difference was just not significant ($p=0.08$). There were no significant changes in AA/DHA-ratio and α-tocopherol (not shown).







*$p<0.05$ versus pre-HI, †$p<0.05$ versus CONT

Figure 7.2 Mean plasma concentrations (± 1SD) of non-protein-bound iron, sulfhydryl groups and malondialdehyde in the control (CONT), NLA-10 and NLA-40 group during the various time points.

7.5 Discussion

The results of the present study suggest that hypoxia-ischemia decreases myocardial function and that nitric oxide synthesis inhibition has neither a positive nor a negative effect on post-HI myocardial performance. The CO showed a significant decrease in all 3 groups at 60 min post-HI, with subsequently only a partial recovery suggesting a subnormal LV-function at this time point. Also SW was decreased at 60 min post-HI, which was significant in both NLA-treated groups. Although the CO and SW were decreased and the very low arterial pH, base excess, and plasma lactate concentration at 2 and 15 min post-HI indicated severe HI in all 3 groups, the indices for LV-myocardial contractility showed only a very limited decrease. This may be explained by the fact that 7 of the 14 studied lambs received adrenaline upon resuscitation, which was necessary to keep them alive. Moreover, subsequent temporary infusion of dopamine in these lambs (to prevent hypotension) may have accounted for this apparent preservation of LV-function, despite the severe hypoxic ischemia. The production of reactive oxygen species was at its highest level at 60 min post-HI, indicated by the significantly increased concentration in plasma NPBI, and the significant decrease of sulfhydryl groups in especially the CONT lambs. The decrease in CO and SW at 60 min post-HI in combination with an increased production of reactive oxygen species at this time point suggests, but does not prove, a causative relationship between changes in cardiac pump performance and reactive oxygen species mediated stress. In this respect, it must be stressed that no differences between groups were detected with regard to any parameter of cardiac function, suggesting neither a positive nor negative effect of nitric oxide synthesis inhibition on post-HI myocardial performance. The significantly higher mean P_{ao} at 60 min in the NLA-40 group as compared to the pre-HI value was not unexpected because nitric oxide synthesis inhibition blocks the vasodilatory action of nitric oxide on the systemic vascular bed[2,12]. It remains however unclear why this effect is only present at 60 min post-HI. Possibly failure of cardiac function, due to myocardial hypoxia-ischemia, as indicated by the need for adrenaline and dopamine to keep half of the lambs alive, may have accounted for the fact that the increase in P_{ao} reached no statistical significance at the other time points.

Nitric oxide has many physiologic roles in the cardiovascular system[1,2,3]. Recently, it has been shown that inhibition of nitric oxide synthesis causes myocardial ischemia in endotoxemic rats[31]. Other animal studies showed a cardioprotective effect of nitric oxide donors on reperfusion-induced endothelial dysfunction and infarct size after myocardial ischemia[32,33]. Moreover, inhibition of nitric oxide synthesis with N^G-

monomethyl-L-arginine (L-NMMA), an arginine analogue comparable with NLA, made it very probable that the basal release of nitric oxide is essential for coronary dilatation and reperfusion of the myocardial tissue after myocardial infarction: inhibition of nitric oxide synthesis caused an increase in LV coronary resistance and a subsequent increase in infarct size in the 8 week old rat[34].

On the other hand there are indications that an enhanced production of nitric oxide plays a role in heart failure in man[35,36]. It has been speculated that post-HI reperfusion of the myocardium causes a sharp increase of endothelial derived nitric oxide (NO•) and reactive oxygen species such as superoxide ($O_2^{\bullet -}$), giving rise to the production of the highly reactive peroxynitrite ($NO\bullet + O_2^{\bullet -} \rightarrow ONOO^-$), which decays to form the extremely toxic hydroxyl radical (OH•), inducing endothelial injury of the coronary microvessels[37,38,39]. Moreover, peroxynitrite can directly degrade sulfhydryl groups which have important anti-oxidative properties[7]. A recent study of Matheis et al. in newborn piglets showed indeed that the nitric oxide pathway played an important role in myocardial reoxygenation injury in these animals[15]. They found that reoxygenation after a period of profound hypoxia caused a decreased contractility and an increased lipid peroxidation of the myocardial tissue. Moreover, nitric oxide synthesis inhibition with the nitric oxide synthase inhibitor (L-NMMA) upon reoxygenation gave a nearly complete protection against myocardial reoxygenation injury and prevented lipid peroxidation.

With respect to the conflicting results, reporting either increases in infarct size after myocardial ischemia or protection against myocardial reoxygenation injury after administration of nitric oxide-synthesis inhibitors, it is important to stress that there is an essential difference between *local* and *global* ischemia, the latter occurring during neonatal hypoxia-ischemia. After local (partial) occlusion of a coronary artery, nitric oxide synthesis may cause vasodilatation and thereby "rescue" some cells in the penumbra surrounding the infarct lesion, indicating a cardioprotective effect. Moreover, the absence of reperfusion and reoxygenation of the infarct lesion itself may prevent the production of highly toxic free oxygen species in the infarction zone. On the other hand, during global ischemia, such as during birth asphyxia, it is important to reduce the nitric oxide production in order to prevent reactive oxygen species mediated oxidative damage, which means that nitric oxide synthesis inhibition after global ischemia may be cardioprotective.

As already stated, the present study showed neither a negative nor a positive effect of post-HI nitric oxide synthesis inhibition on myocardial performance and LV-contractility. However, there was a small but significant positive effect of nitric oxide synthesis inhibition on the redox status in the blood. The formation of the pro-oxidant NPBI and the degradation of sulfhydryl groups was less in the NLA-treated groups, suggesting less reactive oxygen species mediated stress in these animals.

The results of the present study, suggesting that nitric oxide synthesis inhibition has no negative effect on post-HI LV-function, are important with respect to future studies investigating the possible neuroprotective effects of nitric oxide synthesis inhibition in neonates born after severe birth asphyxia.

7.6 References

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8 SUMMARY

8.1 Summary

Perinatal hypoxia-ischemia, also called perinatal asphyxia, is despite improvements in obstetric and perinatal care, still the most important cause of brain injury of the newborn. Estimates, based on figures of other countries, suggest that in the Netherlands approximately 2000 neonates per year will suffer from perinatal hypoxia-ischemia, 900 of which will die and approximately 200 will survive with long term neurologic deficits such as cerebral palsy, mental retardation, learning disabilities and epilepsy. Some of them will be so handicapped that they will be chronically dependent on institutions for mentally and physically disabled. Given these incidence figures it becomes clear that perinatal hypoxia-ischemia is an important health problem with considerable social consequences. This thesis was established to get some insight in the complex pathophysiology of post hypoxic-ischemic brain injury and to search for potential therapies to prevent or reduce the long-term consequences of perinatal hypoxia-ischemia.

The first two studies of this thesis (chapters 2 and 3) describe the cerebral hemodynamics and the plasma concentration of non-protein-bound iron during the first 24 hours of life in perinatally asphyxiated neonates. The next four studies (chapters 4 to 7) describe the effect of post hypoxic-ischemic nitric oxide synthesis inhibition on cerebral hemodynamics and metabolism, redox status, pulmonary artery pressure and oxygen need, and left ventricular function in newborn lambs subjected to severe hypoxia-ischemia.

Chapter 1 gives some background information about the general and hemodynamic pathophysiology of perinatal hypoxia-ischemia. Although some damage may occur during the actual hypoxic-ischemic insult, it is now well established that a substantial proportion of post hypoxic-ischemic brain injury can be attributed to the production of reactive oxygen species, and in particular nitric oxide, upon reperfusion and reoxygenation, thus *after* cessation of the primary hypoxic-ischemic insult. Specific paragraphs are devoted to the role of non-protein-bound iron and nitric oxide in this reactive oxygen species mediated post hypoxic-ischemic brain injury. The final part of chapter 1 summarizes the broad range of the complex metabolic and biochemical sequences of events occurring after the primary hypoxic-ischemic insult and discusses potential targets for (pharmaco) therapeutical intervention.

Chapter 2 describes that postasphyctic cerebral hypoperfusion and decreased cerebral metabolism, as has been reported previously in adults and newborn animals, also

occurs in the perinatally asphyxiated neonate. This issue was studied by monitoring changes in oxyhemoglobin (HbO_2), deoxyhemoglobin (HbR), total hemoglobin (HbO_2+HbR, which represents changes in cerebral blood volume [CBV]) and cytochrome oxidase ($Cytaa_3$, which indicates changes in the oxidation level of this intracerebral mitochondrial enzyme) by means of Near Infrared Spectroscopy (NIRS) in healthy, moderately asphyxiated, and severely asphyxiated neonates. This study showed that only in severely asphyxiated neonates, who subsequently developed neurological abnormalities, postasphyctic cerebral hypoperfusion and decreased cerebral metabolism were present in the first 12 hours of life, suggesting that post hypoxic-ischemic reperfusion injury of the brain during early neonatal life occurs in neonates with severe birth asphyxia.

Chapter 3 describes that, although normal "adult's" plasma doesn't contain any non-protein-bound iron, it appears to be present in plasma of some neonates, especially in those suffering from severe perinatal asphyxia. In this study non-protein-bound iron was detectable in 30% of control, 60% of moderately asphyxiated, and 80% of severely asphyxiated neonates. The non-protein-bound iron concentration was significantly elevated in those severely asphyxiated neonates who subsequently died during the neonatal period or survived with brain damage. In contrast, three of the four severely asphyxiated babies with a normal outcome at 1 year of age appeared to have no detectable non-protein-bound iron in their plasma. Statistical analysis showed that the plasma concentration of non-protein-bound iron during the first 8 hours after birth was inversely related to the neurodevelopmental outcome at one year of age. It was therefore concluded that non-protein-bound iron may play an important role in oxidative damage mediated post-asphyxial brain injury and subsequent neurodevelopmental outcome.

The studies described in chapters 4 to 8 were established to investigate whether post hypoxic-ischemic cerebral reperfusion injury might be reduced by immediate post hypoxic-ischemic inhibition of nitric oxide synthesis by N-ω-nitro-L-arginine (NLA) and whether this was accompanied by any (negative) pulmonary or cardiac side effects. These studies were performed in newborn lambs subjected to severe hypoxia-ischemia, and receiving either a placebo or a low or high dose NLA upon resuscitation.

Chapter 4 describes the effect of immediate post hypoxic-ischemic inhibition of nitric oxide synthesis on cerebral perfusion, metabolism, electrocortical brain activity and histological brain damage of the Purkinje cells of the cerebellum. Normally, post

hypoxic-ischemic brain injury is characterized by a short initial cerebral hyperperfusion, followed by cerebral hypoperfusion, a decreased cerebral metabolic rate of oxygen and a decreased electrocortical brain activity. Immediate post hypoxic-ischemic inhibition of nitric oxide synthesis by a low, as well as a high dose NLA prevented a drop in cerebral metabolic rate of oxygen. The electrocortical brain activity decreased in all groups, but only recovered in the group receiving the low dose NLA. Both NLA-treated groups had less necrotic Purkinje cells and less cerebral edema as compared to the control group, although this difference was not significant. Because of the preservation of the cerebral metabolic rate of oxygen in both NLA-groups, but a recovery of the electrocortical brain activity only in the group receiving the low dose NLA, it was concluded that NLA, and especially a low dose NLA, may reduce post hypoxic-ischemic brain injury.

Chapter 5 describes the effect of immediate post hypoxic-ischemic inhibition of nitric oxide synthesis on plasma pro-oxidants (non-protein-bound iron), lipid peroxidation (malondialdehyde) and anti-oxidative capacity (ratio of ascorbic acid/dehydroascorbic acid: AA/DHA-ratio, α-tocopherol, sulfhydryl groups, allantoin/uric acid ratio and vitamin A) in blood effluent from the brain. Earlier studies have shown that post hypoxic-ischemic reperfusion induces endothelium and neurons to produce excessive amounts of nitric oxide and superoxide, leading to peroxynitrite formation, release of protein-bound metal ions (i.e. iron) and cytotoxic oxidants. In this study immediate post hypoxic-ischemic inhibition of nitric oxide synthesis by a low, as well as a high dose NLA reduced the post hypoxic-ischemic increase of non-protein bound iron and prevented a decrease of the AA/DHA-ratio and α-tocopherol. Moreover malondialdehyde was significantly lower in the NLA-treated groups. We therefore concluded that post hypoxic-ischemic inhibition of nitric oxide synthesis is able to diminish non-protein bound iron increment and preserve anti-oxidant capacity.

Chapter 6 describes the side effects of immediate post hypoxic-ischemic inhibition of nitric oxide synthesis on pulmonary artery pressure and oxygen need. Inhibition of nitric oxide production may reduce post hypoxic-ischemic brain damage, but may also induce pulmonary hypertension by inhibiting endogenous nitric oxide production in the pulmonary vascular bed, resulting in an increased pulmonary artery pressure and oxygen need. Immediate post hypoxic-ischemic inhibition of nitric oxide synthesis by a low, as well as a high dose NLA resulted in a significant increase of pulmonary artery pressure as well as a significantly decreased PaO_2/FIO_2-ratio, suggesting that inhibition of nitric oxide synthesis after perinatal hypoxia-ischemia may compromise pulmonary function by inducing pulmonary hypertension leading to a higher oxygen need.

Chapter 7 describes the side effects of immediate post hypoxic-ischemic inhibition of nitric oxide synthesis on left ventricular function. Previous reports concerning the cardiac effects of post hypoxic-ischemic nitric oxide synthesis inhibition show conflicting results. Cardiac function has been shown to be negatively influenced by an impairment of nitric oxide-induced relaxation of the coronary vessels, whereas on the other hand post hypoxic-ischemic nitric oxide synthesis inhibition may also reduce reactive oxygen species mediated stress to the myocardium. In this study we found that hypoxia-ischemia decreases left ventricular function and that nitric oxide synthesis inhibition has neither a positive nor a negative effect on post-HI left ventricular function. However, there was a positive effect of nitric oxide synthesis inhibition on the redox status of plasma obtained from the right atrium: formation of the pro-oxidant non-protein-bound iron and the degradation of sulfhydryl groups was significantly lower in the NLA-treated animals suggesting less reactive oxygen species mediated stress in these animals. The results of this study, suggesting that nitric oxide synthesis inhibition has no negative effect on left ventricular function and may have a positive effect on reactive oxygen species mediated stress, are important with respect to future studies investigating the possible neuroprotective effects of nitric oxide synthesis inhibition in neonates born after severe birth asphyxia.

8.2 Concluding remarks and future directions

The studies described in this thesis suggest that immediate post hypoxic-ischemic inhibition of nitric oxide synthesis may reduce reactive oxygen species mediated stress with concomitant post hypoxic-ischemic reperfusion injury of the brain. There is currently no reason to fear for negative cardiac side effects, although some caution is warranted with respect to possible negative pulmonary side effects. Further investigations to the potential beneficial effects of immediate post hypoxic-ischemic administration of non-protein-bound iron scavengers or nitric oxide synthesis inhibitors can now be set up. The studies with respect to nitric oxide synthesis inhibitors should especially focus on specific inhibitors of the different forms of nitric oxide synthase. The existing inhibitors nitro-L-arginine methyl ester (L-NAME), N-ω-nitro-L-arginine (NLA) and monomethyl-L-arginine (L-NMMA) inhibit both the constitutive and inducible form of the enzyme, and do not discriminate between the neuronal and endothelial form. Besides the fact that the neuronal, endothelial and inducible form all exhibit different functions, the neuronal and endothelial form are expressed all the time, whereas the inducible form is not expressed until several hours

after a hypoxic-ischemic insult. It is therefore important to search for new NOS inhibitors which can selectively inhibit the neuronal, endothelial or inducible NOS.

The best therapeutical regimen may prove to be a combination of a *high* dose of an nNOS inhibitor with a *low* dose of an eNOS inhibitor immediately after the hypoxic-ischemic insult: a high dose of an nNOS inhibitor to reduce glutamate-induced neurotoxicity, and a low dose of an eNOS inhibitor to reduce early post hypoxic-ischemic hyperperfusion and subsequent "early reperfusion injury". Administration of a high instead of a low dose of an eNOS inhibitor may cause additional ischemia due to massive vasoconstriction. This early administration of a high dose of a nNOS inhibitor and a low dose of an eNOS inhibitor must be accompanied after several hours by a high dose of an iNOS inhibitor to reduce the "late reperfusion injury". Following studies must then be established to determine the optimum dose and dosage scheme.

When finally from these studies new modalities of therapy become available for clinical use, their application to the human neonate should first be scrutinized through appropriate clinical trials adequate to determine their safety and efficacy in the clinical setting. Moreover it must be taken into account that inhibition of nitric oxide synthesis focuses only on a very small part of the extensive pathomechanism of post hypoxic-ischemic brain injury. In all likelihood a combination with other possible therapies, such as those scavenging non-protein-bound iron (chelating drugs, plasma administration, exchange transfusions), scavenging or reducing the production of reactive oxygen species and hypothermia, will finally prove to ensure the most effective result.

9 SAMENVATTING

9.1 Samenvatting

Zuurstoftekort rondom de geboorte, ook wel perinatale asfyxie genoemd, is ondanks grote vooruitgang in de obstetrische en perinatale zorg nog steeds een van de belangrijkste oorzaken van hersenbeschadiging van de pasgeborene. Op basis van gegevens uit andere landen kan berekend worden dat er jaarlijks in Nederland zo'n 2000 baby's geboren worden met perinatale asfyxie. Van deze baby's zullen er ongeveer 900 overlijden en naar schatting 200 ernstige blijvende hersenbeschadiging ontwikkelen in de vorm van spasticiteit, mentale retardatie, ernstige leermoeilijkheden en epilepsie. Een groot deel van deze kinderen is zodanig gehandicapt dat chronische verpleging in instellingen voor meervoudig gehandicapten noodzakelijk is. Het hierboven geschetste beeld maakt duidelijk dat perinatale asfyxie een belangrijk gezondheidsprobleem is met verstrekkende maatschappelijke gevolgen. De in dit proefschrift beschreven studies zijn opgezet om enig inzicht te krijgen in het zeer complexe ontstaansmechanisme van hersenbeschadiging na perinatale asfyxie. Tevens is gezocht naar potentiële behandelingsstrategieën teneinde in de toekomst de hersenschade na perinatale asfyxie te reduceren of zo mogelijk zelfs geheel te voorkomen.

De eerste twee studies van dit proefschrift (hoofdstukken 2 en 3) zijn verricht bij baby's met ernstige perinatale asfyxie en beschrijven de veranderingen in de hersendoorbloeding en plasma-concentratie van niet-eiwitgebonden ijzer gedurende de eerste 24 uur na de geboorte. De volgende vier studies (hoofdstukken 4 tot 7) zijn elk uitgevoerd bij pasgeboren lammeren waarbij in een experimentele setting perinatale asfyxie is nagebootst. Deze studies beschrijven het effect van medicamenteuze inhibitie van de productie van stikstofmonoxide op de doorbloeding en het metabolisme van de hersenen, op de productie van vrije radicalen, alsmede de neveneffecten op de linkerventrikelfunctie van het hart en op de pulmonale arteriële druk en gaswisseling in de longen.

Hoofdstuk 1 geeft enige achtergrondinformatie over de algemene en hemodynamische mechanismen die een rol spelen bij perinatale asfyxie. Ten gevolge van het initiële zuurstoftekort ontstaat beschadiging van de hersencellen. Recente studies hebben echter aangetoond dat deze beschadiging niet stopt op het moment dat er weer voldoende zuurstof aanwezig is. Gedurende de herstelfase, dus na het al opgeheven zijn van het oorspronkelijke zuurstoftekort, treedt additionele schade op, ook wel "reperfusieschade" genoemd. De productie van vrije radicalen, en in het bijzonder de productie van stikstofmonoxide, lijkt hierin een centrale rol te spelen. Er wordt in

aparte paragrafen uitgebreid aandacht besteed aan de rol van niet-eiwitgebonden ijzer en stikstofmonoxide bij het ontstaan van deze zogenaamde "vrije radicalen gemedieerde reperfusieschade". De slotparagraaf geeft een samenvatting van de diverse mechanismen die een rol spelen bij het ontstaan van hersenbeschadiging na perinatale asfyxie en bespreekt de mogelijke aangrijpingspunten voor (medicamenteuze) therapie.

Hoofdstuk 2 beschrijft dat pasgeborenen met ernstige perinatale asfyxie na de geboorte een afname van de doorbloeding en het metabolisme van de hersenen laten zien, hetgeen kenmerkend is voor post-asfyctische cerebrale reperfusieschade. Dit fenomeen is tot dusver alleen nog maar beschreven in volwassenen en pasgeboren dieren. In een groep gezonde pasgeborenen en in twee groepen pasgeborenen met respectievelijk matige en ernstige perinatale asfyxie, is door middel van Near Infrarood Spectroscopie (NIRS) gekeken naar veranderingen in de cerebrale doorbloeding en oxygenatie van het intracerebrale mitochondriale enzym cytochroom-aa_3. In deze studie konden we bij de groep pasgeborenen met ernstige perinatale asfyxie, die vervolgens zijn overleden of blijvende ernstige hersenschade hebben ontwikkeld, een afname van de doorbloeding en de oxygenatie van het enzym cytochroom-aa_3 detecteren gedurende de eerste 12 uur na de geboorte. Deze bevindingen suggereren dat er daadwerkelijk cerebrale reperfusieschade optreedt bij pasgeborenen met ernstige perinatale asfyxie.

Hoofdstuk 3 beschrijft dat plasma van pasgeborenen, in tegenstelling tot plasma van volwassenen, soms niet-eiwitgebonden ijzer bevat, wat betekent dat pasgeborenen extra "vatbaar" zijn voor door vrije radicalen gemedieerde reperfusieschade. Vooral het plasma van asfyctische pasgeborenen bleek veel vaker dan normaal niet-eiwitgebonden ijzer te bevatten. In deze studie was er bij 30% van gezonde pasgeborenen, 60% van pasgeborenen met matig perinatale asfyxie en zelfs bij 80% van pasgeborenen met ernstige perinatale asfyxie niet-eiwitgebonden ijzer in het plasma aanwezig. Daarnaast bleek de concentratie van niet-eiwitgebonden ijzer significant hoger te zijn in de groep ernstige asfyctische pasgeborenen ten opzichte van de groep gezonde pasgeborenen. De concentratie van niet-eiwitgebonden ijzer bleek vooral sterk verhoogd te zijn bij de ernstige asfyctische pasgeborenen die zijn overleden of blijvende ernstige hersenschade hebben ontwikkeld, terwijl bij 3 van de 4 ernstige asfyctische pasgeborenen die volledig normaal waren op de leeftijd van 1 jaar in het geheel géén niet-eiwitgebonden ijzer in het plasma aanwezig was. Statistische analyse toonde verder aan dat de hoogte van de concentratie van niet-eiwitgebonden

ijzer in het plasma gedurende de eerste 8 uur na de geboorte omgekeerd evenredig was met de kans op een normale neurologische ontwikkeling op de leeftijd van één jaar. De conclusie van deze studie was daarom dat niet-eiwitgebonden ijzer een belangrijke rol speelt bij het ontstaan van hersenbeschadiging na perinatale asfyxie.

De studies die beschreven worden in hoofdstuk 4 tot en met 8 zijn opgezet om te onderzoeken of de hersenbeschadiging die optreedt na perinatale asfyxie mogelijk gereduceerd zou kunnen worden door inhibitie van de productie van stikstofmonoxide door middel van vroegtijdige toediening van het medicament N-ω-Nitro-L-Arginine (NLA). Tevens is apart gekeken naar mogelijke bijwerkingen van NLA op de linkerventrikelfunctie van het hart en de pulmonale arteriële druk en gaswisseling in de longen. Al deze studies zijn uitgevoerde in 3 groepen pasgeboren lammeren waarbij in een experimentele setting perinatale asfyxie is nagebootst. Bij de reanimatie kreeg één groep een placebo, één groep een lage en één groep een hoge dosis NLA toegediend.

Hoofdstuk 4 beschrijft welk effect inhibitie van de productie van stikstofmonoxide na perinatale asfyxie heeft op de cerebrale doorbloeding, zuurstofconsumptie en de electrocorticale hersenactiviteit. Eveneens is op histologisch niveau gekeken naar het effect op de Purkinjecellen van het cerebellum. Reperfusieschade na perinatale asfyxie wordt gekenmerkt door een korte initiële cerebrale hyperperfusie, gevolgd door een afname van de cerebrale doorbloeding, metabolisme en de electrocorticale hersenactiviteit. Inhibitie van de productie van stikstofmonoxide door vroegtijdige toediening van zowel een lage als een hoge dosis NLA bleek de hierboven beschreven afname van de zuurstofconsumptie te kunnen voorkomen. De electrocorticale hersenactiviteit daalde in alle drie de groepen, maar herstelde zich alleen in de groep lammeren die een lage dosis NLA kreeg toegediend. Beide met NLA behandelde groepen leken minder hersenoedeem en minder necrotische Purkinjecellen te vertonen in vergelijking met de groep die een placebo had ontvangen. Op basis van het feit dat zowel een lage als een hoge dosis NLA een afname van de zuurstofconsumptie kon voorkomen, maar de electrocorticale hersenactiviteit zich alleen herstelde in de groep lammeren die een lage dosis NLA kreeg toegediend, werd geconcludeerd dat NLA, en in het bijzonder een lage dosis NLA, cerebrale reperfusieschade na perinatale asfyxie lijkt te reduceren.

Hoofdstuk 5 beschrijft welk effect inhibitie van de productie van stikstofmonoxide na perinatale asfyxie heeft op de productie van plasma pro-oxidanten (niet-eiwitgebonden ijzer), vetzuur peroxidatie (malondialdehyde) en anti-oxidatieve capaciteit (ratio gereduceerd/geoxideerd ascorbinezuur, α-tocopherol, sulfhydryl-groepen,

allantoine/urinezuur ratio en vitamine A) in bloed afkomstig van de hersenen. Eerdere studies hebben aangetoond dat er tijdens de reperfusiefase na perinatale asfyxie excessieve hoeveelheden stikstofmonoxide en andere vrije radicalen geproduceerd worden die tezamen kunnen reageren tot nog agressievere vrije radicalen (o.a. peroxinitriet). Deze vrije radicalen kunnen directe schade aanrichten, maar kunnen ook indirect hun toxiciteit uitoefenen via de vorming van niet-eiwitgebonden ijzer. In deze studie bleek dat inhibitie van de productie van stikstofmonoxide door vroegtijdige toediening van zowel een lage als een hoge dosis NLA de stijging van de concentratie niet-eiwitgebonden ijzer kon reduceren en een daling van de anti-oxidanten α-tocopherol en de ratio van gereduceerd/geoxideerd ascorbinezuur kon voorkomen. Bovendien was de mate van vetzuur peroxidatie significant lager in de met NLA behandelde lammeren. Op basis van deze resultaten werd geconcludeerd dat vroegtijdige toediening van NLA na perinatale asfyxie een stijging van de concentratie niet-eiwitgebonden ijzer kan verminderen en een daling van de anti-oxidatieve capaciteit kan voorkomen.

Hoofdstuk 6 beschrijft het effect van inhibitie van de productie van stikstofmonoxide na perinatale asfyxie op de pulmonale arteriële druk en gaswisseling in de longen van de pasgeborene. Naast het feit dat inhibitie van de productie van stikstofmonoxide mogelijk (een deel van de) hersenschade na perinatale asfyxie kan voorkomen, kan het theoretisch ook de productie van stikstofmonoxide in de longen blokkeren, hetgeen zal leiden tot verhoging van de pulmonale arteriële druk en een verhoogde zuurstofbehoefte. In deze studie bleek dat inhibitie van de productie van stikstofmonoxide na perinatale asfyxie door vroegtijdige toediening van zowel een lage als een hoge dosis NLA een significante toename van de pulmonale arteriële druk , alsmede een verhoogde zuurstofbehoefte tot gevolg had. Dit betekent dat inhibitie van de productie van stikstofmonoxide na perinatale asfyxie de pulmonale arteriële druk en gaswisseling in de longen van de pasgeborene mogelijk tijdelijk nadelig kan beïnvloeden.

Hoofdstuk 7 beschrijft het effect van inhibitie van de productie van stikstofmonoxide na perinatale asfyxie op de linkerventrikelfunctie van het hart van de pasgeborene. Eerdere studies betreffende het effect van inhibitie van de productie van stikstofmonoxide op de functie van het hart tonen tegenstrijdige resultaten. Enerzijds wordt de functie van het hart negatief beïnvloed doordat inhibitie van de productie van stikstofmonoxide in de coronairvaten leidt tot vasoconstrictie. Anderzijds kan de functie van het hart positief beïnvloed worden doordat inhibitie van de productie van

stikstofmonoxide kan leiden tot een verminderde productie van vrije radicalen en daardoor minder beschadiging van de hartspiercellen. In deze studie bleek perinatale asfyxie een negatieve invloed te hebben op de linkerventrikelfunctie van het hart. Inhibitie van de productie van stikstofmonoxide na perinatale asfyxie bleek hierop geen invloed te hebben. Daarentegen bleek dat inhibitie van de productie van stikstofmonoxide wel de concentratie niet-eiwitgebonden ijzer reduceerde en een daling van sulfhydryl-groepen voorkwam, wat suggereert dat er minder vrije radicalen geproduceerd werden in de met NLA behandelde lammeren. De resultaten van deze studie suggereren dat vroegtijdige toediening van NLA na perinatale asfyxie geen negatieve invloed heeft op de linkerventrikelfunctie van het hart en mogelijk zelfs een positieve invloed uitoefent op de door vrije radicalen gemedieerde beschadiging van hartspiercellen. Deze bevindingen zijn belangrijk met betrekking tot het opzetten van vervolgstudies naar de toepasbaarheid van NLA in de klinische setting.

9.2 Slotopmerkingen en mogelijkheden voor toekomstig onderzoek

De in dit proefschrift beschreven studies suggereren dat vroegtijdige inhibitie van de productie van stikstofmonoxide na perinatale asfyxie, door toediening van het medicament N-ω-Nitro-L-Arginine, de productie van vrije radicalen vermindert en (een deel van) de cerebrale reperfusieschade kan voorkomen. Er zijn momenteel geen aanwijzingen voor negatieve bijwerkingen van N-ω-Nitro-L-Arginine op de linkerventrikelfunctie van het hart, alhoewel wel rekening gehouden moet worden met mogelijk tijdelijke negatieve neveneffecten op de pulmonale arteriële druk en gaswisseling in de longen. Vervolgstudies naar de effectiviteit van ijzerchelatie en inhibitie van de productie van stikstofmonoxide in zowel de experimentele als klinische setting lijken nu geïndiceerd. De studies met betrekking tot de inhibitie van de productie van stikstofmonoxide zouden zich vooral moeten richten op het ontwikkelen van medicamenten die specifieke vormen van de stikstofmonoxide productie kunnen blokkeren. De bestaande medicamenten, te weten Nitro-L-Arginine methyl ester (L-NAME), N-ω-Nitro-L-Arginine (NLA) en monomethyl-L-Arginine (L-NMMA), blokkeren elk zowel de constitutionele vorm als de induceerbare vorm van het enzym dat stikstofmonoxide produceert en maken bovendien geen onderscheid tussen de constitutionele vorm die zich in het endotheel (eNOS) en de constitutionele vorm die zich in de neuronen (nNOS) bevindt. Behoudens het feit dat de verschillende vormen van het enzym elk verantwoordelijk zijn voor een verschillende functie, is het ook zo dat de constitutionele vorm van het enzym continu aanwezig is, terwijl de induceerbare vorm van het enzym (iNOS) pas enkele uren na het asfyctische insult actief wordt. Het is daarom belangrijk om nieuwe medicamenten te ontwikkelen die

selectief het eNOS, nNOS of iNOS kunnen inhiberen. Mogelijk zal uiteindelijk blijken dat zo snel mogelijke toediening van een combinatietherapie van een *hoge* dosis van een nNOS-inhibitor samen met een *lage* dosis van een eNOS-inhibitor het meest effectief is. Een hoge dosis van een nNOS-inhibitor om de glutamaat en vrije radicalen gemedieerde hersenschade te beperken en een lage dosis van een eNOS-inhibitor om de initiële cerebrale hyperperfusie te beperken en daardoor de zogenaamde "vroege reperfusieschade" te reduceren. Na enkele uren zou deze vroegtijdige toediening van een hoge dosis van een nNOS-inhibitor samen met een lage dosis van een eNOS-inhibitor gevolgd moeten worden door een hoge dosis van een iNOS-inhibitor om daardoor ook de zogenaamde "late reperfusieschade" te reduceren. Vervolgstudies zullen uiteindelijk moeten uitwijzen wat het beste doserings- en tijdsschema is van de toediening van de diverse medicamenten.

Wanneer uiteindelijk ten gevolge van de in dit proefschrift beschreven studies en de hieruit voortkomende vervolgstudies nieuwe medicamenten ontwikkeld worden, zullen ze eerst zeer uitgebreid getest moeten worden op effectiviteit en veiligheid, alvorens toepasbaarheid in een klinische setting mogelijk is. Bovendien moet in acht genomen worden dat inhibitie van de productie van stikstofmonoxide slechts aangrijpt op één onderdeel van het zeer complexe ontstaansmechanisme van perinatale asfyxie. Het is daarom zeer wel mogelijk dat combinatie met andere therapieën uiteindelijk het meest effectief blijkt te zijn. Hierbij moet vooral gedacht worden aan combinatie met therapieën die het niet-eiwitgebonden ijzer in het bloed reduceren (scavengers van niet-eiwitgebonden ijzer, vers plasma transfusies en wisseltransfusies), combinatie met anti-oxidatieve therapieën en combinatie met hypothermie.

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CURRICULUM VITAE

The author of this thesis was born on May 22, 1967 in Den Haag. In 1985 she graduated from the Gemeentelijke Scholengemeenschap Doetinchem and started her medical studies at the University of Leiden. In 1986 she passed her "Propaedeutisch" exam. The basis for her interest in pediatric research was laid in 1988 when she took part in a research project of Prof. dr. J.M.J.J. Vossen on pediatric bone marrow transplantation. In 1989 she took part in an exchange program and went to Izmir to practice Primary Health Care. In 1990 she received an Erasmus Grant which allowed her to perform laboratory research on bone marrow transplantation in Edinburgh. When she came back she passed her "Doctoraal" exam en started with clinical research in the Neonatal Unit of the University Hospital of Leiden under supervision of Prof. Dr. F. van Bel. During a year she performed several studies with Near Infrared Spectroscopy in a broad scale of different groups of patients (perinatally asphyxiated neonates, preterm neonates, neonates receiving surfactant and neonates who underwent cardiac surgery). The results of the studies in the perinatally asphyxiated neonates gave rise to the set-up of further studies and formed the basis of this thesis. From 1992 till 1993 she performed her internships and passed her "arts" exam in September 1993. From October 1993 until April 1994 she worked as a resident in Neonatology at the University Hospital of Leiden. In April 1995 she continued her research with a grant from the Gisela Thier Fund. During one year she performed experimental research on perinatal asphyxia in newborn lambs in the Cardiac Physiology Laboratory of the Department of Cardiology under supervision of Prof. Dr. F. van Bel and Prof. Dr. J. Baan. From June 1996 she performs research on Perinatal Epidemiology at TNO Preventie en Gezondheid in Leiden and in the mean time finished the writing of this thesis.


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