Recovery from severe perinatal hypoxia;ischemia: studies of pathophysiology and treatment in the fetal sheep

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RECOVERY FROM SEVERE PERINATAL HYPOXIA-ISCHAEMIA
Studies of pathophysiology and treatment in the fetal sheep

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RECOVERY FROM SEVERE PERINATAL HYPOXIA-ISCHAEMIA

Studies of pathophysiology and treatment in the fetal sheep

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aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Mr. G.P.M.F. Mols,
ingevolge het besluit van het College van Decanen,
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CHAPTER 1

GENERAL INTRODUCTION

HISTORY

In 1861 William John Little (1810-1894) defended his thesis before the Obstetric Society of London, where he argued his idea, based on 200 cases he accumulated in 20 years practice in orthopedics, that asphyxia (derived from a Greek word meaning pulseless) immediately before or during parturition could cause permanent damage to the central nervous system resulting in 'spasmodic tetanus-like rigidity and distortion of the limbs' (Little 1861; Longo and Ashwal 1993). Little noted associations between the generalised spasticity and varying degrees of prematurity, difficult protracted labour requiring forceps delivery and severe asphyxia with convulsions (Longo and Ashwal 1993). Little postulated that the disabilities were secondary to '...interruption of the proper placental relation of the foetus to the mother and non-substitution of pulmonary respiration, [rather] than from direct mechanical injury' acting on the brains of 'too early and unripe-born foetuses' (Longo and Ashwal 1993). The long-term disabilities and deformities associated with asphyxia (then termed "asphyxia neonatorum") were later termed "cerebral palsy" by William Osler (1849-1919) (Osler 1889). Besides motor handicaps, Osler also noted associations with impaired intellect, speech problems, epilepsy; of which, he found the latter to be the 'most common and distressing symptom' (Longo and Ashwal 1993).

Over time many different definitions for asphyxia have been used. In the 1930's Dr. Eastman defined asphyxia as "an inability of the child to breathe and apnea associated with oxygen deficiency during labour" and he demonstrated that asphyxia was associated with neonatal acidosis (Eastman 1932). Early experimental work studying the mechanisms of asphyxial brain injury was carried out in rhesus monkeys in the laboratory of Windle and Myers in the 1950's and 60's (Windle 1963; Myers 1967). In their studies asphyxiated newborn rhesus monkeys were resuscitated so that a number of them survived. Amongst the survivors, a certain number had neurologic deficits and a developmental pattern resembling cerebral palsy in human survivors of asphyxia. Brain histology showed a pattern of brain injury also observed in the asphyxiated human infant with parasagittal necrosis and "brain swelling" (Brann and Myers 1975).

ASPHYXIA

Asphyxia is now defined as an impaired maternal-fetal respiratory gas exchange resulting in hypoxemia, accompanied by the development of a combined respiratory and metabolic acidosis (Bennet *et al.* 2002). In practice, the key difference between 'hypoxia' and 'asphyxia' in experimental studies is simply severity: generally 'hypoxia' means moderate hypoxia with fetal PaO₂ between 10 and 14 mmHg with no, or trivial metabolic acidosis, in contrast with the severe hypoxia used in studies of asphyxia. Asphyxia may be caused by insufficient umbilical or insufficient uterine blood flow, as can occur following umbilical cord entanglement, uterine rupture, or placental abruption (Bennet *et al.* 2002). It is important to understand that asphyxia only leads to functional and structural organ damage when there is a long enough period of circulatory failure (hypoperfusion/ischaemia) (Parer 1994; Jensen *et al.* 1999; Bennet *et al.* 2002), as discussed in more detail under "Fetal responses to asphyxia", below. Although asphyxia is a global insult, affecting the whole fetus, it is the brain, heart and kidney that are particularly at risk of damage from ischaemia-reperfusion injury associated with asphyxia (Jensen *et al.* 1999; Bennet *et al.* 2002).

Ischaemia-reperfusion injury in the brain and other organs of the fetus or neonate due to asphyxia remains a significant challenge for perinatal medicine. It continues to be associated with a high mortality in both premature and full-term infants, neonatal complications and permanent consequences in survivors including spastic motor deficits (cerebral palsy), mental retardation, language, hearing, vision, behavioural problems and epilepsy in later life (MacDonald *et al.* 1980; Mulligan *et al.* 1980; Vannucci

2000; Volpe 2001b). This of course profoundly affects the child's future life and that of its family and therefore hypoxic-ischaemic injury has major social and health implications (Volpe 2001b).

When does asphyxia lead to brain injury?

The prevalence of biochemically defined fetal asphyxia (i.e. the presence of an oxygen debt as shown by a metabolic acidosis) in deliveries at term is estimated to be around 25 per 1000 live births (Low 2004). Besides acidosis determined from either fetal scalp sampling or umbilical artery blood, the diagnosis of asphyxia is usually inferred clinically from highly non-specific signs such as the presence of abnormal intrapartum fetal heart rate tracings, low Apgar scores (Nelson and Léviton 1991; Low *et al.* 1995b; Roland and Hill 1995). Approximately 15% of biochemically defined cases of asphyxia are considered to be moderate or severe asphyxia, i.e. associated with adverse short-term outcomes such as acute renal failure, newborn encephalopathy and other organ system complications (Low 1997; Low 2004).

There is now consensus that a pH of less than 7.00 with a base deficit >10 or 12 mmol/l on umbilical arterial cord blood represents an approximate threshold at which significant adverse neonatal sequelae, including subsequent neurological dysfunction and death begin to be seen (van den Berg *et al.* 1996; Hankins and Speer 2003). Even when this low pH threshold is used to define significant acidemia, most newborns in this category will be neurologically normal, with no apparent morbidity. For example, more than half of babies born with severe acidosis (pH <7.0 and base deficit >16 mmol/l) do not develop even mild encephalopathy, while although at low frequency encephalopathy can still occur in association with relatively modest acidosis (Low 1997). The development of brain damage is associated most strongly with the severity and duration of fetal hypotension (Mallard *et al.* 1994). As it is not possible to directly measure fetal hypotension, but only other signs of stress such as pH and heart rate via CTG it will remain difficult to predict when asphyxia leads to brain injury.

Fetal responses to asphyxia

The absence of encephalopathy or other complications in many neonates exposed to 'significant' asphyxia must reflect in part the immense adaptive capacity of the fetus to prevent injury from asphyxia, and thus to delay or prevent the onset of hypotension (Low 1997; Bennet *et al.* 2002). Fetal defences have been studied primarily in animal models, such as the fetal sheep and llama and to a lesser extent the chick embryo (Bennet *et al.* 1998; Mulder *et al.* 1998; Llanos *et al.* 2003). Studies in fetal sheep and other models have clearly demonstrated that the fetus can initially compensate for an asphyxial insult and protect the vital organs such as the brain, heart and adrenals (Jensen and Berger 1991; Bennet *et al.* 1999; Bennet *et al.* 2000; Gunn *et al.* 2001). The most important initial defense is redistribution of combined ventricular output away from 'peripheral' organs such as the kidney, gut and limbs to maintain perfusion pressure to the critical organs (Peeters *et al.* 1979; Sheldon *et al.* 1979; Richardson 1989; Jensen and Berger 1991; Ball *et al.* 1994b; Mulder *et al.* 1998; Bennet *et al.* 1999). This is also termed circulatory centralization, a process mediated principally via the chemoreceptor and the sympathetic-adrenomedullary axis (Hanson 1997; Berger and Garnier 2000). Sympathetic activation and catecholamine release cause vasoconstriction in the 'peripheral' organs, resulting in a (relative) ischaemia of the organs affected, which via anaerobic metabolism leads to a buildup of lactate (Hanson 1997; Berger and Garnier 2000). Later, after restoration of oxygenation, the fetus can use lactate and ketone bodies directly as alternative energy substrates (Volpe 2001b).

Local defense mechanisms also have an important role in preventing injury from asphyxia. Critically, in the brain there is an actively mediated reduction in energy metabolism and oxygen consumption in an attempt to match blood flow to tissue requirement (Field *et al.* 1990; Richardson 1993; Parer 1994). Adenosine appears to play a key regulatory role in mediating the reduced cerebral metabolism

during moderate hypoxia (Hunter *et al.* 2003a). Nitric oxide (NO) has been shown to play a role in mediating the local increase in cerebral blood flow (CBF) during hypoxia (Hunter *et al.* 2003b), although its role is less well studied during asphyxia (Hanson 1997).

If the asphyxial insult continues then fetal defense mechanisms will eventually fail. The fetus is then unable to maintain circulatory centralization, and cardiac output gradually falls as the myocardial glycogen stores and mitochondrial ATP concentrations become depleted. These two factors lead to progressive hypotension (Gunn *et al.* 2001). Fetal autoregulation of cerebral blood flow can maintain adequate cerebral perfusion during mild hypotension, however when blood pressure falls below the lower margin of autoregulation (Volpe 2001b; Muller *et al.* 2002), an adequate cerebral perfusion pressure to match blood flow and tissue demand can no longer be maintained (Berger and Garnier 2000). Thus during prolonged asphyxia (severe hypoxia), in contrast to moderate hypoxia, cerebral blood flow eventually falls leading to hypoperfusion (Bennet *et al.* 1998; Bennet *et al.* 1999). This combination of severe hypoxia with hypoperfusion is a classic combination of events, leading to 'hypoxic-ischaemic' injury to the brain, heart, kidney and other organs, and eventually death (Parer 1998; Gunn *et al.* 2001). The pathophysiology of this injury is discussed below. There is now considerable evidence that secondary damaging processes continue following reoxygenation and reperfusion after a period of significant hypoxia-ischaemia (Berger and Garnier 2000; Gunn 2000). Thus hypoxic-ischaemic injury may also be referred to as ischaemia-reperfusion injury.

BRAIN INJURY

Causes of brain injury in infants born at term

There has been much debate as to what proportion of cerebral palsy can be attributed to perinatal asphyxia. Estimates vary from 12-36% in term newborns (Nelson and Leviton 1991; Badawi *et al.* 1998b; Edwards and Nelson 1998; MacLennan 1999; Hagberg *et al.* 2001). Other causes of cerebral palsy in children born at term include infection, ischaemic stroke, cerebral malformation, genetic disorders, maternal thyroid disease and many other conditions (Badawi *et al.* 1998b; Badawi *et al.* 1998a; MacLennan 1999; Felix *et al.* 2000; Cowan *et al.* 2003). Some of these underlying conditions may make the fetus more susceptible to hypoxia during labour and delivery or even be confounded with intrapartum asphyxia (Tuerlings *et al.* 2002; Cowan *et al.* 2003).

Pattern of brain injury in infants born full-term

Two major patterns of injury are typically identified in full-term neonates with hypoxic-ischaemic encephalopathy (Martin and Barkovich 1995). One pattern, commonly associated with prolonged partial asphyxia, is injury to the watershed regions of the parasagittal cortex and subcortical white matter, with relative sparing of the thalami and basal ganglia. The second pattern is a more central pattern of injury, whereby the basal ganglia, thalami, cerebellum, and brain stem are mostly affected, with relative preservation of the cerebral cortex (although rolandic/sensorimotor cortex and hippocampal injury may be present). This pattern is associated with profound, near terminal asphyxia such as can occur in placental abruption (Barkovich *et al.* 1995; Rutherford *et al.* 1995; Yokochi and Fujimoto 1996; Campistol *et al.* 1999; Sie *et al.* 2000; Volpe 2001b; Mercuri *et al.* 2002; Cowan *et al.* 2003; Sargent *et al.* 2004). Although brain injury in neonates at term was previously thought to be predominantly neuronal, there is increasing evidence from MRI studies that subcortical white matter injury is also a significant contributor to neurodevelopmental problems in infant born at full-term and may accompany both patterns described above (Barkovich *et al.* 1995; Yokochi and Fujimoto 1996; Okumura *et al.* 1997; Mercuri *et al.* 1999).

True perinatal stroke is now also becoming increasingly recognised as a cause of neonatal brain injury, although its probably still underestimated because of variation in the presentation and evaluation (Lynch and Nelson 2001; Nelson and Lynch 2004). The incidence is approximately 1 in 4000 term births (Nelson and Lynch 2004). The middle cerebral artery is most commonly affected due to a thromboembolism from an intracranial or extracranial vessel, the heart, or the placenta (Nelson and Lynch 2004). Risk factors include cardiac disorders, coagulation abnormalities, and infection in the perinatal period (Lynch and Nelson 2001; Nelson and Lynch 2004).

Causes of brain injury in prematurely born infants

Compared to infants born at full-term, the incidence of brain injury is much higher in infants born preterm (Volpe 1998a). All births before 37 weeks of gestation are considered preterm (or premature) according to the World Health Organization (WHO) (11 % of all births), however births before 32 weeks' gestation (2 % of all births) account for the majority of neonatal deaths and neurological disorders (Robertson *et al.* 1992; Tucker and McGuire 2004). Broadly, outcomes improve with increasing gestational age; for any given length of gestation survival is reduced in growth retarded infants. Other factors, including ethnicity and gender also influence survival and the risk of neurological impairment (Stevenson *et al.* 2000; Tucker and McGuire 2004).

Technological, pharmacological and organization advances in perinatal medicine such as regionalization of specialist perinatal care have substantially improved survival, with survivors born as early as 23-24 weeks gestation (Allen *et al.* 1993; Goldenberg and Rouse 1998). These advances include antenatal corticosteroid administration, new mechanical ventilation techniques and exogenous-surfactant therapy. For example, survival of infants born at 25 weeks of gestation is now approximately 80% compared to just 5% in the 1960's (Allen *et al.* 1993).

However, this improved survival has not been accompanied by a proportionate improvement in age specific morbidity and mortality. Neurodevelopmental complications such as cerebral palsy, blindness and/or deafness and mental retardation are common in these children (Hagberg *et al.* 1989; Pharoah *et al.* 1996; Clark and Hankins 2003). twelve to thirty-five percent of surviving infants born at 25 weeks' gestation have severe complications (Hack and Fanaroff 2000; Wood *et al.* 2000). The incidence of cerebral palsy in developed countries is around 2 per 1000 life births and has not changed significantly in the last 2 decades (Hagberg *et al.* 2001; Nelson 2003). Approximately 10% of children with cerebral palsy are born extremely preterm (under 28 weeks), 18% are very preterm (28–31 weeks), 15% are moderately preterm (32–36 weeks), and 58% are born at term (>37 weeks), according to a recent study by Hagberg *et al.* (Hagberg *et al.* 2001). More subtle disabilities, such as learning difficulties at school and behavioral difficulties occur in a majority of very premature infants (Hack *et al.* 2002). Thus, despite all advances and improved survival rates the percentage of prematurity-associated neurologic handicaps has not changed significantly over the years (Hack and Fanaroff 2000).

The question arises whether the adverse neurodevelopmental outcome associated with prematurity is primarily due to the gestational age itself or whether outcome depends primarily on the presence of acquired brain lesions. Recent studies indicate that most cases of adverse neurodevelopmental outcome and symptoms of attention deficit hyperactivity disorder can be predicted on the type of the intracranial lesion rather than simply gestational age (Krageloh-Mann *et al.* 1999; Han *et al.* 2002; Vollmer *et al.* 2003; De Vries *et al.* 2004). However, a correlate for cognitive impairment in prematurely born children without mental retardation cannot be identified using conventional imaging techniques (Krageloh-Mann *et al.* 1999), although sophisticated MRI analyses shows this may be related to a reduced cerebral cortical surface development in very premature infants (Ajayi-Obe *et al.* 2000).

The etiology of brain injury in premature infants is complex and multifactorial (Volpe 2001b). Both clinical and pathological data suggest that exposure to hypoxia-ischaemia and potentially infection-inflammation are important factors in the etiology of brain injury in preterm neonates (Volpe 2001b; Jacobsson *et al.* 2002; Kumazaki *et al.* 2002; Salhab and Perlman 2005). Clinical studies using serial EEG (electro-encephalography) or ultrasound have shown that events leading to brain injury may start antenatally, but the majority of events occur during or shortly after birth (Bejar *et al.* 1988; de Vries *et al.* 1998; Hayakawa *et al.* 1999; Hagberg *et al.* 2001). Exposure to moderate or severe asphyxia is much more common in infants born prematurely than at full-term (MacDonald *et al.* 1980; Low *et al.* 1990; Low 2004; Salhab and Perlman 2005) and may begin before labour (Low *et al.* 2003). Exposure to perinatal asphyxia is highly associated with worse mortality and adverse neurodevelopmental outcome (MacDonald *et al.* 1980; Weindling *et al.* 1985; Low *et al.* 1990; Mires *et al.* 1991; Low *et al.* 1992; Gibbs and Weindling 1994; Low *et al.* 1995a; Spinillo *et al.* 1998; Han *et al.* 2002; Groenendaal *et al.* 2003; Victory *et al.* 2003; Salhab and Perlman 2005). Haemodynamic disturbances in the neonatal period such as hypotension also can contribute to the development of brain injury (Volpe 2001b; Martens *et al.* 2003).

Pattern of brain injury in prematurely born infants

In the premature infant, periventricular leukomalacia (PVL) and periventricular /intraventricular haemorrhage (PVH/IVH) are the leading forms of brain injury. PVH/IVH is most common before 28 weeks of gestation, and the severe forms with extension into the parenchyma are associated with adverse outcome (de Vries and Groenendaal 2002). PVL is the major pattern of injury associated with neurological morbidity in surviving premature infants (Volpe 1998b; de Vries and Groenendaal 2002). The incidence of PVL is highest between 28 and 34 weeks (Volpe 1998b). PVL may either be focal necrotic lesions in periventricular white matter (cystic PVL) and/or more diffuse cerebral white matter injury. It is estimated that time of injury to the time of cavitation is 2-3 weeks (de Vries *et al.* 1992). Deep focal periventricular lesions are associated with spastic diplegia and cerebral visual impairment, whereas diffuse PVL is hypothesised to be associated with cognitive and behavioral deficits (Volpe 2001a). The mechanism of this surprising association is still unclear, but is most likely mediated through subsequent impaired development of the grey matter regions, including the cortex and deep nuclei (Inder *et al.* 1999; Volpe 2005). This impaired development may be in part due to the often unappreciated hypoxic-ischaemic injury to the subcortical regions, and partly due to chronic transformations secondary to initial injury (Barkovich and Sargent 1995; Yokochi 1997; Paneth 1999; Lin *et al.* 2001; Volpe 2001b; Volpe 2005).

EXPERIMENTAL ANIMAL STUDIES OF PERINATAL ISCHAEMIA-REPERFUSION INJURY

In order to understand and potentially treat the pathophysiological processes after hypoxia-ischaemia that lead to tissue damage, a range of specialised models are required.

Models of hypoxia-ischaemia

The choice of experimental approach should be driven by the specific research question asked (Gunn *et al.* 1997a). In general, experimental models of perinatal brain injury can be divided into pathophysiological and functional in nature. Pathophysiological models attempt to mimic potential systemic factors in the human that lead to brain injury, such as asphyxia (Hagberg *et al.* 2002). In contrast, functional models focus on the cerebral injury itself, for example by inducing cerebral ischaemia by carotid artery

occlusion. Functional models therefore differ from clinical asphyxia with respect that they lack multi-organ injury, which is present in all the infants with severe asphyxial brain injury and which may influence outcome.

Use of non-human primates is very expensive and has ethical implications, and thus are seldom used in most countries. Small functional animal models are extensively used because they are cheap, but have considerable disadvantages, which include limited access for continuous physiological monitoring (blood pressure, bloodflow, temperature) or blood sampling (Hagberg *et al.* 1997). The widely used Vannucci-Rice model (unilateral carotid occlusion with inhalational hypoxia) produces a stroke like pattern with an essentially bimodal incidence of damage (Hagberg *et al.* 1997; Ashwal and Pearce 2001).

Physiological models: the fetal sheep

Fetal sheep are relatively precocial at birth, i.e. show more advanced development than human infants (Dobbing and Sands 1979), they share many developmental features with the human fetus, making the chronically instrumented fetal sheep preparation (figure 1) an excellent model to study fetal cardiovascular, neural and endocrine (patho)physiologic processes in detail. Many studies in fetal sheep have examined the fetal cardiovascular and cerebrovascular responses to (isocapnic) hypoxia (Sheldon *et al.* 1979; Iwamoto *et al.* 1989; Jensen and Berger 1991; Giussani *et al.* 1997; Hanson 1997; Bennet *et al.* 1998; Hunter *et al.* 2003b). These studies have gained important insights in the fetal responses and the mechanism controlling these responses to isolated fetal hypoxia, however, this insult is not typically associated with neurological or organ injury (Gunn *et al.* 1991). Therefore, several pathophysiological fetal sheep models of asphyxia have been developed whereby asphyxia is induced by inflation of an occluder around the umbilical cord, producing either continuous or intermittent occlusion (Itskovitz

Figure 1: the chronically instrumented fetal sheep preparation.

et al. 1983; Mallard *et al.* 1992; Mallard *et al.* 1994; Richardson *et al.* 1996; De Haan *et al.* 1997; Keunen *et al.* 1997; Bennet *et al.* 1999; Gardner *et al.* 2001). Other models have been developed, for example using an occluder around the maternal uterine or common internal iliac artery (Muijsers *et al.* 1990; Jensen *et al.* 1991; Ball *et al.* 1994a; Rees *et al.* 1997). These models differ in the pattern and severity of brain injury, depending on the fetal gestational age, duration of occlusion, and whether fetal hypotension was induced (Gunn *et al.* 1997a; Gunn *et al.* 2001).

An alternative but more functional ischaemia-reperfusion model in near term fetal sheep, is cerebral ischaemia induced by bilateral occlusion of the carotid arteries for 30 minutes (Williams *et al.* 1990; Gunn *et al.* 1997b). This model induces predominantly parasagittal neuronal and white matter injury, but also neuronal injury to the striatum and hippocampus (Williams *et al.* 1990; Gunn *et al.* 1997b; Guan *et al.* 2001; Petersson *et al.* 2002).

PATHOPHYSIOLOGY OF ISCHAEMIA-REPERFUSION BRAIN INJURY

Using such models of perinatal hypoxia-ischaemia, significant advances have been made in the understanding of the pathophysiological mechanisms leading to brain damage in the last few decades. The key concept that is now overwhelmingly established is that cerebral injury following a hypoxic-ischaemic insult is an evolving process, with cell death occurring well after the initial insult (Kirino 1982; Williams *et al.* 1993; du Plessis and Volpe 2002). This concept is critical as it suggests that treatment can still be effective for some time after the primary event has occurred.

Phases of evolving of cerebral injury following hypoxia-ischemia

Figure 2: Phases of cerebral injury following a severe hypoxic-ischaemic insult, adapted from (Gunn 2000).

Phases of evolving cerebral injury

Studies in near term animals have delineated several important phases of evolving injury. These events are illustrated in figure 2. During the primary phase, i.e. the actual hypoxic-ischaemic event, cerebral energy failure develops as shown by magnetic resonance spectroscopy (MRS) (Lorek *et al.* 1994). There is depolarisation of cells, leading to severe cytotoxic edema, and initiation of a complex cascade of damaging processes including extracellular accumulation of excitatory amino acids ('excitotoxins'), such as glutamate (Williams *et al.* 1991; Abi Raad *et al.* 1999). The resulting "overstimulation" of neuronal glutamate receptors, particularly the N-methyl-D-aspartate (NMDA) receptor leads to an increase in intracellular calcium through receptor-gated ion channels, resulting in metabolic derangements and generation of reactive oxygen species (ROS) and nitric oxide (NO) (Lee *et al.* 2000).

Following reperfusion, there is a rapid recovery of cellular oxidative and energy metabolism, with resolution of the acute cellular hypoxic-depolarization and cell swelling (Williams *et al.* 1991; Abi Raad *et al.* 1999). The initial cell death following reperfusion has a 'necrotic' appearance (Northington *et al.* 2001b). This may be followed by a latent phase, in which there is nearly complete normalization of oxidative cerebral energy metabolism, with similar recovery of cell swelling but depressed electroencephalogram (EEG) activity and a secondary fall in cerebral blood flow (Williams *et al.* 1991; Lorek *et al.* 1994; Abi Raad *et al.* 1999). During this latent phase there is activation of pro-inflammatory cytokines, such as IL-1 and TNF- α (Silverstein *et al.* 1997; Bona *et al.* 1999). It is highly likely that the processes active during the latent period involve activation of the intracytoplasmic phase of programmed cell death (Samejima *et al.* 1998; Earnshaw *et al.* 2000; Kaufmann *et al.* 2000; Susin *et al.* 2000).

The latent phase may be followed by *secondary* deterioration phase 6 to 15 hours after the acute asphyxial event (Gunn 2000). This phase is marked by the onset of seizures, secondary cytotoxic edema as measured by cerebral impedance (Williams *et al.* 1991; Abi Raad *et al.* 1999). Cerebral blood flow in the delayed phase is markedly increased (Abi Raad *et al.* 1999). Studies using NIRS also show a delayed increase in cerebral blood volume, indicating vasodilation (Marks *et al.* 1996). At the cellular level there is an accumulation of toxic metabolites, such as the excitatory amino acid glutamate, impaired intracellular calcium turnover, activation of microglia, iron accumulation and overproduction of free-radicals (Bona *et al.* 1999; Gunn 2000). Cytochrome c concentrations as shown by NIRS progressively fall, indicating impaired mitochondrial respiration (Marks *et al.* 1996; Chang *et al.* 1999), with a corresponding secondary impairment of oxidative metabolism (Lorek *et al.* 1994). In asphyxiated infants the degree of the delayed energy failure closely correlates with neurodevelopmental impairment at 1 and 4 years of age (Roth *et al.* 1997).

Cell death

Ultimately these events lead to delayed cell death. Critical processes in the programmed cell death are in progress from as early as 6 h post-insult, including elevation of the Fas death receptor protein, accumulation of cytosolic cytochrome c, caspase-8 activation, and perinuclear clustering of mitochondria (Northington *et al.* 2001a). Caspase 3 activation, showing the onset of the execution phase of apoptosis, occurs from 24 h post-insult (Nakajima *et al.* 2000). Histologically, delayed cell death may have either an apoptotic or necrotic appearance (Beilharz *et al.* 1995; Colbourne *et al.* 1999b; Northington *et al.* 2001b). However, appearance is not a good guide to whether apoptotic processes are or are not involved, and indeed the distinction between necrosis and apoptosis in the delayed phase does not appear to be as clear as once thought. Instead, an 'apoptotic-necrotic continuum' has recently been described on electron microscopy studies (Martin 2001). Compared to the adult brain, apoptotic mechanisms appear to be more important after hypoxia-ischaemia in the immature brain. As caspase-3 is expressed until at least 7 days post-insult, apoptosis is likely to have a prolonged role in neonatal cerebral injury (Nakajima *et al.* 2000).

Temperature

There is now strong experimental evidence that changes in cerebral temperature either during hypoxia-ischaemia or in the latent phase critically modulate the extent of brain injury (Busto *et al.* 1987). Hyperthermia either during or following an hypoxic-ischaemic event is associated with worse outcome in both animals and humans, while hypothermia generally improves functional and structural outcome (Busto *et al.* 1987; Baena *et al.* 1997; Lieberman *et al.* 2000; Suzuki *et al.* 2000; Tomimatsu *et al.* 2003). An explanation for the epidemiological link between evidence of infection, which typically involves maternal pyrexia and adverse neonatal outcomes may be that high cerebral temperature renders the fetal brain much more susceptible to even mild hypoxia-ischaemia (Tomimatsu *et al.* 2003), as can occur in normal labour and delivery. For example, hyperthermia has been shown to prevent the protective metabolic and cerebral flow responses during hypoxia (Suzuki *et al.* 2000) and to induce earlier and stronger caspase-3 activation following ischaemia (Tomimatsu *et al.* 2003). Alternatively of course elevated levels of cytokines, either as a result of ischaemia or infection/fever may represent a final common pathway leading to brain injury (Shalak and Perlman 2002).

Endocrine and auto/paracrine responses

Apart from secondary damaging processes following ischaemia-reperfusion, systemic and auto/paracrine responses from neurotrophic factors such as insulin growth factor (IGF)-I and transforming growth factor β (TGF β) may play a role in reducing brain injury (Beilharz *et al.* 1998; Gluckman *et al.* 1998; Gluckman *et al.* 2001). Inhibition of apoptosis is one of the known important functions of IGF-I following ischaemia-reperfusion (Guan *et al.* 2003). The role of circulating IGF following ischaemia-reperfusion is less clear. Local induction of insulin-like growth factor binding proteins appear to be involved in the transport of IGF-I to the site of injury (Guan *et al.* 1996). Activation of the hypothalamic-pituitary-adrenal (HPA) axis may have an important cardiovascular role, but adverse effects of cortisol on the development of brain injury has also been suggested (Fassbender *et al.* 1994).

Maturational factors

The initiating mechanisms of brain injury, principally ischaemia and reperfusion, and the final common pathways to cell death are similar in term and preterm infants (Inder and Volpe 2000). Thus the precise mechanisms of the much greater prevalence and unique patterns of brain injury in prematurely born infants are not fully understood. One likely explanation for the increased prevalence is that events that initiate injury, such as hypoxic-ischaemic events occur more frequent in prematurely born infants than infants born at term (MacDonald *et al.* 1980; Low *et al.* 1990; Low 2004). Nevertheless, it has also been postulated that the preterm fetus or infant is intrinsically more vulnerable to hypoxic-ischaemic events due to its immaturity (Volpe 2001b). Several maturational factors influencing overall vulnerability and differences in the pattern of cellular injury have been suggested. These include anatomical, cellular and physiological factors.

Anatomical factors that may be involved in the maturation dependent vulnerability of the preterm fetus include the maturation of the cerebral vascular system (both arterial and venous), myelination, and changes in neurotransmitters and their receptors. The vascular supply to periventricular white matter is in an incomplete state of development in preterm infants, making it a vascular watershed zone, particularly vulnerable to cerebral hypoperfusion (Volpe 2001a). The regions of hypoxic-ischaemic brain injury in term infants correspond more to the mature intervascular watershed zones, including parasagittal cortex and the subcortical white matter (Barkovich and Truwit 1990; Volpe 2001b). Regional vulnerability of the brain may also be related to differences in metabolic rate, i.e. the oxygen

and glucose requirement of certain areas and cell types, such that higher obligate rates of oxidative metabolism may be associated with increased vulnerability. Consistent with this hypothesis, in very premature infants (less than 32 weeks), the greatest blood flow and glucose uptake occurs in the thalami and posterior fossa, with comparatively little cortical activity, whereas in the term infant blood flow and glucose uptake increases in the basal ganglia and perirolandic cortex (sensorimotor cortex) (Barkovich and Hallam 1997). There is some evidence that these metabolic differences may be associated with differences in the expression and function of receptors that contribute to ischaemia-reperfusion injury, such as the NMDA receptors (Mishra *et al.* 2001).

Before the onset of myelination, which in the human brain starts at around 32 weeks of gestation, the cerebral white matter predominantly consists of immature oligodendrocytes (Back *et al.* 2001). Back and colleagues have suggested that the late oligodendrocyte progenitors may be selectively vulnerable to hypoxia-ischaemia and free radicals (Volpe 2001a; Back *et al.* 2002). Nevertheless, it is important to accept that there is evidence from other studies that mature, myelinating oligodendrocytes are at least as vulnerable to hypoxia-ischaemia as neurons (Ikeda *et al.* 1998; Petersson *et al.* 2002).

Physiological factors

Several physiological factors have been proposed to play a role in the increased vulnerability of the preterm fetus to organ injury. It has been hypothesized that the “stress responses” of the preterm fetus are immature compared to the near-term fetus (Iwamoto *et al.* 1989). This is posited to compromise the ability of the preterm fetus to respond to severe hypoxia and to contribute to the high rate of hypotension in premature newborns (Bolt *et al.* 2002a; Bolt *et al.* 2002b; Ng *et al.* 2004). This supposedly immature stress response includes both the catecholamine (Cohen *et al.* 1982; Mulder *et al.* 2000) and the hypothalamus-pituitary-adrenal (HPA) axis response of ACTH and cortisol (Rose *et al.* 1981; Challis and Brooks 1989; Matsuda *et al.* 1992; Fraser *et al.* 2001). On the other hand, preterm newborns have been suggested to be able to mount a considerably more vigorous response to inflammatory stimuli than infants born at term (Dammann and Leviton 2000).

The original studies that suggested this immature stress response examined only moderate hypoxic insults. The preterm fetus has a much greater anaerobic reserve and relatively reduced aerobic requirements compared to the term fetus (Dawes *et al.* 1959; Mott 1961; Shelley 1964), making it more tolerant to hypoxia/asphyxia (Mallard *et al.* 1994). There have been no studies examining the HPA response of the preterm fetus to a severe hypoxic challenge sufficiently as to be expected to elicit a near-maximal HPA response. Further, studies in preterm fetal sheep have demonstrated that the preterm fetus has highly adaptive and relatively mature cardiovascular responses to severe asphyxia (Dawes *et al.* 1963; Mallard *et al.* 1994; Bennet *et al.* 1999; Quaedackers *et al.* 2004), and can survive much more prolonged periods of asphyxia than the near-term fetus (Gunn *et al.* 2001).

Preterm fetuses and infants have a smaller range of cerebral blood flow autoregulation compared to term infants (Papile *et al.* 1985; van de Bor and Walther 1991; Volpe 2001a; Muller *et al.* 2002). Especially in sick newborns the cerebral circulation has been proposed to be almost pressure-passive, meaning that each reduction in blood pressure leads to hypoperfusion and ischaemia in the arterial end zones and border zones in cerebral white matter (Gluckman *et al.* 2005), whereas 10 minutes of umbilical cord occlusion in late-gestation fetal sheep leads to hippocampal damage (Mallard *et al.* 1994) and in term monkeys damage is detected after only 7 minutes (Ranck and Windle 1959). Thus the ability to survive extra-ordinarily prolonged exposure of the brain to hypoxia-ischaemia may paradoxically allow the ultimate development of severe injury to the brain in the preterm fetus (Mallard *et al.* 1994; Gunn *et al.* 2001).

TREATMENT OF HYPOXIC-ISCHAEMIC ENCEPHALOPATHY

Potential treatments

Currently, post-resuscitation treatment of preterm and term neonates at risk for brain injury is only supportive in nature, such as blood pressure support and seizure control (Niermeyer *et al.* 2001). There is a strong need for interventions to reduce brain injury from ischaemia-reperfusion following both term and premature birth. To date there are no approved specific treatments to improve the neurological outcome.

The first hours after asphyxia (the '*latent phase*') appears to represent a key window of opportunity for intervention, prior to the final execution phase of delayed cell death (Gunn 2000). Many different treatment modalities have been or are currently under investigation (Vannucci and Perlman 1997; Dammann and Leviton 2000; Gluckman *et al.* 2001), as listed in table 1. Although drugs to ameliorate brain injury appear effective in animal models of perinatal brain injury, clinical trials have been unsuccessful to date (Gluckman *et al.* 2001).

Table 1: List of potential treatments for HIE under investigation

Hypothermia
Oxygen free radical scavengers, eg. Allopurinol, vitamin K
NO/ NOS inhibitors, eg. Allopurinol, 2-Iminobiotin
Membrane stabilizing agents, eg. Monoganglioside GM-1
Calcium channel blockers, eg. flunarizine
Glutamate Antagonists:
- NMDA receptor antagonists, eg. MK-801, MgSO ₄
- AMPA-kainate receptor antagonists, eg. NBQX
- Metabotropic glutamate receptor antagonists
Anti-inflammatory, eg. IL-1 B receptor antagonist
Growth factors/ anti-apoptotic factors eg. IGF-1, GPE, TGF- β , bFGF
Pro-plasticity

Adapted from: (Vannucci and Perlman 1997; Dammann and Leviton 2000; Gluckman *et al.* 2001; Peeters and van Bel 2001)

Several factors may play a role in the failure of many drugs trials to prevent permanent brain injury. Many previous agents have focussed on treating NMDA-receptor mediated glutamate excitotoxicity. However, clinical use of many NMDA receptor antagonists is limited by adverse effects (Lutsep and Clark 1999). Magnesium sulphate (MgSO₄) is a safer (partial) NMDA receptor antagonist, however it has low potency in the neonatal brain (Chahal *et al.* 1998). In a recent trial, magnesium sulfate given to women immediately before very preterm birth was not effective in reducing the primary end points, i.e. the risk of total pediatric mortality or cerebral palsy among survivors at 2 years of age (Crowther *et al.* 2003).

Another potential factor is that the role of white matter injury in neurodevelopmental outcome was previously under-recognised. Most treatments focussed on treatment of neuronal injury, and may have been less effective for the damaged white matter. For example, NMDA-receptor blockade does not seem to protect against axonal damage (Yam *et al.* 2000). Other neuroprotective agents are only effective during or immediately following ischaemia-reperfusion, limiting their clinical use.

Recently, hypothermia has been demonstrated to improve neurological outcome after circulatory arrest in adults (Bernard *et al.* 2002; Hypothermia After Cardiac Arrest Study Group 2002).

Experimental studies in a number of different animal models have shown good efficacy following ischaemia-reperfusion, even if start of treatment was delayed by several hours after the insult (Gunn 2000). Therefore, hypothermia is now considered one of the most promising treatment modalities to improve neurodevelopmental outcome following neonatal HIE (Du Plessis 1998; Thoresen 1999; Gunn 2000).

HYPOTHERMIA

Hypothermia in neonates is typically defined as a body temperature below 36.5°C. In the studies discussed below, mild hypothermia is defined as a rectal temperature of 34–35 °C and moderate hypothermia as 30–33 °C.

History of hypothermia

The potential therapeutic benefits of cooling were reportedly recognized by Hippocrates for sprains, fractures, tetanus and febrile convulsions (Thoresen 2000). Sir John Floyer (1697) advocated the use of ice cold water to stimulate the onset of respiration in depressed newborn infants (Wyatt and Thoresen 1997). In 1949, Miller was the first to show that survival of newborn guinea pigs under anoxia was increased by hypothermia (Miller 1949). Following on from those observations, Miller and Westin were the first to study the use of hypothermia for “asphyxia neonatorum” (Miller *et al.* 1964; Thoresen 2000). In 1958, they reported induced hypothermia by immersing the 10 severely depressed, term apnoeic infants in a specially constructed cold water bath and stopped when spontaneous respirations commenced or when the rectal temperature approached 27°C. The infants were then dried and allowed to rewarm spontaneously. Only one of the 10 infants died, the remaining 9 infants survived, despite minimum core temperatures of between 23°C and 30°C, and periods of apnoea ranging from 8 to 79 minutes. At follow-up 10 years later, none of the nine infants showed neuromotor impairment or cerebral palsy (Wyatt and Thoresen 1997). Subsequent uncontrolled studies had similar encouraging results. Mortality was the same in all studies, around 12–15%, which was similar to standard care at that time (Thoresen 2000). Russians doctors were the first to describe a more “selective” head cooling technique, using a shower to the head they cooled the head 1.5°C lower than rectal temperature (30°C rectal) (Kopchev 1985).

Despite these positive results, the use of hypothermia following fetal asphyxia fell out of favor because the studies were uncontrolled, and controlled studies in preterm infants reported increased mortality rates with even mild hypothermia (Silverman *et al.* 1958; Buetow and Klein 1964; Day *et al.* 1964).

The mechanisms of action of hypothermia

Scientific interest in the therapeutic properties of hypothermia resurfaced after it was recognised that the temperature of the brain has an important influence on the extent of brain injury following hypoxia-ischaemia (Busto *et al.* 1987; Thoresen and Wyatt 1997; Gunn and Gunn 1998). Recent research in fetal and newborn animal models has focussed on the mechanisms of action of hypothermia and questions of post-ischaemic delay, depth, duration of hypothermia and method of cooling.

Hypothermia *during* hypoxia-ischaemia is likely to work via different mechanisms than delayed, post-ischaemic hypothermia. The protective effects of intra-ischaemic hypothermia is mostly due to a graded reduction in cerebral metabolism which slows cell depolarisation, reduces accumulation of excitotoxic neurotransmitters, and suppresses oxygen free radical release and lipid peroxidation of cell membranes (Gunn and Gunn 1998). The mechanisms of mild delayed hypothermia after severe transient hypoxia-

ischaemia include suppression of post-ischaemic inflammation (Inamasu *et al.* 2000b), including nitric oxide synthesis and induction of microglia (Kumar and Evans 1997), and suppression of apoptosis (Edwards *et al.* 1995; Inamasu *et al.* 2000a). Reducing the duration of post-asphyxial seizures may be an other important mechanism of hypothermia (Thoresen and Whitelaw 2005).22

Optimal timing of hypothermia

Even though cooling during ischaemia or in the immediate resuscitation is effective, it would be almost impossible to test it in clinical practice. Therefore, the effect of delaying hypothermia has been tested at different time points after ischaemic insults in several models, including the fetal sheep and adult gerbil (Colbourne and Corbett 1994; Colbourne and Corbett 1995; Gunn *et al.* 1997b; Gunn *et al.* 1998; Gunn *et al.* 1999a; Colbourne *et al.* 2000). In near term fetal sheep, cooling starting at 90 minutes after reperfusion resulted in dramatic neuroprotection (Gunn *et al.* 1997b). Hypothermia was still partially neuroprotective when started 5.5 hours after reperfusion (6h after start of ischaemia) (Gunn *et al.* 1998), but not neuroprotective when it was initiated at 8.5 h after reperfusion, during the phase of secondary deterioration, indicated by the onset of post-ischaemic seizures (Gunn *et al.* 1999a). It is not known if the window of opportunity for neuroprotection is the same for different areas or cell populations in the brain, such as oligodendrocytes in the white matter.

Depth of hypothermia

The optimal depth of hypothermia required for effective neuronal rescue seems to be between 32 and 34°C (Colbourne *et al.* 1997). Studies by Gunn *et al.* in fetal sheep cooled from 90 minutes after ischaemia show substantial neuroprotection only in fetuses who had a sustained decrease in the extradural temperature to less than 34°C (normal temperature in fetal sheep, 39.5°C) (Gunn *et al.* 1997b). In adult gerbils, cooling to a rectal temperature of 32°C was associated with greater behavioural and histologic neuroprotection than cooling to 34°C (Colbourne and Corbett 1994; Colbourne and Corbett 1995; Colbourne *et al.* 2000). As the core temperature falls below 34°C the risk of complications increases markedly (Gunn and Gunn 1998). The effectiveness of hypothermia may also decrease. For example, in adult dogs, deep hypothermia (15°C) following a cardiac arrest produced detrimental cerebral and cardiac outcomes, whereas mild (34°C) hypothermia improved outcome compared to normothermia (Weinrauch *et al.* 1992). The adverse effects responsible for the impaired effectiveness of greater cooling levels are unknown but are likely to be related to impaired cardiac contractility leading to decreased cardiac output, arterial hypotension, and compromised perfusion (Gunn and Gunn 1998). Consistent with these experimental data, in adults after a cardiac arrest mild hypothermia (34 to 36°C) from 10 minutes until 12 hours after was beneficial (Bernard *et al.* 2002; Hypothermia after Cardiac Arrest Study Group 2002).

Duration of hypothermia

The optimum duration for hypoxic-ischaemic injury may depend on the severity of the injury and the delay before hypothermia is achieved (Colbourne *et al.* 1999a; Gunn 2000). A longer period of cooling may be necessary to compensate for severe injury or a longer delay. Studies in fetal sheep by Gunn *et al.* have used 72 hours of hypothermia, as pilot studies suggested that shorter duration was not effective (Gunn *et al.* 1997b). Nevertheless, shorter durations of hypothermia (even 3 hours) has also been effective in anesthetized neonatal piglets when cooling was started directly following reperfusion (Haaland *et al.* 1997). In gerbils, increasing the duration of hypothermia that started 1 h after ischaemia from 12 to 24 h produced much greater neuroprotection (Colbourne and Corbett 1994). Rewarming after cooling treatment for severe hypoxic-ischaemic encephalopathy is potentially more

complex than induction of cooling, because it might lead to destabilizing changes in neuronal activity (Battin *et al.* 2004). There is evidence for example of rebound seizure activity during rapid rewarming from a 72-hour period of hypothermia in the fetal sheep (Gerrits *et al.* 2005). The optimal rewarming strategy following cooling is unknown, but clinical studies of therapeutic hypothermia typically have rewarmed infants at no more than 0.5°C per hour (Gluckman *et al.* 2005).

Method of cooling

Brain hypothermia can be induced by either cooling the whole body (i.e. lowering core temperature) or by semi-selective cooling of the brain, using a cooling cap around the infant's head. The advantages of whole body cooling are simplicity and that the whole brain, including its deep structures is cooled (Van Leeuwen *et al.* 2000). Conversely, the key disadvantage of whole body cooling is that the risk of systemic complications of hypothermia is likely to be higher (Gunn and Gunn 1998).

Head cooling induces a marked temperature gradient across the brain, with very cool temperatures near the brain's surface and less cool temperatures more deeply in the brain. Mathematical modelling studies suggest that cooling of deep brain structures in humans may not be feasible with selective head cooling (Van Leeuwen *et al.* 2000). However, Tooley *et al.* have shown in the piglet that it is possible by means of a head-cooling cap to cool the basal ganglia substantially more than the body (3 to 5°C) for a 24-hour period while keeping the core temperature mildly hypothermic (Tooley *et al.* 2002) or even normothermic (Tooley *et al.* 2005).

Physiological responses to hypothermia

Induced hypothermia is associated with many cardiovascular and metabolic responses. These responses are important to understand as they may have implications for the therapeutic effects of hypothermia. Hypothermia leads to rapid peripheral vasoconstriction, also termed centralization of blood flow (Gunn and Gunn 1998; Gunn and Battin 2000). This vasoconstriction is mediated via both central and skin thermoreceptors (Gordon and Heath 1986), whereby in man, skin temperatures only contribute 20% to the onset threshold for vasoconstriction during cooling (Cheng *et al.* 1995). Hypothermia also has well known cardiac effects, which include sinus bradycardia, prolonged PR interval, prolonged QT interval and Osborn or J waves visible on electrocardiography, and in some cases cardiac arrhythmias. All cardiac effects of hypothermia are reversible with rewarming (Gunn *et al.* 1999b).

The other major response to hypothermia is increased heat production. This response is typically seen at a similar but not identical temperature threshold to vasoconstriction (Gunn and Battin 2000). Unlike adults, infants preferentially use brown fat (nonshivering thermogenesis) rather than shivering to produce heat (Gunn and Gluckman 1995). Factors that inhibit nonshivering thermogenesis or muscle activity, such as hypoxia and many anesthetic or sedative agents, result in reduced endogenous heat production. Post-asphyxial seizures may also be an important source of both cerebral and peripheral thermogenesis (Jordan 1994; Gonzalez *et al.* 2005).

Recently, a study examining the effects of hypothermia in unsedated piglets found that shivering was associated with elevated blood levels of cortisol, indicating that feeling cold induced a stress response that potentially interfered with the neuroprotective effects of hypothermia (Thoresen *et al.* 2001). Although not conclusive, this study points to the need of studying the stress response in studies of hypothermia, and to evaluate whether sedation might be necessary in infants treated with hypothermia. However, the effects of hypothermia are different in humans (and sheep) as compared to piglets. Human infants and sheep both predominantly use non-shivering thermogenesis and do not shiver until brown fat stores have been depleted (Gunn and Gluckman 1995). Nevertheless, further

studies are warranted to examine whether there is a stress response to hypothermia and whether this impairs the neuroprotective effects of hypothermia. Finally, the effects of hypothermia on other endocrine systems such as IGF and its binding proteins are largely unknown currently. This is of interest as IGF has important functions in metabolic regulation, growth, and organ function and development.

Clinical experience

In the words of late William A. Silverman "The hoary intervention for total-body hypothermia was first mooted 300 years ago. A proper trial (with concurrent controls) of the latter intervention should have been carried out at least 35 years ago: the mills of the gods do, it seems, grind very slowly indeed." (Silverman 1998).

After this considerable delay, hypothermia has now been the subject of several large randomized controlled trials both in adults and newborns to evaluate its efficacy. Trials in adults demonstrated that hypothermia initiated immediately after cardiac arrest improved outcomes (Bernard *et al.* 2002; Hypothermia after Cardiac Arrest Study Group 2002). The first large RCT in term infants with hypoxic-ischaemic encephalopathy (HIE) was the CoolCap trial (Gluckman *et al.* 2005). Seventy-two hours of selective head cooling with mild systemic hypothermia (rectal temperature $34.5 \pm 0.5^\circ \text{C}$) reduced death or disability in the infants with less severe electroencephalographic changes at entry of the trial, but there was no significant benefit in infants with the most severe electroencephalographic changes. Cooling was not associated with any adverse effects. Inder *et al.* demonstrated that systemic hypothermia led to less cortical gray matter injury on magnetic resonance imaging (MRI) (Inder *et al.* 2004). Further randomized-controlled trials are underway (Jacobs *et al.* 2004).

OBJECTIVES OF THE STUDIES IN THIS THESIS

This review has highlighted many aspects of the pathogenesis and treatment of perinatal asphyxia. Many questions remain unanswered. For example, hypothermia is a promising treatment for hypoxic-ischaemic encephalopathy in term neonates (Gunn 2000): however, there is no information on whether it is effective in the preterm brain, and in particular whether delayed hypothermia can reduce white matter loss. Further, there is surprisingly little information on how it affects recovery from severe systemic hypoxia, and none in preterm models.

Thus, overall aim of this thesis was to better understand the endocrine and cardiovascular responses of the fetus to severe hypoxia (asphyxia) and to dissect the effects of rescue therapy with head cooling on neural and systemic recovery from such severe hypoxia.

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CHAPTER 2

CORTISOL AND ACTH RESPONSES TO SEVERE ASPHYXIA IN PRETERM FETAL SHEEP.

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ABSTRACT

It has been hypothesized that the hypothalamic-pituitary-adrenal (HPA) axis is immature in the preterm fetus and that this compromises their ability to adapt to hypoxic stress; however, there are few direct data. We therefore examined the effects of asphyxia on HPA responses in chronically instrumented preterm fetal sheep (104 days of gestation; term is 147 days), allocated to a sham control group ($n = 7$) or 25 min of complete umbilical cord occlusion ($n = 8$), followed by recovery for 72 h. During umbilical cord occlusion there was a rapid rise in ACTH levels (230.4 ± 63.5 versus 14.1 ± 1.8 ng ml⁻¹ in sham controls, 16-fold) and cortisol levels (7.4 ± 4.9 versus 0.2 ± 0.1 ng ml⁻¹, 31-fold), with further increases after release of cord occlusion. ACTH levels were normalized by 24 h, while plasma cortisol levels returned to sham control values 72 h after asphyxia. Fetal arterial blood pressure was elevated in the first 36 h, with a marked increase in femoral vascular resistance, and correlated positively with cortisol levels after asphyxia ($P = 0.05$). In conclusion, the preterm fetus shows a brisk, substantial HPA response to severe hypoxia.

INTRODUCTION

Both human and ovine fetuses show activation of the hypothalamic-pituitary-adrenal (HPA) axis in late gestation (in the ovine fetus from 120 days gestation onwards), which contributes to organ maturation (Challis & Brooks, 1989). Activation of the fetal HPA axis also occurs in response to hypoxic stress, as shown by increased plasma concentrations of ACTH and cortisol, which in turn support acute cardiovascular and metabolic adaptations (Giussani *et al.* 1994c). It has been proposed that these two phenomena are linked, i.e. that the rise in basal cortisol levels (Whittle *et al.* 2001) and increased levels of mRNA for the adrenal ACTH receptor near term (Fraser *et al.* 2001) predict a maturational increase in the responsiveness of the fetal adrenal gland to hypoxic stress and ACTH (Challis & Brooks, 1989).

Consistent with this proposal, the ACTH and cortisol responses of preterm fetuses (before 120 days gestation) to moderate hypoxia (Akagi & Challis, 1990b; Matsuda *et al.* 1992), brief umbilical cord occlusion (Green *et al.* 2000) or hypotension (Rose *et al.* 1978, 1981) were markedly less than those reported in the late gestation fetus (Rose *et al.* 1978, 1981; Jackson *et al.* 1989; Akagi & Challis, 1990b; Richardson *et al.* 1996; Carmichael *et al.* 1997; Unno *et al.* 1997; Fraser *et al.* 2001; Gardner *et al.* 2001). Strikingly, although some studies in preterm fetuses have reported a rise in ACTH, albeit to lower peak levels than in late gestation, they found no rise in cortisol levels (Rose *et al.* 1981; Akagi & Challis, 1990b; Green *et al.* 2000). For example, at 112–116 days gestation, Green *et al.* (2000) found a significant rise in ACTH levels, but no increase in cortisol levels after either the first or seventh episode of a series of 90 s umbilical cord occlusions repeated at an interval of 30 min. These data suggested that it is the adrenocortical response to ACTH that is immature in the preterm fetus (Rose *et al.* 1982; Green *et al.* 2000), possibly due to reduced expression of the adrenal ACTH receptor (Fraser *et al.* 2001). It is hypothesized that this apparent profound adrenocortical immaturity compromises the ability of the preterm fetus to maintain homeostasis during hypoxic stress, contributing to hypotension and other morbidity after birth (Bolt *et al.* 2002; Ng *et al.* 2004). This has important implications, since severe hypoxia is more common in infants born prematurely than at term (Low, 2004), and frequently occurs before the onset of labour (Low *et al.* 2003).

However, an important methodological issue for studies of hypoxic stress is that preterm fetuses have a much greater anaerobic capacity and lower aerobic requirements than at term (Dawes *et al.* 1959; Mott, 1961; Shelley, 1964). These considerations led us to hypothesize that previous experiments in the preterm fetus using mild or brief hypoxic insults may not have been sufficient to elicit a near maximal HPA response, and that responses comparable with that seen at term could be elicited by a severe insult. We therefore examined the time course of changes in the HPA axis after severe asphyxia in the preterm fetus at 70% of gestation (term is 147 days gestation), equivalent to the human fetus of 30–32 weeks of gestation (McIntosh *et al.* 1979).

METHODS

Experimental preparation

All procedures were approved by the Animal Ethics Committee of The University of Auckland. Groups of Suffolk ewes were time mated (three days) with Romney rams in a ratio of 5:1 at fortnightly intervals. The ewes were individually identified by ear tags. Ewes who did not conceive after 48 h with the ram were not re-bred. Pregnancy was confirmed by ultrasound at 40 days. Fifteen singleton fetal sheep were instrumented at 97–99 days of gestation (term is 147 days) as previously described (Bennet *et al.* 1999). Food, but not water was withdrawn 18 h before surgery. Ewes were given 5 ml of Streptocin (procaine

penicillin, 250 000 IU) and dihydrostreptomycin (250 mg ml⁻¹; Stockguard Laboratories Ltd, Hamilton, New Zealand) intramuscularly for prophylaxis 30 min prior to the start of surgery. Anaesthesia was induced by i.v. injection of Alfaxan (alphaxalone, 3mg kg⁻¹; Jurox, Rutherford, NSW, Australia), and general anaesthesia maintained using 2–3% halothane in O₂. Ewes were intubated, and the depth of anaesthesia, maternal heart rate and respiration were constantly monitored by trained anaesthetic staff.

Using sterile techniques, catheters were placed in the left fetal femoral artery and vein, right brachial artery, and the amniotic sac. An ultrasound blood flow probe (size 2R; Transonic Systems Inc., Ithaca, NY, USA) was placed around the right femoral artery to measure femoral blood flow (FBF). A stainless-steel electrode (CoonerWire, Chatsworth, CA, USA) was placed across the fetal chest to measure the fetal electrocardiogram (ECG). An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA, USA; Bennet *et al.* 2000; Quaedackers *et al.* 2004a). All fetal leads were exteriorized through the maternal flank and a maternal long saphenous vein was catheterized to provide access for postoperative care. After surgery, all exteriorized catheters and leads were kept in an enclosed Perspex box suspended from the side of the Ewe's metabolic cage. Antibiotics (gentamicin, 80 mg; Pharmacia and Upjohn, Rydalmere, NSW, Australia) were administered into the amniotic sac prior to closure of the uterus.

Postoperatively all sheep were housed in separate metabolic cages with access to water and food *ad libitum*, together in a temperature-controlled room (16 ± 1°C, humidity 50 ± 10%) with a 12 h:12 h light:dark cycle. A period of 4–6 days postoperative recovery was allowed before experiments commenced, during which time antibiotics were administered to the ewe daily for four days i.v. (benzylpenicillin sodium, 600 mg; Novartis Ltd, Auckland, New Zealand, and gentamicin, 80 mg). Fetal catheters were maintained patent by continuous infusion of heparinized saline (20 U ml⁻¹ at 0.15 ml h⁻¹) and the maternal catheter maintained by daily flushing.

Experimental design

Experiments were conducted at 103–104 days gestation (70% of gestation). Fetal arterial and venous pressures, corrected for maternal movement by subtraction of amniotic fluid pressure, FBF and fetal heart rate (FHR) derived from the fetal ECG were recorded continuously from 12 h before occlusion to 72 h after occlusion. Data were stored to disk by custom software for off-line analysis (Labview for Windows; National Instruments Ltd, Austin, TX, USA). Fetuses were randomly assigned to the sham control ($n = 7$) or the asphyxia group ($n = 8$). Fetal asphyxia was induced by rapid inflation of the umbilical cord occluder for 25 min with sterile saline of a defined volume known to completely inflate the occluder and totally compress the umbilical cord, as determined in pilot experiments with a Transonic flow probe placed around an umbilical vein (Bennet *et al.* 1999). The duration of occlusion was chosen as one that we have previously reported to represent an acute, severe, near-terminal insult but which could be survived without postasphyxial cardiac support (Bennet *et al.* 2000; Quaedackers *et al.* 2004a,b). Successful occlusion was confirmed by observation of a rapid onset of bradycardia with a rise in mean arterial pressure (MAP), and by pH and blood gas measurements.

Fetal arterial blood samples were taken from the brachial catheter at 15 min prior to umbilical cord occlusion or sham occlusion, 5 and 20 min during occlusion or sham occlusion and 2, 4, 6, 10, 24, 48 and 72 h after occlusion or sham occlusion for determination of blood gases, acid–base balance (Ciba-Corning Diagnostics 845 blood gas analyser and co-oximeter; EastWalpole, MA, USA) and for glucose and lactate determination (YSI model 2300; Yellow Springs Instruments, Yellow Springs, OH, USA). Blood samples (1 ml) were collected at the same times (except for 5 min of occlusion), transferred immediately to chilled test tubes and centrifuged at 4°C for 15 min (1811 g). Plasma was stored at -20°C for subsequent adrenocorticotrophic hormone (ACTH) and cortisol assay. After the last

blood sample, ewes and fetuses were killed by an intravenous overdose of pentobarbitone sodium (9 g) to the ewe (Pentobarb 300; Chemstock International, Christchurch, New Zealand).

Hormone analysis

Fetal plasma ACTH and cortisol levels were measured using specific radioimmunoassays (RIA) established and validated for ovine plasma (Fraser *et al.* 1997). Total immunoreactive cortisol concentrations were determined in triplicate after extraction with diethyl ether by an in-house RIA validated for use with maternal and fetal ovine plasma. The antiserum to cortisol was raised in rabbits against Cortisol-3-O-carboxymethyl-oximebovine serum albumin (Cortisol 3-CMO: BSA) and was used at a final dilution of 1:19 200. The cross-reactivity of the antiserum at 50% binding with other relevant steroid related compounds was 3.3% 11-deoxycortisol, 0.18% cortisone, 0.5% corticosterone, 0.015% progesterone and 0.002% 11 α -hydroxyprogesterone. No detectable crossreactivity was observed for 17 α -hydroxypregnenolone, pregnanediol, 21-deoxycortisone, aldosterone, cholesterol or dexamethasone. The lower limit of detection was 10 pg per tube (0.13 ng ml⁻¹); samples containing <0.13 ng ml⁻¹ were given this value for the purposes of analysis. The intra- and interassay coefficients of variations were 3.68 and 3.97%, respectively, at the cortisol concentrations determined in plasma.

Immunoreactive concentrations of ACTH were measured in duplicate using a commercially available ¹²⁵I RIA kit (24130, DiaSorin, Stillwater, MN, USA) previously validated for use with both fetal and maternal ovine plasma. The intra-assay and interassay coefficients of variation were 9.7 and 12.8%, respectively. The mean sensitivity of the ACTH assay was 9.7 pg ml⁻¹; samples containing <9.7 pg ml⁻¹ were given this value for analysis.

Data analysis and statistics

Off-line analysis of the physiological data was performed using customized Labview programs (Labview, National Instruments, Austin, TX, USA). The effect of asphyxia was evaluated by analysis of variance (ANOVA; SPSS v10, SPSS Inc., Chicago, IL, USA). Femoral vascular resistance (FVR) was calculated using the formula (mean arterial pressure – mean venous pressure)/absolute femoral artery blood flow (mmHg min ml⁻¹). The baseline period was taken as the mean of the 12 h before occlusion. Differences between groups were determined by analysis of variance, adjusted using baseline levels as a covariate (ANCOVA). Where a significant effect of treatment group or an interaction between time and group was found, *post hoc* comparisons were undertaken using the least significant difference test. The within-subjects relationship between fetal mean arterial blood pressure and cortisol levels after asphyxia was examined using the method of Bland&Altman (1995). Statistical significance was accepted at $P < 0.05$. Data are presented as means $\pm$ S.E.M.

RESULTS

Blood composition measurements

Twenty-five minutes of umbilical cord occlusion was associated with marked fetal hypoxia, hypercapnia and mixed respiratory and metabolic acidosis (Table 1), which resolved after release of occlusion.

Fetal cardiovascular responses during and immediately after umbilical cord occlusion

There were no significant baseline differences between the sham and asphyxia groups in any parameter measured. At the onset of cord occlusion there was a rapid fall in FHR, with a sustained bradycardia throughout the period of occlusion (nadir at the end of occlusion 57.4 ± 3.8 beats min⁻¹ *versus* sham

Table 1. Fetal arterial pH, blood gas, glucose and lactate values for sham control (S) and asphyxia (A) groups before umbilical cord occlusion (baseline), after 20 min of occlusion or sham occlusion, and 2, 4, 6, 24, 48 and 72 h postocclusion

	Baseline	Asphyxia	2 h	4 h	6 h	24 h	48 h	72 h
pH								
S	7.39 ± 0.00	7.38 ± 0.00	7.39 ± 0.00	7.39 ± 0.00	7.38 ± 0.00	7.38 ± 0.00	7.37 ± 0.00	7.37 ± 0.01
A	7.37 ± 0.00	6.82 ± 0.03*	7.34 ± 0.01	7.40 ± 0.00	7.41 ± 0.00	7.37 ± 0.00	7.38 ± 0.00	7.38 ± 0.00
P_aCO₂ (mmHg)								
S	48.8 ± 1.3	48.3 ± 1.5	46.8 ± 1.0	47.3 ± 0.8	47.8 ± 1.2	45.0 ± 1.7	48.0 ± 1.1	46.3 ± 1.6
A	48.2 ± 1.0	142.2 ± 6.0*	46.8 ± 1.0	47.8 ± 1.2	45.6 ± 1.4	47.4 ± 1.1	47.2 ± 0.9	46.5 ± 1.2
P_aO₂ (mmHg)								
S	22.5 ± 0.9	22.7 ± 0.9	22.1 ± 1.0	22.6 ± 1.0	22.3 ± 1.0	22.2 ± 2.1	23.4 ± 1.5	23.5 ± 1.4
A	23.0 ± 0.9	8.7 ± 0.8*	26.2 ± 1.3*	24.8 ± 1.0	24.8 ± 0.9	26.6 ± 1.3	28.0 ± 1.3*	28.3 ± 3.6*
Glucose (mmol l⁻¹)								
S	1.03 ± 0.10	1.03 ± 0.11	1.01 ± 0.12	1.10 ± 0.06	1.11 ± 0.10	1.20 ± 0.12	1.20 ± 0.10	1.20 ± 0.10
A	1.02 ± 0.10	0.67 ± 0.10*	1.24 ± 0.10	1.30 ± 0.10*	1.40 ± 0.10*	1.50 ± 0.14*	1.30 ± 0.10	1.23 ± 0.10
Lactate (mmol l⁻¹)								
S	0.73 ± 0.10	0.73 ± 0.11	0.66 ± 0.12	0.75 ± 0.04	0.75 ± 0.15	0.64 ± 0.10	0.83 ± 0.11	0.90 ± 0.12
A	0.72 ± 0.03	6.46 ± 0.17*	3.6 ± 0.52*	2.48 ± 0.44*	1.86 ± 0.24*	1.00 ± 0.12*	0.78 ± 0.10	0.88 ± 0.10

Values are expressed as means ± S.E.M. P_aCO₂, fetal arterial partial pressure of CO₂; P_aO₂, fetal arterial partial pressure of O₂. *P < 0.05 compared to sham control.

188.9 ± 3.3 beats min⁻¹, *P* < 0.05; Fig. 1). Following the end of occlusion FHR rapidly increased to a peak of 235.4 ± 5.4 beats min⁻¹ at 8 min postocclusion (*P* < 0.001) before returning to baseline values by 1 h. MAP was initially elevated at the onset of occlusion (peaking at 4 min of occlusion at 57.0 ± 1.0 mmHg *versus* 37.3 ± 0.2 mmHg in sham controls, *P* < 0.001, Fig. 1). MAP then fell, to a nadir of 10.3 ± 0.6 mmHg, *P* < 0.05 [Fig. 1). Following the end of occlusion MAP rapidly recovered, with an initial transient period of hypertension (peaking at 8 min at 49.9 ± 2.7 mmHg, *P* < 0.05) before falling slightly, but remaining elevated through the first hour (*P* < 0.05).

At the onset of occlusion FBF fell rapidly to a nadir of 0.6 ± 0.6 ml min⁻¹ *versus* 10.8 ± 0.2 ml min⁻¹ by 4 min of occlusion (*P* < 0.05, Fig. 1). FBF then transiently rose to approximately 50% of baseline, before falling again to a final nadir of 2.1 ± 0.5 ml min⁻¹ at the end of occlusion. Following the end of occlusion there was an initial increase in FBF back to baseline values by 5 min, followed by a secondary fall (nadir at the end of the first hour postocclusion 3.8 ± 0.7 ml min⁻¹, *P* < 0.05, Fig. 1). FVR was significantly elevated at the onset of occlusion (peaking at 4 min of occlusion at 207.4 ± 70.2 mmHg min ml⁻¹ *versus* 3.7 ± 0.04 mmHg min ml⁻¹, *P* < 0.05, Fig. 1). FVR then returned to baseline values, with a secondary more modest increase in resistance observed during the last 7 min of occlusion (7.4 ± 2.3 mmHg min ml⁻¹ at 25 min of occlusion, *P* < 0.05). Following occlusion, a transient fall in FVR to baseline values during the first 5 min was followed by a significant increase, peaking at 18.0 ± 3.0 mmHg min ml⁻¹ at the end of the first hour (*P* < 0.05, Fig. 1).

Fetal cardiovascular responses after release of cord occlusion

Following reperfusion, there was an initial rapid recovery of FHR with a brief overshoot tachycardia followed by a return to baseline values, although there was a trend for FHR to be elevated initially.

Figure 1. Time sequence of changes in fetal heart rate (FHR), mean arterial blood pressure (MAP), femoral blood flow (FBF) and femoral vascular resistance (FVR) in the asphyxia group from 60 min before occlusion of the umbilical cord until 60 min after release of occlusion. Sham control data are not shown for clarity. The 25 min period of umbilical cord occlusion is shown by the shaded area. Note the initial increase in fetal MAP, associated with a rapid increase in FVR and reduction in FBF. These changes were not sustained. From approximately 6 min of occlusion the increase in FVR was lost, with a relative increase in FBF and a progressive fall in MAP. Profound hypotension was present at the end of the insult. Data are 1 min averages, shown as means $\pm$ S.E.M.; * $P < 0.05$, + $P < 0.01$, † $P < 0.001$ compared to sham controls.

FHR then fell and was significantly suppressed compared with the sham group between 42 and 66 h after occlusion (Fig. 2). In contrast, MAP was significantly elevated overall during the first 36 h after occlusion ($P < 0.05$, ANOVA; Fig. 2), maximal in the first 3 h. FBF was profoundly reduced throughout the entire recovery period compared with the sham group ($P < 0.05$; Fig. 2), while FVR was increased throughout recovery ($P < 0.05$; Fig. 2). There was a marked increase in FVR in the first 3 h after release of occlusion, corresponding with the early increase in MAP. FVR then transiently fell for a few hours (although it remained above values in the sham group), rose to a second peak between 18 and 24 h, then progressively fell, and was only mildly increased compared to sham control group levels in the final 24 h of the study.

Endocrine data

There were no significant baseline differences in ACTH or cortisol levels between the sham and asphyxia groups. After 20 min of cord occlusion there was a significant rise in plasma concentrations of both ACTH ($P < 0.05$, ANOVA) and cortisol ($P < 0.05$). Following reperfusion, plasma ACTH levels peaked between 2 and 4 h in the asphyxia group and returned to control values at 24 h (Fig. 3, top panel). Plasma cortisol levels remained significantly elevated after the end of occlusion compared to

Figure 2. Time sequence of changes in fetal heart rate (FHR), mean arterial blood pressure (MAP), femoral blood flow (FBF) and femoral vascular resistance (FVR) in the sham control (○) and the asphyxia group (●) from 12 h before occlusion until 72 h after release of occlusion. The 25 min period of umbilical cord occlusion occurs at time 0 and is not shown (see Fig. 1). Data are means $\pm$ S.E.M.; * $P < 0.05$ + $P < 0.05$, compared to sham controls.

the sham control group until 72 h after occlusion ($P < 0.05$; Fig. 3, bottom panel). There was a modest correlation between fetal cortisol levels and fetal mean arterial blood pressure in the 72 h after umbilical cord occlusion ($r^2 = 0.15$, $P = 0.05$, within-subjects regression analysis).

DISCUSSION

The present study demonstrates for the first time that exposure to severe hypoxia induced by umbilical cord occlusion was associated with a rapid and marked rise in ACTH followed by increased cortisol levels, in unanaesthetized preterm fetal sheep. Cortisol levels rose substantially, to 31-fold greater than in the sham control group after release of occlusion, compared with approximately 10-fold after severe hypoxia near term (Jackson *et al.* 1989; Gardner *et al.* 2001). The absolute levels were similar to those attained with a pharmacological infusion of 2–3 mg kg⁻¹ day⁻¹ of cortisol (Forhead & Fowden, 2004). Furthermore, this rise was sustained for 48 h after the end of the asphyxial insult. Thus this study strongly refutes the hypothesis that the preterm fetus has an immature HPA response to hypoxic stress.

Figure 3. Fetal plasma ACTH and cortisol levels before, during and after sham occlusion or 25 min of asphyxia induced by occlusion of the umbilical cord. Data are means $\pm$ S.E.M. * $P < 0.05$ compared to sham occlusion. Con, preocclusion values; Occl, values during occlusion.

Activation of the HPA axis is a well-characterized response to stresses such as hypoxia (Jackson *et al.* 1989; Richardson *et al.* 1996; Carmichael *et al.* 1997; Unno *et al.* 1997; Fraser *et al.* 2001) and hypotension (Rose *et al.* 1978) in the near-term and term fetus and neonate. Comparison with studies in earlier gestation are complicated by the many-fold lower basal cortisol levels seen in the preterm fetus (Challis & Brooks, 1989); however, with some caution, the relative rise in cortisol may be used as an index suggesting broadly equivalent physiological significance. On this basis, a four- to 10-fold rise in cortisol levels during hypoxic stress has been observed in near-term fetal sheep (Jackson *et al.* 1989; Unno *et al.* 1997; Gardner *et al.* 2001), whereas no rise in cortisol was found following brief cord occlusion or moderate hypoxaemia or hypotension in preterm fetuses (Rose *et al.* 1981; Akagi & Challis, 1990a; Green *et al.* 2000).

Results from other types of study, however, have raised contradictory evidence. For example, although basal cortisol levels are low in the preterm ovine fetus, the fetal pituitary is highly responsive both to endogenous stimuli such as infusion of corticotrophin releasing hormone and to exogenous stimuli such as exteriorization and haemorrhage (McFarlane *et al.* 1995). However, these studies did not examine the adrenal responses.

In the present study, when the preterm fetus was exposed to a more severe challenge, the ACTH and cortisol responses were far more pronounced than in previous studies of the preterm fetus that used inhalational hypoxia (Richardson *et al.* 1996) or intermittent short umbilical cord occlusion (Green *et al.* 2000). It is likely that the apparent discrepancy between the present findings and previous

reports is related to the far greater anaerobic tolerance of the preterm fetus (Mott, 1961), with higher cardiac glycogen levels than at term (Shelley, 1964). This means that the preterm fetus can survive far more prolonged intervals of severe asphyxia than at term (George *et al.* 2004). Thus, one reason for the attenuated HPA response in previous studies of hypoxia or shorter periods of asphyxia might be simply that, in effect, the insults were only mild or even subthreshold stimuli for the preterm fetus. It is interesting that in a previous study of short (90 s) umbilical cord occlusions in preterm fetuses, which were approximately 10 days older than in the present study and presumably correspondingly more mature, there was a significant rise in ACTH but not cortisol levels on the first day of occlusions (Green *et al.* 2000). After 4 days of continued occlusions in that study, however, a small (2.8-fold) postocclusion rise in cortisol levels was found. This suggests that a critical duration of stimulation is required to trigger the cortisol response of the preterm fetus. Consistent with this hypothesis, at 105–112 days gestation in the fetal sheep, an infusion of ACTH was associated with only a three-fold rise in fetal cortisol levels after 3 h, but 28-fold after 24 h (Carter *et al.* 1998).

The acute cardiovascular responses in the present study also contrast with previous studies of moderate hypoxia. Such studies suggested that the preterm fetus does not reduce peripheral blood flow during hypoxia (Iwamoto *et al.* 1989) and during partial cord compression cannot increase arterial blood pressure (Iwamoto *et al.* 1991), i.e. that the ability to centralize circulation in response to a hypoxic challenge is immature. In contrast, and consistent with previous studies of the renal responses to asphyxia at this gestational age (Quaedackers *et al.* 2004b), we found marked initial hypertension during complete umbilical cord occlusion associated with an initial, actively mediated fall in FBF, as shown by the very rapid and large increase in vascular resistance in the first 6 min after the start of umbilical cord occlusion. In the near-term fetus the initial vasoconstriction during asphyxia is primarily mediated by sympathetic neural activity (Jensen & Lang, 1992) and possibly augmented by release of stress hormones such as arginine vasopressin and renin–angiotensin system (RAS) activation (Raff *et al.* 1991; Giussani *et al.* 1994b; Rosnes *et al.* 1998); limited data suggest that this also occurs in preterm fetuses (Cheung, 1992; Lumbers *et al.* 2001). Finally, the rise in circulating cortisol demonstrated in the present study may also help to support initial hypertension, as discussed below.

The degree of peripheral vasoconstriction observed initially during occlusion in the present study is similar to that measured using the microsphere technique in the near-term fetus (Jensen *et al.* 1987). This response is believed to be an important adaptive mechanism acting to redistribute combined ventricular output from non-essential or 'peripheral' organs to essential organs such as the heart, brain and adrenals (Hanson, 1988). The present data provide further evidence that this initial cardiovascular defence response is 'mature' in the preterm fetus (Gunn *et al.* 2001). However, consistent with studies in the near-term fetus, this initial adaptation was rapidly lost, leading to partial peripheral reperfusion, followed by a nearly pressure-passive fall in parallel with the secondary fall in arterial blood pressure (Jensen *et al.* 1987; Jensen & Lang, 1992). The failure of redistribution of combined ventricular output during asphyxia is not simply a function of maturation, since the response is seen at all ages examined thus far, but the mechanisms that mediate this loss of peripheral vascular tone are unknown. Furthermore, it is not unique to the femoral vascular bed, with similar responses being seen in the gut and kidney (Quaedackers *et al.* 2004a,b). Potentially, loss of vasoconstriction could reflect loss of sympathetic activity. However, this seems improbable given that circulating catecholamines increase markedly during prolonged asphyxia (Gu *et al.* 1985). Similarly, it does not reflect loss of adrenal activity since, in the present study, cortisol levels were high at the end of the period of cord occlusion. Thus these data suggest that some additional factor, such as severe local tissue acidosis, actively inhibits femoral vascular tone during prolonged asphyxia.

The initial HPA axis response during hypoxia in our study suggests an ACTH-driven rise in cortisol levels, with a rapid rise in both hormones. There is some evidence that the fetal lung can also secrete

ACTH during stress, but since the lung clears ACTH at higher concentrations, it is unlikely to be a factor in the present study (Cudd & Wood, 1995). Maternal cortisol can cross the placenta (Wood & Rudolph, 1984). However, the major increase in fetal cortisol levels in the present study occurred during the interval of complete occlusion of the umbilical cord, suggesting that a maternal contribution is improbable.

Later in recovery there was a marked dissociation between fetal ACTH and cortisol levels, with significantly elevated cortisol values at 24 and 48 h in the asphyxia group, while ACTH concentrations were not different from sham control levels. This suggests the possibility of an ACTH-independent adrenal response in that phase, as previously proposed (Gagnon *et al.* 1997). Furthermore, there are data indicating that carotid sinus denervation delays the rise in cortisol levels but not ACTH concentrations (Giussani *et al.* 1994a), suggesting the possibility of partial neural control of cortisol release. However, it might potentially reflect slower clearance of cortisol, although there are few data on relative clearance rates in the preterm fetus. Alternatively, the delayed fall in cortisol levels may simply reflect local adrenal cortisol upregulation which is slower to resolve. Tangalakis *et al.* (1990), for example, have shown that in preterm fetal sheep at a very similar gestational age to the present study, an infusion of ACTH induced an increase in the width of the adrenal cortex and in the levels of mRNA for key steroidogenic enzymes.

The physiological importance of the sustained elevation of fetal cortisol in the recovery period is unknown. As recently demonstrated, sympathetic nervous system activation is the major mediator of the peripheral vasoconstriction and mild increase in MAP after asphyxia in preterm fetal sheep (Quaedackers *et al.* 2004a). The correlation between fetal MAP during the 3 day recovery period with cortisol levels in the present study suggests that the adrenocortical response also has a role in sustaining blood pressure during recovery. This is consistent with the observation that exogenous infusions of cortisol increase fetal arterial blood pressure (Tangalakis *et al.* 1992; Forhead *et al.* 2000a; Hegarty *et al.* 2000; Forhead & Fowden, 2004). The sustained increase in femoral vascular resistance, which had not fully resolved after 72 h, is similar to that which we have reported in other vascular beds at this age (Quaedackers *et al.* 2004a,b). It may be speculated that increased resistance across all peripheral vascular beds is necessary to help support blood pressure because of transiently impaired cardiac contractility secondary to reversible cardiac injury (Barberi *et al.* 1999; Gunn *et al.* 2000).

The mechanisms of the effect of cortisol on blood pressure remain unclear. Glucocorticoids have direct cardiac effects, mediated by augmented coupling of the β -adrenoreceptors to cellular postreceptor signal transduction (Bian *et al.* 1993) and, of particular interest, potentially by augmented sympathetic responses (Chan *et al.* 1991; Segar *et al.* 2001). Consistent with this suggestion, Padbury *et al.* (1995) have shown that neonatal lambs which had received antenatal betamethasone had significantly elevated blood pressure, cardiac output and cardiac contractility, despite similar preload (as assessed by left ventricular end-diastolic pressure). In part this may be due to a significant increase in β -adrenergic receptor dependent myocardial cyclic adenosine monophosphate generation (Stein *et al.* 1993). Furthermore, synthetic glucocorticoid administration is associated with increased vascular resistance (Derks *et al.* 1997; Fletcher *et al.* 2000; Schwab *et al.* 2000). Finally, there is evidence in late gestation that hypercortisolaemia leads to activation of the fetal RAS (Forhead *et al.* 2000b; Zimmermann *et al.* 2003). However, the initial rise in blood pressure was independent of the RAS (Forhead & Fowden, 2004) and we have previously reported that, in the present model, renin levels are reduced during recovery from asphyxia (Quaedackers *et al.* 2004b).

CONCLUSIONS

Profound asphyxia in the preterm fetus led to a very large relative increase in ACTH and cortisol levels during asphyxia, which was comparable with near-term responses, followed by sustained elevation that gradually resolved between 24 and 72 h. These data do not support the concept that there is a phase of adrenocortical underresponsiveness in the preterm fetus. Rather they suggest that the greater anaerobic tolerance of the preterm fetus, compared with the near-term fetus, means that its full range of responses is only activated by more severe insults. The correlation between cortisol levels following severe asphyxia and fetal MAP suggests that, at least in part, the prolonged adrenocortical response may help to sustain fetal blood pressure during a critical period of recovery.

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CHAPTER 3

IMPROVED NEURAL OUTCOME
WITH CEREBRAL HYPOTHERMIA
AFTER ASPHYXIA
IN PRETERM FETAL SHEEP
IS ASSOCIATED WITH SUPPRESSION
OF EEG TRANSIENTS
DURING RECOVERY

Submitted

Roelfsema V, Bennet L, George S, Gunn TR, Quaedackers JS, Dean JM, Gunn AJ

ABSTRACT

Prolonged, moderate cerebral hypothermia is consistently neuroprotective after experimental hypoxia-ischemia; however, there is little information in the preterm brain. Using a model of complete umbilical cord occlusion for 25 min in 0.7 gestation fetal sheep we examined the effects of cerebral hypothermia (fetal extradural temperature reduced from $39.4 \pm 0.1^{\circ}\text{C}$ to $29.5 \pm 2.6^{\circ}\text{C}$) from 90 min to 70 h after the end of occlusion. Asphyxia led to severe acidosis and profound hypotension. In the first 6 h after reperfusion fetal EEG activity was abnormal with a mixture of fast and slow epileptiform transients superimposed on a suppressed background; stereotypical evolving seizures developed from 9.1 ± 5.3 h. Hypothermia was associated with a marked reduction in numbers of epileptiform transients in the first 6 h, and reduced amplitude of overt seizures. After 3 days recovery the EEG spectral frequency, but not total intensity, was increased in the cooled group compared with asphyxia alone. Hypothermia was associated with a significant overall reduction in neuronal loss in the hippocampus and basal ganglia ($P < 0.001$), with suppression of activated caspase-3 and microglia (isolectin B4 positive). In conclusion, neuroprotection with delayed, prolonged head cooling after a severe asphyxial insult in the premature fetus was associated with potent, specific suppression of epileptiform transients in the early recovery phase, but not of numbers of delayed seizures.

Keywords: Asphyxia – prematurity – hypothermia, induced – EEG – epileptiform transients

INTRODUCTION

There is now strong experimental evidence from a range of animal models that a reduction in brain temperature of 2 to 5°C, applied following perinatal hypoxia-ischemia, can improve neuropathological (Bona *et al.* 1998; Edwards *et al.* 1995; Gunn *et al.* 1997; Gunn *et al.* 1998; Roelfsema *et al.* 2004; Sirimanne *et al.* 1996; Tooley *et al.* 2003), cerebral energetic (Nedelcu *et al.* 2000; Thoresen *et al.* 1995), electrophysiological (Gunn *et al.* 1997; Gunn 2000) and functional outcomes (Bona *et al.* 1998; Wagner *et al.* 2002). However, these studies have focused on neuroprotection with hypothermia at term or later. Preterm infants have a very high burden of neurological injury (du Plessis and Volpe 2002). It is unknown whether hypothermia is protective in the very immature brain. Further, preterm infants may be particularly vulnerable to adverse systemic effects during mild hypothermia (Silverman *et al.* 1958).

Although the etiology of preterm brain injury is complex and multifactorial, exposure to perinatal asphyxia is highly associated with worse outcomes (Low *et al.* 1995), is more common in infants born prematurely than at term (Low 2004) and frequently occurs before the onset of labor (Low *et al.* 2003). The typical pattern of neural injury after exposure to severe perinatal hypoxia in preterm infants is distinctive, with severe damage in the basal ganglia and other subcortical regions, but sparing of the cerebral cortex (Barkovich and Truwit 1990; Barkovich and Sargent 1995; de Vries *et al.* 1998; Gilles *et al.* 1998). In the long-term, involvement of subcortical nuclei is a common feature in surviving preterm infants (Argyropoulou *et al.* 2003; Lin *et al.* 2001; Yokochi 1997).

We have recently reported that exposure to severe asphyxia in the preterm fetal sheep leads to a similar pattern of subcortical neural injury with cortical sparing (George *et al.* 2004). Strikingly, while overall EEG activity was profoundly suppressed for many hours after the asphyxial insult, regardless of whether injury later developed or not, epileptiform EEG transient activity during the early recovery phase was seen only in fetuses that went on to develop neural injury (George *et al.* 2004). These EEG transients were low to moderate amplitude events similar to the subtle, brief spikes and waveforms observed in preterm infants (Scher *et al.* 1994). The timing of these events corresponds with a secondary fall in carotid blood flow (Bennet *et al.* 1999; George *et al.* 2004) and a fall in cerebral oxygenation on near-infrared spectroscopy (Bennet *et al.* 1999). These data suggest the hypothesis that these events may directly contribute to evolving injury, similar to adult models of ischemic injury (Golanov and Reis 1999; Hossmann 1996; Wolf *et al.* 1997). Although the effects of mild to moderate hypothermia on overt, stereotypical seizures remain controversial (Gunn *et al.* 1997; Thoresen *et al.* 1999; Tooley *et al.* 2003), local brain cooling can inhibit epileptiform activity (Baldwin and Frost 1956; Karkar *et al.* 2002).

The aim of this study was therefore to test the hypothesis that following hypoxic-ischemic injury induced by profound asphyxia in the 0.7 gestation fetal sheep treatment with cerebral hypothermia, induced 90 min after reperfusion and continued for 3 days, would improve neural outcome and suppress the development of post-asphyxial EEG transients on continuous EEG monitoring. In terms of cerebral maturity the 0.7 gestation fetal sheep is comparable to the human brain at term at 30-32 weeks of gestation (McIntosh *et al.* 1979), just prior to the onset of cortical myelination (Barlow 1969).

MATERIALS & METHODS

Experimental preparation

All procedures were approved by the Animal Ethics Committee of The University of Auckland. Twenty three singleton Romney/Suffolk fetal sheep were instrumented at 97-99 days of gestation (term = 147 days) under general anesthesia (2% halothane in O₂) using sterile techniques. Catheters were placed

in the left femoral artery and vein, right brachial artery and vein, and the amniotic sac. Carotid artery blood flow (CaBF) was measured by a 3S ultrasound blood flow probe (Transonic Systems Inc., Ithaca, NY, USA). Two pairs of electroencephalographic (EEG) electrodes (AS633-5SSF, Cooner wire Co., Chatsworth, CA, USA) were placed on the dura over the parasagittal parietal cortex (5 mm and 15 mm anterior to bregma and 10 mm lateral), with a reference electrode sewn over the occiput. A thermistor (Incu-temp-1, Mallinckrodt Medical Inc., St Louis, MO) was placed over the parasagittal dura 20 mm anterior to bregma, to measure extradural temperature, and the burr holes were sealed and the skin over the fetal skull was secured with cyanoacrylate glue. Electromyographic (EMG) electrodes were placed in the nuchal muscle and electrocardiogram (ECG) electrodes were sewn across the chest to record the fetal ECG. A second thermistor (to measure fetal core body temperature) was placed in the fetal esophagus at the level of the right atrium (Gunn et al. 1997). An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA, USA).

A cooling coil made from silicone tubing (external diameter 7.9 mm, internal diameter 4.8 mm; Silclear, Degania Silicone, Degania Bet, Israel) was attached over the dorsal surface of the scalp and extended over the lateral surface of the cranium down to the level of the external auditory meatus. The fetus was returned to the uterus and all fetal leads were exteriorized through the maternal flank. The maternal long saphenous vein was catheterized to provide access for maternal care and euthanasia. Antibiotics were given to the ewes (5 mls of Streptopen, i.m.) prior to the start of surgery, were administered into the amniotic sac prior to closure of the uterus (80 mg Gentamicin), and after surgery (80 mg Gentamicin i.v. daily for the first 3 days, and 600 mg Crystapen i.v. for 4 days).

After surgery sheep were housed together in separate metabolic cages with access to water and food *ad libitum*. They were kept in a temperature-controlled room ($16 \pm 1^\circ\text{C}$, humidity $50 \pm 10\%$), in a 12 h light/dark cycle. A period of 4–6 days post-operative recovery was allowed. Fetal arterial blood was taken daily from the brachial artery for blood gas analysis for the assessment of fetal health. Catheters were maintained patent by continuous infusion of heparinized saline (20 U ml^{-1} at 0.15 ml h^{-1}).

Experimental design and recordings

Experiments were conducted at 103–104 days gestation. Fetal mean arterial pressure (MAP), corrected for maternal movement by subtraction of amniotic fluid pressure, heart rate (FHR), CaBF, EEG and nuchal EMG activity were recorded continuously from 12 h before the experiment until 72 h afterwards. Data were collected by computer and stored to disk for off-line analysis (Labview for Windows, National Instruments Ltd., Austin, Texas, USA).

Fetuses were randomly assigned to either the asphyxia-normothermia group ($n = 8$), asphyxia-cooling group ($n = 8$) or sham control ($n = 7$). Fetal asphyxia was induced in both groups by rapid inflation of the umbilical cord occluder for 25 min with sterile saline of a defined volume known to completely inflate the occluder. Successful occlusion was confirmed by observation of a rapid flattening of the EEG. In all groups fetal arterial blood were taken at 15 min prior to asphyxia, 5 and 20 min during asphyxia, and 0.5, 2, 4, 6, 24, 48 and 72 h post-asphyxia for blood gas, acid-base balance (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, MA, USA) and for glucose and lactate determination (YSI model 2300, Yellow Springs, Ohio, USA). Intrauterine cooling was performed from 90 min to 70 h after the end of occlusion. Fetuses were then rewarmed for 2 h. Cooling was induced by circulating cold water (10°C) through a coil around the fetal head. Cooling was titrated in the first 2 h to reduce fetal extradural temperature from $39.4 \pm 0.1^\circ\text{C}$ to between 29 and 33°C .

Fetuses were studied for 3 days after the end of occlusion. On completion of the experiment the ewes and fetuses were killed by an overdose of sodium pentobarbitone (9 g i.v. to the ewe: Pentobarb 300, Chemstock International, Christchurch, New Zealand).

Histology Tissue Preparation

Fetal brains were perfusion fixed *in situ* with normal saline followed by 500 ml of 10% phosphate buffered formalin. Following removal from the skull, tissue was fixed for a further 5-6 days before processing and embedding using a standard paraffin tissue preparation (Gunn *et al.* 1997). Neuronal loss was evaluated by light microscopy by an assessor masked to the treatment group on 8 μ m thick coronal sections stained with thionin and acid fuchsin (Gunn *et al.* 1997). The proportion of neurons showing ischemic cell change as shown by nuclear condensation and acid fuchsin (pink) staining of the cytoplasm in multiple pre-assigned areas was scored on a 6 point scale as follows: 0 = no dead neurons; 5 = >0–10%; 30 = >10–50%; 70 = >50–90%; 95 = 90–<100%; and 100 = 100% dead neurons. Average scores were calculated for each region (Gunn *et al.* 1997). Activated microglial cells were labelled on parallel sections using the lectin, Bandeiraea simplicifolia I isolectin-B4 (Sigma, St. Louis, MO, U.S.A.) (Roelfsema *et al.* 2004).

Caspase 3 expression

Staining was performed on coronal sections (6 μ m) at the level of the mid-striatum cut and mounted on chrome alum coated slides. Prior to specific staining all the slides were deparaffinized in xylene twice for 15 min, dehydrated in a series of ethanol (100%, 100%, 95%, 95%, 70%, for two min each), and incubated in 0.1 M phosphate buffered saline (PBS) twice for 5 min. The sections were boiled in 0.1 mM citrate buffer for antigen unmasking. The sections were then pretreated with 1% H₂O₂ in 50% methanol for 20 min, washed in PBS (3x5 min) and incubated with the primary antibody for 2 days at 4°C.

Rabbit anti-Caspase-3 Asp175 was used (cleaved Caspase-3 Antibody, which detects endogenous levels of the large fragment of activated caspase-3 (17-20kDa), Cell Signaling Technology, Beverly, MA, USA). The antibody was diluted 1:1000 in PBS containing normal horse serum/goat serum. The primary antibody was washed off with PBS (3x10 min) and then incubated with anti-rabbit biotinylated immunoglobulin G (Vector laboratories, Burlingame, CA, USA, diluted 1:200) overnight at 4°C. The sections were washed, incubated in avidin-biotin complex (ABC, Vector Laboratories, Peterborough, England, diluted 1:100) in the ratio 1:500 for 3 h at room temperature, washed again in PBS, and then reacted in diaminobenzidine tetrahydrochloride (DAB, Sigma). Sections were then dehydrated in a series of alcohol to xylene washes and overslipped with mounting medium. Control sections were processed in the same way except that the primary antibody was omitted from the incubation solution.

Numbers of activated caspase-3 positive cells were counted by light microscopy at x100 magnification on coronal sections using a 1 cm² grid in pre-selected areas of the dorsolateral aspect of the caudate nucleus and the putamen. Values from the left and right hemisphere were averaged. The cross-sectional area of each nucleus was measured.

Data analysis and statistics

Off-line analysis of electronic data was performed using an analysis program written using Labview for Windows. The nuchal EMG signal was bandpass filtered between 100 Hz and 1 kHz and the signal then integrated using a time constant of 1 sec. The EEG signal was low-pass filtered with a 6th order low-pass Butterworth filter, with a cut-off frequency of 50 Hz. A power spectrum was then calculated from this 256 Hz sampled signal. The EEG intensity and spectral edge were then calculated on the portion of the power spectrum between 1 Hz and 20 Hz, and stored as 1 min averages for the analysis of average EEG changes over time. Data from left and right EEG electrodes were averaged to give mean total EEG activity. For clarity of data display the EEG intensity was log transformed (dB, 20 x log(intensity)), as this transformation gives a better approximation of the normal distribution (Williams

et al. 1990), and normalized. Additionally, the raw EEG signal was processed through a digital FIR low-pass filter with a cut-off frequency of 30 Hz and stored at a sampling rate of 64 Hz for analysis of seizures.

The raw EEG was assessed for epileptiform activity; specifically the presence of spikes and sharp waves (i.e. epileptiform transients (George *et al.* 2004)). A spike was defined as having a sharp outline and duration of less than 70 msec. Sharp waves were assessed as single or repeated mono- or biphasic transients lasting 100 to 250 msec, with an amplitude greater than 10 μ V, typically superimposed on a flat EEG background (Scher 2003; Scher 2002). Overt electrographic seizures were identified visually and defined as the concurrent appearance of sudden, repetitive, evolving stereotyped waveforms in the EEG signal lasting more than 10 sec (Scher 2002).

Treatment effects were evaluated by analysis of variance (ANOVA, SPSS v10, SPSS Inc.), followed by the protected least-significant difference post-hoc test when a significant overall effect was found. For comparisons over time, baseline levels were used as a covariate (ANCOVA). The baseline period was taken as the mean of the 6 hours before occlusion. Cerebral region in the histology score and time series data were treated as repeated measures to allow for repeated sampling. Statistical significance was accepted when $P < 0.05$. Data are mean $\pm$ SD.

Table 1 Fetal arterial pH, blood gases, glucose and lactate values for sham control (C), normothermia asphyxia (N) and hypothermia asphyxia (H) groups 15 min before (baseline), at 20 min during (occl), and 30 min, 2 h, 4 h, 6 h, 24 h, 48 h and 72 h after either sham occlusion or 25 min of umbilical cord occlusion. Data are mean $\pm$ SD. * $P < 0.05$ compared to sham control, # $P < 0.05$ asphyxia normothermia vs hypothermia (ANOVA, followed by the Least Significant Difference post-hoc test for between group comparisons).

		Control	20 min occl	30 min	2 h
pH	C	7.39 $\pm$ 0.0	7.38 $\pm$ 0.0	7.38 $\pm$ 0.0	7.39 $\pm$ 0.0
	N	7.37 $\pm$ 0.0	6.82 $\pm$ 0.0*	7.25 $\pm$ 0.0*	7.34 $\pm$ 0.0*
	H	7.39 $\pm$ 0.0	6.85 $\pm$ 0.0*	7.27 $\pm$ 0.0*	7.39 $\pm$ 0.0#
PaCO ₂ (mmHg)	C	48.8 $\pm$ 4.5	49.1 $\pm$ 4.1	47.8 $\pm$ 4.1	48.1 $\pm$ 4.3
	N	48.2 $\pm$ 2.6	142.2 $\pm$ 16*	45.8 $\pm$ 2.2	46.8 $\pm$ 2.6
	H	47.8 $\pm$ 4.3	138.4 $\pm$ 24*	37.8 $\pm$ 3.5*	37.9 $\pm$ 2.9*,#
PaO ₂ (mmHg)	C	22.5 $\pm$ 2.6	22.7 $\pm$ 2.3	22.3 $\pm$ 2.1	22.1 $\pm$ 2.6
	N	23.0 $\pm$ 2.6	8.7 $\pm$ 2.2*	27.9 $\pm$ 3.8*	26.2 $\pm$ 3.8*
	H	24.7 $\pm$ 3.0	9.1 $\pm$ 4.3*	27.3 $\pm$ 2.0*	24.7 $\pm$ 3.7
O ₂ ct	C	3.6 $\pm$ 0.5	3.5 $\pm$ 0.5	3.6 $\pm$ 0.5	3.4 $\pm$ 0.6
	N	3.5 $\pm$ 0.5	0.5 $\pm$ 0.1*	3.9 $\pm$ 0.5	4.1 $\pm$ 0.5
	H	3.7 $\pm$ 0.4	0.5 $\pm$ 0.2*	3.9 $\pm$ 0.6	4.2 $\pm$ 0.8*
Glucose (mM)	C	1.0 $\pm$ 0.3	1.0 $\pm$ 0.3	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3
	N	1.0 $\pm$ 0.2	0.7 $\pm$ 0.3*	1.3 $\pm$ 0.4	1.2 $\pm$ 0.2
	H	0.9 $\pm$ 0.2	0.5 $\pm$ 0.4*	1.4 $\pm$ 0.5	1.3 $\pm$ 0.3
Lactate (mM)	C	0.8 $\pm$ 0.2	0.8 $\pm$ 0.1	0.8 $\pm$ 0.2	0.8 $\pm$ 0.4
	N	0.6 $\pm$ 0.2*	6.5 $\pm$ 0.5*	4.8 $\pm$ 0.5*	3.6 $\pm$ 1.5*
	H	0.7 $\pm$ 0.1*	5.8 $\pm$ 1.4*	4.7 $\pm$ 1.3*	2.9 $\pm$ 1.6*

RESULTS

Baseline

All fetuses had normal FHR, MAP, blood gases, acid base, glucose and lactate status, and EEG signals before each experiment, according to the standards of our laboratory. Values and statistical comparisons for arterial pH, blood gases, and glucose and lactate levels for the sham control, asphyxia normothermia and asphyxia hypothermia groups are presented in Table 1.

Effect of umbilical occlusion

Umbilical cord occlusion was associated with marked fetal hypoxia and acidosis (table 1) and led to bradycardia, hypotension and cerebral hypoperfusion. Both EEG intensity and nuchal EMG activity were rapidly and profoundly depressed during the entire period of occlusion ($P<0.05$ for each variable; data not shown). There were no significant differences between the animals allocated to the normothermia and the hypothermia group during the occlusion period.

Blood composition in the recovery period

Following release of the umbilical occluder, there was rapid recovery of blood gases, pH and glucose and lactate levels by 4 to 6 h (Table 1). Compared to the sham control group, arterial pO_2 and O_{2ct} were significantly elevated in the two asphyxia groups for most of the recovery period. There was a small reduction in arterial pCO_2 in the cooled group ($P<0.05$) compared to both sham controls and the asphyxia normothermia group, with mildly elevated pH levels ($P<0.05$).

Effect of cooling on brain and body temperature

Head cooling was initiated 90 min after the end of the umbilical cord occlusion and was associated with a significant fall in extradural temperature starting within an hour after the onset of cooling,

4 h	6 h	24 h	48 h	72 h
7.39±0.0	7.38±0.0	7.38±0.0	7.37±0.0	7.37±0.0
7.40±0.0	7.41±0.0*	7.37±0.0	7.38±0.0	7.38±0.0
7.46±0.0*,#	7.44±0.0*,#	7.43±0.0*,#	7.41±0.0*,#	7.43±0.0*,#
49.2±5.2	49.4±5.0	47.5±6.3	49.2±4.1	48.2±6.4
47.7±3.2	45.6±4.0	47.4±2.9	47.1±2.5	46.4±3.2
39.8±4.0*,#	39.5±3.0*,#	38.8±7.3*,#	41.5±2.9*,#	42.9±5.1
22.6±2.6	22.3±2.5	22.2±4.8	23.4±4.0	23.5±3.7
24.8±2.8	24.8±2.5	26.6±3.7*	28.0±3.2*	28.3±3.6
25.1±3.7	24.4±3.2	23.6±2.6	24.5±4.3	27.0±7.1
3.4±0.6	3.4±0.6	3.2±0.6	3.4±0.7	3.7±0.6
3.9±0.8	4.0±0.7	4.1±0.8	4.1±0.7	4.2±0.7
4.5±0.6*	4.5±0.8*	4.2±0.5	3.9±0.6	4.3±0.5
1.1±0.2	1.1±0.2	1.2±0.3	1.2±0.2	1.1±0.3
1.3±0.3	1.4±0.3*	1.5±0.4	1.3±0.2	1.2±0.2
1.4±0.3*	1.5±0.3*	1.4±0.4	1.3±0.4	1.4±0.3
0.8±0.2	0.9±0.3	0.7±0.1	0.8±0.3	0.9±0.3
2.4±1.3*	1.9±0.7*	1.0±0.3*	0.8±0.3	0.9±0.2
1.4±0.8#	1.2±0.5*,#	0.9±0.3	0.7±0.2	0.8±0.2

Figure 1. Time sequence of changes in fetal temperature, electroencephalogram (EEG) intensity (power) and spectral edge (frequency). The 25 min period of umbilical cord occlusion is shown by the solid vertical lines, while cooling is shown by the shaded bar. The top panel shows changes in extradural and core body (esophageal) temperatures in the hypothermia (closed symbols) and asphyxia normothermia (open symbols) groups. The extradural and esophageal temperature were significantly different from normothermia fetuses throughout the cooling period ($P<0.001$, not shown). Bar plus * $P<0.05$ hypothermia vs normothermia; bar plus # $P<0.05$ asphyxia normothermia vs sham control group. Data are mean $\pm$ SD.

reaching a nadir of $29.5 \pm 2.6^{\circ}\text{C}$ (vs $39.3 \pm 0.3^{\circ}\text{C}$; $P<0.05$, Fig 1). Extradural temperature gradually increased during the second and third day of cooling to a mean of $32.3 \pm 2.2^{\circ}\text{C}$ in the last 6 h. During cooling, core body temperature dropped mildly to a nadir of $37.2 \pm 0.8^{\circ}\text{C}$ (vs $39.0 \pm 0.4^{\circ}\text{C}$; $P<0.05$), which was sustained over the whole cooling period.

EEG intensity and spectral edge

EEG intensity was rapidly suppressed at the onset of occlusion in both asphyxia groups, and remained significantly suppressed compared with sham controls following reperfusion for the remainder of the

Figure 2. Time sequence of changes in fetal heart rate (bpm), mean arterial blood pressure (MAP, mmHg) and total carotid blood flow (CaBF), in the hypothermia (▲), asphyxia normothermia (●) and sham control (■) groups. The 25 min period of umbilical cord occlusion is shown by the vertical lines, while cooling is shown by the shaded bar. Data are mean $\pm$ SD, * $P < 0.05$ hypothermia vs asphyxia normothermia. † $P < 0.05$ cooling vs sham asphyxia. # $P < 0.05$ asphyxia normothermia vs sham controls.

experiment ($P < 0.01$). Hypothermia was associated with an immediate fall in EEG intensity, which remained significantly less than in the normothermia asphyxia group until 9 h after occlusion ($P < 0.05$); thereafter there was no significant difference between the two groups.

EEG spectral edge (SE) was also markedly suppressed in both occlusion groups during and after reperfusion with a modest rise in the first 4 h, followed by a fall reaching its nadir at approximately 7 h. SE was not significantly increased in the hypothermia group compared to the normothermia group during the first 12 h of cooling (N.S.). From 12 h onward SE was significantly increased ($P < 0.001$, repeated measures ANOVA) in the hypothermia group compared to the asphyxia normothermia group, attaining sham control values in the final 24 h of the study.

Figure 3. Examples of raw electroencephalographic (EEG) data showing normal discontinuous mixed frequency EEG activity (panel A), epileptiform transients (fast spikes) superimposed on a suppressed EEG background at 3 h post-occlusion (panel B) and marked suppression of EEG transients during hypothermia at 3 h (panel C). The bottom panel quantifies numbers of events per minute in the asphyxia normothermia and hypothermia groups. * $P < 0.05$ hypothermia vs asphyxia normothermia group. Data are mean $\pm$ SD.

Epileptiform activity

The raw EEG signal after reperfusion was highly abnormal showing marked loss of background activity, with frequent high voltage sharp and spike wave transients in the asphyxia group but not sham controls (Fig. 3). These EEG transients peaked between 3 and 5 h after the end of occlusion. No abnormal EEG waveforms, or burst suppression activity was observed in the sham control group at any time. The start of cooling at 90 min was associated with an immediate reduction in numbers of EEG transients ($P < 0.001$, Fig. 3). Although there was a trend for increasing numbers of transients in the hypothermia group after approximately 5 h, the onset of overt EEG seizures at this time in some fetuses meant that EEG transients could no longer be reliably counted.

This interval of fast and sharp wave spike activity was then followed by the onset of high amplitude, low frequency stereotypic evolving seizure activity from 9.1 ± 5.3 h in the asphyxia group vs 10.1 ± 4.7 h in the hypothermia group (N.S.). A total of 66.5 ± 65.5 vs 73.1 ± 70.3 seizure events occurred (N.S.). The amplitude of these events was reduced in the cooling group (130.4 ± 62.8 vs 67.5 ± 20.9 μ V, $P = 0.006$, Mann-Whitney U test). No seizures were observed in sham control fetuses at any time.

The raw EEG recordings were examined in the final hour of recording (i.e. after rewarming the hypothermia group). Both asphyxia groups showed marked suppression of EEG amplitude at that time (see examples Fig. 4). In the asphyxia normothermia group the activity is predominantly low frequency and occasional epileptiform transients were seen. The asphyxia hypothermia group showed a mixture of high and lower frequency activity, but no epileptiform transients.

Figure 4. Examples of raw electroencephalographic (EEG) data taken 72 h after either sham occlusion (panel A) or umbilical cord occlusion followed by normothermia (panel B) or hypothermia (panel C). The sham control shows typical discontinuous mixed frequency EEG activity. EEG amplitude is markedly suppressed in both asphyxia groups. In the asphyxia normothermia group the activity is predominantly low frequency (panel B), whereas in the asphyxia hypothermia group a mixture of high and lower frequency activity is seen (panel C).

CaBF, MAP and FHR

Following reperfusion, there was an initial rapid recovery in MAP, FHR and CaBF. The asphyxia-normothermia group showed a transient tachycardia (maximal at 3 h after occlusion), followed by a gradual fall, and became significantly lower than in sham controls in the final 48 h of the study ($P < 0.05$, ANOVA). In contrast, cooling was associated with a significant reduction in FHR compared to both sham controls ($P < 0.001$, from 5 h until the end of cooling) and the asphyxia normothermia group ($P < 0.05$, from 3 h to 24 h).

MAP was significantly elevated in the first 24 h after the end of occlusion in the normothermia and hypothermia asphyxia groups compared with sham controls ($P < 0.005$, ANOVA). The elevation was maximal at 1 h, and returned to sham control values in the final 48 h. The hypothermia group was not significantly different from the asphyxia normothermia group overall (ANOVA); however, there was an apparently more rapid resolution of the initial elevation of MAP, such that the hypothermia group was not significantly different from sham controls from 3 h to 12 h after occlusion.

CaBF initially returned to sham control values after occlusion, followed by a secondary fall, which was significantly less than sham controls from 90 min to 18 h after occlusion. Thereafter CaBF was not significantly different from sham controls. Hypothermia was associated with a greater reduction in CaBF than in the asphyxia-normothermia group from 2.5 to 8 h ($P < 0.05$). In the final 42 h of the experiment the hypothermia group showed a progressive increase in CaBF, reaching values greater than the asphyxia normothermia group in the final 24 h ($P < 0.05$, ANOVA).

Figure 5. The top panel quantifies neuronal loss in hypothermia-treated and sham cooled fetuses. Mean $\pm$ SD, * = $P < 0.05$ hypothermia vs. sham cooled fetuses. CP (Parasagittal cortex), CL (Lateral or temporal cortex), DG (dentate gyrus), CA (cornu ammonis of the hippocampus), St (Striatum), Th (Thalamus). The panel at the bottom shows photomicrographs of the CA 3 region of the dorsal arm of the hippocampus stained with acid fuschin and thionin staining. Photomicrograph A, the box shows the selected CA 3 region. Photomicrograph B, an example of a sham control showing normal neuronal morphology. Photomicrograph C, example of a sham cooled (asphyxia) fetus showing severe neuronal loss. Photomicrograph D, a fetus treated with hypothermia showing increased numbers of intact cells. The arrows indicate examples of ischemic cell change.

Figure 6. Photomicrographs showing isolectin B-4 staining in the CA 3 region of the dorsal hippocampus. No isolectin B-4 positive cells were seen in sham controls (A). Umbilical cord occlusion was associated with intense induction of isolectin B-4 positive cells (B), which was almost completely suppressed by early initiation of cooling (hypothermia group, C). Scale bar = 50 μ m.

Histology

There was no histological injury or cell loss visible on acid fuchsin and thionin staining in sham control animals. Asphyxia was associated with severe subcortical neuronal loss, particularly in the dorsolateral aspect of the basal ganglia and the cornu ammonis (CA) regions of the dorsal horn of the hippocampus. There was only trivial neuronal loss in the cortical regions, typically only a few cells in the parasagittal gyri. Hypothermia was associated with a significant overall reduction in neuronal loss ($P < 0.001$), which was greatest in the striatum (27.8 ± 6.4 vs $5.5 \pm 1.2\%$) and CA regions of the hippocampus

Figure 7. Top panel: numbers of caspase-3 [Asp175] positive cells counted in a 1 cm² area in the caudate nucleus and putamen were significantly increased after three days recovery from umbilical cord occlusion compared to the sham control group. The hypothermia group showed marked suppression of caspase-3 activation compared to the asphyxia-normothermia group. *, $P<0.001$. Data are mean $\pm$ SD. Bottom panel: Photomicrographs showing examples of caspase-3 positive staining (brown) in the caudate nucleus 3 days after umbilical cord occlusion in 0.7 gestation fetal sheep, with many positive cells in the asphyxia normothermia fetus (left), and suppression in the hypothermia fetus (right). In the asphyxia normothermia fetus note the presence of coarse granular fragments of caspase positive material around some of the positive cells. Scale bar = 50 μ m.

(43.9 ± 10.0 vs $10.1 \pm 4.3\%$, Fig. 5). Sham control animals showed virtually no isolectin B-4 staining in any region. Occlusion was associated with induction of isolectin B4 staining activated microglia, greatest in the CA regions of the hippocampus. This was nearly completely suppressed in the hypothermia group (Fig. 6).

Activated caspase-3 expression in the striatum

Only occasional cleaved 17kD caspase-3 positive cells were seen, in a few sham control animals. Following umbilical cord occlusion in the asphyxia normothermia group, marked activated caspase-3 expression was seen in the caudate nucleus and putamen (Fig. 7). The density of caspase-3 staining varied between cells due to the timing of cleavage of caspase-3. Cells were counted which had evidence of newly cleaved caspase-3, as shown by morphologically dense caspase-3 (Asp175) staining evenly distributed within the ectoplasm (Fig. 7, bottom panel). Hypothermia was associated with significantly reduced numbers of cells expressing activated caspase-3 compared to normothermia ($P < 0.001$, ANOVA).

DISCUSSION

The present study demonstrates that moderate, delayed cerebral hypothermia from 90 minutes and continued until 70 hours after a prolonged episode of umbilical cord occlusion is associated with improved electrophysiological and histological recovery in preterm fetal sheep. Cooling was associated with marked suppression of numbers of early onset epileptiform transients. Although cooling did not prevent the development of subsequent, delayed stereotypic EEG seizures, the amplitude of these events was significantly reduced. After three days recovery from asphyxia, carotid blood flow was significantly greater in the hypothermia group than in the asphyxia alone group, and EEG frequency, although not amplitude, had returned to sham control levels. These hemodynamic and electrophysiological improvements were reflected in greater histological neuronal survival in the striatum and the cornu ammonis regions of the hippocampus, with suppression of induction of activated caspase-3 and of microglia.

It is now well established that hypoxic-ischemic injury is an evolving process in which final cell death may not occur until well after the initial insult (Kirino 1982). It is this delay that has raised the tantalizing possibility that asphyxial damage might be able to be prevented even well after reperfusion. For example, many term infants with hypoxic-ischemic encephalopathy show initial, transient recovery of cerebral oxidative metabolism after birth in a 'latent' phase, followed by secondary deterioration with cerebral energy failure from 6 to 15 hours after birth (Azzopardi *et al.* 1989; Roth *et al.* 1992). Experimentally, at the term equivalent in the fetal sheep, this secondary deterioration is marked by the delayed onset of seizures, accumulation of excitotoxins and redox deterioration (Gunn *et al.* 1997; Marks *et al.* 1996; Tan *et al.* 1996). The severity of this secondary deterioration is closely correlated with neurodevelopmental outcome at 1 and 4 years of age (Roth *et al.* 1992). Critically, there are data that experimental hypothermia may be effective only if it is initiated in this latent phase (Gunn *et al.* 1997; Gunn *et al.* 1999; Thoresen *et al.* 1995).

The key mechanisms that mediate the effects of hypothermia in the latent phase are unclear, but likely include suppression of excitotoxic activity (Thoresen *et al.* 1997), apoptotic events (Edwards *et al.* 1995; Roelfsema *et al.* 2004), and local inflammation (Gunn and Bennet 2002; Roelfsema *et al.* 2004). In the present study, hypothermia potently inhibited the spikes and sharp and slow wave complexes observed in the normothermia group in the latent phase and caused a significant further

suppression of EEG intensity compared with the normothermia group. The immature brain appears to be particularly susceptible to post-hypoxic hyperexcitability (Jensen and Wang 1996). The appearance of epileptiform transients after exposure to asphyxia and their apparent correlation with brain injury is consistent with other studies, which have reported the common presence of fast epileptiform EEG transients in preterm newborns (Scher *et al.* 1994; Vecchierini-Bliveau *et al.* 1996). Although these events can occur in asymptomatic newborns with a normal outcome (Scher *et al.* 1994; Vecchierini-Bliveau *et al.* 1996), overall, the appearance of such activity in preterm infants is strongly associated with poor outcome (Biagioni *et al.* 2000; Hughes and Guerra 1994; Marret *et al.* 1997; Okumura *et al.* 2003; Rowe *et al.* 1985; Vecchierini-Bliveau *et al.* 1996).

Many studies have suggested that excitotoxic activity is particularly important in preterm brain injury, as recently reviewed (du Piessis and Volpe 2002). Adult studies suggest that this may be mediated by an increase in glutamate receptor-mediated excitatory responses following ischemia rather than by high extracellular glutamate concentrations (Kalemenev *et al.* 2002; Mitani *et al.* 1998). The mechanism of suppression of the EEG transients by hypothermia is unknown. However, it is well established that *in vitro* hypothermia reduces the slope of excitatory postsynaptic potentials (Aihara *et al.* 2001) and of glutamate release after depolarisation (Nakashima and Todd 1996) in a temperature dependent manner. Further, focal cerebral cooling can reduce epileptiform activity in animals (Baldwin and Frost 1956) and in patients (Karkar *et al.* 2002) without changing the motor threshold. In our study, this effect seemed to be specific, since continued cooling did not reduce numbers of late onset stereotypic, evolving seizures that were seen from a mean of 9 hours. This is consistent with previous studies, which have reported that hypothermia is associated with either no significant effect on secondary seizures (Gunn *et al.* 1997; Gunn *et al.* 1998) or a modest reduction (Tooley *et al.* 2003).

A likely further potential mechanism is suppression of apoptosis by hypothermia. Apoptotic pathways are suggested to be relatively more important in hypoxic-ischemic brain damage in the neonate compared to adult (Hu *et al.* 2000), and several studies have suggested that hypothermia selectively suppresses morphological apoptotic cell loss in the neonatal brain (Edwards *et al.* 1995; Xu *et al.* 1998). Supporting this concept, mild hypothermia can reduce mitochondrial cytochrome C translocation, a critical step in the intrinsic pathway of apoptosis (Xu *et al.* 2002; Yenari *et al.* 2002), and has been shown to suppress expression of caspase-3 both following hypoxia-ischemia *in vivo* (Fukuda *et al.* 2001; Tomimatsu *et al.* 2001) and serum deprivation *in vitro* (Xu *et al.* 2002). Consistent with these results, the present study confirms a significant suppression by hypothermia of post-asphyxial activated caspase-3 in the striatum.

Alternatively, there is considerable evidence that inflammatory processes, including induction of activated microglia with subsequent release of pro-inflammatory cytokines, contribute to both evolving neuronal and white matter injury (Silverstein *et al.* 1997). Cytokines exacerbate brain injury through various pathways including direct neurotoxicity and induction of apoptosis or by promoting stimulation of capillary endothelial cell pro-inflammatory responses and leukocyte adhesion and infiltration into the ischemic brain (Mallard *et al.* 2003; Silverstein *et al.* 1997). Thus, the present finding that early post-occlusion cerebral hypothermia markedly reduced microglial induction in subcortical regions, suggests that this may contribute to reduced neuronal loss. In the present study, microglial induction was greatest in the hippocampus after exposure to umbilical cord occlusion, and milder in the striatum, likely reflecting the severity of injury in each region. Thus, the effect of hypothermia could be either primary, or secondary to reduced cell loss and local tissue damage leading to a reduced stimulus to further microglial induction (Roelfsema *et al.* 2004).

In order to provide adequate neuroprotection with minimal risk of systemic adverse effects in sick, unstable neonates, ideally only the brain would be cooled. Although this has been demonstrated

experimentally using cardiac bypass procedures (Wass *et al.* 1998), it is clearly impractical in routine practice. Pragmatically, partially selective cerebral cooling can be obtained using a cooling cap applied to the scalp (Gluckman *et al.* 2004). Previous studies of head cooling in the term piglet have shown that a significant gradient between core brain temperature and rectal temperature can be maintained for at least 24 hours (Tooley *et al.* 2002). The current study confirms that a significant gradient is able to be established and maintained between the extradural space and core body temperature using this technique in the preterm fetal sheep.

The present study can only partially address whether this approach can prevent deleterious systemic effects in the apparently uniquely vulnerable preterm infant (Silverman *et al.* 1958); further studies in large newborn species will be required to fully resolve this. Nevertheless, in the present study, FHR was mildly reduced by cooling compared to the normothermia group, consistent with its well established effects on cardiac conduction (Solomon *et al.* 1989), and clinical studies at term (Azzopardi *et al.* 2000; Gluckman *et al.* 2004). MAP was reduced in the first 10 hours in hypothermic compared with normothermic animals, however hypotension did not develop. Furthermore, there was no evidence of significant metabolic acidosis or other adverse changes, except for a mild alkalosis.

Hypothermia was associated with a transient exaggeration of the secondary fall in CaBF, from 2 to 8 hours after asphyxia, compared to normothermic controls, similar to its effect after reversible cerebral ischemia at term (Gunn *et al.* 1997). However, over the last two days of the present study, CaBF progressively increased despite continued cooling and became significantly greater than in the asphyxia normothermia group in the final 24 h. This increase was accompanied by normalization of the frequency of EEG activity to sham control values, whereas frequency remained suppressed in the normothermia group. Together, these data suggest that cerebral function and activity significantly improved during continued cooling.

The results of the present study clearly cannot and should not be extrapolated to all preterm infants, since the etiology of preterm neural injury is extremely complex and has been associated with both antenatal and postnatal events including systemic inflammation, as well as hypoxia-ischemia (du Plessis and Volpe 2002). Nevertheless, data from serial EEG recordings and early MRI imaging suggest that many cases are related to events in the immediate peripartum period (de Vries *et al.* 1998; Hayakawa *et al.* 1999; Okumura *et al.* 2001). Overt perinatal insults are not uncommon, and are associated with 'ominous' fetal heart rate changes (de Vries *et al.* 1998). Pathologically, such asphyxial events are associated with a developmentally defined pattern of neural injury, with cortical sparing and severe damage in subcortical nuclei (Barkovich and Truwit 1990; Barkovich and Sargent 1995; de Vries *et al.* 1998), consistent with that seen in the asphyxia normothermia group in the present study. Thus, although no one experimental model can possibly represent the full spectrum of preterm neural injury, the present findings suggest that at least some cases of preterm neural injury may benefit from hypothermia.

Finally, it is important to recognize that evolving neuronal injury may extend for longer than the 3 days recovery from asphyxia in the present study (Geddes *et al.* 2001). Previous studies using short periods of hypothermia have suggested that protection can be attenuated over time (Trescher *et al.* 1997). However, this may relate to the duration of cooling, since 6 hours of mild cooling in the infant rat after hypoxia-ischemia was not associated with long-term improvement but 72 hours was (Sirimanne *et al.* 1996). Subsequent studies both in the 7 day rat and in adult species have confirmed that prolonged cooling for 24 to 72 hours initiated within 6 hours of injury is associated with persistent behavioral and histological protection (Bona *et al.* 1998; Colbourne *et al.* 1998; Colbourne *et al.* 1999; Nedelcu *et al.* 2000; Wagner *et al.* 2002). Further studies are in progress to evaluate the longer term impact of asphyxia and hypothermia on the preterm brain.

In summary, these data demonstrate that selective cerebral hypothermia after profound asphyxia

can reduce short-term injury of subcortical neuronal structures in the preterm brain. These histological changes were associated with progressive normalization of EEG frequency and carotid blood flow, consistent with possible functional improvements. Hypothermia was not associated with hypotension or acid-base changes, suggesting it may be safe in the preterm infant if carefully controlled. The critical developmental events occurring in the preterm infant, and their potential vulnerability to the adverse systemic effects of hypothermia make it critical to systematically evaluate factors, such as the window of opportunity for starting cooling and the critical depth of hypothermia that determine its effectiveness before human studies can be contemplated.

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CHAPTER 4

THE EFFECT OF MILD HYPOTHERMIA ON INSULIN-LIKE GROWTH FACTORS AFTER SEVERE ASPHYXIA IN THE PRETERM FETAL SHEEP

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ABSTRACT

Objectives: Persistent reductions in insulin-like growth factor I (IGF-I) levels in the preterm infant are strongly associated with increased risk of retinopathy and other complications, and may result from exposure to severe hypoxia. The effects of a potential new treatment for hypoxic-ischemic encephalopathy, cerebral hypothermia, on the responses of the IGF axis to hypoxia are unknown. The aim of this study was to examine the effects of prenatal asphyxia and cerebral hypothermia on changes in IGF-I and -II, IGF-binding protein 1 (IGFBP-1), and insulin levels.

Methods: Fetal sheep at 0.7 of gestation underwent either sham asphyxia and sham cooling ($n = 7$), asphyxia induced by 25 minutes of complete umbilical cord occlusion alone ($n = 8$), or asphyxia and head cooling ($n = 8$, extradural temperature $29.5 \pm 2.6^\circ\text{C}$ [vs $39.4 \pm 0.3^\circ\text{C}$; $P .05$]). Fetuses were studied for 3 days post-insult, during which time fetal blood samples were taken for endocrine measurements.

Results: There were no IGF axis changes during occlusion. Plasma IGF-I levels significantly decreased between 6 hours and 48 hours after asphyxia and IGF-II levels by 10 hours, in both asphyxia groups. IGFBP-1 rose from 4 hours, reaching a peak at 10 hours and returning to control values by 48 hours in the normothermia group, and by 24 hours in the hypothermia group. Insulin levels decreased between 2 hours and 10 hours after asphyxia in both asphyxia groups, and subsequently normalized.

Conclusions: This study demonstrates for the first time that transient asphyxia in the preterm sheep fetus is associated with a significant decrease in IGF-II as well as IGF-I during recovery, and that these responses are not altered by mild systemic hypothermia.

Key words: Asphyxia, prematurity, hypothermia, insulin-like growth factors.

INTRODUCTION

There is now strong experimental evidence that cerebral hypothermia initiated during the early hours after perinatal hypoxia-ischemia can prevent delayed cell death and improve neural recovery.¹ While clinical trials are currently underway in the term infant,² hypothermia has not been evaluated in the preterm infant, despite their much higher burden of neurologic impairment and systemic complications.³ It has been suggested from studies at term that hypothermia can cause adverse endocrine and metabolic effects that may reduce its neuroprotective effects and impair recovery⁴; however, there are no data from preterm paradigms. In recent years, there has been increasing interest in the key role insulin-like growth factors (IGFs) may play in metabolic regulation, growth, and organ function and development in preterm infants.⁵⁻⁸ Preterm birth is associated with a rapid fall in serum IGF-I levels.^{9,10} Persistent reductions in IGF-I levels are associated with an increased risk for later retinopathy, and other complications of prematurity, particularly in infants born around the critical organ development period of 30–33 weeks.^{11,12} Although the etiology of preterm injury is highly complex and multifactorial,¹³ hypoxia-ischemia is one of the many factors that may complicate the courses of preterm infants,¹³ and it may contribute to adverse outcomes. Hypoxia-ischemia can occur before, during, or after birth^{14,15} and it may exacerbate the low levels of IGF-I in premature infants.¹⁶ It was the purpose of the current study, therefore, to determine the IGF, insulin, and IGF-binding protein 1 (IGFBP-1) responses of the preterm fetal sheep to severe acute hypoxia in utero and to evaluate whether these responses are modified by cerebral hypothermia at an age corresponding to human neuronal maturation of 30–32 weeks.¹⁷

MATERIALS AND METHODS

Experimental Preparation

All procedures were approved by the Animal Ethics Committee of The University of Auckland. Twenty three time-mated singleton Romney/Suffolk fetal sheep were operated on at 98–99 days of gestation (term 147 days). Food, but not water, was withdrawn 18 hours before surgery. Ewes were given 5 mL of *Streptopen* (Procaine Penicillin [250,000 IU] and Dihydrostreptomycin [250 mg · mL⁻¹; Stockguard Labs Ltd, Hamilton, New Zealand]) intramuscularly for prophylaxis 30 minutes prior to the start of surgery. Anesthesia was induced by intravenous (IV) injection of Alfaxan (3 mg · kg⁻¹; Alphaxalone, Jurox, Rutherford, NSW, Australia), and general anesthesia maintained using 2–3% halothane in O₂. Ewes were not ventilated and the depth of anesthesia, maternal heart rate, and respiration were constantly monitored by trained anesthetic staff.

All surgical procedures were performed using sterile techniques.¹⁸ Polyvinyl catheters were placed in the left fetal femoral artery and vein, right brachial artery, and the amniotic sac. A stainless steel electrode (Cooner Wire, Chatsworth, CA) was placed across the fetal chest to measure the fetal electrocardiogram (ECG). A thermistor (Incu-Temp-1, Mallinckrodt Medical Inc, St Louis, MO) was placed over the parasagittal dura 20 mm anterior to the bregma; the burr holes were sealed, and the skin over the fetal skull was closed using cyanoacrylate glue. A cooling cap made from a coil of silicone tubing (external diameter, 7.9 mm; internal diameter, 4.8 mm; Degania Silicone, Degania, Bet, Emek Hayarden, Israel) was attached over the dorsal surface of the scalp and extended over the lateral surface of the cranium down to the level of the external auditory meatus. An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA). A second thermistor (to measure fetal core body temperature) was placed in the fetal esophagus at the level of the right

atrium. All fetal leads were exteriorized through the maternal flank and a maternal long saphenous vein was catheterized to provide access for postoperative care and euthanasia. Antibiotics (80 mg Gentamicin; Pharmacia and Upjohn, Rydalmere, NSW, Australia) were administered into the amniotic sac prior to closure of the uterus.

Postoperatively, all sheep were housed together in separate metabolic cages with access to water and food ad libitum. They were kept in a temperature-controlled room ($16 \pm 1^\circ\text{C}$, humidity 50% 10%), on a 12-hour light/dark cycle. A period of 4–6 days postoperative recovery was allowed before experiments commenced, during which time antibiotics were administered IV to the ewe daily for 4 days (600 mg Benzylpenicillin Sodium; Novartis Ltd, Auckland, New Zealand, and 80 mg Gentamicin). Fetal catheters were maintained patent by continuous infusion of heparinized saline (20 U/mL at 0.15 mL/h) and the maternal catheter maintained by daily flushing.

Experimental Design

Experiments were conducted at 103–104 days gestation (0.7 of gestation). Mean arterial pressure (MAP), corrected for maternal movement by subtraction of amniotic fluid pressure, and the fetal ECG were recorded continuously from 12 hours before occlusion to 72 hours after occlusion. Data were stored to disk by custom software for off-line analysis (Labview for Windows; National Instruments Ltd, Austin, TX). Fetuses were randomly assigned to one of three groups: sham controls (sham asphyxia and sham cooling, $n = 7$), asphyxia group (asphyxia with sham cooling, $n = 8$), and hypothermia group (asphyxia with induced hypothermia, $n = 8$). In the asphyxia groups, fetal asphyxia was induced by rapid inflation of the umbilical cord occluder for 25 minutes with sterile saline of a defined volume known to completely inflate the occluder as determined in pilot experiments with a Transonic flow probe placed around an umbilical vein.¹⁹ The duration of occlusion was chosen as one that we have previously reported to represent an acute, severe, near-terminal insult but which could be survived without post-asphyxial cardiac support.^{18,20} Successful occlusion was confirmed by observation of a rapid onset of bradycardia with a rise in MAP, and by pH and blood gas measurements.

Figure 1. Time course of changes in fetal core body temperature (squares) and extradural temperature (circles) in the normothermic (open symbols) and hypothermic asphyxia (closed symbols) groups. Data are mean $\pm$ SEM.

In the hypothermia group, intrauterine cooling was initiated 90 minutes after the end of asphyxia and continued until 2 hours before the end of the study (72 hours after asphyxia or sham asphyxia). Cooling was induced by circulating cold water (10C) through the silicone coil around the fetal head. Cooling was titrated in the first 2 hours to reduce fetal extradural temperature from $39.4 \pm 0.1^{\circ}\text{C}$ to between 29 and 33C (Figure 1).

Fetal arterial blood samples were taken 15 minutes prior to asphyxia or sham asphyxia, 5 and 20 minutes during asphyxia or sham asphyxia, and 2, 4, 6, 10, 24, 48, and 72 hours post-asphyxia or sham asphyxia for blood gas, acid-base balance (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter; Boston, MA) and for glucose and lactate determination (YSI model 2300; Yellow Springs, OH). In the hypothermia group, the 72-hour blood sample was taken after rewarming for 2 hours. Blood was transferred immediately upon collection to chilled test tubes and centrifuged at 4C (3000 rpm) for 15 minutes. Plasma was stored at 20C for subsequent hormone analysis (IGF-I, IGF-II, IGFBP-1, insulin). After the last blood sample ewes and fetuses were killed by an IV overdose of pentobarbitone sodium (9 g) to the ewe (Pentobarb 300; Chemstock International, Christchurch, New Zealand).

Data Analysis and Statistics

Fetal plasma IGF-I, IGF-II, IGFBP-1, and insulin levels were measured using specific ovine radioimmunoassays (RIA) established and validated in this laboratory.¹⁶ Statistical analyses were performed using SPSS version 10 (SPSS Inc, Chicago, IL). Differences between groups were determined by analysis of variance, adjusted using baseline levels as a covariate (ANCOVA); the endocrine time series data were treated as repeated measures to allow for repeated sampling. Where a significant effect of treatment group or an interaction with group was found, post hoc comparisons were undertaken using the least significant difference test. Statistical significance was accepted at $P < .05$.

Figure 2. Fetal plasma concentration changes in IGF-I and IGF-II before, during (sample taken at 20 minutes of a 25-minute occlusion insult) and after (2, 4, 6, 10, 24, 48, and 72 hours), in sham-controls (open bars), asphyxia with normothermia (dark bars) or asphyxia with hypothermia started 90 minutes after the insult and continued until 2 hours before the 72-hour sample (hatched bars). Data are mean $\pm$ SEM. * $P < .05$ compared to sham controls.

RESULTS

Blood Composition Measurements

Data for fetal arterial pH, blood gas, and glucose levels are presented in Table 1.

Effect of Cooling on Brain and Body Temperature

Head cooling caused a significant fall in extradural temperature starting within 1 hour after the onset of cooling, reaching a nadir of $29.5 \pm 2.6^\circ\text{C}$ (vs $39.4 \pm 0.3^\circ\text{C}$; $P < .0001$, ANOVA). Extradural temperature gradually increased during the second and third day of cooling to a mean of $32.3 \pm 2.2^\circ\text{C}$ by 72 hours. During cooling, core body temperature dropped to a nadir of $37.2 \pm 0.8^\circ\text{C}$ (vs $39.3 \pm 0.1^\circ\text{C}$; $P < .05$) and averaged $37.5 \pm 0.7^\circ\text{C}$ over the whole cooling period (Figure 1).

Endocrine Data

There was no significant difference between groups for any endocrine measure during the baseline period. There were no significant changes during asphyxia. IGF-I levels showed a significant effect of treatment group ($P = .006$, repeated measures ANOVA), with a significant interaction between time and group ($P = .001$). There was a significant reduction in circulating plasma concentrations of IGF-I after asphyxia from 6 to 48 hours in the asphyxia and hypothermia groups ($P < .05$ for both groups compared to sham controls, Figure 2), with no significant difference between the asphyxia and hypothermia groups. There was no overall effect of group on IGF-II levels, but an interaction between asphyxia and time ($P < .05$), with a fall in IGF-II levels at 10 hours in the asphyxia-normothermia group and at 6 and 10 hours in the hypothermia group ($P < .05$ compared to sham control, Figure 2). However, there was no significant difference between the normothermia and hypothermia groups.

Table 1. Fetal arterial pH, blood gas, glucose, and lactate values for Sham Control, Asphyxia plus normothermia, and Asphyxia plus hypothermia groups before umbilical cord occlusion (baseline), after 20 minutes of occlusion or Sham occlusion (asphyxia), and 2, 4, 6, 24, 48, and 72 hours after release of occlusion.

		Baseline	Asphyxia	2 hours
pH	S	7.39 ± 0.00	7.38 ± 0.00	7.39 ± 0.00
	A	7.37 ± 0.00	$6.82 \pm 0.03^*$	7.34 ± 0.01
	H	7.39 ± 0.00	$6.85 \pm 0.02^*$	7.39 ± 0.00
PaCO ₂ (mm Hg)	S	48.8 ± 1.3	48.3 ± 1.5	46.8 ± 1.0
	A	48.2 ± 1.0	$142.2 \pm 6.0^*$	46.8 ± 1.0
	H	48.5 ± 1.6	$139.0 \pm 9.0^*$	$38.0 \pm 1.0^*$
PaO ₂ (mm Hg)	S	22.5 ± 0.9	22.7 ± 0.9	22.1 ± 1.0
	A	23.0 ± 0.9	$8.7 \pm 0.8^*$	$26.2 \pm 1.3^*$
	H	24.7 ± 1.1	$9.1 \pm 1.6^*$	24.7 ± 1.3
Glucose (mM)	S	1.03 ± 0.10	1.03 ± 0.11	1.01 ± 0.12
	A	1.02 ± 0.10	$0.67 \pm 0.10^*$	1.24 ± 0.10
	H	0.95 ± 0.10	$0.53 \pm 0.14^*$	1.30 ± 0.10
Lactate (mM)	S	0.73 ± 0.10	0.73 ± 0.11	0.66 ± 0.12
	A	0.72 ± 0.03	$6.46 \pm 0.17^*$	$3.6 \pm 0.52^*$
	H	0.70 ± 0.03	$6.11 \pm 0.52^*$	$2.90 \pm 6.0^*$

Values are mean $\pm$ SEM. S sham control; A asphyxia plus normothermia; H hypothermia. * $P < .05$ compared to sham control.

Changes in IGFBP-1 concentrations showed a significant effect of treatment group ($P = .001$) with a significant interaction between time and treatment group ($P = .003$), such that concentrations of IGFBP-1 increased dramatically from 4 hours in both asphyxia groups, and remained significantly elevated until 24 hours in the asphyxia group and until 10 hours in the hypothermia group ($P < .05$, Figure 3). Plasma insulin levels showed a significant overall effect of treatment group ($P < .05$) but no interaction between treatment and time ($P = .3$). There was a significant fall in plasma concentrations of insulin between 2 and 10 hours after asphyxia in both asphyxia groups ($P < .05$, Figure 3), with no significant difference between the normothermic and hypothermic asphyxia groups.

DISCUSSION

The present study demonstrated that prenatal exposure to asphyxia is associated with a rapid fall in circulating levels of insulin, IGF-I and IGF-II in unanesthetized preterm fetal sheep. These changes were associated with a dramatic rise in IGFBP-1 levels, which would help to further reduce bioavailable circulating IGF-I. The data demonstrate that cerebral cooling with mild systemic hypothermia did not significantly alter the pattern of changes in circulating IGF-I and IGF-II, IGFBP-1, and insulin after an acute severe asphyxia. Further, the current study demonstrates for the first time, in contrast with previous data,¹⁶ that exposure to asphyxia can be associated with a transient reduction in IGF-II levels. The present study suggests that following acute severe hypoxia, IGFs and binding proteins play a key role in metabolic regulation in the preterm sheep fetus. Previous studies in the fetal sheep have shown that an infusion of IGF-I with insulin is highly anabolic.²¹ Thus, the reductions of both circulating IGF-I and insulin levels in the present study may facilitate peripheral protein catabolism. The present study suggests that IGF-II may also contribute to acute metabolic regulation, with circulating IGF-II levels falling at 6 hours and significantly suppressed by 10 hours after occlusion, albeit more modestly than IGF-I. In the fetus, IGF-II has been classically seen as constitutive, and IGF-I more labile. Large environmental changes, such as severe starvation or very prolonged hypoxia, were

4 hours	6 hours	24 hours	48 hours	72 hours
7.39±0.00	7.38±0.00	7.38±0.00	7.37±0.00	7.37±0.01
7.40±0.00	7.41±0.00	7.37±0.00	7.38±0.00	7.38±0.00
7.46±0.01*	7.44±0.00*	7.43±0.01*	7.41±0.00*	7.43±0.00*
47.3±0.8	47.8±1.2	45.0±1.7	48.0±1.1	46.3±1.6
47.8±1.2	45.6±1.4	47.4±1.1	47.2±0.9	46.5±1.2
40.0±1.0*	40.0±1.1*	39.0±3.0*	42.5±1.0*	42.9±2.0
22.6±1.0	22.3±1.0	22.2±2.1	23.4±1.5	23.5±1.4
24.8±1.0	24.8±0.9	26.6±1.3	28.0±1.3*	28.3±3.6*
25.1±1.4	24.4±1.1	23.6±0.9	24.5±1.5	27.0±2.7
1.10±0.06	1.11±0.10	1.20±0.12	1.20±0.10	1.20±0.10
1.30±0.10*	1.40±0.10*	1.50±0.14*	1.30±0.10	1.23±0.10
1.40±0.12*	1.52±0.10*	1.43±0.13	1.30±0.13	1.42±0.10
0.75±0.04	0.75±0.15	0.64±0.10	0.83±0.11	0.90±0.12
2.48±0.44*	1.86±0.24*	1.00±0.12*	0.78±0.10	0.88±0.10
1.40±0.32*	1.21±0.16*	0.92±0.12	0.73±0.10	0.80±0.10

Figure 3. Fetal plasma concentration changes in IGFBP-1 and insulin levels before, during (sample taken at 20 minutes of a 25-minute occlusion insult) and after (2, 4, 6, 10, 24, 48, and 72 hours), in sham-controls (open bars), asphyxia with normothermia (dark bars), or asphyxia with hypothermia started 90 minutes after the insult and continued until 2 hours before the 72-hour sample (hatched bars). Data are mean $\pm$ SEM. * $P < .05$ compared to sham controls.

required before circulating concentrations of IGF-II changed significantly.^{22,23} Recent studies, however, suggest an important role for IGF-II in modulating both the placental supply and fetal demand for nutrition.^{24,25} Thus, the present finding of relatively acute changes in IGF-II levels may reflect the dynamic metabolic adaptations required after severe asphyxia.

The bioavailability and activity of the IGFs is further regulated by changes in the IGFBPs and the IGF receptors. For example, although there are limited data, hypoxia can modulate IGF-I receptor levels in some tissues.²⁶ The increase in IGFBP-1 levels after asphyxia may augment the impact of reduced circulating IGF-I levels by reducing its bioavailability. Several mechanisms are likely to have contributed to the increase in IGFBP-1. In vitro, expression of IGFBP-1 in the liver has been shown to be stimulated by hypoxia.²⁷ The intron 1 region of the IGFBP-1 gene contains an hypoxia response element (HRE), which can be induced either directly by hypoxia or indirectly through release of hypoxia-inducible factor-1 (HIF-1).²⁸ In the present study, IGFBP-1 started to rise from 4 hours after umbilical cord occlusion, at a time when the hypoxia was resolved completely, consistent with the observed delay in vitro between the onset of hypoxia and the subsequent rise in IGFBP-1.²⁸

In addition, insulin is an important modulator of IGFBP-1 levels,²⁹ such that fetal IGFBP-1 levels are inversely related to insulin concentrations.³⁰ Consistent with this, our current findings show that reduced insulin levels after asphyxia preceded the rise in IGFBP-1 levels. We have recently demonstrated in the 0.7 gestation fetus that sympathetic tone is significantly elevated after asphyxia.²⁰ This increase in sympathetic activity may also be important because the hypoxic inhibition of insulin secretion is mediated by an α_2 -adrenergic mechanism,³¹ which in turn stimulates IGFBP-1 production.³² In adults, IGFBP-1 is cleared from the circulation by the kidney and impaired renal function results in high circulating IGFBP-1 levels³³; however, this is unlikely to be important in the fetus as at this

gestation there is only a small, transient reduction in glomerular filtration rate following asphyxia.¹⁸ The present study demonstrates for the first time that induced head cooling with mild systemic hypothermia did not affect the changes in plasma IGF-I levels or IGF-II after asphyxia. IGFBP-1 levels resolved by 24 hours in the hypothermic asphyxia group but remained elevated in the normothermic asphyxia group, even though insulin levels had returned to normal in both groups. This was associated with higher blood glucose levels in the normothermia asphyxia group, and may reflect the hyperglycemic effects of IGFBP1.³⁴ We did observe a mild metabolic alkalosis during cooling, which may be related to stimulation of maternal breathing by cooling.³⁵

Compared with a previous report in younger fetuses (0.6 of gestation) the present study demonstrated a less pronounced fall in IGF-I (50% vs 80%) and an acute fall in IGF-II levels in contrast with a small secondary rise in the days following asphyxia in the younger fetuses.¹⁸ The insults at each gestation are comparable and near terminal. The extent to which these differences reflect differences in metabolic demand and degree of injury between the two ages, maturational differences in sympathetic tone,²⁰ or renin-angiotensin system activity³⁶ remains to be determined.

In conclusion, this study demonstrates that therapeutic head-cooling in the preterm fetus is not associated with major changes in the responses of circulating concentrations of IGFs after asphyxia. The overall profile of the changes in IGFs and insulin is consistent with facilitation of catabolism. Intriguingly, this study suggests for the first time that by 0.7 of gestation changes in IGF-II may also contribute, in addition to IGF-I, to regulation of fetal metabolism during the acute phase of post-asphyxial recovery in utero.

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CHAPTER 5

WINDOW OF OPPORTUNITY OF CEREBRAL HYPOTHERMIA FOR POSTISCHAEMIC WHITE MATTER INJURY IN THE NEAR-TERM FETAL SHEEP.

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ABSTRACT

Summary: Postresuscitation cerebral hypothermia is consistently neuroprotective in experimental preparations; however, its effects on white matter injury are poorly understood. Using a model of reversible cerebral ischemia in unanesthetized nearterm fetal sheep, we examined the effects of cerebral hypothermia (fetal extradural temperature reduced from $39.4 \pm 0.1^{\circ}\text{C}$ to between 30 and 33°C), induced at different times after reperfusion and continued for 72 hours after ischemia, on injury in the parasagittal white matter 5 days after ischemia. Cooling started within 90 minutes of reperfusion was associated with a significant increase in bioactive oligodendrocytes in the intragyral white matter compared with sham cooling (41 ± 20 vs 18 ± 11 per field, $P < 0.05$), increased myelin basic protein density and reduced expression of activated caspase-3 (14 ± 12 vs 91 ± 51 , $P < 0.05$). Reactive microglia were profoundly suppressed compared with sham cooling (4 ± 6 vs 38 ± 18 per field, $P < 0.05$) with no effect on numbers of astrocytes. When cooling was delayed until 5.5 hours after reperfusion there was no significant effect on loss of oligodendrocytes (24 ± 12 per field). In conclusion, hypothermia can effectively protect white matter after ischemia, but only if initiated early after the insult. Protection was closely associated with reduced expression of both activated caspase-3 and of reactive microglia.

Key Words: Hypothermia—White matter—Brain injury.

INTRODUCTION

Although white matter injury is well known to be the dominant cause of neural handicap in premature infants (Inder et al., 1999), there is increasing evidence from magnetic resonance imaging studies that it is also a significant contributor to neurodevelopmental problems at term (Barkovich et al., 1995; Mercuri et al., 1999; Okumura et al., 1997). Experimentally, mature, myelinating oligodendrocytes are at least as vulnerable to ischemic injury as neurons (Cao et al., 2003; Guan et al., 2000; Ikeda et al., 1998; Loeliger et al., 2003; Pantoni et al., 1996; Petersson et al., 2002). Indeed, after asphyxia in the near-term fetal sheep, the mildest pathologic findings were vacuolation and loss of myelin in white matter, rather than neuronal death (Ikeda et al., 1998).

There is now strong clinical and experimental evidence that a prolonged period of moderate cerebral hypothermia initiated within a few hours after severe hypoxia-ischemia can markedly reduce subsequent neuronal loss and improve behavioral recovery (Bernard et al., 2002; Colbourne and Corbett, 1995; Gunn et al., 1997, 1998b; The Hypothermia after Cardiac Arrest Study Group, 2002; Tooley et al., 2003). Although there are few data on perinatal white matter damage, early initiation of hypothermia can ameliorate posttraumatic spinal cord injury (Inamasu et al., 2003), and there is increasing evidence that many of the effects of cooling are relevant to the proposed mechanisms of hypoxic-ischemic oligodendrocyte loss.

Recent studies have shown that the pathogenesis of postischemic demyelination includes a substantial element of delayed or secondary loss (Cao et al., 2003), likely mediated by apoptosis and microglia activation (Hagberg et al., 2002; Shibata et al., 2000). Apoptosis is a significant component of postasphyxial cell death in the developing brain (Edwards et al., 1997; Scott and Hegyi, 1997). Although multiple pathways are likely to be involved in such postischemic apoptosis, caspase-3, one of the family of cysteine proteases, is reported to play a crucial role in the immature brain (Hu et al., 2000; Johnson et al., 1999; Zhu et al., 2003), and specifically in loss of oligodendrocytes (Cao et al., 2003; Shibata et al., 2000). Several reports suggest that hypothermia can specifically suppress the development of apoptotic injury (Edwards et al., 1995; Xu et al., 1998).

In addition, there is increasing evidence that cytokine induction and the associated inflammatory reaction are major contributors to perinatal brain injury (Hagberg et al., 2002). In particular, microglia activated by interferon-gamma induce contact-dependent oligodendroglial death (Nicholas et al., 2003). Hypothermia potently reduces cytokine release and microglial activation both *in vivo* and *in vitro* (Gibbons et al., 2003; Inamasu et al., 2000; Wang et al., 2002).

The effects of cooling on neuronal loss are known to be highly dependent on the timing of initiation of cooling, with rapid loss of effect as delay is increased (Gunn and Bennet, 2002). This relationship has not been established for perinatal white matter injury. Therefore, in the present study we examined the effect of time of initiation of moderate cerebral hypothermia for 3 days after ischemia on white matter damage after transient cerebral ischemia in near-term (0.85 gestation) fetal sheep. In terms of cerebral maturity and myelination, this stage is comparable to the human brain at term (McIntosh et al., 1979). The effects of hypothermia on postischemic white matter apoptosis and the inflammatory reaction after 5 days recovery from ischemia were assessed using proteolipid protein (PLP) mRNA to identify bioactive oligodendrocytes and immunohistochemistry to identify other glial cell types.

MATERIALS AND METHODS

Experimental preparation

All procedures were approved by the Animal Ethics Committee of the University of Auckland. Singleton Romney/Suffolk fetal sheep, while under general anesthesia (2% halothane in O₂), were instrumented at 117 to 124 days of gestation (term = 147 days) using sterile techniques. Ewes were given 5 mL of Streptopen (Stockguard Labs Ltd., Hamilton, New Zealand) intramuscularly before the start of surgery. Polyvinyl catheters were placed in the brachial arteries, a brachial vein, and the amniotic sac. Electrocardiogram (ECG) electrodes were sewn across the chest to record the fetal electrocardiogram. A thermistor was placed in the fetal esophagus at the level of the right atrium to measure fetal core body temperature (Gunn et al., 1997). The vertebral-occipital anastomoses were ligated bilaterally to restrict vertebral blood supply to the carotid arteries. A double-ballooned inflatable occluder was placed around each carotid artery (Gunn et al., 1997). Two pairs of electroencephalographic electrodes (AS633-5SSF, Cooner Wire Co., Chatsworth, CA, U.S.A.) were placed on the dura over the parasagittal parietal cortex (5 mm and 15 mm anterior to bregma and 10 mm lateral), with a reference electrode sewn over the occiput. Cortical impedance was measured using a third pair of electrodes (AS633-3SSF, Cooner Wire Co.) placed over the dura, 5 mm lateral to the electroencephalographic electrodes. A second thermistor (Incu-temp-1; Mallinckrodt Medical, St. Louis, MO, U.S.A.) was placed over the parasagittal dura 20 mm anterior to bregma and the burr holes were sealed and the skin over the fetal skull was secured with cyanoacrylate glue.

A cooling coil made from silicone tubing (external diameter, 7.9 mm; internal diameter, 4.8 mm; Silclear, Degania Silicone, Degania Bet, Israel) was attached over the dorsal surface of the scalp and extended over the lateral surface of the cranium down to the level of the external auditory meatus. The fetus was returned to the uterus and 80 mg of gentamicin was administered into the amniotic sac before closure of the uterus. All fetal leads were exteriorized through the maternal flank. The maternal long saphenous vein was catheterized for administration of antibiotics and for euthanasia. A thermistor was inserted with the catheter to permit measurement of maternal temperature.

After surgery, sheep were housed together in separate metabolic cages with access to water and food *ad libitum*. They were kept in a temperature-controlled room ($16 \pm 1^\circ\text{C}$, humidity $50\% \pm 10\%$), in a 12-hour light/dark cycle. A period of 3 to 5 days' postoperative recovery was allowed, during which the ewe was injected with daily intravenous (i.v.) gentamicin (80 mg gentamicin, Pharmacia & Upjohn, Perth, Australia). Fetal arterial blood was taken daily from the brachial artery, for blood gas analysis. Catheters were maintained patent by continuous infusion of heparinized saline (40 U.mL^{-1} at 0.2 mL.h^{-1}).

Experimental design and recordings

Fetuses were randomly assigned to one of four groups: sham control, ischemia plus sham cooling, or ischemia plus cooling, starting either 2 hours (2-hour cooling group) or 6 hours (6 hour cooling group) after the start of ischemia. Fetal mean arterial pressure, corrected for maternal movement by subtraction of amniotic fluid pressure, fetal heart rate, carotid artery blood flow, fetal electroencephalogram, spectral edge, and impedance were recorded continuously from 12 hours before the experiment until 5 days afterwards. Data were stored at 1-minute intervals to disk by custom software for off-line analysis (Labview for Windows, National Instruments Ltd, Austin, TX, U.S.A.).

Cerebral ischemia was induced by rapid inflation of the carotid artery occluders bilaterally for 30 minutes with sterile saline. Successful occlusion was confirmed by observation of a rapid, sustained flattening of the electroencephalogram. Cooling was started either 2 or 6 hours after the onset of the

ischemic period (i.e., 90 minutes vs. 5.5 hours after the initiation of reperfusion) and was continued until 72 hours after occlusion. Fetal arterial blood was taken 60 minutes before carotid occlusion, and then at 35 minutes after the start of occlusion (i.e., 5 minutes after the end of occlusion), and 2, 4, 6, 12, 24, 48, 72, 96, and 120 hours and analyzed for pH and blood gas determination (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, East Walpole, MA, U.S.A.) and for glucose and lactate measurements (YSI model 2300, Yellow Springs, OH, U.S.A.).

On completion of the experiment at 120 hours, the ewe and fetus were killed by an overdose of pentobarbitone sodium given IV to the ewe. The fetal brain was perfusion fixed with saline followed by 10% neutral buffered formalin, stored overnight for fixation in 10% neutral buffered formalin, then embedded in paraffin using a standard paraffin tissue preparation.

The pH, blood gas data, and glucose and lactate measurements, carotid blood flow and electrophysiologic changes, and neuronal loss scores have been published in a subset of these animals (Gunn et al., 1997, 1998b).

Immunohistochemistry

Immunohistochemical staining was performed on coronal sections (6 μ m), at the level of the parietal cortex, cut and mounted on chrome alum-coated slides. Before specific staining, all the slides were deparaffinized in xylene, twice for 15 minutes, dehydrated in a series of ethanol (100%, 100%, 95%, 95%, 70%, for 2 minutes each), and incubated in 0.1 mol/L phosphate buffered saline (PBS) twice for 5 minutes. The sections were pretreated with 1% H₂O₂ in 50% methanol for 20 minutes, washed in PBS (3 x 5 minutes) and incubated with the primary antibody for 2 days at 4°C. The following primary antibodies and dilutions were used: mouse anti-myelin basic protein (MBP, Roche, Mannheim, Germany; 1:200); isolectin B-4 (Sigma, St. Louis, MO, U.S.A.; 1:100); mouse anti-glial fibrillary acidic protein (Sigma; 1:200); mouse-proliferating cell nuclear antigen PC10 (PCNA; DAKO, Denmark, diluted 1:100); rabbit anti-Caspase-3 Asp175 (cleaved Caspase-3 Antibody, detects endogenous levels of the large fragment of activated caspase-3 (17–20kDa), Cell Signaling Technology, Beverly, MA, U.S.A., diluted 1:1,000). The antibodies were diluted in PBS containing normal horse serum/goat serum. The primary antibodies were washed off with PBS (3 x 10 minutes) and then incubated with anti-mouse/anti-goat biotinylated immunoglobulin G (Vector Laboratories, Burlingame, CA, U.S.A., diluted 1:200) overnight at 4°C. The sections were washed, incubated in avidin-biotin complex (ABC, Vector Laboratories, Peterborough, England, diluted 1:100) in the ratio

1:500 for 3 hours at room temperature, washed again in PBS, and then reacted in diaminobenzidine tetrahydrochloride (DAB, Sigma). Sections were then dehydrated in a series of alcohol to xylene washes and coverslipped with mounting medium. Control sections were processed in the same way except that the primary antibody was omitted from the incubation solution.

In situ hybridization

In situ hybridization, using a sheep PLP RNA probe, was used to identify bioactive oligodendrocytes, as previously described (Guan et al., 2001).

Analysis and statistics

Scoring of oligodendrocytes, astrocytes, microglia, and density of myelin was performed on coronal sections by light microscopy at x200 magnification. Within these sections, cells were counted using a fixed grid (0.42 x 0.21 mm), in three areas in the intragyral white matter of the parasagittal cortex, and one in the corona radiata, of both hemispheres (Fig. 1). The density of MBP from the same areas and their background was measured using image analysis (Sigmascan, SPSS, Chicago, IL, U.S.A.). The difference between the

Figure 1. Photomicrograph showing the regions used to count different cell types and to assess the average density of myelin in the intragyral white matter and the corona radiata. Three regions in the intragyral white matter (squares 1, 2, and 3) and one area in the corona radiata (square 4), in both hemispheres, were used for assessment.

MBP density and the background reading from adjacent gray matter was calculated and used for data analysis. The number of cells positive for glial fibrillary acidic protein, IB-4, PLP, and PCNA and the MBP density were averaged from the three selected intragyral areas. Because of the relatively low density of caspase-3 (Asp 175) positively stained cells, the measurement represents the total number of caspase-3 (Asp 175)-positive cells present in the white matter tract of the first parasagittal gyrus.

The sham cooling (ischemia) group showed marked tissue swelling in the intragyral regions, with a significant increase in intragyral white matter areas compared with sham controls. To correct for decreased cell and staining density because of tissue swelling, cell counts and MBP staining density measurements in the sham cooling group were adjusted by the relative increase in white matter area (the ratio of intragyral white matter area in sham cooled fetuses to the sham control group) before statistical analysis. There was no significant tissue swelling in the two hypothermia groups; therefore the counts and staining density were not adjusted. Treatment effects were evaluated by analysis of variance (SPSS v10, SPSS Inc.), followed by the protected least-significant difference post-hoc test when a significant overall effect was found. Significance was accepted at $P < 0.05$. Data are mean $\pm$ SD.

RESULTS

Myelin Basic Protein

Myelin basic protein immunohistochemistry showed specific staining of myelin sheaths in the intragyral and corona radiata without obvious staining of cell bodies (Figs. 2a and 2e). Cerebral ischemia was associated with a severe loss of MBP density compared with sham controls (Figs. 2b and 2f, and Fig. 3, $P < 0.05$), which was greatest in the parasagittal intragyral white matter ($P < 0.05$). Morphologically, the normally parallel microstructure of MBP was disorganized and fragmented. The intragyral white matter tracts were markedly swollen (Figs. 2b and 2f), with a significant increase of the intragyral white matter area compared with sham controls (12.70 ± 2.25 vs 7.11 ± 1.63 mm², $P < 0.05$).

Both cooling groups showed reduced loss of MBP staining density in the intragyral white matter and corona radiata after 5 days recovery compared with the shamcooled ischemia group, which was greatest in the 2-hour group (Figs. 2c, 2d, 2g, 2h, and Fig. 3, $P < 0.05$). On visual assessment, MBP staining was denser, more compact and less fragmented than in the sham-cooled (ischemia) group (Figs. 2g and 2h). Consistent with this, the area of the intragyral white matter area was significantly

Figure 2. Photomicrographs showing the distribution (a, b, c, d, scale bar = 1 mm) and the morphologic appearance of myelin basic protein (MBP) (e, f, g, h, scale bar = 50 μ m) in sham controls (a and e), ischemia with sham cooling (b and f), and ischemia followed by cooling commenced at either 2 hours (2-hour cooling group, c and g) or 6 hours (6-hour cooling group, d & h) after the start of ischemia. In sham control fetal sheep, the white matter tracts were densely labeled with MBP, which was greatest in the corona radiata (a). Morphologically, the MBP staining fibers are compact and parallel the white matter tracts (e). After ischemia, the density of MBP staining was significantly reduced in the sham cooled group, with severe loss in the intragryal regions of the white matter tracts accompanied by marked tissue swelling (b). MBP immunopositive staining associated with the white matter fibers was reduced, fragmented, and less compact compared with the sham controls (f). Early initiation of cooling (2-hour group) was associated with nearly complete normalization of average MBP density (c). Morphologically, cooling also partially restored both the distribution and compactness of MBP-positive nerve fibers (g). Late initiation of cooling (6-hour cooling group) was associated with an intermediate degree of loss of MBP staining (d). Morphologically, this group showed some continuing edema and fragmentation of the MBP-positive nerve fibers, but to a lesser extent than in the sham cooled fetuses (h).

reduced in both the 2- and 6-hour cooling groups compared to ischemia alone ($P < 0.05$, 8.30 ± 243 and 9.02 ± 3.29 mm², respectively) and not significantly different from sham controls.

Proteolipid protein mRNA expression

In the sham control group, PLP mRNA staining was densely distributed from the corona radiata area toward the intragryal regions of the parasagittal white matter tracts. Proteolipid protein staining was localized in the periphery of the cytoplasm of oligodendrocytes in a bipolar distribution, as previously described (Guan et al., 2001). Ischemia was associated with a significant reduction in the number of the PLP staining oligodendrocytes compared to sham controls ($P < 0.05$; bottom panel, Fig. 3); the loss was greater in intragryal white matter tracts compared with the corona radiata ($P < 0.05$). The 2-

Figure 3. Density of myelin basic protein (MBP, top) immunostaining and numbers of proteolipid protein (PLP, bottom) mRNA-positive cells in parasagittal white matter. Ischemia resulted in a severe loss of MBP density (top) after 5 days recovery compared to the sham control group. Both the 2-hour and the 6-hour cooling groups showed a significant increase in average MBP density after ischemia compared to sham cooled (ischemia) fetuses, both in the intragyrar white matter and the corona radiata. There was a significant loss of PLP mRNA-containing cells (bottom) in the sham cooling (ischemia) group compared to sham controls both in the intragyrar white matter and the corona radiata. The 2-hour but not the 6-hour cooling group showed a significant increase in PLP mRNA-positive cells compared to the sham cooling (ischemia) group. # $P < 0.05$ vs sham controls, * $P < 0.05$ vs ischemia with sham cooling. Data are mean $\pm$ SD.

hour, but not the 6-hour, cooling group showed a significantly greater number of PLP mRNA-positive cells than in the sham cooling (ischemia) group in both white matter regions ($P < 0.05$). Numbers of PLP mRNA cells in the 2-hour cooling group were still less than in sham controls in the intragyrar white matter ($P < 0.05$) but similar to sham controls in the corona radiata.

Astrocytes and microglia

Glial fibrillary acidic protein-positive astrocytes were localized throughout the fetal white matter tracts of sham control animals (Fig. 4a). Double labeling of glial fibrillary acidic protein with PLP mRNA showed a close association between astrocytes and oligodendrocytes in the white matter (data not shown). Ischemia was associated with an apparent reduction in numbers of astrocytes in the intragyrar white matter (Fig. 4b) but not the corona radiata (Fig. 5). However, this was not statistically significant after correction for tissue swelling in this group (Fig. 5). There was no significant effect of cooling (Figs. 4c, 4d, and Fig. 5).

Sham control animals showed virtually no isolectin B-4 staining in the intragyrar regions or corona radiata (Fig. 4e). Ischemia was associated with a significant increase of isolectin B-4 staining activated microglia in the intragyrar and the corona radiata regions (Fig. 4f and Fig. 5). Both the 2-hour and 6-hour cooling groups showed a significant reduction in numbers of microglia compared with the sham cooled (ischemia) group (Figs. 4g and 4h, respectively, $P < 0.05$) in both white matter regions, which was relatively greater in the 2-hour cooling group (Fig. 5).

Activated caspase-3 expression

Only occasional cleaved 17-kD caspase-3 positive cells were seen in a few sham control animals. After ischemia in the sham-cooled (ischemia) and both cooling groups, there was a significant increase in activated caspase-3 expression in the parasagittal cerebral white matter (Fig. 6). The density of caspase-

Figure 4. Photomicrographs showing glial fibrillary acidic protein immunostaining (a–d) and isolectin B-4 staining (e–h) in the intragyrar white matter. The sham control group showed abundant glial fibrillary acidic protein staining (a). There was an apparent reduction in numbers of glial fibrillary acidic protein-positive cells after ischemia (b), because of severe tissue swelling. Both the 2-hour and 6-hour cooling groups showed numbers of glial fibrillary acidic protein-positive cells that were similar to the sham controls (c and d). No isolectin B-4 positive cells were seen in sham controls (e). Ischemia was associated with intense induction of isolectin B-4 positive cells (f), which was almost completely suppressed by early initiation of cooling (2-hour cooling group) (g). The 6-hour cooling group showed intermediate expression of isolectin B-4 positive cells (h). Scale bar = 50 μ m.

Figure 5. Numbers of isolectin B-4 (Iso-B4, top) and glial fibrillary acidic protein [GFAP, bottom] immunopositive cells in predefined areas of the intragyrar cerebral white matter and parasagittal corona radiata. The number of Iso-B4-positive cells was significantly increased after ischemic injury compared to the sham controls. Treatment with either early (2-hour group) or late (6-hour group) initiation of hypothermia significantly suppressed isolectin B-4 positive cells compared to the sham cooled group, although the degree of suppression was less with late cooling. Corrected for the area of the intragyrar white matter tracts, there was no effect of ischemia on numbers of GFAP cells (bottom), and no effect of hypothermia. # $P < 0.05$ vs sham controls, * $P < 0.05$ vs ischemia with sham cooling. Data are mean $\pm$ SD.

Figure 6. The total number of caspase-3 (Asp175)-positive cells in the parasagittal cerebral white matter tract (top) was significantly increased after 5 days' recovery from cerebral ischemia compared to the sham control group. The 2-hour cooling group showed near-complete suppression of caspase-3 activation compared to the sham cooled group, with a much smaller effect in the 6-hour cooling group. Ischemia reduced the number of proliferating cell nuclear antigen (PCNA, bottom)-positive cells in the intragyrus white matter tract. There was no significant additional effect of hypothermia. # $P < 0.05$ vs sham controls, * $P < 0.05$ vs ischemia with sham cooling. Data are mean $\pm$ SD.

3 staining varied between cells because of the timing of cleavage of caspase-3. Cells were counted that had evidence of newly cleaved caspase-3, as shown by morphologically dense caspase-3 (Asp175) staining evenly distributed within the ectoplasm, with coarse granular fragments around the cells (Fig. 7). Thionine counterstaining showed condensed chromatin fragments within the caspase-3 (Asp175)-positive cells. Both cooling groups showed significantly reduced numbers of cells expressing activated caspase-3 compared to sham cooling; the reduction in the 2-hour cooling group was greater than in the 6-hour cooling group (Fig. 6, $P < 0.05$).

Proliferating cell nuclear antigen expression

Figure 6 shows changes in cell proliferation in the white matter tracts assessed by PCNA immunohistochemistry. There were abundant PCNA labeling cells in sham control animals. Ischemia was associated with a significant reduction in the number of PCNA labeling cells compared to sham control ($P < 0.05$). The number of PCNA labeled cells was not significantly different between sham cooling (ischemia) and the two cooling groups.

DISCUSSION

The present study demonstrates that moderate, delayed cerebral hypothermia is associated with potent, delay-dependent suppression of postischemic cerebral demyelination in the near-term fetal sheep. Moderate hypothermia was begun 90 minutes after reperfusion (2-hour cooling group) and continued until 3 days after insult, and was associated with greater oligodendrocyte survival and myelin density and with reduced tissue edema in the parasagittal white matter tracts when evaluated 5 days after the ischemic insult. Oligodendrocyte protection was lost when initiation of cooling was delayed until 5.5 hours after reperfusion. The effects of hypothermia were strongly associated with suppression of both activated caspase-3 expression and the microglial reaction.

Figure 7. Photomicrograph showing an example of caspase-3-positive staining (brown) in the intragryal white matter tracts after cerebral ischemia in the near-term fetal sheep. Note the nuclear condensation within the caspase-3 (Asp175)-positive cells and coarse granular fragments of caspase-positive material around the cells.

Proteolipid protein (PLP) gene expression has been used as a sensitive marker to detect bioactive oligodendrocytes at the translation stage of myelination (Guan et al., 2001; Mandai et al., 1997). Expression of PLP in the central nervous system is classically reported only in differentiated, myelinating oligodendrocytes, although trace amounts may be found earlier in development (Schindler et al., 1990). *in vitro* and *in vivo* evidence now clearly demonstrate that both mature and immature oligodendrocytes are exquisitely vulnerable to hypoxic-ischemic injury (Back et al., 2001; Pantoni et al., 1996; Skoff et al., 2001). Although *in vitro* myelinating oligodendrocytes are reported to be relatively resistant to excitotoxicity compared to immature oligodendrocytes (Rosenberg et al., 2003), there is increasing evidence that white matter injury is also a significant contributor to neurodevelopmental disability after hypoxic-ischemic encephalopathy at term (Mercuri et al., 1999; Okumura et al., 1997; Rutherford et al., 1998). Because cooling is already being tested clinically as a potential neuroprotective treatment for newborn infants exposed to perinatal asphyxia (Gunn et al., 1998a; Shankaran et al., 2002), it is important to ascertain whether cooling is effective in reducing white matter loss at term.

We have previously reported, in the same experimental model as the present study, that improved neuronal loss and electrophysiologic recovery with cerebral hypothermia are dependent on the timing of initiation of cooling after reperfusion. There is dramatic improvement if moderate cooling is initiated within a few hours of severe ischemia (Gunn et al., 1997), but only partial protection if cooling is delayed until 6 hours from the start of ischemia (5.5 hours after reperfusion) (Gunn et al., 1998b). Clinically, the development of seizures in the recovery period after asphyxia is one of the major prognostic factors associated with an adverse outcome (Caravale et al., 2003), and most clinicians would prefer to treat only infants who demonstrate seizures. However, experimentally, when cooling was delayed until after seizures were established (8.5 hours after reperfusion), there was no electrophysiologic or overall histologic improvement (Gunn et al., 1999).

These studies demonstrated the importance of starting cooling during the latent phase of recovery (approximately the first 6 hours in this paradigm), before the phase of secondary deterioration with seizures and cytotoxic edema (Gunn et al., 1999). This finding supports the hypothesis that there are events during early recovery, well after resolution of the insult itself, which contribute significantly to the final severity of injury. The present data show that hypothermia is associated with an even more acute time dependence for improvement in oligodendrocyte loss than for neuronal loss, such that

cooling delayed by 5.5 hours after reperfusion did not improve numbers of oligodendrocytes after 5 days recovery. Thus, the current study further highlights the considerable importance of early initiation of cooling for cerebral protection.

The mechanisms of this rapid loss of protection of both oligodendrocytes and neurons with hypothermia with increasing delay are unclear. One potential mechanism is the timing of induction of caspase-mediated cell death. *in vitro*, cytoplasmic activation of caspases occurs in the latent phase, before the active or "execution" phase of programmed cell death involving intranuclear mechanisms (Samejima et al., 1998). Apoptotic pathways are suggested to have a relatively more important role in ischemic brain damage in the neonate compared to the adult (Hu et al., 2000), particularly in mediating posthypoxic loss of oligodendrocytes (Shibata et al., 2000). Similarly, we have reported that ischemia in the near-term fetal sheep leads to a marked increase in cells expressing activated caspase-3, and that this colocalized exclusively with PLP mRNA expression (Cao et al., 2003).

Several studies have suggested that hypothermia acts to reduce morphologic apoptotic cell loss in the neonatal brain (Edwards et al., 1995; Xu et al., 1998). Supporting this concept, mild hypothermia can reduce mitochondrial cytochrome C translocation, a critical step in the intrinsic pathway of apoptosis (Xu et al., 2002; Yenari et al., 2002), and has been shown to suppress expression of caspase-3 both after hypoxia-ischemia *in vivo* (Fukuda et al., 2001; Tomimatsu et al., 2001) and serum deprivation *in vitro* (Xu et al., 2002). Consistent with these results, the present study confirms a significant time of initiation-dependent suppression by hypothermia of postischemic activated caspase-3 in the cerebral white matter.

However, quantitatively, caspase-3 expression after 5 days' recovery can only account for a minority of oligodendrocyte cell loss, with a relatively low density of caspase-positive cells observed relative to the extent of loss of PLP-labeled cells. Speculatively, this discrepancy is likely related to the timing of histologic evaluation, with the preponderance of activation of caspases occurring much earlier after ischemia (Northington et al., 2001). A recent study has demonstrated that in the early stages of the development of cerebral necrosis, neurons display a number of features of early apoptosis, including cytoplasmic and nuclear condensation and specific caspase activation (Benchoua et al., 2001). Furthermore, in addition to caspase-mediated mechanisms, caspase independent apoptotic pathways, such as apoptosis inducing factor, contribute to delayed cell death and to necrotic processes (Leist and Jaattela, 2001; Sanchez-Gomez et al., 2003). Often, more than one of these pathways seem to be activated simultaneously, and cell fate is then determined by the relative speed of each process (Leist and Jaattela, 2001).

Alternatively, suppression of secondary inflammatory processes after hypoxic-ischemic insults may be an important mechanism of protection with hypothermia. There is considerable evidence that such processes, including induction of activated microglia with subsequent release of proinflammatory cytokines, contribute to both evolving neuronal and white matter injury (Silverstein et al., 1997). Cytokines exacerbate brain injury through various pathways including direct neurotoxicity and induction of apoptosis or by promoting stimulation of capillary endothelial cell proinflammatory responses and leukocyte adhesion and infiltration into the ischemic brain (Mallard et al., 2003; Silverstein et al., 1997).

However, the role of microglia in the brain is complex. Under physiologic conditions, microglia may actually help promote oligodendrocyte survival (Nicholas et al., 2003; Rabchevsky and Streit, 1997), but once activated, microglia induce contact-dependent oligodendroglial death *in vitro* (Nicholas et al., 2003). Thus, the present finding that early postischemic hypothermia profoundly reduced microglial induction suggests that this may be a significant contributor to improved oligodendrocyte survival. The effect on microglia was specific, because there was no effect of hypothermia on numbers of astrocytes. Clearly this effect could be either primary, or secondary to reduced cell loss and local

tissue damage, leading to a reduced stimulus to further microglial induction. The markedly reduced effect of late cooling on microglia closely paralleled the loss of effect on oligodendrocyte numbers, despite an identical period of recovery after rewarming to the early cooling group. This time dependence strongly suggests a significant secondary element.

Nevertheless, there is good evidence that postischemic cooling directly suppresses the inflammatory reaction. *in vitro*, hypothermia potently inhibits proliferation and superoxide and nitric oxide (NO) production by cultured microglia (Si et al., 1997), with a greater effect on inducible NO production than on other sources (Gibbons et al., 2003). Similarly, to the present study, postischemic hypothermia delays microglial activation after transient focal ischemia in the adult rat (Inamasu et al., 2000). Thus, a direct effect of early cooling on microglia cannot be ruled out. It is striking that in the present study, late cooling in the 6-hour group was associated with reduced tissue swelling and with substantial preservation of myelin, despite no significant effect on numbers of oligodendrocytes. This raises the possibility that a major effect of suppression of microglial induction by hypothermia may not have been on cell survival per se, but rather to reduce local effects of microglia on the extracellular matrix and degradation of myelin.

The present findings that early initiation of cerebral cooling improved oligodendrocyte survival with reduced microglial activation and reduced total proliferation provide an interesting contrast with our previous report that early IGF-1 treatment also improves oligodendrocyte survival, but with significantly increased glial induction and overall proliferation (Cao et al., 2003). This difference is consistent with *in vitro* data showing that hypothermia acts to broadly suppress the cell death program (Bossenmeyer-Pourie et al., 2000), whereas IGF-1-mediated receptor activity actively initiates cellular programs including anti-apoptotic pathways and proliferation (Mason et al., 2000; McMorris et al., 1986). The changes in proliferation, as measured by PCNA immunohistochemistry, are consistent with this interpretation. In the control group there was abundant PCNA expression in the white matter tracts, which was markedly reduced after ischemia. Given the severity of cell death, it is likely that the residual proliferation in the sham cooling (ischemia) group reflected almost entirely microglia. In contrast, the persistent reduction in PCNA expression after hypothermia despite increased oligodendrocyte survival must reflect general suppression of glial proliferation (Lee et al., 2002), not just microglia (Si et al., 1997).

Finally, evolving neuronal and white matter injury may extend for longer than the 5-day recovery from ischemia in the present study (Geddes et al., 2001). The continued presence of activated caspase-3 at this time also suggests ongoing injury, and thus it is possible that the observed white matter protection will reduce over time. Nevertheless, there was markedly reduced caspase-3 expression as well as greater oligodendrocyte survival in the 2-hour group 48 hours after rewarming, suggesting a reduction in ongoing damage. Consistent with this, in adult rodents persistent neuronal protection with hypothermia has been found 2 to 6 months after ischemia (Colbourne and Corbett, 1995; Corbett et al., 2000), although clearly further longer term outcome studies are necessary to confirm the present findings.

CONCLUSION

Prolonged moderate cerebral hypothermia can prevent postischemic white matter damage in the near-term fetal sheep, but there is rapid attenuation of this effect with increasing delay in the initiation of cooling. Furthermore, hypothermic white matter protection was closely associated with reduced activation of both caspase-3 and microglia. These data strongly support the critical importance of early initiation of cooling in clinical trials.

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CHAPTER 6

THE GROWTH HORMONE
AND INSULIN-LIKE
GROWTH FACTOR AXIS:
ITS MANIPULATION
FOR THE BENEFIT
OF GROWTH DISORDERS
IN RENAL FAILURE.

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ABSTRACT.

Renal failure is associated with dramatic changes in the growth hormone/insulin-like growth factor (GH/IGF) axis. In children, this results in growth retardation, which is treated with injections of recombinant human GH (rhGH). Given the many recent advances in the knowledge of the components of the GH/IGF axis, it is timely to review the role of GH in renal failure and to discuss likely new treatments for growth failure. Renal failure is not a state of GH deficiency but a state of GH and IGF resistance, making other approaches to manipulating the GH axis more logical than treatment with rhGH alone. Although in children rhGH is safe, in critically ill adults it can be lethal. As the mechanisms of these lethal actions of rhGH are unknown, caution is advised when using rhGH outside approved indications. In renal failure, an optimal balance between safety and efficacy for growth may be achieved with the use of the combination of rhGH and rhIGF-I, as animal studies have shown synergistic growth responses. However, inhibition of the GH axis, with the use of GH antagonists, is likely to be tested clinically given the beneficial effects of GH antagonists on renal function in animal models of renal disease. Manipulating IGF-I by either administering rhIGF-I or its binding proteins or increasing IGF-I bioavailability with the use of IGF displacers could prove to be a safer and more effective alternative to the use of rhGH in renal failure. In the future, both rhGH and rhIGF-I likely will be included in growth-promoting hormone cocktails tailored to correct specific growth disorders.

INTRODUCTION

Chronic renal failure (CRF) is associated with many severe metabolic and hormonal derangements, including alterations in the growth hormone/insulin-like growth factor-I (GH/IGF-I) axis, which is an important contributor to the growth disorders of renal failure. This review focuses on recent discoveries and insights into the GH/IGF axis and examines potential novel treatment modalities for renal-related growth disorders.

Normal Regulation of the GH Axis

The GH/IGF axis is a key endocrine modulator of postnatal growth and metabolism (Figure 1). The normal regulation of the axis is well understood as are some of the disruptions induced by renal failure. The axis involves the peripheral effector peptide hormones GH, IGF-I, and IGF-II and a regulatory feedback loop (reviewed in references 1–3).

GH is secreted by the anterior pituitary gland in a pulsatile manner under the acute stimulatory effects of the hypothalamic peptide GH-releasing hormone (GHRH) and the inhibitory effects of somatostatin. The recent discovery of ghrelin, a naturally occurring GH-releasing peptide (GHRP) that is expressed in both the stomach and the hypothalamus and is present in the blood of rats and humans, suggests that this molecule is involved in the hormonal regulation of GH release (4). The presence of ghrelin in the stomach suggests that it may play a role in the nutritional regulation of the GH/IGF axis. However, many hormones affect GH release, including insulin, so the true functions of gut ghrelin remain to be determined.

Once GH is released from the pituitary gland, it circulates in the blood to increase IGF-I production in many tissues, leading to a rise in blood IGF-I, which provides a long-term inhibition of further pituitary GH secretion (2). Similarly, the administration of IGF-I reduces GH secretion; conversely, genetically engineered IGF-I null mutant mice show markedly increased GH secretion (5).

GH and IGF-I Physiology

In the 1970s and 1980s, the somatomedin hypothesis proposed that IGF-I (formerly termed somatomedin C) mediated all of the effects of GH (1). Liver-generated IGF-I, the major contributor to IGF-I levels in the blood, was proposed to be crucial to the growth-promoting actions of GH. However, IGF-I is produced by many tissues of both epithelial and mesenchymal origin during both fetal and adult life (6). Because GH receptors occur in many tissues and some of the effects of GH do not involve the generation of IGF-I, it was necessary to modify the somatomedin hypothesis. It was proposed, therefore, that GH could act directly on many tissues, including cartilage and kidney, and produce IGF-I locally (7).

Recent studies in knockout mice have supported this modified hypothesis. As predicted by the somatomedin hypothesis, IGF-I $-/-$ knockout mice are dramatically growth retarded (8). Depending on their genetic background, up to 95% of IGF-I $-/-$ dwarf mice die shortly after birth. Knockout mice that do survive show a markedly reduced growth rate compared with wild-type mice and have an adult weight of approximately 30% of normal (8). In contrast, liver-specific (Cre/loxP) IGF-I knockout mice, in which only liver-generated IGF-I is reduced, have low blood levels of IGF-I but are viable and show normal growth (9).

This result was surprising given the belief that liver-generated endocrine IGF-I is important for body growth. GH supplementation in mice in which IGF-I is knocked out in all tissues does not increase growth (5). These studies suggest that although IGF-I is essential for normal postnatal body growth, liver-generated circulating IGF-I is not crucial for normal growth. In contrast, these experiments show

the importance of local GH actions and/or IGF-I production, for example on bone growth (Figure 1). It is possible that liver-specific IGF-I knockout mice grow normally because the bioactivity of IGF-I in the blood is unchanged, despite that total levels of circulating IGF-I are decreased markedly. Conversely, the high GH levels in these mice argues for a reduced bioactivity of blood IGF-I.

In blood, approximately 97% of the IGF are bound to six IGF-binding proteins (IGFBP-1 to IGFBP-6), with the remaining IGF-I either in a bioactive-free fraction (approximately 1%) or in an easily dissociable form (10). Most IGF-I in the circulation is bound in a 150-kD complex of IGF-I, IGFBP-3 and a third protein, the acid-labile subunit. This ternary IGF complex is a storage form of IGF in blood and has a half-life of several hours (1). The binding of IGF-I to IGFBP limits the bioactivity of IGF-I, as bound IGF-I probably cannot activate the IGF-I receptor.

Figure 1. The somatotrophic axis. The synthesis and release of growth hormone (GH) from the pituitary are controlled by the hypothalamic hormones GH-releasing hormone (GHRH) and somatostatin (SRIF), which in turn are regulated by feedback (dashed lines) from blood GH and insulin-like growth factor-I (IGF-I) concentrations. The recently discovered endogenous GH-releasing peptide, called ghrelin, also stimulates GH release. Circulating GH acts directly on many organs to stimulate IGF-I production, with IGF-I production in the liver providing the main source of blood IGF-I. Most of the IGF-I in the circulation is bound to IGF-binding protein-3 (IGFBP-3) in a ternary complex with acid-labile subunit (ALS); a smaller fraction is bound to the five other IGFBP. A small fraction of the total IGF-I in blood is in a bioactive-free fraction. In the kidney, IGF-I increases renal plasma flow and GFR, whereas on bone it acts on the epiphyseal plate, which leads to longitudinal bone growth. As illustrated, GH also has direct effects on many organs, including kidney and cartilage, which can be independent of IGF-I action.

This inhibition of bioactivity, especially that by IGFBP-1 and -2, has been shown in several experiments; for example, the administration of IGFBP-1 blocks the effects of both GH and IGF-I on body growth (11), and IGFBP-1 and IGFBP-2 transgenic mice are growth retarded yet have normal total IGF-I concentrations in blood (12). Therefore, in renal failure, IGF-I bioactivity in blood is probably low as circulating concentrations of IGFBP-1 and -2 are elevated (13,14).

GH/IGF Axis and the Kidney

The GH/IGF/IGFBP system is present in the kidney and is important to kidney structure and function. GH receptors are expressed in proximal tubules and thick ascending limb, whereas IGF-I receptors are found predominantly in the glomerulus and proximal and distal tubules (15). Both GH and IGF-I increase renal plasma flow and GFR. These effects of GH are likely to be mediated by IGF-I, as IGF-I increases these parameters within minutes to hours, whereas the effects of GH are delayed until IGF-I levels rise (15).

The role of IGF-I in renal development is thought to be minor as general kidney morphology of complete IGF-I $-/-$ mice is normal (16). It is of interest to produce a kidney-specific IGF-I deletion, as it would show the relative importance of local IGF-I to kidney growth and function. Targeted gene deletion in the kidney now is feasible (17). Furthermore, in such an IGF-deleted mouse, recovery from renal injury would be of particular interest.

Although most actions of GH are mediated by IGF-I, and GH and IGF-I share several activities, there are actions of GH and IGF-I that are very different. For example, mice that are transgenic for IGF-I develop glomerular hypertrophy, whereas mice that are transgenic for GH develop glomerular sclerosis (18). Therefore, on the kidney, GH and IGF-I can have quite different actions.

Cancer Risk

Epidemiologic studies have associated the IGF axis as a risk factor for several common cancers. Multiple prospective case-control studies have found that elevated serum IGF-I levels and low IGFBP-3 levels are associated with an increased risk of prostate, colorectal, breast, and lung cancers (19,20). It is important to note that a causal role of IGF-I in the pathogenesis of cancer has not been established and that the interpretation of these new data are the subject of much debate.

FETAL PROGRAMMING, GH/IGF, AND THE KIDNEY

Large epidemiologic studies have found a strong correlation between low birth weight and the subsequent incidence of obesity, diabetes, hypertension, and renal disease during adult life (the so-called Barker hypothesis) (21). There also is evidence that intrauterine growth restriction (IUGR) results in altered programming of the GH/IGF system, altered programming of the renin-angiotensin system, and/or altered gene expression, causing abnormal renal and cardiovascular physiology (22,23). Renal abnormalities include structural changes such as a low nephron count, which eventually may underlie the development of adult hypertension (23,24).

Abnormalities in the GH/IGF axis have been associated with growth failure after IUGR and also could lead to the above-described long-term health sequelae. It remains to be established whether therapeutic interventions after IUGR aimed at deprogramming the GH/IGF axis or other regulatory systems early in life will affect the long-term outcomes of metabolic, cardiovascular, and renal disease.

CHANGES IN GH, IGF, AND IGFBP DURING RENAL FAILURE

CRF is associated with several derangements in the GH/IGF/IGFBP axis (Figure 2), including in children an increased pulsatile release of GH and reduced metabolic clearance rate of GH, resulting in a rise in circulating GH concentrations (25,26). This should result in high IGF-I concentrations, but in uremia IGF-I synthesis in the liver is reduced, which results in normal concentrations of circulating

Figure 2. Deranged somatotrophic axis in chronic renal failure. The GH/IGF-I axis in chronic renal failure (CRF) is changed markedly compared with the normal axis, shown in Figure 1. In CRF, the total concentrations of the hormones in the GH/IGF-I axis are not reduced, but there is reduced effectiveness of endogenous GH and IGF-I, which probably plays a major role in reducing linear bone growth. The reduced effectiveness of endogenous IGF-I likely is due to decreased levels of free, bioactive IGF-I as levels of circulating inhibitory IGFBP are increased. In addition, less IGF-I is circulating in the complex with ALS and IGFBP-3 as a result of increased proteolysis of IGFBP-3. Together, these lead to decreased IGF-I receptor activation and a decreased feedback to the hypothalamus and pituitary. Low free IGF-I and high IGFBP-1 and -2 levels probably contribute to a reduced renal function and lead to a reduced stature. The direct effects of GH on bone, which are poorly understood, also are blunted.

IGF-I (27). However, IGF-I bioavailability probably is reduced in renal failure as a result of increased plasma levels of IGFBP-1, -2, -4, and -6 (15,28). In CRF, there are increased amounts of low molecular weight IGFBP-3 fragments, which have a reduced affinity for IGF-I and which accumulate because of reduced renal clearance (13).

The increased levels of IGFBP-1 and -2 in CRF are correlated inversely with residual GFR and with height (13). Free IGF-I levels, however, are correlated positively with renal function (14). Therefore, the high IGFBP-1 and IGFBP-2 levels probably contribute to the resistance to the metabolic and growth-promoting properties of GH and IGF-I in renal failure.

There is some evidence for receptor-mediated GH and IGF resistance in CRF. In experimental CRF, there is evidence of a decreased GH receptor abundance in tibial growth plates (29) and liver (30) and a defect in IGF signaling in muscle (31). However, the weight of evidence favors a key involvement of the IGFBP in the GH and IGF resistance that can occur in renal failure. Thus, renal failure is not a state of GH or IGF-I deficiency but a state in which the regulation and bioavailability of components of the GH/IGF system are altered.

THERAPEUTIC MANIPULATION OF THE GH/IGF AXIS IN RENAL FAILURE

Growth Hormone

Growth. Recombinant human GH (rhGH) is approved for the treatment of growth failure in children with CRF. A daily dosage of 0.05 mg/kg body wt given by subcutaneous injection is recommended (32).

Figure 3. Mechanism of action of an IGF displacer compound. A novel concept for manipulating the IGF axis is shown. The IGF system is regulated by interactions among IGF-I, IGFBP, and the IGF-1 receptor (IGF1-R). IGFBP bind IGF-I with high affinity, so very little IGF-I is available normally to bind to and activate IGF receptors. The introduction of an IGF displacer (IGF-D) will both prevent IGF-I binding to an IGFBP and displace IGF-I that is bound to an IGFBP. The IGF displacer therefore will act as an indirect IGF agonist by increasing the amount of IGF-I that is available for binding to IGF receptors. This strategy could be particularly useful in renal failure, in which there are excess IGFBP.

Using this dose, two large multicenter clinical trials in CRF patients showed that rhGH treatment can improve statural growth (33,34). Recent studies showed that rhGH treatment is most effective when it is started at an early age and that the growth response is affected by the degree of renal impairment (35).

Although treatment with rhGH clearly stimulates body growth in children with renal failure, it is possible that rhGH may adversely affect renal function in some situations. For example, GH has been suggested to play a role in the development of glomerulosclerosis in mice (18), but there is no proof in children that rhGH has deleterious effects on renal function or, when given before renal transplantation, on graft function (36). However, rhGH treatment is not an ideal treatment for the growth disorders associated with CRF in children because a state of GH excess already exists in CRF.

RhGH therapy also has been proposed for the treatment of growth failure after renal transplantation, as catch-up growth does not occur in up to 75% of these patients (37). Guest et al. (38) showed in a prospective randomized study that rhGH therapy after renal transplantation tended to increase the number of acute biopsy-proven rejections (9 rejections in 44 rhGH-treated patients versus 4 of 46 control patients), although this was related to a previous history of rejection. RhGH treatment after renal transplantation still must be considered experimental (39).

Anabolic and Cardiovascular Effects. Studies have suggested a beneficial effect of rhGH treatment on left ventricular mass in patients with dilated cardiomyopathy, but this has not always been associated with an improved clinical outcome (40,41). In nine adult hemodialysis patients (median age, 48.6 yr), rhGH treatment (4 IU/m² per d for 6 mo) significantly increased lean body mass, reduced fat mass (42), and significantly increased left ventricular muscle mass but had no effect on ejection fraction, BP, or maximum exercise capacity (43). One rhGH-treated patient died as a result of severe pneumonia. Johannsson et al. (44) showed that rhGH administration (66 µg/kg given three times a week for 6 mo) in 10 elderly patients (mean age, 73 yr) who were on maintenance dialysis increased serum albumin and increased muscle strength. Two patients in the rhGH group died, whereas no placebo-treated patients died during the treatment period.

The use of rhGH to treat catabolism in dialysis patients also is of doubtful value given the lack of effect on clinical outcome and the adverse side effects of rhGH in intensive care patients (45).

Sustained-Release GH

New formulations of rhGH, to allow more convenient administration regimens, have been tested in animals (46,47).

One form, an injectable sustained-release formulation of rhGH in erodable polylactate polyglycolate microspheres, was approved recently by the FDA for use in pediatric GH-deficient patients. Two multicenter, open-label clinical studies in prepubertal GH-deficient children showed that this formulation of rhGH caused significant catch-up growth (48). Patients who were maintained on rhGH depot for 12 mo showed a mean growth rate of 7.8 ± 1.9 cm/yr, which was lower compared with the first-year growth rate of 10.0 ± 3.1 cm/yr that could be expected if the patients were given daily injections of rhGH (32).

Sustained-release rhGH has not yet been studied in patients with renal insufficiency. A potential issue in these patients is that the clearance of GH is slowed, which could lead to GH accumulation. In addition, different effects of GH can be produced by intermittent or continuous GH administration (46). For example, compared with daily GH injections, continuous GH administration in rodents produces an initial rapid response in body growth, which wanes rapidly. Long-term studies in humans comparing intermittent exposure using daily GH injections with continual exposure using the depot formulation may be needed to allay such efficacy concerns. In our opinion, the efficacy of this new formulation of rhGH needs to be established in long-term controlled studies, especially for use in pediatric CRF patients.

Adverse Effects of GH

RhGH treatment has been proposed as an anabolic therapy for catabolic critically ill patients (49). However, two large multicenter, double-blind, placebo-controlled Phase III clinical trials showed very clearly ($P < 0.001$) that rhGH (0.10 mg/kg body wt) doubled the overall mortality of critically ill patients from approximately 20 to 40% (45). The dosage of rhGH in the critically ill patients was double the recommended dosage for growth disorders in pediatric CRF.

It now is recommended that rhGH treatment not be initiated in patients with an acute critical illness. The cause of the increase in mortality is unknown; therefore, great caution needs to be exercised in the use of rhGH in adults outside the currently approved use in GH deficiency. There is no clear recommendation on the course of action to take when a patient who is already receiving rhGH treatment develops an acute illness. However, it seems reasonable to cease rhGH treatment in both children and adults if an acute illness occurs, as there are no parameters available to gauge the risk of an adverse outcome if treatment is continued. This is partly because the adverse effects of rhGH may be via either the direct or the indirect effects of GH. Initial experimental studies in animals suggest that metabolic or cytokine responses may be involved in these adverse effects (50). There has been much recent debate on the safety of manipulating the GH/IGF axis in acute critical illness (51).

Other adverse reactions to GH treatment in CRF include an increased risk of benign intracranial hypertension (52). The recent suggestion of an increased incidence of type II diabetes mellitus in children who are treated with GH also indicates that rhGH-treated patients deserve close monitoring (53). It seems prudent in pediatric CRF to start GH treatment with a half-dose, which should be ramped up to the therapeutic dose within 1 to 2 mo.

The use of rhIGF-I or rhIGF-I plus rhGH in catabolic states such as end-stage renal disease (ESRD), critical illness, and AIDS has not been associated with an increased mortality (54,55). These data reinforce our theme of differences between the physiology and the pharmacology of rhGH and rhIGF-I treatment.

Insulin-Like Growth Factor-I

Although rhIGF-I seems to be a logical treatment for growth failure in pediatric CRF, because of the increased IGFBP in this condition, clinical studies with rhIGF-I have not been performed in children outside of patients with GH insensitivity syndrome (GHIS). Studies in animal models of CRF (5/6 nephrectomy) show that the growth response after rhIGF-I treatment is almost comparable to that after GH treatment (56). Besides effects on stature, rhIGF-I has direct anabolic effects and improves renal function. In normal subjects, rhIGF-I rapidly increases GFR and renal plasma flow by approximately 30% (57).

Acute Renal Failure. No drug has been approved as a stimulator of renal function in acute renal failure (ARF) (58). However, there is an obvious need for new treatments, as morbidity and mortality remain high after ARF. In animal models, rhIGF-I can improve both functional and histologic recovery after renal ischemia and reperfusion damage (59). These beneficial effects may be due to a variety of actions, such as increasing anabolism and hemodynamic effects in the glomerulus and the preglomerular vessels. RhIGF-I decreases both afferent and efferent arteriolar resistance in the glomerulus and increases renal blood flow by dilation of preglomerular vessels. These effects most likely are mediated by nitric oxide and vasodilatory prostaglandins (15). Recent studies also have shown that rhIGF-I can reduce the inflammatory process after renal ischemia and reperfusion by inhibiting apoptosis (60). Early timing of treatment may be crucial for these effects, although other studies have shown that rhIGF-I treatment can enhance renal repair when administered up to 24 h after injury (59).

These advantageous effects of rhIGF-I administration in animals have led to two randomized, double-blind, placebo-controlled trials (61). Franklin et al. (62) examined the effects of rhIGF-I on recovery of renal function in 58 patients who underwent vascular surgery of the renal artery or the aorta, and Hirschberg et al. (63) tested rhIGF-I in 72 intensive care unit patients with confirmed ARF from different causes. Overall, both studies failed to show clinically significant effects on renal function or outcome.

The disappointing results of these clinical studies in ARF are in contrast to the very positive results in equivalent animal models. These differences are difficult to explain. It seems unlikely that there will be additional studies to test rhIGF-I in human ARF, although the lack of other candidate drugs suggests that IGF-I does deserve additional testing in humans.

Chronic Renal Failure. No drug has been approved for use in patients with CRF and ESRD to stimulate renal function or to delay the need for dialysis (64). The positive effects of rhIGF-I on renal function in healthy subjects led to several studies in CRF patients. Initial studies with high doses of rhIGF-I (100 µg/kg twice a day) increased renal function for several days but caused serious side effects (65,66). Recently, studies by Vijayan et al. (67) showed in patients with ESRD that efficacy could be maintained and side effects could be reduced with the use of an intermittent treatment regimen (4 d on treatment, 3 d off treatment) for rhIGF-I (50 µg/kg per d). This approach was well tolerated and resulted in a sustained improvement in renal function, which may be related to the IGFBP's being relatively unaffected by this mode of delivery. In other human studies, large rises in IGFBP-2 seem to be related to overdosing and the occurrence of side effects (68).

Additional rhIGF-I treatment studies, particularly in CRF in children, seem justified. However, the current limited interest by pharmaceutical companies and the lack of availability of clinical grade rhIGF-I make this unlikely.

Combination Treatment with GH Plus IGF-I or IGF-I Plus IGFBP-3

Studies of combinations of hormones, in animals with the use of GH plus rhIGF-I (29,56) or in humans with rhIGF-I plus IGFBP-3 (69), suggest that there may be advantages in terms of efficacy and safety for combination therapy. The combination of rhGH plus rhIGF-I has been tested in catabolic states in

adults (55) but has not been tested as a growth-promoting agent in pediatric patients. Animal studies suggest that combined treatment is more effective as a growth-promoting agent in experimental uremia (29,56). In catabolic humans, an initial study of the combination of rhGH plus rhIGF-I (both given once daily) suggested a beneficial anabolic effect (55). However, a second study, in which the rhGH was given twice daily (and at half the dose), failed to show a beneficial effect in patients with AIDS-associated wasting (54). The combination of rhIGF-I plus IGFBP-3 is being tested in humans (69), but as yet no growth studies in pediatric patients have been reported, perhaps because only an intravenous formulation has been available. The combination of rhGH plus rhIGF-I remains the most attractive untested therapeutic option for treating pediatric growth disorders. In the near term, human studies with rhGH plus rhIGF-I seem unlikely, as pharmaceutical companies do not seem to be interested in exploiting this combination.

IGF Displacers

The concept of activating the IGF axis, or increasing endogenous IGF-I bioactivity, has been proposed as a potential treatment modality. The idea is that by displacing bound IGF-I from IGF-binding proteins, the levels of "free" IGF-I should increase and thereby activate IGF receptors (Figure 3). Two independent studies (70,71) have confirmed this hypothesis with the use of two different IGF-I analogs (Leu^{24,59,60},Ala³¹hIGF-I and Leu²⁴,Ala³¹hIGF-I), which bind with high affinity to the IGFBP but do not activate directly the IGF receptor. One study showed that the administration of a "receptor inactive" IGF displacer in the rat increased body weight, increased kidney weight, and decreased creatinine and blood urea nitrogen, despite that total rat IGF-I levels fell dramatically (71). In extensions of this work, phage display has been used to discover synthetic peptides that bind to IGFBP (71).

In renal failure, in which free IGF bioactivity is low as a result of elevated IGFBP levels, the administration of IGF displacer peptides seems to be a logical way of increasing the bioavailability of IGF-I (72). This is a more physiologic approach to manipulating the GH/IGF axis in renal failure and should have fewer side effects than the administration of GH or IGF-I. Unfortunately, these peptides do not bind to rodent IGFBP, so their efficacy in animals is difficult to establish. However, because renal failure is characterized by excess IGFBP and not a deficiency of IGF, the concept of IGF displacement as a treatment option in renal failure seems promising. The concept of IGF displacement has been proved in animals, so a drug candidate with the desired characteristics now is needed so that efficacy can be tested in humans with renal disease.

GH-Releasing Hormone

It has been shown that GHRH can induce a growth response in rats (73) and in humans. Thorner et al. (74) studied the efficacy and safety of GHRH (GHRH-(1-29), 30 µg/kg per d) therapy in 110 previously untreated prepubertal GH-deficient children. Treatment for up to 1 yr in this multicenter, open-label study increased height velocity from approximately 4 cm/yr to 8 cm/yr for the first 6 mo and 7 cm/yr thereafter, although not all children were responsive. It seems unlikely that GHRH will replace GH as the treatment of choice for pediatric growth disorders. This is partly because GHRH must be given by injection, whereas a newer class of compounds, the GH secretagogues (GHS), which also release GH, can be given orally (75).

GH-Releasing Peptides

GHRP or GHS are small synthetic molecules that can stimulate the release of GH from the pituitary (75). GHRP are highly specific for GH release and can be more effective at releasing GH than GHRH. However, compared with GHRH, the GHRP have little effect on GH synthesis and storage, which may

limit their efficacy. The GHS receptor (GHS-R) has been cloned (76), and an endogenous ligand was discovered recently and named ghrelin (4). Ghrelin is expressed predominantly in the stomach and other tissues, including the kidney (77).

Therapeutic applications for GHRP have been studied, but no clear efficacy end point has been established. Potentially, GHRP could be used in situations in which rhGH is currently approved. A requirement for the use of GHRP is that the hypothalamo-pituitary function be intact (78). It has been proposed that GHRP may be preferential to rhGH treatment in some conditions, as GHRP can induce a more physiologic pulsatile GH secretion that is self-regulating via GH and IGF-I feedback.

Small-molecule GHRP can be given orally, would be less expensive than rhGH treatment, and therefore seem attractive for treating some forms of growth failure. After the discovery of ghrelin and its receptor, it is possible that new indications for GHRP will be discovered. In renal failure, in which GH secretion is already increased, it seems unlikely that GHRP would be a preferred form of therapy.

GH-Receptor Antagonists and Somatostatin Analogues

Several studies that used animal models of diabetes mellitus found a relationship between elements of the GH/IGF axis and both early and late renal deterioration (79). Diabetes-associated kidney damage is reduced in genetically modified animals that either have a disrupted GH receptor gene or overexpress a GH antagonist (80,81). GH antagonists, which are GH mutant molecules that bind only to one GH receptor, prevent GH receptor dimerization and receptor activation. The administration of GH antagonists has been shown to prevent renal disease in animal models of diabetic nephropathy (82). Similar effects were seen when endogenous GH was suppressed with the use of long-acting somatostatin analogues (79).

CONCLUSIONS

The success of recombinant DNA technology has made GH widely available for the treatment of growth disorders. Advances in our understanding of the endocrinology of growth have produced new ways of manipulating the GH/IGF axis in renal failure. This has led to new potential treatment modalities that could affect body growth, anabolism, renal function, and even renal disease.

One reason that new treatment regimens are needed is that renal failure is not a disorder of GH deficiency but a derangement of the GH/IGF/IGFBP system, resulting in decreased effects of endogenous GH and IGF-I. The need for new treatments has been highlighted recently by the questioning of the safety of rhGH treatment in critically ill adults. Two placebo-controlled, double-blind clinical trials showed that rhGH treatment in adults had lethal, acute side effects (45). The causes of the lethal effects of rhGH or the organs involved are as yet ill-defined, making it very difficult to proceed with some indications for rhGH treatment, such as congestive heart failure.

In pediatric patients, rhGH is a safe treatment. However, because we do not yet understand why rhGH treatment had lethal effects in critically ill patients, there is no way to identify patients who are at risk or markers that can be used to predict when adverse consequences will ensue from rhGH treatment.

Most of the effects of GH depend on its pattern of administration. Therefore, the lethal side effects of rhGH also may depend on its pattern of administration. Research is needed to establish the relative safety of different means of manipulating GH levels, such as depot rhGH preparation or GHS. It is possible that stimulating endogenous pulses of GH, with the use of a GH releaser, is safer than giving injections of a GH preparation.

Molecules that act as antagonists at the GH receptor show efficacy in preventing renal damage in animal models of diabetic nephropathy and are likely to be tested in humans.

An obvious alternative to the use of GH or compounds that release GH for growth promotion is the manipulation of the IGF-I axis. Unfortunately, rhIGF-I treatment has not been tested for growth promotion in pediatric CRF patients, in whom it may increase renal function and delay the need for dialysis or transplantation. Safety has been a concern for rhIGF-I treatment. An intermittent pattern of rhIGF-I administration seems to produce a sustained renal response with reduced side effects in adult CRF patients. It is likely that an optimal balance between safety and efficacy on body growth can be achieved with the use of the combination treatment of rhGH and rhIGF-I, particularly in renal failure, as animal studies have shown that in combination they cause at least additive growth responses. However, the future use of rhIGF-I in clinical studies is uncertain, given the current limited interest by pharmaceutical companies.

Binding proteins for IGF-I are targets for drug development. The administration of IGFBP-3 plus rhIGF-I is being tested in humans, but it seems unlikely that this combination will be used widely to promote growth in children. New strategies aimed at increasing endogenous levels of free, bioactive IGF-I show promise as high IGFBP levels probably mediate most of the relative IGF resistance in renal failure. A mutated form of IGF-I that binds to IGFBP but not to IGF receptors (Figure 3) caused growth promotion and affected renal structure and function in animals. Novel peptides, termed IGF displacers, which bind to IGFBP and displace IGF-I, also have been developed. It is hoped that a molecule of this type eventually will be tested in humans.

The success of rhGH as a drug has led to a large research effort and has produced a long list of new approaches to manipulating the GH/IGF axis in the laboratory and the clinic. In the future, it is likely that combinations of hormones will be used to adjust or correct the endocrine abnormalities that cause disordered growth. Children with CRF and children with isolated GH deficiency both show reduced stature. However, their GH/IGF axes are disordered in very different ways. At present, rhGH treatment is used in both cases. It seems logical that a child with CRF and a child with isolated GH deficiency should receive different and tailor-made treatments to correct their GH/IGF system. For the foreseeable future, GH will remain the cornerstone of treatment, but in the future, treatment will be tailored with other compounds, such as IGF-I, being added in a cocktail to produce an optimal growth response.

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CHAPTER 7

SUMMARY
AND
FUTURE PERSPECTIVES

SUMMARY

The aim of this thesis was to better understand the endocrine and cardiovascular responses of the fetus to severe hypoxia (asphyxia) and to examine the effects of rescue therapy with head cooling on neural and systemic recovery from such severe hypoxia.

Chapter 1 discusses the clinical and experimental background to this thesis. Ischaemia-reperfusion injury remains a significant challenge for perinatal medicine today for both term and preterm infants (Volpe 2001; Shalak and Perlman 2004; Salhab and Perlman 2005). Severe asphyxia can initiate a cascade of events in the brain and other organs, leading to organ and brain injury, which can result in life-long neurodevelopmental handicaps for the child. During hypoxia-ischaemia there is cellular energy failure, accumulation of excitotoxins due to increased neurotransmitter release and inhibition of re-uptake, an efflux of K^+ but a corresponding influx of Na^+ and water, increased free intracellular Ca^{2+} , severe oxidative stress, which lead to cell swelling and induction of cell death pathways.

Recovery from asphyxia can be divided into several phases of evolving injury. After the initial period of reperfusion, there is a **latent** phase, in which there is nearly complete normalisation of oxidative cerebral energy metabolism, but electroencephalogram (EEG) activity is depressed and there is a secondary fall in cerebral blood flow (Williams et al. 1991; Lorek et al. 1994; Abi Raad et al. 1999). The latent phase may last for many hours, up to 15 h in some studies, before being followed by a phase of **secondary** deterioration (Gunn 2000). This phase is marked clinically and experimentally by the onset of seizures, delayed cytotoxic edema (cell swelling), extracellular accumulation of excitotoxins, failure of oxidative metabolism, and induction of inflammatory responses, and ultimately delayed apoptotic and necrotic cell death.

Treatment strategies to prevent the (long-term) consequences of ischaemia-reperfusion are highly necessary for both term and preterm infants. Although there is no precise way of determining the exact "window of opportunity" for neuroprotective treatment, broadly the events occurring in the latent phase after hypoxia-ischaemia appear to be potentially amenable to neuroprotective strategies, whereas generally treatments begun after the onset of the secondary deterioration seem to have very little or no benefit (Gunn 2000). Therapeutic hypothermia is currently the most promising candidate for neonatal hypoxic-ischaemic encephalopathy (Gunn 2000).

Before new treatment strategies are studied in human infants it is vital to understand systemic effects both of the underlying hypoxic-ischaemic insult and of the treatment, and not just the effects on the brain. Thus one goal of my thesis was to better understand the systemic effects of cooling to ensure that there are no adverse systemic effects that might compromise its neuroprotective benefits, especially for preterm neonates.

The chronically instrumented fetal sheep model of both global asphyxia and cerebral ischaemia-reperfusion has several great advantages above other experimental paradigms such as rodent models. In fetal sheep it is possible to continuously monitor cerebral and systemic physiologic parameters such as temperature, blood flow, blood pressure and electro-encephalography in utero, with the mother serving as a ideal stable incubator (Gunn et al. 1997). Furthermore it allows repeated blood sampling for example to study endocrine changes and post-mortem histological examination. The sheep brain, like the human brain is gyrencephalic (in contrast to the lissencephalic rat brain) and shares many developmental features with the human infant.

This is clearly a compromise as fetal and neonatal metabolism differ in several aspects. However, this approach provides a primary insult, which occurs under fetal conditions, and avoids hypothermia and anaesthesia, which can interact with drug treatment. In terms of cerebral maturity the 0.7 gestation fetal sheep is comparable to the human brain at 30-32 weeks of gestation, whereas the near-term, 0.8-0.85 gestation fetus is equivalent to the term infant (McIntosh et al. 1979).

Chapter 2

It is widely believed that one reason for the increased rate of neural injury after preterm birth is that the ability of the preterm fetus to defend itself from ischaemia-reperfusion is not as well developed as at term. In particular, it has been hypothesized that the hypothalamus-pituitary-adrenal (HPA) responses to stresses such as asphyxia are immature in the preterm fetus and that this contributes to the frequent development of subsequent hypotension in the perinatal period. These experimental studies were undertaken using relatively mild insults. However, we now know that compared to the near-term fetus, survival of the premature fetus is much more prolonged during a severe asphyxial insult (Gunn et al. 2001). We therefore examined the effects of prolonged, severe asphyxia induced by complete umbilical cord occlusion on HPA responses in chronically instrumented preterm fetal sheep (at 0.7 of gestation) for 3 days.

Profound asphyxia in the preterm fetus led to a very large relative increase in ACTH and cortisol levels during asphyxia. Although the absolute levels of cortisol were much lower than at term, the pattern and magnitude of the relative changes were consistent with near-term responses. In the recovery phase there was a sustained elevation that gradually resolved between 24 and 72 h. There was a modest correlation between cortisol levels in the recovery phase and fetal mean arterial pressure suggesting that the adrenocortical response helps to sustain fetal blood pressure during a critical period of recovery.

Our conclusion is that the adrenocortical response of the preterm fetus is not immature. Rather our data suggest that the greater anaerobic tolerance of the preterm fetus, compared with near-term, means that its full range of responses is only activated by more severe insults.

Chapter 3

Prolonged, moderate cerebral hypothermia has been shown to be able to reduce neural injury in a wide range of experimental ischaemia-reperfusion paradigms, but not yet in a model of premature brain injury. Using a chronically instrumented preterm (0.7 gestation) fetal sheep model, we examined the effects of cerebral hypothermia starting in the early recovery phase (90 min after asphyxia) and continued for 3 days, on neuronal loss and epileptiform activity.

We found that delayed, prolonged head cooling after a severe asphyxial insult in the premature fetus was neuroprotective in the hippocampus and basal ganglia, and suppressed activated caspase-3 (apoptosis) and microglia (inflammation). Neuroprotection was associated with potent, specific suppression of epileptiform transients in the early recovery phase, but not with number of delayed seizures. The histological improvement was associated with progressive normalisation of EEG frequency and carotid blood flow after reperfusion, consistent with possible functional improvements. Hypothermia was not associated with significant side-effects such as hypotension or acid-base changes, suggesting it may be safe in the preterm infant if carefully controlled.

Thus, our data demonstrate that selective cerebral hypothermia after profound asphyxia can reduce short-term injury of subcortical neuronal structures in the preterm brain. In view of the critical developmental events occurring in the preterm infant, and their potential vulnerability to the adverse systemic effects of hypothermia it will be critical to systematically evaluate the factors, such as delay before initiated cooling and the depth of hypothermia, that determine its effectiveness before human studies can be contemplated.

Chapter 4 and 6

Insulin-like growth factor-I (IGF-I) is a potent endogenous neuroprotective and metabolic and growth factor. The IGF-I axis involves the peripheral effector peptide hormones GH, IGF-I, and IGF-II and a regulatory feedback loop. In blood, approximately 97% of the IGF are bound to six IGF-binding proteins

(IGFBP-1 to IGFBP-6), with the remaining IGF-I either in a bioactive-free fraction (approximately 1%) or in an easily dissociable form. The binding of IGF-I to IGFBP (especially IGFBP-1) limits the bioactivity of IGF-I, as bound IGF-I probably cannot activate the IGF-I receptor. Manipulating IGF-I by either administering IGF-I or its binding proteins or increasing IGF-I bioavailability with the use of IGF displacers could prove to be a safer and more effective alternative to growth hormone or IGF-I treatment, for example in paediatric renal failure or ischaemia-reperfusion injury.

The effects of asphyxia and hypothermia on circulating IGF-I are currently poorly understood, and may affect infants recovery from asphyxia. Our results show that the overall profile of changes in IGFs (and insulin) after asphyxia are highly consistent with facilitation of catabolic metabolism. While the primary IGF involved is clearly IGF-I, intriguingly, our study also suggests for the first time that by 0.7 of gestation IGF-II may also contribute to regulation of fetal metabolism during the acute recovery phase following asphyxia.

This study also demonstrates that therapeutic head-cooling in the preterm fetus is not associated with major changes in the responses of circulating concentrations of IGFs after asphyxia, suggesting reassuringly that hypothermia does not affect the metabolic adaptation mediated by circulating IGFs.

Chapter 5

Post-resuscitation cerebral hypothermia is consistently neuroprotective in experimental preparations. Less is understood about the effects of hypothermia on white matter injury (oligodendrocytes and myelin). This is of some importance since one proposed reason for the consistent clinical failure of putative neuroprotective drugs has been their failure to protect white matter as well as neurons. Using a model of reversible cerebral ischaemia in unanesthetised near-term fetal sheep, we examined the effects of cerebral hypothermia (fetal extradural temperature reduced from $39.4 \pm 0.1^\circ\text{C}$ to between 30 and 33°C), induced at different times after reperfusion and continued for three days after ischaemia, on injury in the parasagittal white matter 5 days after ischaemia.

Cooling started within 90 minutes of reperfusion was associated with a significant increase in bioactive oligodendrocytes in the intragryal white matter compared with sham cooling, increased myelin basic protein density and reduced expression of activated caspase-3. Reactive microglia were profoundly suppressed compared with sham cooling with no effect on numbers of astrocytes. When cooling was delayed until 5.5 hours after reperfusion there was no significant effect on loss of oligodendrocytes.

We conclude that prolonged moderate cerebral hypothermia can prevent post-ischaemic white matter damage in the near-term fetal sheep, but there is rapid attenuation of this effect with increasing delay in the initiation of cooling. Protection of the white matter was closely associated with reduced expression of both activated caspase-3 and of reactive microglia. These data strongly support the critical importance of early initiation of cooling in clinical trials.

FUTURE PERSPECTIVE

Prevention

Obviously, prevention of injury of course is better than treatment. On one hand, improvements in routine obstetric care, to minimise the risk of hypoxia-ischaemia in both term and preterm infants, are possible. There is now some evidence that innovations in monitoring and early intervention can further improve detection of intrapartum asphyxia and thus reduce the rate of neonatal encephalopathy (Amer-Wahlin et al. 2001; Noren et al. 2003). However, it is improbable that complete prevention of neonatal hypoxic-ischaemic encephalopathy could ever be achieved, and thus there will always be a need for

rescue treatment. As many agents have been shown to be only effective when treatment is started during hypoxia-ischaemia, an elegant approach would be to start treatment in at risk pregnancies before labour, for example by administering neuroprotective agents to the mother. For example, there is some evidence that magnesium sulphate, which is a partial NMDA receptor antagonist, given to women immediately before very preterm birth may improve some neurodevelopmental outcomes in the children, although in this trial there was no overall statistically significant effect (Crowther et al. 2003).

Therapeutic hypothermia

As discussed in this thesis, hypothermia is currently one of the most promising interventions for neonatal hypoxic-ischaemic encephalopathy. In many different animal models it has consistently been shown to improve structural and functional outcome, and the pharmacological parameters for effective use have been reasonably well defined. Based on these experimental data randomised controlled clinical trials using either selective cerebral hypothermia ("CoolCap trial") or whole-body hypothermia have just been completed or are in progress (Jacobs et al. 2004; Shankaran et al. 2004; Gluckman et al. 2005). Results from the CoolCap trial suggested that the combination of head cooling with mild systemic hypothermia reduced death or disability in the infants with less severe electroencephalographic changes at entry of the trial. However, cooling using this protocol was not significantly beneficial in infants with the most severe electroencephalographic changes before the onset of cooling. These data strongly suggest that further research is important to find better ways of selecting newborn infants who may benefit most from treatment. Currently (amplitude integrated)-EEG appears to have the best potential selection capacities (Groenendaal and de Vries 2000), but further studies are needed to validate the CoolCap trial experience and to define the most robust, generally applicable criteria.

Many more questions remain about the best ways of optimizing clinical neuroprotection with hypothermia. For example, it is unclear whether head cooling is better than whole body. It is likely that this will be a balance between risks and benefits, and we may speculate that there may prove to be better therapeutic effects of whole body cooling on subcortical structures at the expense of an increased risk of complications from the deeper systemic hypothermia used in whole body cooling. Further, the real width of the so called "window of opportunity" after birth, the, optimal depth and duration of cooling and of the speed of warming babies up after cooling are all highly unclear, and likely depend on factors such as the severity of injury, its precise timing before birth, the type of insult (e.g. single profound episode of anoxia vs. repeated shorter episodes), the age and maturity of the infant, the presence of pre-existing growth retardation and so forth. Our results summarised above suggest that hypothermia may be potentially an effective intervention to reduce neurological morbidity in preterm infants. Given the well established finding of increased mortality in infants less than 1500 g who were allowed to get mildly hypothermic, much more basic research is needed before hypothermia could be considered for this uniquely vulnerable group.

Combination therapy

Although hypothermia reduces the massive cell death the first week after ischaemia-reperfusion injury, protection is only partial, and there will often still be permanent structural and functional injury to the neonatal brain. One way of further improving protection would be to combine hypothermia with other neuroprotective agents. For example, the combination of hypothermia and NMDA-receptor antagonist xenon has recently been shown to have additive neuroprotective effects (Ma et al. 2005). Alternatively, mild early hypothermia can be used to prolong the window of opportunity for treatment with neuroprotective peptides such as insulin-like growth factor (IGF)-I (Guan et al. 2000). IGF-I has to be

given directly into the cerebral ventricles; thus combination treatment of hypothermia with an IGF-I displacer may be a more clinically acceptable approach (Loddick et al. 1998; Mackay et al. 2003).

Plasticity

Finally, it may also be fruitful to combine therapeutic hypothermia with factors that improve brain recovery. The neonatal brain has very high levels of endogenous growth factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and IGF-I that are further upregulated in the recovery from injury (Beilharz et al. 1998; Lee et al. 2000). These growth factors are potently anti-apoptotic, but can also stimulate the generation of new cells (both neurons and oligodendrocytes) and promote axon sprouting within and surrounding damaged areas (Zaidi et al. 2004). The endogenous adaptive responses following brain injury are termed plasticity. They include elimination of neurons through apoptosis and pruning of synapses. It is proposed that ischaemia-induced neurogenesis and plasticity contributes to the long-term recovery of function (Sharp et al. 2002). There must be some potential risk that excessive regeneration and plasticity may lead to maladaptive brain circuits. Nevertheless, the magnitude of this response in the brain compares poorly with the degree of regeneration and functional repair seen in other organs, for example the liver, and there is now intriguing evidence from adult and infant rats that environmental stimulation can enhance neurogenesis and improve recovery from various insults. Thus it seems likely that there is real potential for behavioural or drug treatments to augment neural regeneration and plasticity and thus improve long-term functional recovery from perinatal hypoxic-ischemic encephalopathy (Young et al. 1999; Faverjon et al. 2002).

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SAMENVATTING

Het doel van dit proefschrift was om de hormonale en cardiovasculaire effecten van asfyxie (ernstig zuurstof tekort leidend tot acidose en verminderde weefsel doorbloeding) te bestuderen en om de therapeutische effecten van koeling van het hoofd te bestuderen in premature en à terme foetale schapen.

In **hoofdstuk 1** wordt de klinische en experimentele achtergrond van dit proefschrift besproken. Hersenschade ten gevolge van asfyxie is nog steeds een veel voorkomend probleem bij zowel à terme als prematuur geboren zuigelingen. Neuronen in de grijze stof van de hersenen zijn zeer kwetsbare cellen. Voor hun functie zijn ze afhankelijk van cellen in hun omgeving (o.a. glia cellen). Het lichaam is er alles aan gelegen tijdens een periode van zuurstoftekort zo lang mogelijk de doorbloeding van de hersenen in stand te houden, zelfs ten koste van de doorbloeding van andere organen zoals de nieren. Indien er tijdens langdurige asfyxie toch een cerebraal cellulair energietekort optreedt, zullen cellen in de grijze en witte stof dood gaan. Voor een deel zal deze celdood acuut optreden (necrose). Een ander deel raakt beschadigd of verliest contacten met de omgeving door de schade in de witte stof. Beschadigde cellen zullen uren tot dagen na het insult alsnog sterven, meestal middels activatie van een cascade van zelfvernietigingseiwitten. Dit proces wordt ook wel geprogrammeerde celdood of apoptose genoemd.

Klinisch kan de herstelperiode na asfyxie in verschillende fases verdeeld worden. In de eerste fase, ook wel de latente fase genoemd, treedt herstel op van oxidatief metabolisme. Het electroencefalografie signaal (EEG) is onderdrukt. Na 6-15 uur treedt er een fase op van verslechtering, de secundaire fase. Het begin van deze fase wordt meestal gemarkeerd door het ontstaan van epileptiforme activiteit op het EEG, het ontstaan van hersenzwelling, de intrede van ontstekingscellen en celdood. Na enkele dagen komt de tertiaire fase van stabilisatie. Het is waarschijnlijk dat in deze fase nog meer celdood plaatsvindt.

Het is de laatste jaren duidelijk geworden dat niet alleen schade aan de grijze stof belangrijk is voor de klinische prognose, maar dat ook schade aan de witte stof een belangrijke rol speelt.

Inmiddels hebben vele studies aangetoond dat in de latente fase interventies mogelijk zijn om de mate van grijze stof schade te beperken. Momenteel is er nog geen klinisch bewezen effectieve behandeling om de lange termijn effecten van asfyxie te beperken. Voor zowel premature als à terme pasgeborenen is een effectieve behandeling om handicaps te voorkomen zeer gewenst. Therapeutisch koelen van de hersenen (hypothermie) is een van de meest veelbelovende behandelingsstrategieën ter voorkoming van permanente hersenschade bij pasgeborenen. Het is echter nog onduidelijk of deze therapie ook effectief is voor de witte stof en bij prematuren.

Alvorens nieuwe behandelingen kunnen worden getest op pasgeboren kinderen is het belangrijk om niet alleen naar de effecten op de hersenen te kijken maar tevens naar de systemische effecten. Een van de doelstellingen van dit proefschrift was derhalve om de systemische effecten (o.a. bloeddruk, hersendoorbloeding en hormonale effecten) van koelen in de premature foetus te bestuderen.

Het chronisch geïnstrumenteerde foetale schapen model is een model dat al vele jaren wordt gebruikt voor studies van foetale fysiologie. Op vele relevante aspecten komt het foetale schaap overeen met de mens, ook qua ontwikkeling en structuur van de hersenen. De hersenen van een foetaal schaap op 70% van de totale zwangerschapsduur komen qua ontwikkeling overeen met humane hersenen van 30-32 weken zwangerschapsduur, terwijl schapen hersenen op 85% van de zwangerschapsduur overeenkomen met de à terme humane pasgeborene.

Het is in het schapenmodel mogelijk om continue metingen te doen van hersenfunctie (EEG) en fysiologische parameters zoals temperatuur, bloeddruk en weefsel doorbloeding. Aangezien de foetus

in utero blijft, kan de ooi gezien worden als een ideale, stabiele couveuze. Tevens is het mogelijk op verschillende momenten bloedmonsters af te nemen, en na afloop van de experimenten de weefsels nader te analyseren. In het chronisch geïnstrumenteerde foetale schapenmodel is het mogelijk om selectief de doorbloeding van de hersenen tijdelijk stil te leggen (ischemie) door occlusie van de bloedvaten naar de hersenen, maar ook om de navelstreng tijdelijk af te sluiten, waardoor er een algeheel zuurstof tekort optreedt. Beide methoden leiden tot patronen van hersenschade die relevant zijn voor humane perinatale hersenschade.

Hoofdstuk 2

Het prematuur geboren kind heeft een verhoogd risico op hersenschade. Een van de veronderstelde potentiële mechanismen hiervan is dat de prematuur minder goede beschermingsmechanismen heeft tegen episoden van zuurstofgebrek (asfyxie).

Met name de hypothalamus-hypofyse-bijnier as reactie op stress zoals asfyxie is potentieel immatuur, wat zou kunnen bijdragen tot het ontstaan van hypotensieve episoden. Echter, de studies die suggereerden dat de cortisol stress respons verminderd is in prematuren hebben relatief milde insulten gebruikt. Premature foetale schapen hebben een grotere tolerantie voor asfyxie en zijn in staat 2x zo lange insulten te overleven vergeleken met à terme foetussen, derhalve is het mogelijk dat voorgaande studies de foetale stressreactie niet maximaal gestimuleerd hebben. Om de effecten van ernstige asfyxie op de ACTH-cortisol as te bestuderen hebben wij het chronisch geïnstrumenteerde premature foetale schapen model gebruikt.

Onze studie liet zien dat de premature foetus geen immature hypothalamus-hypofyse-bijnier functie heeft. Echter, door de grote anaërobe tolerantie van de premature foetus vergeleken met een à terme foetus, hoeft de premature foetus pas in een veel latere fase de stressrespons maximaal te activeren. Er was een verband tussen cortisolwaarden en bloeddruk hetgeen suggereert dat cortisol helpt om de bloeddruk stabiel te houden tijdens de kritische herstelperiode na ernstige asfyxie.

Hoofdstuk 3

Verschillende experimentele modellen van hersenschade hebben aangetoond dat koelen van de hersenen een effectieve manier is om de grijze stof te beschermen. Er zijn echter nog geen studies verricht naar de gevolgen van hypothermie voor hersenschade bij prematuren. Dit is relevant omdat prematuren mogelijk gevoeliger zijn voor de nadelige effecten van hypothermie en omdat het patroon van hersenschade verschilt tussen prematuren en à terme zuigelingen.

In het chronisch geïnstrumenteerde premature schapenmodel (70% van de zwangerschap, overeenkomend met 30-32 weken humaan) hebben we het effect bestudeerd van het koelen van de hersenen na een ernstig asfyctisch insult. Koelen was geassocieerd met een daling van epileptiforme activiteit. Koelen beschermt tegen het verlies van neuronen in de hippocampus en basale ganglia, en was geassocieerd met minder apoptose en microglia activatie.

We concluderen dat koelen na ernstige asfyxie in de premature foetus beschermt tegen hersenschade, zonder dat potentieel schadelijke effecten worden gezien, zoals hypotensie of verstoring van het zuur-base evenwicht.

Hoofdstuk 4 en 6

Insulin-like growth factor-I (IGF-I) is een krachtige endogene groeifactor met neuroprotectieve en metabole functies. De rol van groeifactor IGF-II is minder bekend. De aanmaak van IGF-I wordt gestimuleerd door groeihormoon (GH) uit de hypofyse. In het bloed is ongeveer 97% van het IGF gebonden aan speciale bindingseiwitten (IGFBP 1-6). Het overige kleine deel is in de actieve vrije vorm

of los gebonden. Met name IGFBP-1 voorkomt dat IGF-I zich kan binden aan de IGF receptor.

Er is nog weinig bekend over de effecten van asfyxie op circulerend IGF en bindingseiwitten. We hebben daarom de effecten van asfyxie en therapeutische hypothermie onderzocht op veranderingen in IGF en IGFBP-1. Onze resultaten laten na asfyxie een daling van IGF-I en stijging van IGFBP-1 zien, waarschijnlijk om een catabole staat te faciliteren.

In hoofdstuk 6 wordt een nieuwe methode beschreven om de vrije fractie van IGF te verhogen. Behalve het direct toedienen van IGF-I lijkt het mogelijk een stof toe te dienen die IGF los maakt van het bindingseiwit om zo de biologische beschikbaarheid te verhogen. Zo'n stof is mogelijk eenvoudiger toe te dienen en mogelijk veiliger dan directe toediening van IGF-I.

Potentiële therapeutische opties van IGF displacers zijn groeistoornissen bij kinderen waarbij er een verhoogde hoeveelheid IGFBP-1 is, zoals bij nierfalen voorkomt. Daarnaast zijn IGF- displacers mogelijk werkzaam bij hersenschade op basis van ischemie en reperfusie. De studie uit hoofdstuk 4 suggereert dat de werking van therapeutische hypothermie niet het gevolg is van door effecten op circulerend IGF-I en dat milde systemische hypothermie de metabole aanpassingen na asfyxie niet verstoort.

Hoofdstuk 5

Vele experimentele studies hebben aangetoond dat koelen van de hersenen de grijze stof (neuronen) beschermt na ischemie-reperfusie. Er is echter minder bekend over de effecten van hersenkoeling op de overige hersencellen, zoals de witte stof (myeline en oligodendrocyten) en de steuncellen (glia).

Aangezien het ter voorkoming van neurologische schade niet alleen belangrijk is om de grijze stof te beschermen, dient een potentieel succesvolle behandeling ook de witte stof te beschermen. Voor deze studie hebben we gebruik gemaakt van à terme chronisch geïnstrumenteerde schapen. Hersenschade werd aangebracht door het tijdelijk afsluiten van de arteria carotis beiderzijds. De hersenen werden gekoeld tot tussen 30 en 33 graden Celcius. Koelen werd op verschillende tijden na ischemie gestart en gecontinueerd voor 3 dagen.

Wanneer het koelen 90 minuten na herstel van de circulatie werd gestart bleek er een significant grotere dichtheid van myeline en betere overleving van oligodendrocyten te zijn in vergelijking met de controle groep zonder koeling. Tevens was er minder apoptose in de witte stof. Koelen zorgde voor een forse onderdrukking van microglia cellen, terwijl er geen significant effect was op astrocyten. Wanneer koelen pas 5,5 uur na ischemie werd gestart bleek er geen significant beschermend effect te zijn op de witte stof oligodendrocyten.

We concluderen dat het koelen van de hersenen niet alleen beschermend werkt voor de grijze stof maar ook voor de witte stof. Echter, des te later de aanvang van het koelen, hoe minder effectief de bescherming. Bescherming van de witte stof was geassocieerd met een verminderde expressie van geactiveerd caspase-3 (apoptose) en van microglia cellen (inflammatie). De data ondersteunen het belang van het vroeg beginnen met koelen in de klinische studies.

TOEKOMST PERSPECTIEF

Koelen van de hersenen is een veel belovende therapie ter voorkoming van hersenschade voor à terme zuigelingen, maar mogelijk ook voor premature zuigelingen. Gerandomiseerde klinische studies zoals de "CoolCap trial" hebben nu ook aangetoond dat hypothermie effectief mogelijk is, maar met name in zuigelingen met matig ernstige hersenschade. Naast het vaststellen van selectiecriteria voor kinderen die mogelijk baat zullen hebben bij behandeling met hypothermie zullen nog verschillende vragen beantwoord moeten worden voordat koelen een standaard therapie zal worden op de afdeling

neonatologie. De maximale tijd waarna koelen nog gestart kan worden na de geboorte, de mate van koelen en de optimale duur van koelen vergen verder onderzoek. Alvorens koelen ook voor premature zuigelingen getest kan worden zijn meer studies naar veiligheid en effectiviteit gewenst. Behalve interventie door middel van koelen alleen, zullen er ook studies gedaan moeten worden naar combinatie therapieën, zoals koelen en behandeling met groeifactoren zoals IGF-I.

PUBLICATIONS

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First author

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Participation

Todd MM et al., for the Intraoperative Hypothermia for Aneurysm Surgery Trial (IHAST) Investigators (including V. Roelfsema). Mild intraoperative hypothermia during surgery for intracranial aneurysm. *New England Journal of Medicine*. 2005; 352: 135-45.

ABBREVIATIONS

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ACTH	Adrenocorticotrophic hormone
aEEG	amplitude-integrated electro-encephalogram
AGA	Appropriate for gestational age
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ARF	Acute renal failure
BE	Base excess
BDNF	Brain-derived neurotrophic factor
CA	Cornu Ammonis, part of the hippocampus
CaBF	Carotid artery blood flow
CBF	Cerebral blood flow
CFM	Cerebral function monitor
CNS	Central nervous system
CRF	Chronic renal failure
DAB	Diaminobenzidine tetrahydrochloride
DWI	Diffusion-weighted imaging (MRI technique)
EEG	Electro-encephalogram
ELBW	Extremely low birth weight (<1000gram)
EMG	Electro-myograph
FHR	Fetal heart rate
GFAP	Glial Fibrillary Acidic Protein (marker of astrocytes)
GH	Growth hormone
HI	Hypoxia-ischaemia
HIE	Hypoxic Ischaemic Encephalopathy
HPA	Hypothalamic-Pituitary-Adrenal axis
IB-4	Isolectin B4 (marker of microglia)
ICA	Internal carotid artery
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IV	intravenous
IVH	Intraventricular haemorrhage
K+	Potassium
LBW	Low birth weight (<2500 gram)
MAP	Mean arterial pressure
MBP	Myelin Basic Protein (marker of myelin)
MCA	Middle cerebral artery
MgSO ₄	Magnesium sulphate
mmHg	Millimeters of mercury
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRS	Magnetic resonance spectroscopy
Na+	Sodium
NGF	Nerve growth factor
NICU	Newborn intensive care unit

Abbreviations

NIRS	Near infrared spectroscopy
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
O ₂	Oxygen
PBS	Phosphate Buffered Saline
PaO ₂	Partial pressure of oxygen
PCNA	Proliferating nuclear antigen
PLP	Proteolipid protein
PVH	Periventricular hemorrhage
PVL	Periventricular leukomalacia
RCT	Randomised controlled trial
Rh	Recombinant human
RNA	Ribo-Nucleic Acid
ROS	Reactive oxygen species
SGA	Small for gestational age
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase end labelling
VEGF	Vascular endothelial growth factor
VLBW	Very low birth weight (<1500gram)

CURRICULUM VITAE

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Vincent Roelfsema werd op 9 september 1976 geboren te Eindhoven. In 1994 behaalde hij zijn middelbare school diploma Atheneum aan het Bisschop Bekkers College in Eindhoven om vervolgens te beginnen aan de opleiding Geneeskunde aan de Universiteit Maastricht.

Tijdens zijn studie was hij actief lid van studentenvereniging Koko en in het bestuur van het Maastricht Medical Student Research Conference (MMSRC). In 1998 deed hij zijn wetenschapsstage en keuze coschap in het Starship Childrens Hospital bij Prof. Erik Heineman. Na het behalen van het doctoraal examen onderbrak Vincent zijn studie Geneeskunde om samen met Josine terug naar Nieuw Zeeland te gaan om verder onderzoek te doen aan de University of Auckland en het Liggins Institute. Dit onderzoek was in samenwerking met Professor J. de Haan van het onderzoeksinstituut Groei en Ontwikkeling (GROW), Universiteit van Maastricht.

Na een verblijf van bijna 4 jaar in Auckland, Nieuw Zeeland, keerden ze terug naar Maastricht om de co-schappen af te ronden. In september 2004 werd het artsexamen afgelegd met het predikaat "Cum Laude". Sinds oktober 2004 werkt Vincent als AGNIO kindergeneeskunde in het VieCuri Ziekenhuis te Venlo. In juli 2005 is hij begonnen aan de opleiding tot kinderarts.

1988-1994	Atheneum, Bisschop Bekkers College, Eindhoven, The Netherlands
1994-1999	Master degree Medicine, Maastricht University. "Clear pass".
1999-2002	Research Centre for Developmental Medicine and Biology, University of Auckland, New Zealand, Liggins Institute, University of Auckland, New Zealand in collaboration with the research institute Growth and Development (GROW), Maastricht University, The Netherlands.
2002-2004	Internships and Medical degree. "Cum Laude".
2004-2005	Department of paediatrics, VieCuri ziekenhuis, Venlo
2005-present	Residency in Paediatrics

Awards

2000 Paykel travel grant

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