Perinatal brain damage : possible therapeutic approaches to hypoxic-ischemic cerebral injury, studied in fetal animals

PERINATAL BRAIN DAMAGE –

POSSIBLE THERAPEUTIC APPROACHES TO HYPOXIC-ISCHEMIC CEREBRAL INJURY, STUDIED IN FETAL ANIMALS

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Contents

Abbreviations

ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
ADP	adenosine-diphosphate
AMP	adenosine-monophosphate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolpropionacid
AOCC	agonist-operated calcium channels
ATP	adenosine-5´-triphosphate
BE	base-excess
BP	blood pressure
CA	Cornu ammonis, part of the hippocampus
cAMP	cyclic adenosine-3´,5´-monophosphate
Ca^{2+}	calcium
CBF	cerebral blood flow
cGMP	cyclic guanosine-3´,5´-monophosphate
EDRF	endothelium-derived-relaxing-factor
FFA	free fatty acids
FHR	fetal heart rate
GTP	guanosine-5´-triphosphate
HIE	hypoxic-ischemic encephalopathy
IL	interleukin
IUGR	intrauterine growth retardation
L-NAME	N^{ω}-Nitro-L-arginine methyl ester (NOS inhibitor)
L-NNA	N^{ω}-Nitro-L-arginine (NOS inhibitor)
LPS	lipopolysaccharide
MABP	mean arterial blood pressure
Mg^{2+}	magnesium
$MgSO_4$	magnesium-sulfate
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitrix oxide synthase
OGD	oxygen-glucose deprivation
PAF	platelet aggregating factor
PSR	protein synthesis rate
SO_2 (%)	fetal arterial oxygen saturation
TNF-α	tumor necrosis factor-alpha
VSCC	voltage-sensitive calcium channels
XDH	xanthine dehydrogenase
XO	xanthine oxidase

Chapter 1

GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

1.1 INTRODUCTION

Perinatal brain injury is a major contributor to perinatal morbidity and mortality (Volpe 1995). In Germany alone approximately one thousand children per year experience brain damage from perinatal hypoxic-ischemic insults. A considerable number of these children will develop cerebral palsy. The resulting impact on the children affected and their families is considerable and their subsequent care requires a high level of commitment and co-operation between pediatricians, child neurologists, physio-, speech- and psychotherapists, and other specialists. Despite the severe clinical and socio-economic significance, the efficacy of present clinical strategies is too low to reduce the incidence of perinatal hypoxic-ischemic brain damage significantly. However, an increasing number of experimental studies describe the pathophysiological mechanisms that are involved in perinatal brain injury. Based on these pathophysiological mechanisms a variety of excellent neuroprotective strategies have been developed in various animal models (for review: Berger and Garnier 1999). Obstetricians and neonatologists now face the important task of testing these neuroprotective strategies in clinical trials.

The following chapter presents our current understanding of the pathophysiology of hypoxic-ischemic brain damage in mature neonates. The situation in premature neonates is discussed separately wherever necessary. The review of the literature starts with the causes of ischemic brain lesion, especially intrauterine asphyxia and hypoxia of the fetus, and their effects on the cardiovascular system and cerebral perfusion. Asphyxia is defined as the clinical condition of impaired exchange of respiatory gases. Biochemically, asphyxia indicates the combination of oxygen lack with acidemia. Oxygen lack without an accompanying acidemia is called hypoxia (low oxygen tension) or hypoxemia (low percent saturated hemoglobin).

The typical neuropathological findings arising from reduced perfusion of the fetal brain are described. Also of key importance are the cellular mechanisms that are triggered by an ischemic insult. These will be discussed in detail, with particular emphasis on alterations of energy metabolism, intracellular calcium accumulation, the release of excitatory amino acids and protein biosynthesis. Focal cerebral ischemia is characterized by an ischemic core and a peri-infarct region known as ischemic penumbra. The ischemic penumbra is defined as a moderately hypoperfused region that retains structural integrity but has lost function. In animal models of ischaemic stroke, this region is prone to recurrent anoxic depolarization and will become infarcted if reperfusion does not occur (for review: Heiss and Graf 1994). As shown in previous studies, glutamate is released in tremendous amounts from the infarct core into the extracellular space after focal cerebral ischemia.

Increases of up to 80 times above baseline levels have been observed (Hillered et al. 1989). The released glutamate activates the neuronal NO-synthase via calcium influx through NMDA-regulated calcium channels (East and Garthwaite 1991). In focal ischemia this pathway may be of greater importance for the development of neuronal cell damage than in global cerebral ischemia, since in the latter type of ischemia only a moderate and short-lasting increase in glutamate release has been observed (Hagberg et al. 1987).

A considerable portion of neuronal cell damage first occurs during the reperfusion phase following an ischemic insult. The formation of oxygen radicals, induction of the nitric oxide system, inflammatory reactions and apoptosis will therefore be discussed in depth in this context. Finally, therapeutic concepts will be presented that have been developed out of our understanding of these pathophysiological processes and have been tested in animal experiments. Of these, postischemic induction of mild cerebral hypothermia, the application of the calcium-antagonist flunarizine and the administration of magnesium appear to be of the greatest clinical relevance.

1.2 OBJECTIVES OF THE STUDY

In experimental animal models of focal and global cerebral ischemia, flunarizine has shown to be neuroprotective when given before, but not after, the insult (Van Reempts et al. 1983; Silverstein et al. 1986; Gunn et al. 1989; Chumas et al. 1993; Gunn et al. 1994). In some of these studies flunarizine was applied at relatively high doses (i.e., 30 mg/kg body weight) before hypoxia-ischemia. The high dose regimens used had severe cardiovascular side-effects. The purpose of the studies given in *Chapters 4 and 5* was, therefore, to find a therapeutic regimen that does not alter the fetal cardiovascular responses to intrauterine asphyxia but reduces cerebral damage after global cerebral ischemia in fetal sheep.

Lubeluzole, the S-isomer of a novel 3,4-difluoro benzothiazole, has been shown to reduce ischemic neuronal cell damage in a variety of in-vitro as well as in-vivo studies (Aranowski et al. 1996; Buchkremer-Ratzmann et al. 1997; Culmsee et al. 1998; De Ryck et al. 1995, 2000; Haseldonckx et al 1997). The present knowledge on the safety profile and efficiency of lubeluzole seems to be promising enough to warrant further studies in immature animals. Preliminary experiments in our laboratory have demonstrated transient alterations in heart rate as well as arterial hypertension after intravenous application of this compound in late-gestation fetal sheep. Thus, it was thought that any possible

neuroprotective effect of this drug might be counteracted by an impairment of fetal cardiovascular control during hypoxia. The mature fetal sheep reacts to an episode of acute hypoxia with bradycardia and redistribution of combined ventricular output to the central and vital organs, such as the heart, brain, and adrenal glands at the expense of peripheral organs, including lungs, kidneys, gastrointestinal tract, and carcass, containing skin, bones, and muscle (Jensen et al. 1987. This crucial mechanism protects the fetal brain from neuronal injury by increasing cerebral perfusion when oxygen is in short supply. The aim of the present study was therefore to examine in detail whether lubeluzole affects circulatory responses to acute asphyxia in fetal sheep near term (*Chapter 6*). Furthermore, the cerebroprotective properties of lubeluzole were studied in a fetal sheep model of global cerebral ischemia. In this model we determined the time course of changes in organ blood flow during and after cerebral ischemia using the microspheres method. Using this model, we were able to account for drug-related changes in cerebral blood flow that might affect neuronal cell damage (*Chapter 7*).

Since the retrospective analysis of Nelson and Grether (1995) showing that magnesium, a well-known tocolytic agent, also has neuroprotective properties, its use in perinatology is very encouraging. However, the existing data is very non-uniform. Both protective and non-protective effects have been reported with different timing of administration at different stages of development. Furthermore, the physiological mechanisms through which a possible neuroprotective effect of magnesium could be mediated are not well understood. Therefore, the effects of magnesium on metabolic disturbances in fetal brain during and after ischemia were investigated, while excluding its effects on the fetal cardiovascular system. For this purpose we used the in-vitro system of oxygen-glucose deprivation (OGD) in hippocampal tissue slices prepared from mature guinea pig fetuses. Metabolic disturbances were assessed by measuring changes in energy metabolism and protein synthesis. In addition we determined cGMP concentrations in the tissue slices after OGD, as a measure of NO-production, to clarify whether a possible neuprotective effect of magnesium is mediated in part through the NO-system (*Chapter 8*).

In vitro models of hypoxia-ischemia have some advantages over *in vivo* models, including the ability to control or eliminate such variables as blood flow, temperature, ionic environment, nutrient availability, and the ability to serially assess injury or injury surrogates, such as impaired energy metabolism and prolonged inhibition of protein synthesis, respectively. However, multiple *in vitro* models used for examining neuroprotective properties of drugs have limitations that may restrict their ability to predict neuroprotective efficacy in intact animals. For example, in dissociated cell cultures, the anatomic relations of neurons, glia, and synapses are lost and sensitivity to anoxia or

hypoxia-ischemia is much reduced compared with intact animals. In acutely prepared and studied brain slices, anatomic integrity is preserved. Moreover, the hippocampal slice model of cerebral oxygen-glucose deprivation retains the sensitivity to anoxia.

The cerebroprotective properties of mild cerebral hypothermia are discussed in *chapter* 9. There is a growing body of evidence from animal experiments and clinical studies in asphyxiated newborns that mild hypothermia (selective head cooling) induced during or even after cerebral ischemia may protect the fetal and neonatal brain from neuronal cell damage (Gunn et al. 1997;1998a,b). However, the exact interrelation between the postischemic time delay and the degree of mild hypothermia by which neuroprotective effects on ischemic insults of different severity can be achieved has not yet been systematically elucidated. To clarify this point we studied the interaction between these variables in a recently modified hippocampal tissue slice model. We used hippocampal tissue since it is well known that this kind of brain tissue shows a high metabolic sensitivity. Thus, it was shown that already 5 min ischemia are sufficient to produce irreversible inhibition of protein synthesis, followed by delayed neuronal death, in the selectively vulnerable CA1 sector of the hippocampus, while other parts of the brain are much less vulnerable (Bodsch et al. 1985).

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Chapter 2

PERINATAL BRAIN DAMAGE: UNDERLYING MECHANISMS AND NEUROPROTECTIVE STRATEGIES. A REVIEW OF THE LITERATURE.

2.1 CAUSES OF HYPOXIC-ISCHEMIC BRAIN LESIONS IN NEONATES

With a few exceptions, acute hypoxic-ischemic brain lesions in neonates are caused by severe intrauterine asphyxia (Volpe 1995). This was recognised as early as 1844 when Sir William John Little, a London orthopaedic surgeon, published his lectures on *Deformities of the Human Frame*, in which he noted the association between severe asphyxia during delivery with convulsions. Intrauterine asphyxia is usually brought about by an acute reduction in the uterine or umbilical circulation (Jensen and Berger 1991), which in turn can be caused by abruptio placentae, contracture of the uterus, vena cava occlusion syndrome, compression of the umbilical cord etc.

2.2 CIRCULATORY CENTRALISATION AND CEREBRAL PERFUSION

During hypoxemia and asphyxia the distribution of the combined ventricular output changes in the mature fetus much the way it does in the adult. There is a circulatory centralization of blood flow in favour of the brain, heart, and adrenals, and at the expense of the peripheral organs, including lungs, kidneys, gastrointestinal tract, and carcass (for review: Jensen and Berger 1991). The lowered oxygen and raised carbon dioxide partial pressures lead to vasodilatation of the cerebral vascular bed causing an increased cerebral blood flow. This affects the brainstem in particular, while the bood flow to the white matter of the brain is hardly increased at all (Lou et al. 1985; Ashwal et al. 1984). Depending on the extent of the oxygen deficit and the maturity of the fetus, this cerebral hyperperfusion can reach 2-3 times the original rate of blood flow. If the oxygen deficit persists the anaerobic energy reserves of the heart become exhausted. The cardiac output and the mean arterial blood pressure fall. At mean arterial blood pressures of below 25-30 mmHg there is an increasing loss of cerebral autoregulation, and a consequent reduction of the cerebral blood flow (Lou et al. 1979). This affects the parasagittal region of the cerebrum and the white matter most of all. Immature fetuses seem to be particularly endangered by their limited ability to increase the cerebral circulation through vasodilatation.

If cerebral autoregulation is lost, cerebral blood flow varies with arterial blood pressure, but this is not always true. For instance, during acute asphyxia, caused by arrest of uterine

blood flow, cerebral vascular resistance increases and hence cerebral blood flow does not increase inspite of a steep increase in arterial blood pressure. Thus, under these very acute conditions, autoregulation is intact, even though arterial oxygen content is poor (Jensen and Berger 1991).

If the supply of oxygen to the fetus can be improved, cerebral hyperperfusion is brought about by the progressive postasphyxial increase in cardiac output (Jensen and Berger 1991). This hyperperfusion can be demonstrated in experiments using animal models of isolated cerebral ischemia (Fig. 1) (Berger et al. 1996c). Vasodilatation induced by acidosis in cerebral tissues and a reduction of blood viscosity at higher rates of blood flow have been put forward as possible causes of such hyperperfusion. The initial hyperperfusion of the brain is followed directly by a phase of hypoperfusion (Fig. 1) (Berger et al. 1996c; Rosenberg et al. 1989).

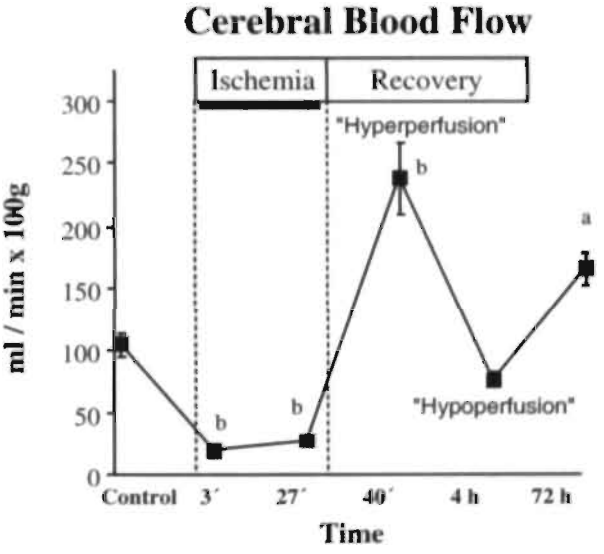

Fig. 1. Blood flow to the cerebrum (ml/min x 100g tissue) in fetal sheep near term before, during and after global cerebral ischemia of 30 min duration. Cerebral ischemia was induced by occluding both carotid arteries. Results are given as means ± SD (a: $P < 0.01$, b: $P < 0.001$ (ischemia / recovery vs. control) (Berger et al. 1996c).

Postischemic hypoperfusion may be caused by oxygen radicals formed during the reperfusion phase after ischemia. Rosenberg and co-workers demonstrated that this phenomenon can be prevented by inhibiting the synthesis of oxygen radicals after ischemia (Rosenberg et al. 1989). In addition, a so-called no-reflow phenomenon can be observed after severe cerebral ischemia. This failure of reperfusion in various brain areas is a consequence of the greater viscosity of stagnant blood, compression of the smallest blood vessels through swelling of the perivascular glial cells, formation of endothelial microvilli, increased intracerebral pressure, postischemic arterial hypotension and increased intravascular coagulation. The extent of the no-reflow phenomenon depends on the duration and type of cerebral ischemia. It is most pronounced when the vessels are engorged with blood after venous congestion (Hossmann 1993). Directly after postischemic hypoperfusion the cerebral blood flow recovers or overshoots into a second phase of hyperperfusion (Fig. 1) (Berger et al. 1996c; Pryds et al. 1990). Since this hyperperfusion is often accompanied by an isoelectric encephalogram, it is regarded as an extremely unfavourable prognostic factor (Pryds et al. 1990).

2.3 *NEUROPATHOLOGY OF HYPOXIC-ISCHEMIC BRAIN* LESIONS

There are essentially six forms of hypoxic-ischemic brain lesion (Table 1): selective neuronal cell damage, status marmoratus, parasagittal brain damage, periventricular leucomalacia, intraventricular or periventricular haemorrhage and focal or multifocal ischemic brain lesions (Berger and Garnier 1999; Volpe 1995).

In mature fetuses, selective neuronal cell damage is found most frequently in the cerebral cortex, hippocampus, cerebellum and the anterior horn cells of the spinal cord (Friede 1966; Larroche 1986; Norman 1978; Volpe 1995). As shown in animal experiments, the damage occurs after ischemia of only 10 min (Williams 1992). Within the cortex, the border zones between the major cerebral arteries are the worst affected. The cell damage is mostly parasagittal and more marked in the sulci than in the gyri, i.e. the pattern of distribution is strongly dependent on perfusion. The neurones show the most damage while the oligodendrocytes, astroglia and microglia remain largely unscathed (Volpe 1995). In contrat to the neuropathological manifestations in the term infant, in the premature infant periventricular oligodendroglial/white matter injury predominates. Our understanding of the biochemical events leading from the hypoxic-ischemic insult to the neuronal injury of

the term infant is much better than our understanding of the events leading to the oligodendroglial injury of the premature infant. The pathophysiological mechanisms leading to the different pattern in neuronal and oligodendroglial injury will be discussed in the following sections.

Status marmoratus, which is observed in only 5% of children with hypoxic-ischemic brain lesions, chiefly affects the basal ganglia and the thalamus. The complete picture of the disease does not emerge until 8 months after birth although the insult begins to take effect during the perinatal period. Status marmoratus is characterised by loss of neurones, gliosis and hypermyelination. The increased number of myelinated astrocytic cell processes and their abnormal distribution give the structures affected, especially the putamen, a marbled appearance (Friede 1966; Rorke 1992).

Table 1. Hypoxic-Ischemic Brain Damage in the Fetus and Neonate.

Neurologic lesion	**Topographic localization**
Selective neuronal necrosis	cortex cerebri cerebellum hippocampus anterior horn cells of the spinal cord
Status marmoratus	basal ganglia thalamus
Parasagittal cerebral injury	cortex cerebri and subcortical substantia alba
Periventricular leucomalacia	substantia alba
Intra-, periventricular hemorrhage	germinal matrix substantia alba ventricles
Focal / multifocal ischemic brain damage	cortex cerebri and subcortical substantia alba

Parasagittal brain damage caused by cerebral ischemia is mostly reported in mature neonates (Friede 1966; Larroche 1986; Norman 1978; Volpe 1995) and affects the parietal and occipital regions in particular. The damage usually arises through insufficient perfusion of the border zones between the main cerebral arteries during cerebral ischemia. The extent of the brain lesions was found to be closely dependent on the duration and severity of the cerebral ischemia (Berger et al. 1996c; Williams et al. 1992). Interestingly, in the cortex, sulci are more badly damaged than the gyri. This arises from the special way in which the blood vessels in the cortex and surrounding white matter develop. When the sulci take shape and deepen in mature neonates, the penetrating blood vessels branching out from the meningeal arteries are forced into a hairpin bend as they cross the border from grey matter into white matter. This produces a triangular area within the white matter at the base of the sulci through which hardly any vessels pass. Thus any reduction in the perfusion of this region causes most damage to the sulci of the cortex. This pattern of damage seems to correspond to that observed clinically in cases of subcortical leucomalacia (Takashima et al. 1978).

Periventricular leucomalacia is characterised by damage to the white matter dorsal and lateral to the lateral ventricle (Larroche 1986; Norman 1978). It occurs most frequently in immature fetuses and chiefly affects the radiatio occipitalis at the trigonum of the lateral ventricle and the white matter around the foramen of Monro. At 6 to 12 hours after an ischemic insult necrotic foci can be observed in these areas (Banker et al. 1962). These are characterised by swelling and rupture of neuronal axons. Necrotic oligodendrocytes are also found, especially ones undergoing differentiation or taking part in myelinisation. Over the next 24 to 48 hours activated microglia are seen more and more frequently. In 25% of cases, periventricular leucomalacia is accompanied by parenchymatous haemorrhaging (Paneth et al. 1990). As the disease progresses small cysts develop out of the necrotic foci that can be identified by ultrasonography (de Vries 1988; Paneth et al. 1990). As gliosis progresses the cysts become smaller. The lack of myelinisation owing to the destruction of the oligodendrocytes and an enlargement of the lateral ventricle then become the most prominent features of the disease (Dambska et al. 1989; Rorke 1992; Takashima et al. 1978). Periventricular leucomalacia around the Radiatio occipitalis at the trigonum of the lateral ventricle and in the white matter around the foramen of Monro arises through vascular problems. Especially in immature fetuses, the ability to increase blood flow by vasodilatation during and after a period of arterial hypotension appears to be extremely limited in these brain areas (Szymonowicz et al. 1990). After the 32nd week of pregnancy the vascularisation of these vulnerable areas is considerably increased and the incidence of periventricular leucomalacia thereby reduced.

Intra- or periventricular haemorrhage is another typical lesion of the immature neonate brain (Volpe 1995). It originates in the vascular bed of the germinal matrix, a brain region that gradually shrinks until it has almost completely disappeared in the mature fetus (Hambleton et al. 1976; Moody et al. 1994; Nakawara et al. 1990). Blood vessels in this brain region burst easily owing to a delay in basal lamina deposition and organization as well as delayed endothelial cell tight junction formation (Ment et al. 1995). Sub- and post-partum fluctuations in cerebral blood flow can therefore lead to rupture of these vessels causing intra- or periventricular haemorrhage (Berger et al. 1997; Funato et al. 1992; Goldberg et al. 1990; Jensen et al. 1992; Milligram et al. 1980). Microscopic studies of the periventricular hemorrhagic necrosis indicate that the lesion is often a hemorrhagic infarction (Volpe 1997). There is increasing evidence from recent clinical and experimental data that the hemorrhagic component of the infarction tends to be most concentrated near the ventricular angle where the medullary veins draining the cerebral white matter become confluent and ultimately join the terminal vein in the subependymal region. Thus, it appears likely that periventricular hemorrhagic necrosis occuring in association with large IVH is, in fact, a venous infarction (for review: Volpe 1997).

Focal or multifocal brain damage usually occurs within areas supplied by one or more of the main cerebral arteries. This form of insult is not normally observed before the 28th week of pregnancy. The incidence then rises with increasing maturity of the fetus (Barmada et al. 1979). Focal or multifocal brain lesions are often the result of infections, trauma or twin births, especially monochoriotic ones (Bejar et al. 1990; Patten et al. 1989; Scheller and Nelson 1992). It is thought that thromboplastic material or emboli from a miscarried co-twin sometimes occludes the cerebrovascular circulation of the living twin. Brain damage may also be caused by anemia or polycythemia and subsequent cardiac insufficiency and cerebral hypoperfusion arising from a feto-fetal transfusion. Alternatively, focal or multifocal brain damage can arise from systemic arterial hypotension, so that there is little distinction between this and other forms of brain damage such as selective neuronal cell damage, status marmoratus, parasagittal brain damage or periventricular leucomalacia (for review: Berger and Garnier 1999; Volpe 1995).

In this context it is important to note, that the fetal and neonatal brain possesses a enormous possibility for repair. The epidermal growth factor (EGF) family is an important regulator for tissue development and repair. Prominent members of the EGF family are transforming growth factor-alpha (TGF-alpha) and heparin-binding EGF (HB-EGF). These polypeptides, produced by neurons and glial cells, play an important role in the

development of the nervous system, stimulating proliferation, migration, and differentiation of neuronal, glial, and Schwann precursor cells. These peptides are also neurotrophic, enhancing survival and inhibiting apoptosis of post-mitotic neurons, probably acting directly through receptors on neurons, or indirectly via stimulating glial proliferation and glial synthesis of other molecules such as neurotrophic factors. TGF-alpha, EGF, and neuregulins are involved in mediating glial-neuronal and axonal-glial interactions, regulating nerve injury responses, and participating in injury-associated astrocytic gliosis, brain tumors, and other disorders of the nerve system (for review: Xian and Zhou 2002).

2.4 ENERGY METABOLISM AND CALCIUM HOMEOSTASIS

The normal functioning of the brain is essentially dependent on an adequate oxygen supply to maintain energy metabolism. Whereas, during moderate hypoxemia, the fetus is able to maintain adequate levels of ATP by speeding up the rate of anaerobic glycolysis (Berger et al. 1993; 1994; 1997b), a severe reduction of the fetal oxygen supply will lead to a breakdown of energy metabolism in the cerebral cortex within a few minutes (Berger et al. 1991; 1992). The ionic gradients for Na^+, K^+ and Ca^{2+} across the cell membranes can no longer be regulated since the Na^+/K^+-pump stops working through lack of energy. The membrane potential approaches 0 mV (Hansen 1985). The energy depleted cell takes up Na^+, and the subsequent fall in membrane potential induces an influx of Cl^- ions. This intracellular accumulation of Na^+ and Cl^- ions leads to swelling of the cells as water flows in through osmosis. Cell edema is therefore an inevitable consequence of cellular energy deficiency (Siesjö et al. 1992).

In addition, loss of membrane potential leads to a massive influx of calcium down the extreme extra-/intracellular concentration gradient. It is currently thought that the excessive increase in intracellular calcium levels, the so-called calcium-overload, leads to cell damage by activating proteases, lipases and endonucleases (Siesjö et al. 1992). Some of the cellular mechanisms that are activated by the calcium influx occurring during ischemia are shown in Figure 2.: alteration of the arachidonic acid cycle affecting prostaglandin synthesis, disturbances of gene expression and protein synthesis and increased production of free radicals and obstruction of the axonal transport system through disaggregation of microtubuli.

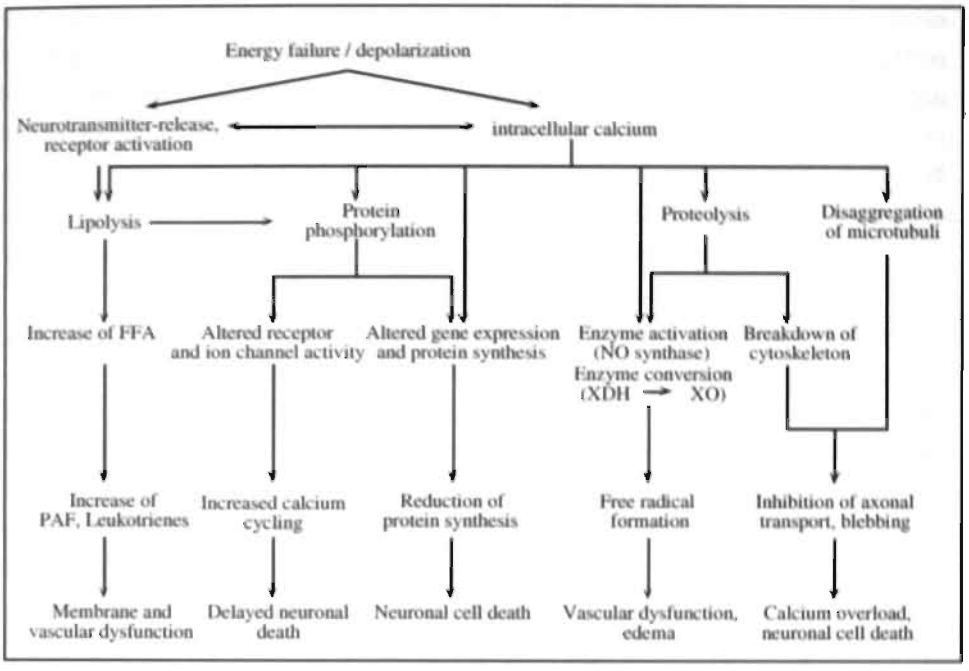

Figure 2. Diagramm illustrating the primary effects of depolarization / receptor activation and the secondary effects of an increased intracellular calcium concentration (modified from Siesjö et al. 1992). FFA, free fatty acids; NO, nitric oxide; PAF, platelet aggregating factor; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

2.5 EXCITATORY NEUROTRANSMITTERS

As early as 1969 Olney succeeded in demonstrating that neuronal cell death could be induced by the exogenous application of glutamate, an excitatory neurotransmitter. In subsequent years, this observation was confirmed in both immature and adult animals of various species including primates (Olney et al. 1969). In 1984, Rothman showed that glutamate antagonists could prevent anoxic cell death in hippocampal tissue cultures. That same year, Benveniste et al. (1984) reported an excessive release of glutamate into the extracellular space during cerebral ischemia *in vivo*, from which they concluded that glutamate might play an important role in neuronal cell death following ischemia (Palmer et al. 1990; Rothman 1984).

Glutamate activates postsynaptic receptors that form ionic channels permeable to cations (Fig. 3) (Schoepfer et al. 1994). The NMDA-receptor regulates a calcium channel, the metabotropic receptors induce an emptying of intracellular calcium stores while the AMPA/KA receptors open a voltage-dependent calcium channel by membrane depolarisation. The increase in free calcium within the cell activates proteases, lipases and endonucleases that then initiate processes leading to cell death (Choi 1988; Siesjö and Bengtsson 1989).

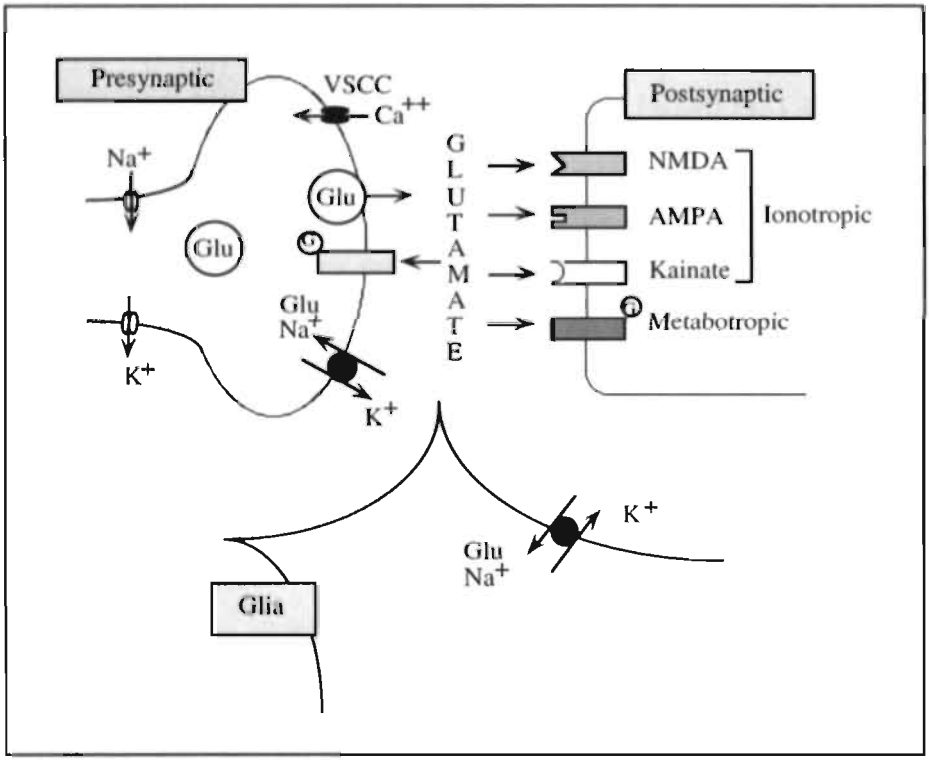

Figure 3. Determinants of glutamate-mediated synaptic transmission. The release of vesicular glutamate (exocytosis) is triggered by action potential invasion of the presynaptic terminal with subsequent depolarization and influx of Ca^{2+} through voltage-sensitive Ca^{2+}-channels (VSCC). Glutamate released into the synaptic cleft activates postsynaptic ionotropic receptors and pre- or postsynaptic metabotropic receptors (i.e. coupled to G-proteins). High-affinity, Na^+-dependent glutamate uptake in presynaptic and glial membranes contributes to termination of glutamate actions (modified after Obrenovitch et al. 1996).

There is no longer any doubt that glutamate release plays a critical role in neuronal cell death after *focal cerebral ischemia* such as that caused by an arterial embolus. Glutamate antagonists have been shown to exert a strong neuroprotective effect against hypoxic-ischemic brain damage in adult (Kochhar et al. 1988; Park et al. 1988; Uematsu et al. 1991) and even in neonatal animals (Andine et al. 1988; Ford et al. 1989; Gilland et al. 1994; Hattori et al. 1989; McDonald et al. 1987; Nozaki and Beal 1992). In neonatal rats it was shown that glutamate release during and after an hypoxic-ischemic insult could evoke epileptogenic activity and that this effect was dependent on the maturity of the brain. In rats, the most marked effect was observed 10 to 12 days after birth (Jensen et al. 1991). The reason for this seems to be a developmental change in the composition of the glutamate receptor which increases the neurone's permeability to calcium (Jensen et al. 1995; Jensen 1997). Furthermore, the levels of GABA, one of the most important inhibitory neurotransmitters, in neuronal tissue are very low at this stage of development (Coyle and Enna 1976). In addition, the formation of LTP's (long term potentiations), that play an important role in synaptic plasticity and hence in learning processes, may be disturbed by the induced epileptogenic activity (Bliss et al. 1993). Long-term neurological damage is the inevitable consequence in the children affected.

From a clinical and experimental point of view it is important to subdivide cerebral ischemia in two major forms: (1) global ischemia which is relatively shortlasting and followed by recirculation, and (2) focal ischemia of the stroke type, encompassing a densely ischemic core ('focus') and a less densely ischemic perifocal, penumbra zone (for review: Siesjö et al. 1992). As shown in adult animals epileptogenic impulses in the vicinity of a brain infarct cause a considerable rise in metabolic activity. In an inadequately perfused section of brain tissue such as the penumbra surrounding an infarct, this can rapidly lead to an imbalance between cell metabolism and blood circulation, resulting in brain damage (Heiss and Graf 1994). Cells in the perifocal penumbra are threatened by two events: irregularly occuring ionic transients, and a gradual compromise of the capillary circulation. According to this, focal ischemia differs from global cerebral ischemia in that flow is usually so slow in the focus, and the chances for quick reperfusion so slim that the area will usually become infarcted. Furthermore, the penumbra is usually considered to have flow rates sufficiently high to make the region essentially viable, at least during the first couple of hours (for review: Siesjö et al. 1992). If reperfusion occurs after longer periods, the ischemic focus may be infarcted while the penumbra zone shows either selective neuronal necrosis or areas of infarction

In global cerebral ischemia, such as that caused by cardiac insufficiency, the situation is quite different to that in focal cerebral ischemia. As shown in adult animals it is far less

clear whether glutamate is directly involved in neuronal cell death (Aitken et al. 1988; Albers et al. 1992). As Hossmannn points out in his 1994 review article, a number of observations argue against any major involvement of glutamate in processes leading to neuronal cell death after global ischemia: (1) Neither the pattern of glutamate release during ischemia nor the cerebral distribution of glutamate receptors matches the regional manifestation of brain damage after global ischemia. (2) Glutamate toxicity in cell cultures from vulnerable brain areas was found to be no higher than in cultures from non-vulnerable regions. (3) In contrast to the effects of *in vitro* ischemia, application of glutamate to cell cultures or hippocampal tissue slices caused no prolonged inhibition of protein synthesis.

Since then, the possibility of glutamate's playing a key role in the induction of brain damage either during or directly after global ischemia, even in the immature brain, has been effectively excluded by the following observations: Application of glutamate or glutamate antagonists to hippocampal slices from guinea pig fetuses did not affect postischemic protein biosynthesis, a parameter used as an early marker of neuronal cell death (Fig. 4) (Berger et al. 1997c). However, it is possible that later, during the reperfusion phase after cerebral ischemia, glutamate-induced epileptogenic activity does cause brain damage. This possibility will be discussed further on.

2.6 PROTEIN BIOSYNTHESIS

As animal experiments show, inhibition of protein synthesis plays a key role in the postischemic processes leading to neuronal cell damage (Hossmann et al. 1992). Protein synthesis is reduced both during ischemia and in the early postischemic phase in vulnerable and non-vulnerable brain areas (Kleihues et al. 1975). At the end of the ischemic period, protein synthesis in non-vulnerable regions recovers to pre-ischemic levels, while in vulnerable regions it remains inhibited (Bodsch et al. 1985). Thus the prolonged inhibition of protein synthesis appears to be an early indicator of subsequent neuronal cell death (Hossmann et al. 1992). This observation ties in with the results of experiments demonstrating the neuroprotective effect of hypothermia or barbiturates after cerebral ischemia (Widmann et al. 1993; Xie et al. 1989). Shortly after cerebral ischemia, the usual inhibition of protein synthesis set in, however, the recovery phase in the normally vulnerable areas was now much shorter, and was accompanied by far less pronounced neuronal cell damage. Similar findings were reported in connection with developmental

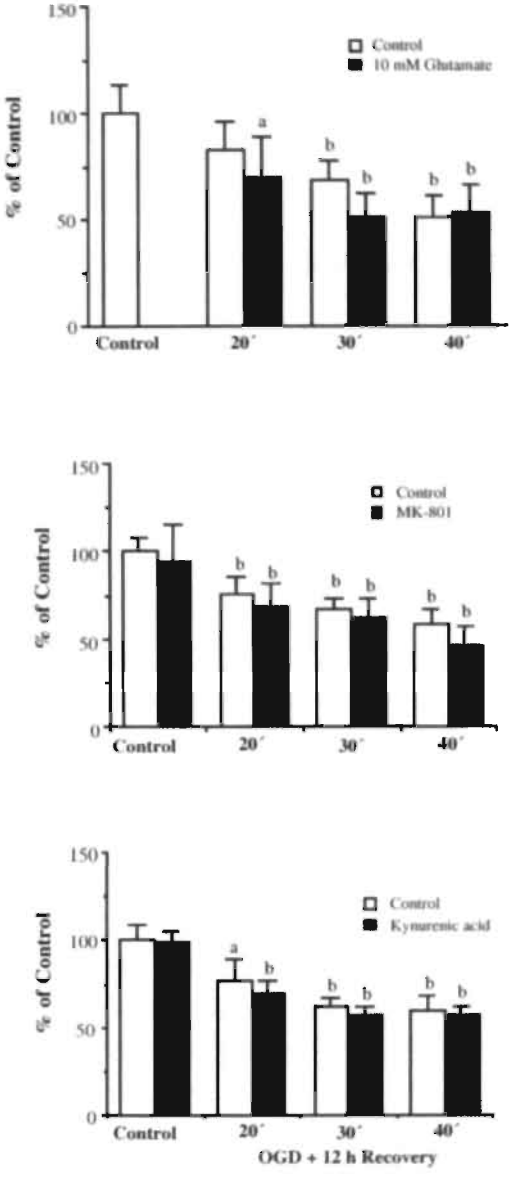

Fig. 4. Protein synthesis rate in hippocampal slices from mature fetal guinea pigs 12 h after oxygen-glucose deprivation (OGD). The OGD lasted between 20 and 40 min (20´, 30´, 40´). Protein synthesis rate was not affected neither by application of glutamate nor by glutamate antagonists (MK-801, Kynurenic acid). Values are given as mean ± SD. Statistical analysis was preformed by ANOVA followed by Scheffé´s F-test (a $P < 0.05$, b $P < 0.01$ (OGD vs. control)) (reproduced with permission, Berger et al. 1997c).

variations in the response of the brain to ischemic insults. Protein synthesis in the fetal brain was found to recover much faster from ischemic insults than that in adult brains (Berger et al. 1996a). The prolonged inhibition of protein synthesis is therefore an early indicator and possibly also one of the causes of neuronal cell damage arising after ischemia (Hossmann et al. 1992).

2.7 SECONDARY CELL DAMAGE DURING REPERFUSION

In cerebral tissue capable of regeneration after an ischemic insult, energy metabolism can be seen to recover rapidly (Berger et al. 1996a; Hossmann et al. 1992). A few hours later, however, the energy status is diminished once again in the affected tissue (Blumberg et al. 1997; Penrice et al. 1997). Simultaneously, a secondary cell edema develops, followed a little later by epileptogenic activity that can be monitored on EEG. These events are quite probably brought about or modulated by oxygen radicals, nitric oxide, inflammatory reactions and excitatory amino acids, particularly glutamate.

Oxygen radicals

During cerebral ischemia, the cut back in oxidative phosphorylation rapidly diminishes reserves of high-energy phosphates. Within a few minutes considerable amounts of adenosine and hypoxanthine accumulate in the cerebrospinal fluid. During reperfusion these metabolic products are metabolised further by xanthine oxidase to produce xanthine and uric acid (de Haan et al. 1993; McCord et al. 1985). Especially, the breakdown of hypoxanthine by xanthine oxidase in the presence of oxygen, produces a flood of superoxide radicals. These are then converted by superoxide dismutase to hydrogen peroxide (Fridovich 1978; 1983). By the Haber-Weiss reaction shown below, hydrogen peroxide and tissue iron can then combine to form hydroxyl radicals.

Numerous studies have shown that oxygen radicals play an important role in processes leading to neuronal cell damage (Traystman et al. 1991; for review: Halliwell et al. 1989). In adult animals various degrees of neuroprotection against ischemic insults can be achieved through the inhibition of xanthine oxidase or by application of oxygen radical scavengers and iron chelators (Beck et al. 1991; Biegon et al. 1995; Cao et al. 1995; Hall et al. 1991; Lin et al. 1991; Martz et al. 1989; Mink et al. 1991; Patt et al. 1990). Oxygen radicals also appear to be involved in mechanisms underlying neuronal cell death in

immature animals. The rate of lipid peroxidation was found to be considerably increased after hypoxia in fetal guinea pigs and newborn lambs (Abdel-Rahman et al. 1995; Goplerud et al. 1992; Mishra and Delivoria-Papadopoulos 1989). The longer the gestational age, the greater this increase was. Furthermore, marked production of oxygen radicals was observed after hypoxia both *in vitro*, in cultures of fetal neurones, and *in vivo*, in neonatal mice (Hasegawa et al. 1993; Oillet et al. 1996). There is also evidence that the infarct volume can be reduced in a model of focal ischemia in neonatal rats by application of allopurinol, an inhibitor of xanthine oxidase and oxygen radical scavengers (Palmer et al. 1990).

Nitric oxide

During cerebral ischemia, a massive influx of intracellular calcium takes place through various channels, regulated, among other things, by the neurotransmitter glutamate (Choi 1992; Siesjö and Bengtsson 1989). The rise in intracellular calcium activates NO-synthase (East and Garthwaite 1991; Garthwaite 1991) , which produces NO, citrulline and water from arginine, NADPH and oxygen. There is also an accumulation of cGMP (Beckman et al. 1992). Since there is no oxygen available during ischemia, NO cannot be synthesized until the reperfusion phase.

NO-Synthase

$$\text{Arginine} + \text{NADPH} + \text{H}^+ + \text{O}_2 \quad \Rightarrow \quad \text{NO} + \text{Citrulline} + \text{NADP}^+ + \text{H}_2\text{O}$$

Likewise, large numbers of superoxide radicals are produced by xanthine oxidase and via other pathways in the mitochondria during and, to an even greater extent, after ischemia (Lynch et al. 1978). During reperfusion, NO and superoxide radicals combine to produce peroxynitrite, leading to the formation of more potent radicals. Destruction of the tissue is the inevitable result (Beckman et al. 1992).

Investigations of the action of inhibitors of NO-synthase in models of cerebral ischemia in adult animals have yielded highly variable results (Caldwell et al. 1994; Dalkara et al. 1994; Dawson et al. 1994; Hamada et al. 1995; Kuluz et al. 1993; Nagafiji et al. 1993; Nishikawa et al. 1993; Zhang et al. 1994). This can be explained by the fact that the neuroprotective effect of NO-synthase blockers after ischemia, that is brought about by a lowering of NO production and consequent reduction of the build-up of potent radicals, is counteracted by a marked vasoconstriction induced by the fall in NO concentration in

endothelial cells (Dambska et al. 1989). Thus Moskowitz and co-workers found markedly smaller infarct loci after occlusion of the A. cerebri media in mice whose expression of the neuronal form of NO-synthase had been blocked than in the wild type of the animal (Huang et al. 1994). The same group was also able to protect the brain from ischemic insults by application of selective blockers of neuronal NO-synthase (Dambska et al. 1989).

To date hardly any studies have investigated the importance of nitric oxide in neuronal cell death in neonates or fetuses. After a hypoxic-ischemic insult in neonatal rats, a greater number of neurones were found to contain NO-synthase (Higuchi et al. 1996). The activity of this NO-synthase, however, appeared to be diminished (Jensen 1997). Furthermore, two peaks of NO production were detected in this animal model: one during hypoxia and the other one during the reoxygenation period. The neuronal and the inducible form of NO-synthase seems to be differently involved in this process (Higuchi et al. 1998). Some authors succeeded in preventing ischemic lesions in the brains of immature animals through application of NO-blockers (Ashwal et al. 1984; Hamada et al. 1994; Trifiletti et al. 1992), while other research teams were unable to achieve this effect or observed, instead, a worsening of the damage (Marks et al. 1996; Spanggord et al. 1996). As already mentioned, this discrepancy may have arisen from the different effects of NO-blockers on vascular endothelia and neurones. In our investigations of the effect of blocking NO-synthase we therefore by-passed the cardiovascular system, by carrying out experiments on hippocampal slices (Berger et al. 1998b). Although postischemic NO-production could be completely blocked with NO-inhibitors, this intervention had no influence on the postischemic inhibition of protein biosynthesis, a parameter used as an early indicator of neuronal cell death. Whether or not NO is directly involved in the pathogenesis of neuronal cell death following ischemia in fetuses therefore remains an open question.

Glutamate

Williams and co-workers observed epileptiform activity in mature sheep fetuses about 8 hours after 30 min of global cerebral ischemia that reached a peak 10 hours after the ischemic period (Williams et al. 1991). They were able to completely inhibit this epileptiform activity by application of the glutamate antagonist MK-801, and show that the resulting brain damage was markedly reduced in the treated animals (Tan et al. 1992). This suggests that a secondary wave of glutamate release or an imbalance between excitatory and inhibitory neurotransmitters during reperfusion may induce epileptiform bursts of neuronal activity that can lead to an uncoupling of cell metabolism and blood flow. This would automatically impair pathways of energy metabolism and cause a secondary wave

of cell damage (Hossmann 1994).

2.8 INFECTION RELATED CEREBRAL INJURY

As demonstrated by a variety of recent studies, inflammatory reactions not only aggravate secondary neuronal damage after cerebral ischemia, but may also affect the immature brain directly. Thus, after chorioamnionitis the incidence of immature babies suffering from periventricular leucomalacia and peri- or intraventricular hemorrhage is significantly increased (Berger et al. 1997; Jensen et al. 1992; Yoon et al. 2000; Wu and Colford 2000). However, it remains unclear whether fetal brain damage following endotoxemia is the result of cerebral hypoperfusion caused by circulatory decentralization or whether it is caused by a direct effect of endotoxins on cerebral tissue (for review: Dammann and Leviton 1997).

The effects of sytemically applied lipopolysaccharides (LPS) on circulatory responses were studied in chronically instrumented immature fetal sheep (0.7 of gestation) (Fig. 5) (Garnier et al. 2001). Within 1 h after i.v. injection of LPS (*E.* coli; 53 ± 3 µg per kg fetal weight) there was a marked fall in arterial oxygen saturation (-46%) and pH (7.36±0.03 vs. 7.16±0.11). Whereas blood flow to the placenta severely decreased, that to the carcass rose. During a short period of superimposed intrauterine asphyxia circulatory dezentralization occured. During the immediate recovery period oxygen delivery to the cerebrum was minimal. It is obvious that under such circumstances fetal hypoxic-ischemic brain injury may occur.

Recently, we studied the effects of very low doses of LPS (*E.* coli; 100 ng/kg fetal BW) on fetal cardiovascular function and resulting neuropathology. Systemically administered LPS caused a substantial and longlasting decrease in umbilical blood flow resulting in sustained fetal hypoxemia without acidemia (Garnier et al. 2002). FHR and MAP increased at 4-5 hrs after LPS and was elevated during 15 hrs after LPS. Placental blood flow began to fall 1 hr after LPS and was minimal (-30%) at 4-5 hours after LPS, while placental vascular resistance rose by 70% during this period. Thereafter, placental blood flow slowly returned to control values at 12-16 hrs after LPS. Histological examination revealed groups of inflammatory cell infiltrations in the periventricular white matter in LPS treated fetuses. On electronmicroscopical evaluation these cells appeared to be polymorphonuclear leukocytes (neutrophils; PMNs) and activated microglia. Moreover, an increased rate of apoptotic cells was detected after LPS treatment in the periventricular white matter.

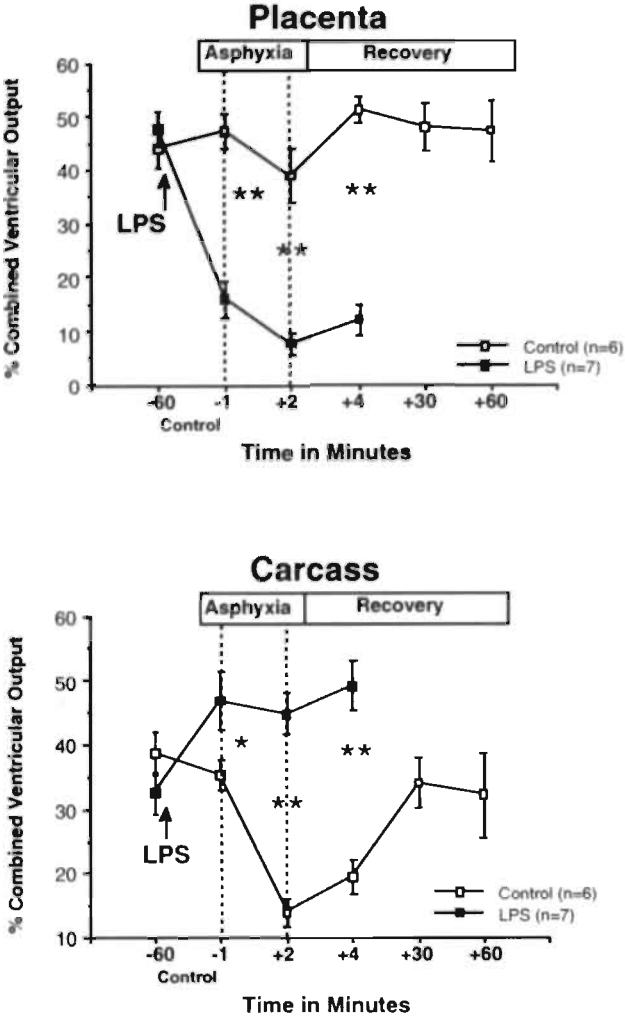

Figure 5. Combined ventricular output directed to the placenta and carcass in control (n = 6) and LPS treated (n = 7) chronically catheterized immature fetal sheep (0.7 of gestation) before, during and after arrest of uterine blood flow for 2 min. In LPS treated fetuses there was a significant fall in the percentage of combined ventricular output directed to the placenta while that directed to the carcass significantly increased. This was not seen in controls. During arrest of uterine blood flow the portion distributed to the carcass remained elevated in fetuses of the study group ($P<0.001$). Within 60 min after induction of asphyxia 5 out of 7 LPS treated fetuses died, whereas control fetuses completely recovered during this period. Values are given as means ± SEM. The data were analysed within and between groups using a two-way ANOVA followed by Games-Howell post-hoc test (*$P<0.05$, **$P<0.01$) (Garnier et al. 2001)

PMNs are blood-born inflammatory cells with potent oxidative and proteolytic potential that are usually the first line of defense against invading pathogens. Their ability to exit blood vessels and migrate rapidly to extravascular sites in tissues is crucial for succesfull elimination of bacterial, parasitic, and viral infections (for review: Wagner and Roth, 1999). Activation of PMNs is frequenttly implicated in the promulgation of deleterious inflammatory processes, including tissue injury associated with exposure to endotoxin.

The direct cytotoxic effect of LPS *in vivo* was examined in a neonatal rat model. Rat pups were subjected to an hypoxic-ischemic insult using a combination of a common carotid artery ligation (Levine model) and hypoxic exposure for 60 min. One hour before the insult the rats received either NaCl or 5 µg LPS into the cisterna magna. LPS significantly increased the resulting neuronal cell damage in the cerebral cortex (Coumans et al. 2002).

2.9 APOPTOSIS AND POSTISCHEMIC GENOME EXPRESSION

It is still unclear whether secondary cell death after ischemia is necrotic or apoptotic. The latter condition is characterised by a shrinking of the cell, blebbing of the cell membrane, condensation of chromatin and DNA fragmentation induced by a calcium-dependent endonuclease (Fig. 6) (Busciglio et al. 1995). In DNA electrophoresis this fragmentation can be recognised by a typical DNA ladder (McManus et al. 1993).

In neuronal cell cultures, apoptosis can be prevented by postischemic inhibition of protein synthesis using cycloheximide, or inhibition of RNA synthesis with actinomycin or through inhibition of endonuclease with aurin tricarboxylic acid. In addition, the amount of apoptotic cell death was reduced by inhibition of caspases in neonatal rats after a hypoxic-ischemic insult (Cheng et al. 1998). These findings all point towards the existence of a built-in cellular suicide programme (Ratan et al. 1994; Rosenbaum et al. 1994). It is also possible that the form of secondary cell death following ischemia is determined by the severity of the primary insult. Thus Dragunow and co-worker were able to demonstrate that delayed cell death in immature rat brains subjected to a 15-min period of hypoxic-ischemia was of an apoptotic nature, while after a 60-min insult the neuronal damage was predominantly necrotic (Dragunow et al. 1994). Other investigators have also reported correlations between the severity of the insult and the extent of apoptotic cell death (Li et al. 1995; Mehmet et al. 1994).

As has been shown in numerous studies, including some on immature animals, cerebral

ischemia can induce the expression of a whole series of proto-oncogenes (Blumenfeld et al. 1992; Ferriero et al. 1990; Munell et al. 1994). Proto-oncogenes themselves code for proteins that act as transscription factors and regulate the expression of genes modulating cell growth and differentiation. They are also termed 'immediate early genes' since they are expressed within a few minutes of an insult. These include c-fos, c-jun, jun-B, jun-D. The transscriptional activity of proteins of the fos-family is caused by a heterodimer formation with proteins of the jun-family (Longo et al. 1997). Fos- and jun-proteins can also form dimers with proteins of the ATF- and CREB families and thereby increase their promotor affinity (Hai and Curran 1991).

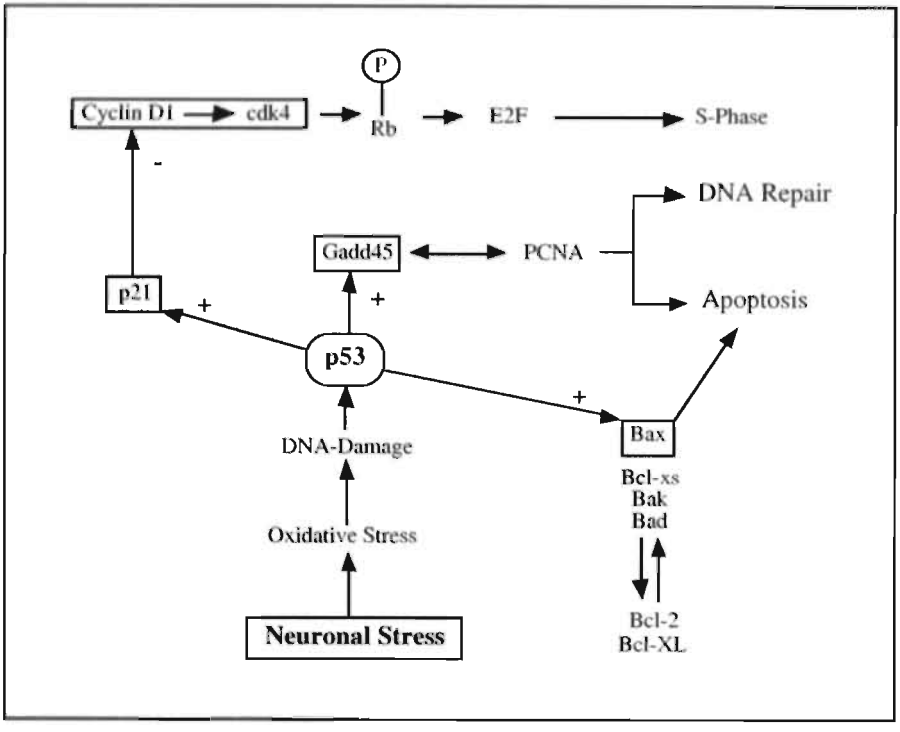

Figure 6. Diagramm illustrating the central role of p53 in mediating DNA repair or apoptosis in neurons during and after cerebral ischemia (modified after Khrestchatisky et al. 1996). Gadd45, growth-arrest-and-DNA-damage-inducible; cdk4, cyclin D-dependent kinase; for further abbreviations see text.

As already mentioned, transscription factors control the expression of genes participating

in cell growth and differentiation. Depending on the severity of the insult, these factors are therefore capable of initiating processes leading to apoptotic cell death or triggering a recovery programme. Recent research findings have indicated that the proto-oncogenes and cell cycle-dependent proteins such as cyclin D1 (Timsit et al. 1996; Wiessner et al. 1996), and tumor suppressor genes such as p53 are critically involved in this control function.

Depending upon the developmental stage of the injured brain and the extent of cell damage on the one hand, and upon damage-induced p53 expression on the other, neurons may attempt cell cycle entry, a process that will involve a certain amount of DNA repair, or may only attempt transscription-coupled DNA repair. The cell death decision may result from the impossibility to proceed with both processes. Indeed, it has recently been shown *in vitro* that the p53 transscription factor, besides its role in halting replication while favoring repair, attenuates Bcl-2 expression, and is a direct transscriptional activator of the Bax gene, whose product is shown to induce apoptosis (Miyashita et al. 1994; for review: Reed et al. 1994).

2.10 PERINATAL NEUROPROTECTION

Despite the critical clinical and socio-economic consequences of perinatal brain damage, no effective therapeutic strategies have yet been developed. However, as already mentioned, some promising possibilities have been revealed through animal experiments that could be developed and tested in clinical studies. As discussed in the sections above, the cascade of subsequent events to neuronal death involves accumulation of cytosolic calcium and activation of a variety of calcium-mediated deleterious events, including generation of free radicals. It is important to note that this cascade of events leading to neuronal and oligodendroglial death occur over hours after termination of the hypxic-ischemic insult. The exciting implication concerning therapeutic management is that interruption of this deleterious cascade even after termination of the insult could prevent or ameliorate the brain injury in hypoxic-ischemic disease (for review: Berger and Garnier 1999; Vanucci and Perlman 1997; Volpe 2001). In the following sections, current therapeutic concepts will be described by which neuroprotection has been achieved in animal models.

2.10.1 PHARMACOLOGICAL INTERVENTION

Now that the pathophysiological mechanisms underlying neuronal cell damage are better understood, diverse possibilities present themselves for pharmacological intervention. Interest is currently focused on the administration of oxygen radical scavengers, NO inhibitors, glutamate antagonists, calcium antagonists, growth factors and anticytokines. Table 2 presents potential neuroprotective drugs currently under investigation (modified according to Tuor et al. 1996).

Flunarizine

In experimental animal models of focal and global cerebral ischemia, flunarizine, a class IV calcium channel blocker, has shown to be neuroprotective when given before, but not after, the insult (Van Reempts et al. 1983; Silverstein et al. 1986; Gunn et al. 1989; Chumas et al. 1993; Gunn et al. 1994). In some of these studies flunarizine was applied at relatively high doses (i.e., 30 mg/kg body weight) before hypoxia. Ipsilateral infarction of the brain after ligation of one carotid artery could be almost totally prevented in animals receiving such treatment (Van Reempts et al. 1983; Silverstein et al. 1986; Gunn et al. 1989; Chumas et al. 1993). Similar results have been described by Gunn and co-workers (1994) in a fetal sheep model. However, the high dose regimens used by these authors had severe cardiovascular side-effects. In a low dose treatment protocol (flunarizine, 0.5 mg/kg fetal body weight), de Haan and co-workers (1993) found no significant changes in regional cerebral blood flow in severely asphyxiated fetal sheep.

Lubeluzole

Lubeluzole, the S-isomer of a novel 3,4-difluoro benzothiazole, has been shown to reduce ischemic neuronal cell damage in a variety of *in vitro* as well as *in vivo* studies. The neuroprotective property of lubeluzole may result from various effects on neuronal tissue. First, lubeluzole has been shown to block ischemia-induced increases in extracellular levels of glutamate and may therefore reduce excitotoxic cell injury (Scheller et al. 1997). Second, lubeluzole inhibits glutamate-stimulated nitric oxide production (Lesage et al. 1996). Nitric oxide combines with superoxide anions to synthesize peroxynitrite, a compound that spontaneously decomposes to form hydroxyl radicals, nitrogen dioxide and NO^{2+}. All these radicals are able to destroy cell membranes and various intracellular structures. Third, lubeluzole has been shown to activate voltage-sensitive Ca^{2+} channels in isolated rat dorsal root ganglion cells thus possibly reducing the tremendous intracellular

Table 2: Pharmacological Intervention on Hypoxic-Ischemic Brain Damage in Various Models of Hypoxia-Ischemia (modified after Tuor et al. 1996)

Treatment class	Treatment details	Age/species	Hypoxic/ischemic insult	Time of treatment with respect to insult	Neuro-protection/ pathology	Reference
VSCC´s anatgonists	Flunarizine (30mg/kg)	7 days / rat	UCO + 2 h 8% O_2	pre	partial	Silverstein et al. 1986
	Flunarizine (30mg/kg)	7 days / rat	UCO + 3 h 8% O_2	pre	partial	Chumas et al. 1993
	Flunarizine (30mg/kg)	21 days / rat	UCO + 2 h 8% O_2	pre	partial	Gunn et al. 1989
	Flunarizine (9mg/kg)	fetal sheep	30 min BCO (+VOAO)	pre	partial	Gunn et al. 1994
	Flunarizine (1mg/kg)	fetal sheep	30 Min. BCO (+VOAO)	pre	partial	Berger et al. 1998
	Nimodipine (70µg/kg or 0,5mg/kg)	7 days / rat	UCO + 3 h 8% O_2	pre	no effect	Chumas et al. 1993
	Nimodipine (0,5mg/kg)	0-3 days / pig	30 min BCO + hypotonia & 15 min 6% O_2	post	no effect	Le Blanc et al. 1991
NMDA anatgonist	MK-801 (10mg/kg)	7 days / rat	BCO + 1 h 8% O_2	pre	total	Hattori et al. 1989
	MK-801 (10mg/kg)	7 days / rat	BCO + 1 h 8% O_2	post	partial	Hattori et al. 1989
	MK-801 (1mg/kg)	7 days / rat	UCO + 3 h 8% O_2	pre, intra	partial	McDonald et al. 1987
	MK-801 (10mg/kg)	7 days / rat	UCO + 2 h 8% O_2	pre, intra	partial	Ford et al. 1989
	MK-801 (0,3 bzw. 0,5mg/kg)	7 days / rat	UCO + 1,5 h 7,6% O_2	post (0 h)	partial	Hagberg et al. 1994
	MK-801 (0,75mg/kg)	7 days / rat	UCO + 1,5 h 7,6% O_2	post (0 h)	no effect	Hagberg et al. 1994
	MK-801 (3mg/kg)	0-3 days / pig	30 min BCO + hypotonia & 15 min 6% O_2	post (0 h)	no effect	Le Blanc et al. 1991

Table 2: continued...

NMDA anatgonist (continued)	MK-801 (0,3 mg/kg + Felbamate (300mg/kg)	Schaffet 7 days / rat	30 min global ischemia BCO + 1 h 6,5% O_2	post (6-36 h) post	partial partial	Tan et al. 1992 Wasterlain et al. 1993
AMPA antagonist	NBQX (20+20 mg/kg)	7 days / rat	UCO + 1,5 h 7,6% O_2	post (0 +1 h)	partial	Hagberg et al. 1994
Glutamate release inhibitor	BW1003C87 (10mg/kg)	7 days / rat	UCO + 1,5 h 7,7% O_2	pre	partial	Gilland et al. 1994
Nonspecific glutamate antagonist	Kynurenic acid (300mg/kg)	7 days / rat	UCO + 2 h 7,7% O_2	post	partial	Altman et al. 1984
	Kynurenic acid (200-300mg/kg)	7 days / rat	UCo + 1,5 h 8% O_2	pre (1 h)	partial	Nozaki et al. 1992
Antioxidant enzymes	PEG-SOD + PEG-Catalase (10.000 U/kg)	0-3 days / pig	30 min BCO + hypotonia u. 15 Min. 6% O_2	post	no effect	Le Blanc et al. 1993
Iron chelator	Deferoxamine (100mg/kg)	7 days / rat	UCO + 2,25 h 8% O_2	post (5 min)	partial	Palmer et al. 1994
Free radical scavengers	Allopurinol (135mg/kg)	7 days / rat	UCO + 3 h 8% O_2	pre or post (15 min)	partial	Palmer et al. 1990,1993
	U74006F (7,5mg/kg)	7 days / rat	UCO + 2 h 7,7% O_2	post or pre & post	partial	Bagenholm et al. 1995
	U74689F (10mg/kg)	7 days / rat	UCO + 3 h 8% O_2	pre & post pre & post	no effect	Chumas et al. 1993
NO synthase inhibitors	Nitro-L-Arginine (2mg/kg)	7 days / rat	UCO + 2,5 h 8% O_2	pre & post	partial / no effect	Hamada et al. 1994
	Nitro-L-Arginine (50-100mg/kg)	7 days / rat	UCO + 8% O_2	pre	partial	Trifiletti et al. 1992
Glucocorticoids	Dexamethasone (0,01-0,5mg/kg/Tag)	7 days / rat	UCO + 3 h 8% O_2	pre	total	Barks et al. 1991
	Dexamethasone (0,1mg/kg)	7 days / rat	UCO + 3 h 8% O_2	pre	total / no effect partial	Barks et al. 1991 Chumas et al 1993

Table 2: continued...

Glucocorticoids (cont.)	Methylprednisolone (0,7mg/kg)	7 days / rat	UCO + 3 h 8% O_2	pre (24 h)	partial	Tuor et al. 1995
	Corticosterone (40mg/kg)	7 days / rat	UCO + 2 h 8% O_2	pre (24 + 5 h)	partial	Tuor & del Bigio 1995
	Dexamethasone (0,1mg/kg)	14 days / rat	UCO + 1 h 8% O_2	pre (24 + 5 h)	partial	Tuor et al. 1995
	Dexamethasone (0,1mg/kg)	1 month / rat	UCO + 30 min 8% O_2	pre (24 + 5 h)	no effect	Tuor et al. 1995
Antiinflammatory	Interleukin-1-receptor-antagonist (100mg/kg)	7 days / rat	UCO + 2 h 7,5% O_2	pre & post	partial	Martin et al. 1994
	Osteogenic protein-1 (50µg)	12 days / rat	BCO + 20 min 8% O_2	pre	partial	Perides et al. 1995
	Antineutrophil serum	7 days / rat	UCO + 2,25 h 8% O_2	pre	partial	Palmer et al. 1995
Growth factor	bFGF (100µg/kg)	7 days / rat	UCO + 1,5 h 8% O_2	pre (30 min)	partial	Nozaki et al. 1993
Gangliosides	GM1 (50mg/kg/Tag)	7 days / rat	2 h 7% O_2	pre & post	partial	Hadjiconstantinou et al. 1990
	GM1 (30mg/kg/Tag)	fetal sheep	30 min ischemia	pre	partial	Tan et al. 1993
Anticonvulsants	Zonisamide (75mg/kg)	7 days / rat	UCO + 2,5 h 8% O_2	pre	partial	Hajakawa et al. 1994
	Phenytoin (50mg/kg)	7 days / rat	UCO + 2,5 h 8% O_2	pre	partial	Hajakawa et al. 1994
Inhibition of caspases	boc-aspartyl-fluoromethyl-ketone	7 days / rat	UCO + 2 h 7,5% O_2	post	partial	Cheng et al. 1998

UCO: unilateral occlusion of carotid arteries, BCO: bilateral occlusion of carotid arteries, VOAO: occlusion of the vertebro-occipital anastomoses, BP: arterial blood pressure, bFGF: basic fibroblast growth factor

influx of this ion during an ischemic insult (Marannes and De Prins 2000). This so-called calcium overload leads to cell damage by activating proteases, lipases and endonucleases (Siesjö and Bengtsson 1989). Fourth, lubeluzole decreased DNA fragmentation and annexin-V binding in primary hippocampal neurons (Maiese and Vincent 2000a,b). Since these two phenomena are specific markers of apoptosis, lubeluzole might protect neurons from ischemic injury through its inhibitory effects on pathophysiological pathways that trigger the cellular suicide programme. However, these last three lubeluzole-mediated effects have so far only been observed in neuronal tissue *in vitro*. Their *in vivo* significance has still to be confirmed.

Magnesium

In the clinical setting, $MgSO_4$ has been used widely in obstetric practice for over 60 years. A retrospective epidemiologic study by Nelson and Grether (1995) suggested that premature fetuses whose mothers received $MgSO_4$ for the treatment of preeclampsia or as a tocolytic agent are less likely to develop cerebral palsy compared to gestational age–matched group of fetuses not exposed to the drug. Almost identical results were recently obtained in a retrospective study carried out by Schendel and co-workers (Schendel et al. 1996).

Magnesium is a naturally occurring NMDA receptor antagonist that blocks the neuronal influx of Ca^{2+} within the ion channel (McDonald et al. 1990). Experimental studies in newborn animals have shown a neuroprotective effect of magnesium sulfate ($MgSO_4$). $MgSO_4$ reduced brain injury in 7-day old rats when administered 15 minutes after cerebral injection of NMDA (McDonald et al. 1990). However, experimental studies on application of magnesium in various perinatal models of cerebral hypoxia/ischemia have as yet been inconclusive. While a variety of investigations have shown clear neuroprotective effects of magnesium (Altura and Altura 1981; Choi 1990; Hallak et al. 2000; Hoffman et al. 1994; Maulik et al. 1999; Mishra and Delivoria-Papadopoulos 1992; Nowak 1984; Sameshima et al. 1999; Thordstein et al. 1993), this could not be confirmed by others (de Haan et al. 1997; Penrice et al. 1997). Postischemic treatment in particular was seen to be ineffective or even to widen the extent of the resulting damage in the neonatal rat model (Levine-model) (Sameshima et al. 1999). As already discussed by Sameshima and co-workers this exacerbation of ischemic damage may arise from magnesium-induced hypotension (Sameshima et al. 1999). Using the same animal model Vanucci and Ringel reported almost complete ischemia in the ipsilateral brain hemisphere during 2 h of hypoxia (Ringel et al. 1991; Vanucci et al. 1998). Although they did not measure regional blood flow after hypoxia-ischemia, reperfusion to the brain hemisphere could possibly occur during an

early stage of recovery. Since magnesium can alter vascular tone by blocking neuromuscular junctions, treatment with magnesium after ischemia may quantitatively change blood flow to brain regions during the reperfusion period and result in more severe brain damage.

2.10.2 PHYSIOLOGICAL INTERVENTION

Hypothermia

The induction of mild hypothermia has raised interesting possibilities for neuroprotection from cerebral ischemia (for review: Maher and Hachinski 1993). Various publications dating back to the 1950s, have described the therapeutic benefits of hypothermia in brains subjected to a wide variety of insults including brain trauma (Parkhouse 1957; Sedzimir 1959), cerebral haemorrhage (Howell et al. 1956), cardiac arrest (Benson et al. 1959), carbon monoxide poisoning (Craig et al. 1959), neonatal asphyxia (Westin et al. 1959) and seizures (Brown and McGarry 1961). Based on these findings, routine induction of hypothermia was introduced early on in heart and brain surgery to protect the brain in the event of iatrogenic intraoperative cardiac arrest (Boterell et al. 1956; Drake et al. 1964; Lewis and Taufic 1953; Lougheed et al. 1955; Negrin 1961). Over the last few years, induction of mild hypothermia has been examined once again as a means of protecting the brain from ischemically induced damage. Experimental studies on adult animals have shown that lowering of the brain temperature by 3-4°C during global cerebral ischemia reduces neuronal cell damage dramatically (Busto et al. 1987; Coimbra et al. 1994; Green et al. 1992; Welsh et al. 1990; Widmann et al. 1993). Furthermore, the treated animals were found to perform better than controls in subsequent learning and behavioural tests (Green et al. 1992).

In hippocampal slices from mature guinea pig fetuses induction of mild hypothermia considerably improved the postischemic recovery of protein synthesis and energy metabolism (Berger et al. 1996b; 1998a). In a recently published study, Gunn and co-workers described the effects of moderate hypothermia in sheep fetuses subjected to severe global cerebral ischemia in utero (Gunn et al. 1997). Hypothermia was initiated during the reperfusion phase, 90 min after induction of 30 mins of ischemia, in a 4-vessel occulusion model, and maintained for 72 hours. By this method it was possible to reduce the extent of neuronal cell damage in areas of the cortex cerebri by up to 60% (Gunn et al. 1997). Even if hypothermia was started not before several hours after ischemia, neuroprotection could be observed in various animal model (Gunn et al. 1998). Based on these results, many

authors now consider the induction of hypothermia during and particularly after a hpoxic-ischemic insult to be an effective therapeutic strategy (Busto et al. 1989; Gunn et al1997). In fact, recent clinical safety studies have demonstrated that induction of mild hypoiermia in newborn infants after perinatal asphyxia has no harmful side-effects (Gunn et a 1998; Azzopardi et al. 2000).

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Chapter 3

MATERIAL AND METHODS

3.1 SHEEP EXPERIMENTS (Chapters 4 to 7)

3.1.1 Animal preparation

Surgery was performed in 46 sheep of known mating dates between 121 and 134 days of gestation ('asphyxia-model' 132 ± 1 days; 'ischemia-model' 125 ± 1 days; term is at 147 days). All ewes were anesthetized by subarachnoid injection of 8 mL of 0.75% (w/v) bupivacaine at the lower spine, and were operated under sterile conditions. Polyvinyl catheters were placed into a maternal iliac artery and vein through tibial vessels. The ewe's abdominal wall was opened in the midline and a snare was placed around the descending aorta below the renal artery, which was then used to arrest uterine and ovarian blood flow during the experiment ('asphyxia-model'; *Chapters 4 and 6*). Care was taken not to include any nerves. The uterus was incised in an area free of cotyledons. Fetal catheters were inserted after local anesthesia (prilocaine HCL 1.0%) via the pedal vein of each hindlimb into the inferior vena cava and into the fetal ascending aorta via both brachial arteries. Polyvinyl catheters used had an outer diameter of 1.5 mm and an inner diameter of 0.75 mm.

In the 'ischemia-model'(*Chapters 5 and 7*) the second uterine incision was performed over the fetal snout. Head and neck of the fetus were exteriorized. To prevent the fetus from breathing, its head was covered by a water-filled (39°C) rubber glove. Furthermore, both fetal common carotid arteries were prepared under local anaesthesia. Cerebral ischemia was induced by occluding the carotid arteries bilaterally below the thyroid and above the lingual artery for 30 min. Thus, blood flow to the cerebrum via anastomoses between the carotid and vertebral arteries was arrested. After global cerebral ischemia, a catheter was inserted into the amniotic cavity, and the second intrauterine incision was closed in two layers. Lost amniotic fluid was replaced by warm (39°C) saline. All catheters were filled with heparin (1.000 IU/mL), plugged, and passed s.c. to the ewe's flank, where they were exteriorized and protected by a pouch sewn to the skin. On the day of surgery and each day thereafter, the ewe received 2 million units of penicillin G (Grünenthal, Germany) and 80 mg of gentamycin sulfate (Merck, Germany), half i.v. and half into the amniotic cavity. The fetuses were allowed to recover for 2 days.

3.1.2 Experimental protocols (Chapters 4 and 6)

After control measurements of fetal blood gases and acid-base balance as well as physiological variables fetuses were treated either with flunarizine (*Chapter 4*) or lubeluzole (*Chapter 6*) intravenously, while fetuses of the control group received solvent. The drugs were donated by Janssen Pharmaceutica, Beerse, Belgium. Detailed information

on administration, pharmacology, and effects of these drugs are described in the chapters mentioned above.

Each experiment started with a baseline period of at least 1 hour. Sixty minutes after drug administration (at 0 min) uterine blood flow was arrested by a single occlusion of the descending aorta for 2 min to induce acute fetal asphyxia. To determine drug related effects of flunarizine or lubeluzole on the time course of circulatory centralization before, during and after acute asphyxia, blood flow to fetal organs and the distribution of combined ventricular output were measured by injecting six batches of differently labeled isotope microspheres (^{141}Ce, ^{114}In, ^{113}Sn, ^{103}Ru, ^{95}Nb and ^{46}Sc, 16 µm diam., New England Nuclear) into the inferior vena cava (Heymann et al. 1977; Rudolph and Heymann 1967). Depending on the activity, 0.7 to 2.8 million microspheres per batch were applied. Microspheres were injected at 75 s before asphyxia (control measurement), at 1 (+1´) and 2 (+2´) min during arrest of uterine blood flow, and at 1 (+3´), 2 (+4´), and 28 (+30´) min after release of the snare (recovery period). Reference blood samples were withdrawn from both a carotid and a femoral artery at a rate of 2.5 mL/min. Sampling was continuous for 270 s during control, occlusion and the immediate recovery period (-75´´, +1´, +2´, +3´, +4´). Separate samples were taken for 75 s during the 28-min recovery measurement. The volume of blood withdrawn was about 25 mL and was simultaneously replaced by maternal blood maintained at 39°C in a waterbath.

When using the microspheres method to measure blood flow at 1-2 min intervals the sampling time for reference blood must be adequate and the number of microspheres still circulating 30 to 40 s after each injection must be negligible. As shown previously these conditions are fulfilled in the present experimental model (Jensen et al. 1987).

3.1.3 Experimental Protocols (Chapters 5 and 7)

About 30 min before preparation of the carotid arteries fetuses received either flunarizine or lubeluzole intravenously, while an equal volume of the solvent was administered to the remaining fetuses. The drugs were donated by Janssen Pharmaceutica, Beerse, Belgium. The dosage applied was within the therapeutic range (Todd and Benfield 1989). The experiments were done in a randomized fashion. The operators were not blind to the nature of injection. Sixty min after drug administration global cerebral ischemia was started. To determine the time course of changes in fetal cerebral blood flow before (-15 min), during (+3 min and +27 min), and after (+10 min, +3 h, +72 h) 30 min of global cerebral ischemia, six batches of microspheres labelled with different isotopes (^{141}Ce, ^{114}In, ^{113}Sn,

^{103}Ru, ^{95}Nb and ^{46}Sc, 16 μm diam., New England Nuclear) were injected into the inferior vena cava, while reference samples from the brachial artery were withdrawn at a rate of 2.5 mL/min for 90 s (Rudolph and Heymann 1967; Jensen et al. 1987; Berger et al. 1996, 1998). The microspheres, suspended in 10% dextran containing 0.01% Tween 80, were sonicated and checked for size, shape and aggregation. Depending on the specific activity, 1.2-1.8 million microspheres per batch were injected. The number of injected microspheres was large enough to ensure both an adequate number of microspheres per sample and valid blood flow measurements during cerebral ischemia (Buckberg et al. 1971; Jensen et al. 1987). Specific calculations revealed that about 400 microspheres were trapped in low flow cerebral areas during ischemia. Thus, for theoretical considerations the blood flow estimates in these areas are within 5 % of the true values (Buckberg et al. 1971). During and shortly after injection of the microspheres no significant changes in fetal heart rate or arterial blood pressure were found. The volume of blood withdrawn was about 22.5 mL and was simultaneously replaced by maternal blood maintained at 39°C in a water bath.

During and shortly after global cerebral ischemia fetal heart rate, ascending aortic and intrauterine pressure were contionously recorded. After having injected the fourth batch of microspheres (at +40 min) all catheters were closed and secured as described above, the abdominal wall was closed and the ewe was brought back to the metabolic cage, where the fifth injection (at +3 h) was performed. For technical reasons there were no pressure measurements at this point in time. Before each injection, blood samples were obtained from the brachial artery to measure blood gases, oxygen saturation of hemoglobin, and acid-base balance. At the end of the experiment (at +72 h) the ewe was given a lethal dose of sodium pentobarbitone and saturated potassium chloride intravenously, while the fetus was perfused with 300 mL of formalin (15 %, wt/vol, saline).

3.1.4 Measurements

Fetal heart rate and arterial blood pressure, amniotic fluid pressure, and maternal arterial blood pressure were recorded on a polygraph (Hellige, Germany) during the experiment. Complete occlusion of the ewe's descending aorta by the snare throughout the 2-min study period was confirmed by the fall in pressure distal to the obstruction (*Chapters 4 and 6*). Before blood flow was measured a blood sample was taken from the descending aorta and analysed for blood gases, acid-base balance (278 Blood Gas System, Ciba Corning, Frankfurt, Germany), hemoglobin concentration, oxygen saturation (OSM 2 Hemoximeter, Radiometer, Copenhagen, Denmark), glucose, lactate, catecholamine and flunarizine concentrations. Plasma concentrations of both catecholamines and flunarizine and brain

tissue concentrations of flunarizine were determined by reversed phase ion-pair High Performance Liquid Chromatography with electrochemical detection (HPLC-ECD), with a detection limit of 5 pg, an intra-assay variance below 5%, and an inter-assay variance below 10%. A detailed description of the HPLC-ECD catecholamine assay is given elsewhere (Jelinek and Jensen 1991; Tegtmeier et al. 1987).

At the end of the experiment a lethal dose of sodium pentobarbitone was given to the ewe and the fetuses were perfused with 300 mL of formalin (15%, w/v, saline). Fetal organs and cotyledons were weighed and placed in vials, which were filled to the same height to reduce variations in geometry. The intestines were separated from the mesentery, opened, and cleared of contents. Paired organs (lungs, kidneys, and adrenals) were counted separately, as were the right and left sides of the cerebrum and of the brainstem regions. No significant preferential streaming of microspheres was found. Specimens of skin and muscle were taken from the hips and shoulders of each side. There were no differences in blood flow between the intact side and the side on which the femoral or brachial arteries were catheterized. Upper and lower carcass were carbonized and the aliquots filled into vials.

The applied solid-state semi-conductor germanium (Ge) gamma counter had a high energy resolution of about 3 keV and was connected to a multichannel (2048) pulse height analyzer (ND 62, Nuclear Data Inc., Illinois, USA). The results were normalized with respect to time and sample weight.

3.1.5 Calculations

'Asphyxia-model'

Fetal combined ventricular output and blood flow to the various organs were calculated from counts of the injected nuclide recovered in fetal organs or placenta, from counts in the appropriate reference samples, and from the withdrawal rate of the reference sample (Heymann et al. 1977; Rudolph and Heymann 1967). Portal venous blood flow was calculated by summing the actual blood flow to all gastro-intestinal organs, including stomach, intestines, mesentery, pancreas, and spleen. The percentage of combined ventricular output distributed to a given organ was calculated from the absolute blood flow to that organ and the combined ventricular output. The vascular resistance was calculated by dividing arterial blood pressure (corrected for amniotic fluid pressure) by blood flow and was expressed in mmHg/mL/min/100g of tissue. Blood flow inferior to 1 mL/min/100g was treated as 1. Umbilical vascular resistance (mmHg/mL/min/kg fetal

weight) was calculated by dividing the perfusion pressure of the umbilical circulation (arterial blood pressure minus the estimated umbilical venous blood pressure before (11 mmHg) and during (17 mmHg) reduction in uterine blood flow (Jensen et al. 1991)) by umbilical blood flow.

'Ischemia-model'

Fetal cerebral blood flow and the extent of neuronal cell damage were assessed in identical brain specimens. To determine fetal cerebral blood flow using the microsphere method, the fetal brain was removed and fixed in formalin for at least seven days. Afterwards the cerebrum was separated from the basal ganglia and divided in four frontal sections (rostral, pericentral, postcentral, occipital) with a thickness of about 1.5 cm (Fig. 7). The right and left parts of these four sections were further subdivided into four equally sized segments (sagittal 1 and 2, lateral 1 and 2) each weighing 1-2 g. In addition to these 32 cerebral specimens, the caudate nucleus, thalamus, hippocampus, tegmentum-colliculi-pons, cerebellum, and medulla oblongata were separated. These brain structures were placed into vials, which were filled to the same height to reduce variations in geometry during gamma counting. The results were normalized with respect to time and sample weight.

After cerebral blood flow analysis the specimens of the fetal brain were removed from the counting vials and embedded in paraffin. Coronal subserial sections of 10 µm were obtained and then stained with cresyl violet/fuchsin. Every 40th section was mounted to evaluate the extent of neuronal cell damage. Neuronal cell damage was assessed at a magnification of 250x. The histological score of each cerebral specimen was calculated by averaging the scores of all visual fields analysed from 3 sections of that specimen. The number of scored visual fields per specimen ranged between 400 and 500 each. The scores from corresponding specimens from the right and left hemisphere were averaged. Neurons with ischemic cell damage were identified according to the criteria of Brown and Brierley (1971). Neuronal cell damage in each microscopical visual field was quantified as follows: 0-5 % damage (score 1), 5-50 % damage (score 2), 50-95 % damage (score 3), 95-99 % damage (score 4), 100 % damage (score 5).

3.1.6 Statistics

Values are given as means ± standard deviation. The data were analyzed for intragroup and intergrouo differences by two-way multivariate analysis of variance (MANOVA) fo repeated measures. The Games-Howell was used as a *post hoc* testing procedure.

Statistical analysis was performed by the Super Anova Statistical Package (Abacus Inc., Ca., USA).

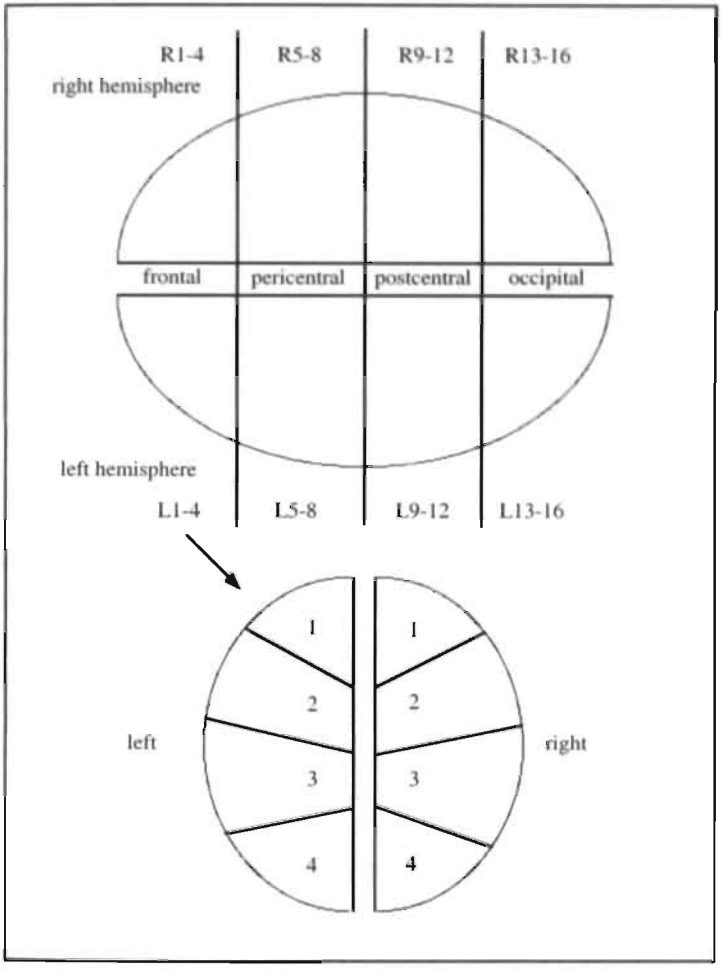

Fig. 7. Schematic illustration of the subdivided cerebrum.

3.2 HIPPOCAMPAL TISSUE SLICE MODEL (Chapters 8 and 9)

3.2.1 Animal preparation

Experiments were performed on mature guinea pig fetuses (E 60). In this species term is at 68 days. For the present study we preferred tissue slices from the hippocampal area, since this region is known to be very sensitive to hypoxic-ischemic insults in comparison vith other parts of the brain (Hossmann et al. 1992; Penny et al. 1974).

Guinea pig dams were anesthetized with halothane and 64 fetuses were delivered by cesarean section. Until further preparation the fetal heads were kept in ice-water. The skull was then cut along the midline with scissors and removed with forceps. After removal of the skull the brainstem was divided below the cerebellum. The hemispheres were lifted with a spatula and the cranial nerves and the blood vessels were cut to allow the brain to fall gently into a beaker containing ice-cold artificial cerebrospinal fluid (aCSF) (0°C). This temperature was strictly maintained throughout the slice preparation period, since preliminary experiments have shown that preservation of cellular metabolism is improved under these conditions. Standard aCSF (Djuricic et al. 1994) contained (in mM): NaCl, 111; $NaHCO_3$, 26; KCl, 3; KH_2PO_4, 1.4; $CaCl_2$, 1.2; $MgSO_4$, 1.3; glucose, 10 (standard) or 2 (low glucose; in low glucose aCSF NaCl was adjusted to 115 mM); and N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 5. The aCSF was supplemented with amino acids (µM) (Djuricic et al. 1994): alanine, 34; arginine, 22; aspartate, 0.13; citrulline, 5.7; cystine, 0.12; histidine, 13; lysine, 27.7; methionine, 3.4; ornithine, 3.8; phenylalanine, 9.1; serine, 28.5; taurine, 8; threonine, 32; tryptophan, 1.5; tyrosine, 8.3; valine, 12.8. To prevent bacterial contamination 10 mg / L each of streptomycin and erythromycin were added to the aCSF. The aCSF was equilibrated with a gas mixture of O_2 / CO_2 (95% / 5%) for at least 60 min prior to the experiments. The pH of the aCSF was adjusted to 7.4 with 1 M NaOH or 1 M HCl. For further preparation the fetal brain was transferred from the beaker onto a cooling plate (0°C) covered with filter paper. The filter paper was moistened with aCSF. The brain was divided into its two halves and the hemispheres were placed with the lateral side down. The brain stem was lifted with a spatula, while the occipital cortex was gently pushed downwards with another spatula until the whole medial and inferior side of the hippocampal formation could be seen. Both ends of the structure were then freed and the hippocampus was rolled upwards and backwards towards the occipital pole by inserting spatulas on both sides under its superior surface. The hippocampal formation now lay with the dentate area down and the alveus up. The hippocampi were then transferred again to chilled standard aCSF until they were cut into slices of 500 (µm thickness on a tissue slicer (Rademacher, Cologne, Germany). Before use the tissue slicer had been pre-cooled in a refrigerator. The slices were transferred from

the chopper onto a nylon mesh with a painting brush. The nylon mesh was stretched across a plastic ring and immersed in standard aCSF. The incubation took place in a temperature controlled (37°C) flow-through chamber, gassed with O_2/ CO_2 (95% / 5%) for 90 min. The flow rate of the aCSF was set to 1 mL/min. Care was taken to ensure that the upper side of the slices was fully exposed to the warm humidified gas mixture, while the underside was lying in aCSF. After the slices had been allowed to recover from the initial preparation stress for 90 min, the flow rate through the chamber was increased to 3 mL/min and standard aCSF with low glucose (2 mM) was pumped through for 30 min. This incubation of slices in low glucose aCSF was introduced to produce a prompt depletion of high energy phosphates during Oxygen/glucose deprivation (OGD) (Djuricic et al. 1994).

Oxygen/glucose-deprived aCSF was prepared in the same manner as standard aCSF except for the addition of HEPES and glucose. HEPES was removed from oxygen/glucose-deprived aCSF, since it provides additional buffering capacity. This could influence the decrease in pH during OGD and alter the tissue response to OGD. Slight changes in osmolarity owing to the removal of glucose and HEPES were compensated by adjusting the NaCl concentration. Before transferring slices to the anoxic chamber, glucose was washed out of the tissue by immersion of the slices in oxygenated, aglycemic aCSF for 2 min. The post-OGD period was initiated by transferring slices to standard aCSF (flow rate 1 mL/min) with O_2 / CO_2 (95%/5%).

In contrast to previously described hippocampal slice models, we used an interface technique in which the upper side of the slices was fully exposed to the warm humidified gas mixture while the underside was lying in aCSF. In addition, the slices were kept in a flow-through chamber and the aCSF was supplemented with amino acids. Using this technique, energy metabolism and protein synthesis can be kept constant for up to 24 h as shown in a variety of previous studies (Berger et al. 1996; 1998; Djuricic et al. 1994). This is especially true of slices from mature fetuses, since they are much less vulnerable to the preparation procedure than those from adult animals (Berger et al. 1996; 1998). As shown in a recent study, hippocampal slices from mature fetal guinea pigs are completely depleted of ATP after 20 to 40 min of OGD. Within 24 h a partial recovery in the tissue concentration of ATP can be seen. The extent of the recovery ranges from 40 to 90 % of control levels depending on the duration of OGD (Berger et al. 1996; 1998). The histological status of the tissue had been investigated in some pilot experiments. Severe deterioration of the tissue could be excluded after an incubation period of up to 24 h.

3.2.2 Experimental protocols

The experimental protocol included a 210 min preincubation phase (180 min in 10 mM glucose and 30 min in 2 mM glucose aCSF), an ischemic phase (10-40 min) and a recovery phase (12 h starting from the end of OGD). A separate incubation chamber, equilibrated with 95% N_2/ 5% CO_2, was used for the induction of OGD. In contrast to the standard aCSF, the ischemic aCSF contained no glucose or HEPES. HEPES was omitted, because its buffering capacity may influence the fall in pH accompanying OGD. Before the tissue slices were transferred to the anoxic incubation chamber, they were washed in aglycemic aCSF in order to lower the glucose levels in the tissue still further. During OGD the tissue slices were completely submerged in the aCSF. No additional aCSF was pumped through the chamber during this period (flow rate: 0 mL/min). In the postischemic phase the tissue slices were transferred back to standard aCSF (flow rate: 1 mL/min) and equilibrated with carbogen (95% O_2/ 5% CO_2). After a recovery period of 12 h the slices were sampled to measure the tissue concentrations of adenine nucleotides and protein synthesis.

Mild hypothermia (Chapter 8) was induced immediately, 2 h or 4 h after OGD by lowering the incubation temperature to 34°C or 31°C, respectively. The hypothermic period lasted for 12 h. At the end of the experiments tissue concentrations of adenylates and protein synthesis were determined in slices of the control and intervention groups. Each experiment (normothermia vs. hypothermia) consisted of a normothermic control group and a hypothermic group without OGD. In addition, there were three normothermic and three hypothermic groups of slices that underwent OGD (20–40 min).

3.2.3 Measurements

The concentrations of adenylates in the tissue slices were measured in the supernatant of neutralized tissue homogenates by high-pressure liquid chromatography after extraction with perchloric acid (Berger et al. 1996). The adenylate energy charge (AEC), a measure of the balance between energy consumption and energy production was calculated as follows (Atkinson 1968): AEC = ([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP]). The protein content of the tissue slices was measured by the Lowry method (Lowry et al. 1951).

The rate of incorporation of ^{14}C-leucine into tissue proteins was taken as a measure of protein synthesis rate (PSR). For these readings, 30 min before the conclusion of the experiments the tissue slices were incubated in standard-aCSF supplemented with 5 mCi/mL L-(^{14}C)-leucine (Amersham Buchler, Braunschweig, Germany; specific activity:

54 mCi/mMol). The slices remained there for 30 min and were homogenized in trichloroacetic acid. The radioactivity of the precipitate was measured by liquid scintillation spectrometry after proteins had been dissolved in 1 M NaOH (Berger et al. 1996).

In a further set of experiments we investigated whether a possible neuroprotective effect of magnesium might be mediated in part through the NO-system. Here we determined the tissue concentrations of cGMP 10 min after an OGD-period of between 10 and 40 min using a RIA (NEN, Bad Homburg, Germany). For these measurements tissue slices were frozen in liquid nitrogen and extracted with perchloric acid. As in the first set of experiments, the magnesium concentration in the incubation medium of the study groups was raised from 1.3 mM to 3.9 mM either 2 h before or immediately after OGD. To confirm that elevated tissue concentrations of cGMP really reflect increased NO-production, we inhibited NO-synthase with the specific blocker N-nitro-L-arginine (L-NNA). L-NNA (100 µM) was added to the incubation medium 30 min prior to, during and 10 min after OGD. By these measures the increase of cGMP tissue concentration 10 min after OGD could be completely suppressed. This observation confirms previous studies showing the same effect (Berger et al. 1998; Paschen 1995).

3.2.4 Statistics

The values are presented as means ± standard deviation. The data for each experimental group was obtained from 4 to 5 tissue slices. We restricted statistical analysis to each single experiment, which consisted of 8 groups of slices, i.e. a normothermic control group, a hypothermic group without OGD as well as three normothermic and three hypothermic groups of slices that underwent OGD (20-40 min). There were no repeat studies in a single preparation and no data was discarded. Significant differences within and between groups were assessed using ANOVA and Scheffé's F-test.

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Chapter 4

LOW DOSE FLUNARIZINE DOES NOT AFFECT SHORT TERM FETAL CIRCULATORY RESPONSES TO ACUTE ASPHYXIA IN SHEEP NEAR TERM

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ABSTRACT

Asphyxia is one of the major causes of perinatal brain damage and neuronal loss that may result in psycho-motor deficits during later development. As shown previously the immature brain can be protected from ischemic injury by flunarizine, a class IV calcium antagonist. However, cardiovascular side-effects of flunarizine, when applied at dosages used in those studies, have been reported. Recently, we were able to demonstrate that even by injecting flunarizine at a far lower dosage (1 mg/kg estimated body weight) neuronal cell damage, caused by occlusion of both carotid arteries for 30 min, can be reduced in fetal sheep near term. The aim of the present study was, therefore, to examine whether low dose flunarizine affects fetal cardiovascular responses to acute asphyxia in sheep near term. Ten fetal sheep were chronically instrumented at a mean gestational age of 132 ± 1 days (term is at 147 days). Fetuses from the study group received a bolus injection of flunarizine (1mg/kg estimated fetal weight) 60 min before asphyxia, while the solvent was administered to the fetuses from the control group. Organ blood flows, physiological variables and plasma concentrations of catecholamines were measured before, during and after a single occlusion of uterine blood flow for 2 min, i.e., at 0, 1, 2, 3, 4, and 30 min. Before asphyxia distribution of combined ventricular output, physiological variables as well as concentrations of catecholamines in fetuses from the control group were in the normal range for chronically prepared fetal sheep near term. During acute asphyxia there was a redistribution of cardiac output towards the central organs accompanied by a pronounced bradycardia and a rapid increase in arterial blood pressure. After asphyxia circulatory centralization did not resolve quite as rapidly as it developed, but was almost completely recovered at 30 min after the insult. There were nearly no differences in the time course of physiological and cardiovascular variables measured before, during and after acute intrauterine asphyxia between control and study group. From the present study we conclude that low dose flunarizine does not affect short term fetal circulatory responses to acute asphyxia in sheep near term.

INTRODUCTION

Asphyxia is one of the major causes of perinatal brain damage that may result in psychomotor deficits during later development (Volpe, 1995). During hypoxia-ischemia a variety of pathological mechanisms are triggering intracellular accumulation of calcium that may induce neuronal cell death (Krieglstein, 1988). Pharmacological interventions that act by limiting intracellular calcium influx during hypoxia-ischemia seems to be effective in reducing neuronal degeneration (Krieglstein and Oberpichler-Schwenk, 1994). In previous studies pretreatment by flunarizine, a class IV calcium antagonist, has been shown to protect the brain of immature animals from hypoxic-ischemic injury (Chumas et al., 1993; Gunn et al., 1989; Gunn et al., 1994; Silverstein et al., 1986; Van Reempts et al., 1993). However, the actual dose of flunarizine must be considered, because dose-dependent cardiovascular side-effects have been observed. After infusion of 45 mg flunarizine Gunn and colleagues reported severe hypotension and bradycardia followed by fetal demise in 3 of 8 fetal sheep near term (Gunn et al., 1994). Flunarizine was also toxic when given to sick fetuses (Gunn et al., 1988). These pharmacological side-effects preclude the inititation of clinical studies. However, it has been shown previously that the therapeutic dosage of flunarizine is far below that used in the studies mentioned above (Todd et al., 1989). By i.v. injection of flunarizine at low dosage (1mg/kg estimated body weight) fetal sheep near term could be protected from neuronal cell damage caused by occlusion of both carotid arteries for 30 min (Berger et al., 1998). The aim of the present study was, therefore, to examine whether low dose flunarizine effects the circulatory and neurohumoral responses to acute asphyxia in fetal sheep near term. In addition, we determined the time course of flunarizine concentrations in fetal plasma and brain under these experimental conditions. A brief account of this study has been published elsewhere (Berger et al., 1998).

METHODS

Animal preparation

Ten fetal sheep were chronically prepared at a mean gestational age of 132±1 days (term is at 147 days). Mean gestational age of untreated fetuses was somewhat higher than that of treated fetuses (129±1 vs. 133±2). The surgical preparation has been described previously (Jensen et al, 1987). Briefly, all ewes were anesthetized by subarachnoid injection of 8 ml of 0.75% (w/v) bupivacaine at the lower spine, and were operated on under sterile conditions. Polyvinyl catheters were placed into a maternal iliac artery and vein through tibial vessels. The ewe's abdominal wall was opened in the midline and a snare was placed around the descending aorta below the renal artery, which was then used to arrest uterine

and ovarian blood flow during the experiment. Care was taken not to include any nerves. Fetal catheters were inserted and maintained as described previously (Jensen et al., 1987). An additional catheter was placed into the amniotic cavity. All catheters were filled with heparin (1,000 IU/ml), plugged, and passed subcutaneously to the ewe's flank, where they were exteriorized and protected by a pouch sewn to the skin. On the day of surgery and each day thereafter, the ewe received 2 million units of penicillin G (Grünenthal, Germany) and 80 mg of gentamycin sulfate (Merck, Germany), half intravenously and half into the amniotic cavity. The fetuses were allowed to recover for 2 days before being studied.

Experimental protocol

After control measurements of the physiological variables, 5 out of 10 fetuses were treated by a bolus injection (at -60 min) of flunarizine (1mg/kg estimated fetal weight), while the remaining received the solvent. The drugs were donated by Janssen Pharmaceutica, Beerse, Belgium. Sixty minutes later (at 0 min) uterine blood flow was arrested by a single occlusion of the descending aorta for 2 min to induce acute fetal asphyxia. To determine the effects of flunarizine on the time course of circulatory centralization before, during and after acute asphyxia, blood flow to fetal organs and the distribution of combined ventricular output were measured by injecting six batches of differently labeled isotope microspheres (^{141}Ce, ^{114}In, ^{113}Sn, ^{103}Ru, ^{95}Nb & ^{46}Sc, 16 µm diam., New England Nuclear) into the inferior vena cava (Heymann et al., 1977; Rudolph and Heymann, 1967). Asphyxia was caused by arrest of uterine blood flow with the snare. The first 5 microsphere injections were made at short intervals, i.e. 75 s before (control measurement, at -1 min), at 1 and 2 min during the arrest of uterine blood flow, and at 1, 2, and 28 min after the release of the snare (recovery period). The experimental protocols were approved by the appropriate institutional review committee and meet the guidelines of the responsible governmental agency.

Measurements

Fetal heart rate and arterial blood pressure, amniotic fluid pressure, and maternal arterial blood pressure were recorded on a polygraph (Hellige, Germany) during the experiment. Complete occlusion of the ewe's descending aorta by the snare throughout the 2-min study period was confirmed by the fall in pressure distal to the obstruction. Before blood flow was measured a blood sample was taken from the descending aorta and analysed for blood gases, acid-base balance (278 Blood Gas System, Ciba Corning, Frankfurt, Germany), hemoglobin concentration, oxygen saturation (OSM 2 Hemoximeter, Radiometer,

Copenhagen, Denmark), glucose, lactate, catecholamine and flunarizine concentrations. Plasma concentrations of both catecholamines and flunarizine and brain tissue concentrations of flunarizine were determined by reversed phase ion-pair High Performance Liquid Chromatography with electrochemical detection (HPLC-ECD), with a detection limit of 5 pg, an intra-assay variance below 5%, and an inter-assay variance below 10%. A detailed description of the HPLC-ECD catecholamine assay is given elsewhere (Jelinek and Jensen, 1991; Tegtmeier et al., 1987).

After the experiment a lethal dose of sodium pentobarbitone was given to the ewe and the fetuses were perfused with 300 ml of formalin (15 %, w/v, saline). Fetal organs and cotyledons were weighed and placed in vials, which were filled to the same height to reduce variations in geometry. The intestines were separated from the mesentery, opened, and cleared of contents. Paired organs (lungs, kidneys, and adrenals) were counted separately, as were the right and left sides of the cerebrum and of the brainstem regions. No significant preferential streaming of microspheres was found. Specimens of skin and muscle were taken from the hips and shoulders of each side. There were no differences in blood flow between the intact side and the side on which the femoral or brachial arteries were catheterized. Upper and lower carcass were carbonized and the aliquots filled into vials. The applied solid-state semi-conductor germanium (Ge) gamma counter had a high energy resolution of about 3 keV and was connected to a multichannel (2048) pulse height analyzer (ND 62, Nuclear Data Inc., Illinois, USA). The results were normalized with respect to time and sample weight.

Calculations

Fetal combined ventricular output and blood flow to the various organs were calculated from counts of the injected nuclide recovered in fetal organs or placenta, from counts in the appropriate reference samples, and from the withdrawal rate of the reference sample (Heymann et al., 1977; Rudolph and Heymann, 1967). Portal venous blood flow was calculated by summing the actual blood flow to all gastro-intestinal organs, including stomach, intestines, mesentery, pancreas, and spleen. The percentage of combined ventricular output distributed to a given organ was calculated from the absolute blood flow to that organ and the combined ventricular output. The vascular resistance was calculated by dividing arterial blood pressure (corrected for amniotic fluid pressure) by blood flow and was expressed in mmHg/ml/min/100g of tissue. Blood flow inferior to 1 ml/min/100g was treated as 1. Umbilical vascular resistance (mmHg/ml/min/kg fetal weight) was calculated by dividing the perfusion pressure of the umbilical circulation (arterial blood pressure minus the estimated umbilical venous blood pressure before (11 mmHg) and

during (17 mmHg) reduction in uterine blood flow (Jensen, Roman and Rudolph, 1991)) by umbilical blood flow.

Statistics

Results are given as means±SEM. The data were analysed for intra- and intergroup differences by two-way multivariate analysis of variance for repeated measures. Games-Howell-test was used as a post-hoc testing procedure. Statistical analysis was performed by Super Anova Statistical Package (Abacus, Inc. Ca., USA).

RESULTS

Control group

In the control period combined ventricular output, heart rate, arterial blood pressure, blood gases, pH, glucose, lactate and plasma concentrations of catecholamines were in the normal range for chronically prepared fetal sheep near term (Jensen and Berger, 1991). Arrest of uterine blood flow for 2 min decreased fetal heart rate, arterial O_2 saturation of hemoglobin, and pH, and increased fetal arterial blood pressure, pCO_2, lactate, and plasma concentrations of catecholamines (Tables 1 and 2).

After 1 min of asphyxia combined ventricular output fell by 48%, however, the portion distributed to placenta and heart increased by 57% and 431%, respectively, whereas that to both the adrenals and total brain did not change significantly (Tables 2 and 4). There was an uneven distribution of combined ventricular output to the various regions of the brain during asphyxia. The portion distributed to some of the lower areas of the brain increased progressively after 1 min asphyxia, whereas that to the cerebrum did not change, and that to the choroid plexus decreased (data not shown).

After 2 min of asphyxia combined ventricular output was reduced by 56% (Table 2), but its redistribution towards central organs resulted in significant increases in blood flow to the heart (78%), midbrain (60%), medulla (91%), no significant change in blood flow to the adrenals and cerebrum, and a decrease in that to the choroid plexus (Table 3).

Table 1. Acid-Base Balance, Blood Gases, and Catecholamine Concentrations in Control and Treated (Flunarizine) Fetuses Near Term.

Groups	Control	Asphyxia 1		Asphyxia 2		Recovery 3		Recovery 4		Recovery 30	
pH											
C	7,40 ± 0,01	7,38 ± 0,00		7,30 ± 0,00	d	7,28 ± 0,00	e	7,30 ± 0,00	d	7,33 ± 0,02	
F	7,39 ± 0,03	7,36 ± 0,02		7,28 ± 0,03	e	7,26 ± 0,03	e	7,28 ± 0,02	d	7,32 ± 0,04	
Oxygen Saturation (%)											
C	54,0 ± 3,6	14,2 ± 3,9	f	4,8 ± 1,8	f	25,7 ± 5,2	f	43,2 ± 4,0		48,8 ± 4,9	
F	54,5 ± 4,8	14,0 ± 2,9	f	3,4 ± 0,4	f	26,8 ± 4,2	f	38,4 ± 3,0	e	47,3 ± 4,5	
pO_2 (mmHg)											
C	21,8 ± 1,2	10,2 ± 1,2	f	5,5 ± 1,0	f	16,9 ± 2,2	d	23,6 ± 1,0		24,3 ± 1,3	
F	21,3 ± 1,8	9,5 ± 1,4	f	3,9 ± 0,3	f	15,9 ± 1,4	d	19,7 ± 1,3		21,9 ± 1,9	
pCO_2 (mmHg)											
C	47,4 ± 1,2	51,4 ± 1,3	f	62,8 ± 2,8	f	63,9 ± 4,1	f	57,9 ± 3,0		50,0 ± 1,4	
F	47,3 ± 1,1	50,4 ± 1,2	f	64,8 ± 3,1	f	62,9 ± 2,9	f	56,8 ± 2,0	e	47,7 ± 2,0	
Glucose (mg/ml)											
C	21,4 ± 2,0	21,8 ± 1,9		19,0 ± 1,0		21,0 ± 1,6		25,4 ± 2,0		28,8 ± 2,7	d
F	21,0 ± 1,3	21,8 ± 2,6		16,8 ± 2,1		18,0 ± 2,1		23,5 ± 2,5		28,4 ± 4,3	d
Lactate (mmol/l)											
C	1,64 ± 0,10	1,74 ± 0,10		2,78 ± 0,20		3,63 ± 0,30		3,69 ± 0,00		4,42 ± 0,37	d
F	2,80 ± 0,74	2,90 ± 0,74		3,95 ± 0,90		4,83 ± 0,77		4,50 ± 0,66		5,91 ± 1,49	d
Norepinephrine (pg/ml)											
C	341 ± 67	7150 ± 2452		50089 ± 12568	f	33263 ± 10507	e	12635 ± 3791		2014 ± 786	
F	815 ± 479	12510 ± 4421		73256 ± 15863	a,f	43646 ± 10064	f	26233 ± 11605	d	6425 ± 5621	
Epinephrine (pg/ml)											
C	47 ± 16	1171 ± 184		31794 ± 6207	f	14561 ± 3906	e	7549 ± 1702		903 ± 682	
F	47 ± 18	2626 ± 1141		34815 ± 6440	f	17164 ± 4420	f	10993 ± 4578	d	2838 ± 2776	
Dopamine (pg/ml)											
C	58 ± 17	247 ± 94		1402 ± 637	d	749 ± 439		254 ± 109		150 ± 74	
F	63 ± 26	825 ± 357		4085 ± 1014	c,f	1592 ± 486	e	660 ± 312		432 ± 372	

a<0.05, b<0.01, c<0.001 significant between groups; d<0.05, e<0.01, f<0.001 significant versus control within groups
C= control fetuses (n=5), F= treated (flunarizine) fetuses (n=5)

In the immediate recovery period, i.e. 1 and 2 min after arrest of uterine blood flow had been released, arterial blood pressure was still high (Table 2) and blood flow to the heart and all parts of the brain except for the choroid plexus increased (Table 3). At 30 min, i.e. after 28 min recovery, these values were not different from control.

In all peripheral organs of the upper and lower body segment, including carcass, skeletal muscle, total gastro-intestinal tract and portal vein, kidneys, spleen, scalp, and body skin, the proportion of combined ventricular output and actual blood flow decreased to very low values during asphyxia, and recovered gradually thereafter (Tables 2 and 3). During asphyxia a pronounced increase in plasma concentrations of catecholamines could be observed that returned to control at the end of the recovery period (Table 1).

Table 2. Changes of Physiologic Variables and Blood Flows in Control and Treated (Flunarizine) Fetuses Near Term.

Groups	Control	Asphyxia 1	Asphyxia 2	Recovery 3	Recovery 4	Recovery 30
Fetal Heart Rate (bpm)						
C	161,2 ± 9,9	100,4 ± 7,9 d	105,6 ± 13,7 d	152,2 ± 35,6	158,4 ± 17,0	196,5 ± 44,6
F	153,8 ± 12,7	76,4 ± 13,9 e	87,8 ± 14,8 d	150,1 ± 28,0	137,6 ± 4,9	178,8 ± 16,8
Arterial blood pressure (mmHg)						
C	47,5 ± 2,4	57,7 ± 2,8	71,7 ± 5,9 d	80,2 ± 5,6 f	73,9 ± 3,2 e	48,5 ± 2,5
F	49,6 ± 2,7	50,8 ± 4,6	69,0 ± 4,6 d	74,1 ± 11,2 d	78,1 ± 1,9 f	64,8 ± 11,9
		Blood flows (ml/min x kg fetus)				
Cardiac Output						
C	536,5 ± 23,5	278,0 ± 22,0 e	237,9 ± 23,6 e	430,8 ± 63,2	540,8 ± 26,5	621,2 ± 101,0
F	506,5 ± 57,6	296,4 ± 29,2 d	290,1 ± 49,2 d	564,7 ± 142,1	510,3 ± 89,5	580,8 ± 43,1
Placenta						
C	207,1 ± 9,8	167,1 ± 14,9	88,5 ± 30,8 e	179,2 ± 48,2	195,6 ± 31,4	187,6 ± 50,7
F	238,0 ± 30,0	196,8 ± 14,5	116,9 ± 17,1 e	168,3 ± 39,9	164,6 ± 18,5	249,1 ± 11,2
Fetal Body						
C	329,5 ± 19,8	110,9 ± 17,7 f	149,4 ± 17,0 e	251,5 ± 16,8	255,2 ± 18,7	386,7 ± 79,0
F	268,6 ± 28,4	99,7 ± 15,4 d	173,2 ± 36,0	396,5 ± 122,5	345,8 ± 82,3	331,7 ± 35,5
Lung						
C	29,7 ± 8,0	6,7 ± 2,5	24,6 ± 10,7	46,1 ± 11,6	43,6 ± 7,7	112,8 ± 58,6
F	18,8 ± 7,3	7,8 ± 4,1	60,4 ± 30,6	187,0 ± 113,6 a,d	164,7 ± 88,0 d	66,4 ± 24,4
		%Cardiac Output				
Placenta						
C	38,7 ± 1,7	60,7 ± 4,9 e	34,4 ± 9,2	38,5 ± 5,5	42,7 ± 4,8	39,2 ± 3,4
F	46,7 ± 1,2	67,1 ± 7,0 e	41,5 ± 3,7	31,2 ± 5,0 d	34,5 ± 4,2	43,5 ± 2,4
Fetal Body						
C	61,3 ± 1,7	39,3 ± 4,9 e	65,6 ± 9,2	61,5 ± 5,5	57,3 ± 4,8	60,8 ± 3,4
F	53,3 ± 1,2	32,9 ± 2,0 e	58,5 ± 3,6	68,8 ± 5,0 d	65,5 ± 4,2	56,5 ± 2,4
Lung						
C	7,5 ± 1,7	2,7 ± 1,1	13,5 ± 4,6	14,7 ± 4,0	12,8 ± 1,9	22,7 ± 7,6
F	3,6 ± 1,1	2,5 ± 1,2	17,5 ± 7,1	25,2 ± 9,1 e	25,9 ± 10,6 e	11,2 ± 4,2

a<0.05, b<0.01, c<0.001 significant between groups; d<0.05, e<0.01, f<0.001 significant versus control within groups
C= control fetuses (n=5), F= treated (flunarizine) fetuses (n=5)

Table 3. Organ Blood Flows in Control and Treated (Flunarizine) Fetuses Near Term

		Blood flow (ml/min x 100g)															
	Group	Control		Asphyxia 1'		Asphyxia 2'		Recovery 3'		Recovery 4'		Recovery 30'		P
Heart	C	216 ± 31		616 ± 131	e	385 ± 118	f	905 ± 144	f	693 ± 104	e	419 ± 103		
	F	207 ± 26		432 ± 105		749 ± 65	f	752 ± 112	f	634 ± 88	e	525 ± 97	d	
Brain	C	206 ± 12		200 ± 24		231 ± 20		362 ± 25	f	345 ± 15	e	188 ± 24		0,05
	F	128 ± 21		127 ± 22		159 ± 25		282 ± 51	f	265 ± 51	e	189 ± 43		
Cerebrum	C	187 ± 13		159 ± 18		182 ± 13		311 ± 24	f	297 ± 17	f	167 ± 21		
	F	129 ± 9	a	108 ± 7		129 ± 19		262 ± 29	f	248 ± 29	f	186 ± 23		
Cerebellum	C	237 ± 8		270 ± 30		302 ± 26		463 ± 41	f	446 ± 18	f	241 ± 40		
	F	168 ± 37		190 ± 28		236 ± 28		354 ± 41	af	353 ± 56	f	263 ± 61		
Brainstem	C	282 ± 17		385 ± 52		461 ± 50	d	594 ± 30	f	567 ± 26	f	267 ± 33		
	F	205 ± 28		294 ± 33		390 ± 24	d	578 ± 94	f	538 ± 61	f	331 ± 77		
Choroid plexus	C	556 ± 82		79 ± 13	f	72 ± 4	f	130 ± 25	f	121 ± 25	f	460 ± 97		
	F	576 ± 81		78 ± 16	f	90 ± 20	f	213 ± 57	e	300 ± 115	d	722 ± 209	a	
Hippocampus	C	176 ± 19		197 ± 26		239 ± 21		336 ± 20	f	313 ± 29	e	201 ± 33		
	F	121 ± 12		126 ± 14		173 ± 21		344 ± 54	f	317 ± 34	f	168 ± 43		
Medulla	C	282 ± 22		456 ± 78	d	539 ± 74	e	650 ± 31	f	631 ± 43	f	263 ± 29		
	F	217 ± 32		375 ± 47		503 ± 38	e	603 ± 74	f	612 ± 87	f	411 ± 85	d	
Midbrain	C	323 ± 20		432 ± 60		515 ± 59	d	650 ± 40	f	620 ± 33	f	282 ± 40		
	F	226 ± 32		312 ± 36		412 ± 41	d	638 ± 123	f	579 ± 59	f	343 ± 83		
Adrenal	C	241 ± 64		338 ± 125		532 ± 200		546 ± 156		539 ± 126		444 ± 157		
	F	470 ± 122		448 ± 110		642 ± 112		677 ± 266		653 ± 170		500 ± 123		
Kidney	C	180 ± 16		31 ± 5	f	10 ± 4	f	40 ± 9	f	69 ± 7	f	163 ± 18		
	F	198 ± 22		47 ± 13	f	6 ± 2	f	63 ± 15	f	57 ± 5	f	171 ± 15		
GI tract	C	83 ± 8		2,3 ± 0,8	f	6,4 ± 2,5	f	39,5 ± 7	e	50,3 ± 3	d	82,1 ± 20,2		
	F	69 ± 22		3,1 ± 1,5	f	4,6 ± 1,2	f	79,4 ± 15	a	52,6 ± 12		67,9 ± 16,6		
Liver, arterial	C	5 ± 2		2,1 ± 1,2		7,6 ± 3,4		7,1 ± 2,7		5,3 ± 1,6		4,4 ± 1,7		
	F	15 ± 6		1,7 ± 0,7	d	5,0 ± 3,9		12,8 ± 5,8		15,3 ± 6,4		5,9 ± 3,2		
Portal Vein	C	87 ± 8		2,3 ± 0,7	f	6,4 ± 2,4	f	39,9 ± 8	e	51,7 ± 1,6	d	90,7 ± 20,5		
	F	82 ± 21		3,0 ± 1,3	f	4,4 ± 1,2	f	80,2 ± 15	a	55,8 ± 10		81,4 ± 19,4		
Spleen	C	215 ± 116		0,4 ± 0,2	e	4,3 ± 1,1	e	36,3 ± 22	e	67,3 ± 32	d	252 ± 59,1		
	F	207 ± 60		2,2 ± 1,2	e	3,3 ± 1,9	e	86,7 ± 24		66,5 ± 22	d	222 ± 64,4		
Skeletal muscle	C	19 ± 3		1,3 ± 0,4	f	2,3 ± 0,5	f	2,6 ± 0,9	f	3,8 ± 0,8	f	12,0 ± 3,4	d	
	F	13 ± 3		0,3 ± 0,1	f	0,5 ± 0,2	f	8,2 ± 3,5		1,5 ± 0,5	f	9,7 ± 2,1		
Body skin	C	35 ± 1		1,5 ± 0,8	f	4,5 ± 1,1	f	17,7 ± 3,8	f	19,0 ± 2,6	f	22,4 ± 4,7	e	
	F	20 ± 5		0,9 ± 0,2	f	0,9 ± 0,3	f	11,5 ± 2,7	d	9,9 ± 3,3	a,d	17,8 ± 4,3		0,05
Scalp	C	47 ± 8		26,0 ± 10	d	13,3 ± 3,9	f	9,5 ± 1,8	f	12,0 ± 1,5	f	16,2 ± 3,1	f	
	F	32 ± 8		9,0 ± 2,0	a,e	3,7 ± 1,3	f	9,9 ± 1,2	e	3,4 ± 2,2	f	35,9 ± 9,4	a	
Carcass	C	27 ± 2		3,5 ± 0,6	f	3,2 ± 0,3	f	9,3 ± 1,2	f	10,9 ± 0,7	f	16,9 ± 2,6	f	
	F	20 ± 3		2,3 ± 0,2	f	3,2 ± 0,6	f	8,3 ± 1,2	f	7,0 ± 1,3	f	18,9 ± 4,0		

a<0.05, b<0.01, c<0.001 significant between groups; d<0.05, e<0.01, f<0.001 significant versus control within groups
C= control fetuses (n=5), F= treated (flunarizine) fetuses (n=5)

Study group

Under control conditions there were no significant differences in physiological variables and combined cardiac output between groups (Tables 1-3). Blood flow to the cerebrum, skeletal muscle, body skin and carcass as well as distribution of combined cardiac output to the gastrointestinal tract were somewhat lower in the treated fetuses (Table 3). Responses of physiological and cardiovascular variables to acute intrauterine asphyxia were almost identical in both groups except for blood flow to the scalp, distribution of the combined cardiac output to the cerebrum and scalp, and vascular resistance of the placenta (Tables 2,3). The increase in plasma concentrations of catecholamines in treated fetuses was more pronounced than in the control group (Table 1). Above all the dopamine concentration during asphyxia was significantly higher in the treated fetuses. During the immediate recovery period there were hardly any differences between groups in physiological variables, blood flow and distribution of cardiac output to the organs studied (Table 1-3). Variable differences could only be observed in blood flow and distribution of combined cardiac output to the lower body, lung, cerebellum, gastrointestinal tract, portal vein, skeletal muscle and body skin. This was also true for the late recovery period (Tables 2,3).

Pharmacokinetic studies

Plasma concentrations of flunarizine increased after bolus injection to 121±5 µg/L serum at 2.5 min followed by a exponential decline to 22±1,3 µg/L over the next 2 h (Fig. 1). There was an accumulation of flunarizine in the various regions of the brain up to levels of 800 ng/g tissue (Fig. 2).

DISCUSSION

The central finding of the present study is that short term circulatory responses to acute asphyxia are not impaired by low dose flunarizine in fetal sheep near term. Before asphyxia distribution of combined ventricular output, physiological variables as well as concentrations of catecholamines in fetuses from the control group were in the normal range for chronically prepared fetal sheep near term. During acute asphyxia there was a redistribution of cardiac output towards the central organs accompanied by a pronounced bradycardia and a progressive increase in arterial blood pressure.

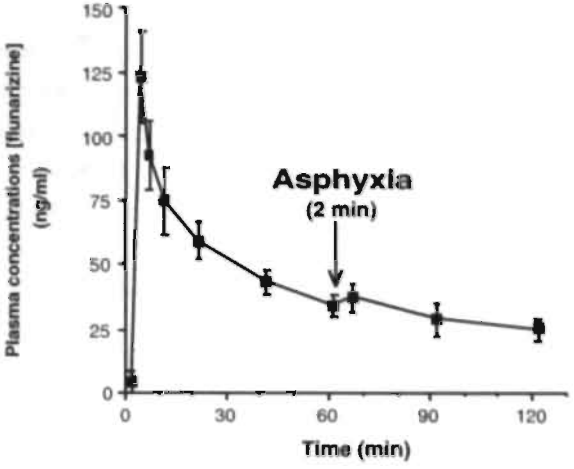

Figure 1. Plasma concentrations of flunarizine (ng/ml) before, during and after arrest of uterine blood flow for 2 min. Fetuses (n=9) received flunarizine (1 mg/kg estimated fetal weight) intravenously 60 min before acute asphyxia.

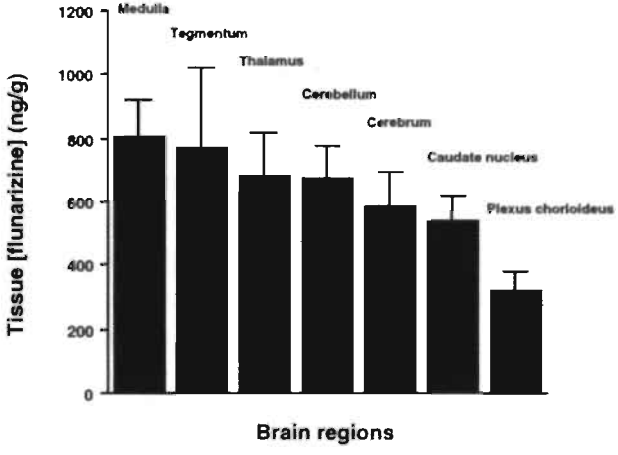

Figure 2. Accumulation of flunarizine - administered 60 min before arrest of uterine blood flow for 2 min - in various brain regions of the fetal sheep 120 min after acute asphyxia (columns are means ± SEM; n=7).

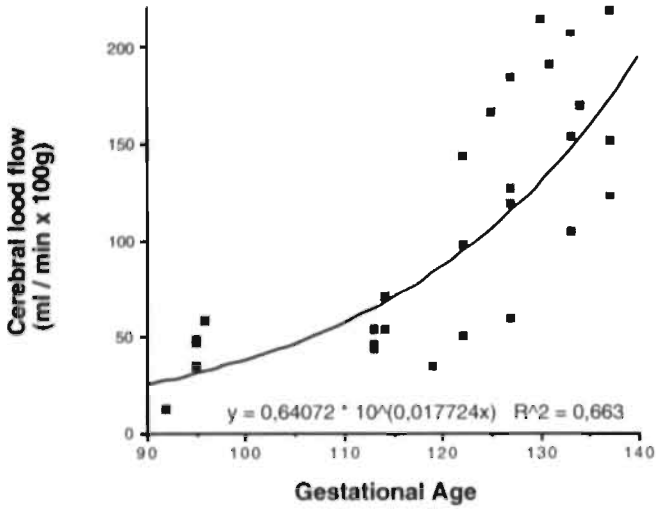

Figure 3. Relation between gestational age and cerebral blood flow in fetal sheep (n=28).

After asphyxia circulatory centralization did not resolve quite as rapidly as it developed, but was almost completely recovered at 30 min after the insult. There were almost no differences between groups in the time course of physiological and cardiovascular variables measured before, during and after acute intrauterine asphyxia. Furthermore, it should be emphasized that in both the treated and untreated fetuses cerebral blood flow did not increase during asphyxia inspite of an increase in arterial blood pressure, suggesting that during acute asphyxia cerebral autoregulation was maintained and not affected by low dose flunarizine. It was not the aim of the present study to examine the effects of flunarizine on neuronal survival in fetal sheep after intrauterine asphyxia, since it is well known that more severe hypoxic-ischemic insults are necessary to induce neuronal necrosis (Berger et al., 1998). In fact we were able to show in a previous paper that cerebral oxygen delivery below 3 ml O_2/100 g/min has to be maintained for 30 min to achieve a rate of neuronal cell damage up to 50 % in parasagittal regions (Berger et al., 1996). However, before flunarizine may be considered to be tested in a clinical trial in a first approach possible detrimental side-effects on cardiovascular responses to acute asphyxial episodes must be excluded. Therefore, in this study we only looked at the effects of 2 min asphyxia on circulatory centralization. Further studies should address possible

adverse effects of flunarizine on cardiovascular function during and after prolonged asphyxia.

In the present study there was a tendency to higher plasma catecholamine concentrations during asphyxia in the treated group of animal that does not seem to have any impact on fetal circulatory centralization. However, one might speculate that a vasoconstriction caused by an increased catecholamine release in the treated group during asphyxia could have been masked by a flunarizine induced vasodilatation. As known from studies in adult animals dopamine initially causes a vasoconstriction in the renal circulation induced by an alpha-adrenergic mechanism and later on a sustained vasodilatation mediated by specific dopamine receptors. Similar responses have been reported from the cerebral circulation (for review see Edvinsson and MacKenzie, 1977). Hence, it cannot be fully excluded whether a vasodilatation caused by flunarizine may have been antagonized a vasoconstriction induced by increased dopamine release in the treated group. However, at present there are no data available to clarify whether the observed differences in dopamine release between control and study group are of biological relevance.

Before asphyxia cerebral blood flow in treated animals was significantly lower than in the control group. After detailed analysis this could be largely attributed to a slight difference in the gestational age between fetuses from control and study group (133±2 d vs. 129±1 d). Figure 3 shows cerebral blood flow of 28 fetal sheep of different gestational age that have been operated at our laboratory during the same period in time as the animals from the present study. Indeed, there is a considerable increase in cerebral blood flow around 130 d of gestation that might explain the differences measured between control and study group. Irrespective of gestational age as a determinant of cerebral blood flow an influence of low dose flunarizine on cerebral circulation under control conditions has been excluded by two other studies in fetal sheep (de Haan et al., 1993; Berger et al., 1998). In the present study tissue levels of flunarizine determined in different parts of the brain were lower than those reported previously in adults after applying identical concentrations of flunarizine (Tegtmeier et al., 1987). However, in fetal animals the loss of the drug via diffusion across the placental barrier into the maternal compartment has to be taken into account. Nevertheless the tissue concentrations reached in our study were well within the neuroprotective therapeutic range (Todd et al., 1989).

From the present study we conclude that low dose flunarizine does not significantly affect short term circulatory responses to acute asphyxia in sheep near term. Since low dose flunarizine protects the fetal brain from ischemic injury in sheep, its clinical use should be

reconsidered, particularly in fetuses that are at risk of hypoxic-ischemic encephalopathy (Berger et al., 1998).

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Chapter 5

LOW DOSE FLUNARIZINE PROTECTS THE FETAL BRAIN FROM ISCHEMIC INJURY IN SHEEP

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ABSTRACT

Flunarizine, a calcium channel blocker, reduced cerebral damage caused by hypoxic-ischemic insults in neonatal rats and in fetal sheep near term. However, the high dose regimen used in these studies produced cardiovascular side-effects that might have counteracted the neuroprotective properties of flunarizine. Therefore, the neuroprotective effect was tested in a low dose protocol (1mg/kg estimated BW). Twelve fetal sheep near term were instrumented chronically. Six fetuses were pretreated with 1 mg flunarizine per kg estimated body weight 1 h before ischemia, while the remainder (n = 6) received solvent. Cerebral ischemia was induced by occluding both carotid arteries for 30 min. To exclude the possibility that the neuroprotective effects of flunarizine were caused by cerebrovascular alterations we measured cerebral blood flow by injecting radio-labelled microspheres before (- 1 h), during (+3 min and +27 min) and after (+40 min, +3 h and +72 h) cerebral ischemia. At the end of the experiment (+72 h) the ewe was given a lethal dose of sodium pentobarbitone and saturated potassium chloride i.v., and the fetal brain was perfused with formalin. Neuronal cell damage was assessed in various brain structures by light microscopy after cresyl violet/fuchsin staining using a scoring system (0-5% damage (1), 5-50% damage (2), 50-95% damage (3), 95-99% damage (4), 100% damage (5)). In ten other fetal sheep effects of low dose flunarizine on circulatory centralization caused by acute asphyxia could be excluded. In the treated group neuronal cell damage was reduced significantly in many cerebral areas to varying degrees (range - control group: 1.03-2.14 vs. range - treated group: 1.00-1.13; $P<0.05$ to $P<0.001$). There were only minor differences in blood flow to the various brain structures between groups. We conclude that pretreatment with low dose flunarizine protects the brain of fetal sheep near term from ischemic injury. This neuroprotective effect is not mediated by changes in cerebral blood flow. We further conclude that low dose flunarizine may be clinically useful as a treatment providing fetal neuroprotection, particularly since the fetal cardiovascular side effects are minimal.

INTRODUCTION

Hypoxic-ischemic cerebral damage is an important contributor to perinatal mortality and morbidity including long-term neurological sequelae in term and preterm fetuses (1). Over the last decade many therapeutic strategies have been developed to reduce neuronal damage caused by ischemic insults in neonatal and adult animals. These have included application of calcium antagonists, glutamate antagonists, oxygen radical scavengers, and nitric oxide synthetase blockers (for review, see Krieglstein *et al.* (2)). Perinatal use of neuroprotective strategies may be particularly successful, since pretreatment of individuals at risk of hypoxic-ischemic encephalopathy might be possible. There is evidence that flunarizine, a class IV calcium channel blocker, is more effective as a neuroprotective drug in pretreatment (3-7) than in post-treatment protocols (8). However, the high dose regimen (30–45 mg/ kg body weight) used in those studies had severe cardiovascular side-effects (7,9). The present study was therefore designed to test whether ischemic insults in fetal sheep near term could be also reduced by a low dose flunarizine protocol. To account for drug-related changes in cerebral blood flow that might affect neuronal cell damage we measured cerebral blood flow by the microsphere method.

METHODS

The experimental model and measurement of the associated variables have been described in detail previously (10).

Animal preparation

Twelve fetal sheep were chronically prepared at a gestational age of 125 days (term is at 147 days). All ewes were anesthetized by subarachnoid injection of 8 ml of 0.75% (w/v) bupivacaine at the lower spine, and were operated under sterile conditions. Polyvinyl catheters were placed in a maternal iliac artery and vein through tibial vessels. The ewe's abdominal wall was opened in the midline and through a small uterine incision, the fetal hindlimbs were exposed. Using local anesthesia with 1.0% (w/v) prilocaine HCl, polyvinyl catheters were inserted via the pedal vein of each hindlimb into the inferior vena cava. The uterine incision was closed and a second uterine incision was made over the fetal snout. Head and neck of the fetus were exteriorized. To prevent the fetus from breathing, its head was covered by a water-filled rubber glove. Catheters were inserted into both fetal brachial arteries. Furthermore, both fetal common carotid arteries were prepared. Cerebral ischemia was induced by occluding the carotid arteries on both sides simultaneously below the thyroid and above the lingual artery for 30 min as described recently (10). Thus, blood flow to the cerebrum via anastomoses between the carotid and vertebral arteries was

arrested. After cerebral ischemia a catheter was placed into the amniotic cavity, and the second intrauterine incision was closed. All catheters were filled with heparin (1000 IU/ml), plugged, and passed subcutaneously to the ewe's flank, where they were exteriorized and protected by a pouch sewn to the skin. On the day of surgery and each day thereafter, the ewe received 2 million units of penicillin G (Grünenthal, Germany) and 80 mg of gentamycin sulfate (Merck, Germany), half intravenously and half into the amniotic cavity.

Experimental Protocol

About 30 min before preparation of the carotid arteries 6 fetuses received a bolus of flunarizine (1 mg/kg estimated body weight) intravenously, while an equal volume of the solvent was administered to the remaining fetuses. The drugs were donated by Janssen Pharmaceutica, Beerse, Belgium. The dosage applied was within the therapeutic range (11). The experiments were done in a randomized fashion. The operators were not blind to the nature of injection. Sixty min after flunarizine injection cerebral ischemia was started. To determine the time course of changes in fetal cerebral blood flow before (-15 min), during (+3 min and +27 min), and after (+10 min, +3 h, +72 h) 30 min of cerebral ischemia, six batches of microspheres labelled with different isotopes (^{141}Ce, ^{114}In, ^{113}Sn, ^{103}Ru, ^{95}Nb and ^{46}Sc, 16 µm diam., New England Nuclear) were injected into the inferior vena cava, while reference samples from the brachial artery were withdrawn at a rate of 2.5 ml/min for 90 s (12). During and shortly after cerebral ischemia, fetal heart rate, and ascending aortic and intrauterine pressure were recorded simultaneously. After having injected the fourth batch of microspheres (+10 min) all catheters were closed and secured as described above, the abdominal wall was closed and the ewe was brought back to the metabolic cage, where the fifth injection (+3 h) was given. For organizational reasons there were no pressure measurements at this point in time. Before each injection blood samples were obtained from the brachial artery to measure blood gases, oxygen saturation of hemoglobin, and acid-base balance. At the end of the experiment (+72 h) the ewe was given a lethal dose of sodium pentobarbitone and saturated potassium chloride intravenously, and the fetus was perfused with 300 ml of formalin (15 %, w/v, saline).

The experimental protocols were approved by the appropriate institutional review committee and met the guidelines of the governmental agency responsible.

Measurements

Ascending aortic and intrauterine pressure, and fetal heart rate were recorded on a polygraph (Hellige, Germany). Blood gases and pH were measured in an automatic blood gas analyzer (278 Blood Gas System, Ciba Corning, Frankfurt, Germany), and base excess was calculated. Hemoglobin concentration and oxygen saturation of hemoglobin were measured photometrically (OSM 2 Hemoximeter, Radiometer, Copenhagen, Denmark) in duplicate. Fetal cerebral blood flow and the extent of neuronal cell damage were assessed in identical brain specimens. To determine fetal cerebral blood flow using the microsphere method, the fetal brain was removed and fixed in formalin for at least seven days. Afterwards the cerebrum was separated from the basal ganglia and divided in four frontal sections (rostral, pericentral, postcentral, occipital) with a thickness of about 1.5 cm. The right and left parts of these four sections were further subdivided into four equally sized segments (sagittal 1 and 2, lateral 1 and 2) each weighing 1-2 g. In addition to these 32 cerebral specimens, the caudate nucleus, thalamus, hippocampus, tegmentum-colliculi-pons, cerebellum, and medulla oblongata were separated. These brain structures were placed into vials, which were filled to the same height to reduce variations in geometry during gamma counting. The solid-state semi-conductor germanium (Ge) gamma counter used had a high energy resolution of about 3 keV and was connected to a multichannel (2048) pulse height analyzer (ND 62, Nuclear Data Inc., Illinois, USA). The results were normalized with respect to time and sample weight.

After cerebral blood flow analysis the specimens of the fetal brain were removed from the counting vials and embedded in paraffin. Coronal subserial sections of 10 µm were obtained and then stained with cresyl violet/fuchsin. Every 40th section was mounted to evaluate the extent of neuronal cell damage. Neuronal cell damage was assessed at a magnification of 250x. Neurons with ischemic cell damage were identified according to the criteria of Brown and Brierley (13). Neuronal cell damage in each microscopical visual field was quantified as follows: 0 - 5 % damage (score 1), 5 - 50 % damage (score 2), 50 - 95 % damage (score 3), 95 - 99 % damage (score 4), 100 % damage (score 5).

Calculations

Fetal cerebral blood flow was calculated from counts of the injected nuclide recovered in the fetal cerebrum and the appropriate reference sample, and from the withdrawal rate of the reference sample (12). The histological score of each cerebral specimen was calculated by averaging the scores of all visual fields analysed from 3 sections of that specimen. The number of scored visual fields per specimen ranged between 400 and 500 each. The scores from corresponding specimens from the right and left hemisphere were averaged.

Effects of low-dose flunarizine on fetal circulatory centralization

Since flunarizine, a class IV calcium antagonist, is a potent vasodilator, the neuroprotective effects of this drug may be counteracted by its cardiovascular side-effects. To clarify this point we subjected 10 chronically instrumented sheep fetuses (gestational age: 132±1 days) to acute intrauterine asphyxia (14). Fetuses from the study group received flunarizine (1 mg/kg estimated body weight) intravenously 60 min before asphyxia, while solvent was administered to the fetuses of the control group. Organ blood flows, physiological variables and plasma concentrations of catecholamines were measured before, during and after arrest of uterine blood flow for 2 min, i.e., at 0, 1, 2, 3, 4, and 30 min. Before asphyxia, the distribution of combined ventricular output, physiological variables and concentrations of catecholamines in fetuses from the control group were within the normal range for chronically prepared fetal sheep near term. During acute asphyxia there was a redistribution of cardiac output towards the central organs (Fig. 1) accompanied by a pronounced bradycardia and a progressive increase in arterial blood pressure. Fetuses of the study group had higher plasma catecholamine levels than the control group. After asphyxia, circulatory centralization did not resolve quite as rapidly as it had developed, but was almost completely recovered at 30 min after the insult. There were hardly any differences between treated animals and controls in the time course of physiological and cardiovascular variables measured before, during and after acute intrauterine asphyxia (Fig. 1). Low dose flunarizine does not, therefore, seem to affect fetal circulatory responses to acute asphyxia in sheep near term.

Statistics

Values are given as means ± SD. Statistical differences in physiological variables, cerebral blood flow, and neuronal cell damage within and between groups were evaluated using a 2-way analysis of variance followed by Games-Howell *post-hoc* test. Owing to the problem of multiple test performing the resulting P-values have to be regarded as descriptive.

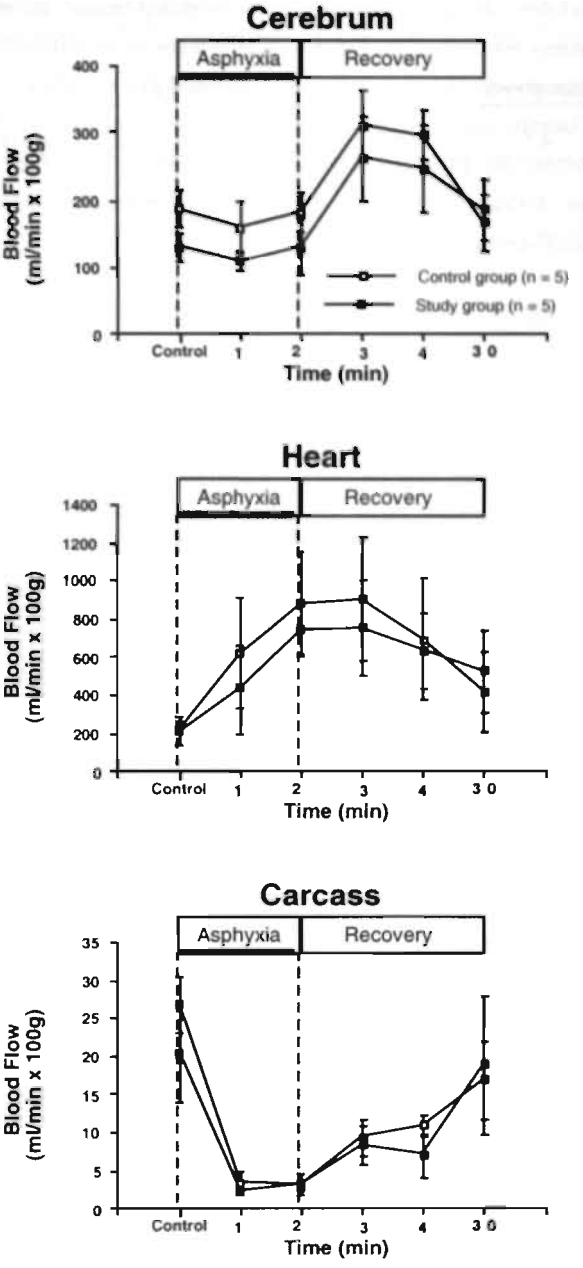

Figure 1. Blood flow to the cerebrum, heart, and carcass (mL/min x 100 g) in fetal sheep near term before, during, and after arrest of uterine blood flow for 2 min, i.e. at 0, 1, 2, 3, 4, and 30 min. Fetuses from the study group (filled squares; $n = 5$) received flunarizine (1 mg/kg estimated fetal BW) i.v. 60 min before asphyxia, whereas solvent was administered to the fetuses of the control group (open squares; $n = 5$).

RESULTS

The physiological variables of the control and the study group before, during and after 30 min of cerebral ischemia are shown in Table 1. There were no significant differences in these variables between groups. At control the arterial blood pH, the plasma concentrations of both glucose and lactate, and the mean arterial blood pressure were slightly above the normal range for chronically prepared fetal sheep (15). However, after ischemia these variables normalized. Furthermore, there were no time-dependent differences in the remaining physiological variables (Table 1).

No differences in blood flow to the 32 specimens from the fetal cerebrum could be observed between treated and control groups. Table 2 shows the mean blood flows to these specimens. During ischemia cerebral blood flow was reduced to less than 20 ml/100g/min. In the immediate recovery period there was hyperperfusion (+10 min) followed by a tendency towards hypoperfusion later on (+3 h). Finally, at 72 h after ischemia, cerebral blood flow was higher than control rates. This time course of changes in cerebral blood flow could be observed in almost all brain structures.

In Table 3 the histological scores for the 16 corresponding areas from both sides of the cerebrum are shown. The most pronounced neuronal cell damage was detected in the parasagittal regions, whereas in the more lateral part of the cortex only minor neuronal damage occured. Ischemic injury could be observed in 172 out of 192 cerebral specimens of the control group, but in only 69 of 192 study group samples. Damage to deeper brain structures could be detected only in a few areas, mainly in the hippocampus and cerebellum (Table 4). Neuronal cell damage was found more often in controls than in treated animals in the hippocampus (10/12 vs. 4/12) and cerebellum (6/12 vs. 1/12). Interestingly, there was a tremendous reduction in neuronal cell damage in almost all parts of the cerebrum after pretreatment with flunarizine (Table 3).

Table 1. Physiological Variables Before, During and After 30 Minutes of Cerebral Ischemia with (F) and without (C) Pretreatment with Flunarizine

		Ischemia		Recovery		
	Control	**3 min**	**27 min**	**10 min**	**3 h**	**72 h**
pH						
C	7.34 ± 0.03	7.35 ± 0.03	7.34 ± 0.05	7.33 ± 0.05	7.36 ± 0.02	7.39 ± 0.02
F	7.29 ± 0.05	7.30 ± 0.04	7.32 ± 0.04	7.28 ± 0.06	7.30 ± 0.06	7.38 ± 0.02 c
pO_2 (mmHg)						
C	21.9 ± 2.7	21.3 ± 4.4	22.0 ± 3.3	21.6 ± 2.8	25.1 ± 1.7	22.8 ± 2.7
F	21.6 ± 2.9	21.4 ± 3.7	20.9 ± 4.5	19.5 ± 2.2	19.9 ± 2.6	21.1 ± 3.4
pCO_2 (mmHg)						
C	45.7 ± 3.3	44.4 ± 5.8	44.1 ± 4.0	43.5 ± 4.1	46.7 ± 2.1	46.8 ± 2.7
F	48.5 ± 5.2	45.6 ± 4.1	45.7 ± 3.9	46.5 ± 3.2	48.0 ± 2.4	46.0 ± 3.0
Oxygen Saturation (%)						
C	67.4 ± 9.8	65.2 ± 15.6	67.6 ± 12.5	65.3 ± 10.2	77.2 ± 4.3	71.7 ± 10.1
F	60.0 ± 9.8	58.1 ± 10.7	59.3 ± 12.8	54.3 ± 9.4	56.8 ± 10.9	66.0 ± 10.1
Glucose (mg/ml)						
C	57.8 ± 19.2	59.0 ± 14.6	61.7 ± 17.7	57.7 ± 20.7	32.3 ± 12.6 a	17.8 ± 4.3 c
F	70.2 ± 18.0	63.8 ± 18.4	67.3 ± 21.5	65.3 ± 25.7	41.5 ± 19.1 a	19.5 ± 9.4 c
Lactate (mmol/l)						
C	3.83 ± 0.82	3.75 ± 0.98	4.65 ± 1.77	5.15 ± 1.52	3.73 ± 0.60	1.30 ± 0.18 a
F	3.65 ± 0.93	3.50 ± 1.16	3.87 ± 1.33	4.73 ± 2.04	4.68 ± 2.36	1.40 ± 0.31 a
Fetal Heart Rate (min^{-1})						
C	168 ± 15	191 ± 19	197 ± 31	199 ± 35	n.m.	161 ± 10
F	147 ± 29	171 ± 56	195 ± 55 a	170 ± 33	n.m.	162 ± 16
Mean Arterial Pressure (mmHg)						
C	51 ± 11	64 ± 10 a	58 ± 13	53 ± 9	n.m.	41 ± 3
F	54 ± 7	68 ± 11 a	67 ± 12 a	50 ± 14	n.m.	42 ± 10

Values are given as means ± SD. Significant differences within groups are indicated by a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ and between groups by d: $p < 0.05$; e: $p < 0.01$; f: $p < 0.001$.
n.m. = not measured

Table 2. Regional Cerebral Blood Flow (ml/100g tissue/min) Before, During and After 30 Minutes of Cerebral Ischemia with (F) and without (C) Pretreatment with Flunarizine

		Ischemia		Recovery		
	Control	**3 min**	**27 min**	**10 min**	**3 h**	**72 h**
Cerebrum						
C	100.6 ± 27.1	15.5 ± 8.3 c	19.6 ± 9.1 c	206.5 ± 65.7 c	68.2 ± 16.7	160.9 ± 22.6 b
F	88.5 ± 19.0	11.0 ± 2.1 c	17.0 ± 7.2 c	168.0 ± 50.8 c	84.5 ± 20.9	147.5 ± 64.5 b
Caudate nucleus						
C	125.8 ± 34.2	34.4 ± 29.7 b	35.5 ± 15.1 b	257.0 ± 94.7 c	99.1 ± 32.1	223.9 ± 66.3 b
F	124.2 ± 33.1	24.9 ± 17.2 b	37.4 ± 16.6 a	187.2 ± 67.7 d	107.9 ± 36.6	195.7 ± 85.8 a
Thalamus						
C	157.4 ± 51.0	27.7 ± 17.0 b	46.1 ± 18.8 b	432.0 ± 185.8 c	113.4 ± 25.4	250.5 ± 35.6 a
F	164.9 ± 40.3	20.6 ± 11.2 c	34.6 ± 15.9 b	299.0 ± 97.7 b,e	158.5 ± 50.8	216.2 ± 94.2
Hippocampus						
C	90.5 ± 19.7	18.9 ± 14.8 b	31.6 ± 16.3 b	219.5 ± 85.6 c	65.5 ± 14.4	173.0 ± 31.2 c
F	93.1 ± 26.3	23.1 ± 14.5 b	24.9 ± 6.6 b	177.3 ± 39.6 c	88.1 ± 22.3	158.8 ± 71.8 b
Colliculi, Tegmentum, Pons						
C	196.5 ± 57.1	27.4 ± 14.3 b	48.8 ± 18.1 b	434.8 ± 229.7 c	140.1 ± 41.8	315.7 ± 64.6 a
F	196.0 ± 51.6	16.3 ± 8.8 b	44.5 ± 15.7 b	327.5 ± 171.7 a	198.7 ± 43.5	256.8 ± 104.7
Cerebellum						
C	155.9 ± 69.3	27.2 ± 21.5 b	45.0 ± 20.8 a	420.5 ± 156.0 c	163.9 ± 74.1	273.2 ± 87.2 b
F	152.0 ± 29.3	28.0 ± 20.3 b	40.5 ± 18.7 a	298.3 ± 83.0 b,e	184.8 ± 32.4	251.2 ± 110.2 a
Medulla						
C	207.6 ± 59.0	64.5 ± 29.6 b	64.2 ± 32.1 b	210.7 ± 96.6	204.6 ± 79.4	372.4 ± 73.7 b
F	198.6 ± 67.8	54.0 ± 19.2 b	63.2 ± 31.9 a	297.3 ± 223.3	216.3 ± 66.5	274.6 ± 126.8

Values are given as means ± SD. Significant differences within groups are indicated by a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ and between groups by d: $p < 0.05$; e: $p < 0.01$; f: $p < 0.001$.

Table 3. Neuronal Cell Damage in the Cerebrum After 30 Min of Ischemia with (F) and without (C) Pretreatment with Flunarizine

	Segments			
	Parasagittal		Lateral	
	1	2	1	2
Rostral				
C	1.42 ± 0.52	1.26 ± 0.26	1.36 ± 0.55	1.48 ± 0.78
F	1.03 ± 0.05*	1.02 ± 0.03	1.05 ± 0.11	1.10 ± 0.23
Pericentral				
C	2.11 ± 0.84	1.50 ± 0.38	1.48 ± 0.70	1.09 ± 0.19
F	1.12 ± 0.18***	1.06 ± 0.12 *	1.00 ± 0.01 †	1.00 ± 0.01
Postcentral				
C	2.14 ± 1.10	1.90 ± 0.96	1.62 ± 0.78	1.03 ± 0.04
F	1.11 ± 0.22***	1.13 ± 0.21**	1.01 ± 0.01*	1.01 ± 0.02
Occipital				
C	1.65 ± 0.94	1.77 ± 1.02	1.67 ± 0.88	1.53 ± 0.82
F	1.00 ± 0.00*	1.01 ± 0.01**	1.02 ± 0.05*	1.01 ± 0.02†

Values are given as means ± SD. Significant differences between groups are indicated by: †: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Table 4. Neuronal Cell Damage in various Areas of the Diencephalon, Mesencephalon and Brain Stem After 30 Min of Ischemia with (F) and without (C) Pretreatment with Flunarizine

Caudate nucleus	C	1.02 ± 0.05
	F	1.04 ± 0.09
Thalamus	C	1.00 ± 0.00
	F	1.00 ± 0.00
Hippocampus	C	1.54 ± 0.65
	F	1.28 ± 0.43
Tegmentum, Colliculli, Pons	C	1.00 ± 0.00
	F	1.01 ± 0.02
Cerebellum	C	1.26 ± 0.33
	F	1.01 ± 0.03
Medulla	C	1.00 ± 0.00
	F	1.00 ± 0.00

DISCUSSION

As shown in previous studies pretreatment of immature animals with flunarizine seems to reduce ischemic neuronal cell damage dramatically (3-7). In some of these studies neonatal rats were subjected to hypoxia after ligation of one carotid artery (3-6). Flunarizine was applied at a relatively high concentration (30 mg/kg BW) before hypoxia. Ipsilateral infarction of the brain could be almost totally prevented in animals receiving such treatment (3-6). Similar results have been described by Gunn and co-workers using a fetal sheep model (7). However, the high dose regimens used by these authors had severe cardiovascular side-effects (7,9). In the present study we were able to show that pretreatment of fetal sheep near term with low dose flunarizine significantly reduces cerebral damage after ischemia. Since we have also provided evidence that low dose flunarizine does not alter the response of the fetal cardiovascular system to intrauterine asphyxia, this therapeutic regimen appears to be safe and may qualify for clinical trials. Because the transport of flunarizine across the placental membrane barrier is very fast due to its lipophilic properties (11), direct application to the fetus by chordocentesis would not be necessary. Thus, women carrying babies at risk of hypoxic-ischemic encephalopathy, i.e. IUGR or preterm fetuses, could be given low dose flunarizine intravenously before birth.

The observed neuroprotective effect of flunarizine in fetal sheep appears to be at variance with some studies in adult animals (16-19). These discrepancies may arise from differences in experimental design. The anticonvulsive properties of flunarizine should also be taken into consideration here. Unlike adult studies, the fetuses in intrauterine experiments are subjected not only to the ischemic insult itself, but also to postischemic seizure activity (20,21). Flunarizine is in part an anticonvulsant (11). Therefore, in studies in which seizure activity is part of the experiment, the neuroprotective effects may be more pronounced. This may explain the overt differences between experiments in adult and fetal animals. An interesting finding of this study is that flunarizine increases plasma catecholamine levels during acute asphyxia. Since elevated catecholamine levels seem to reduce ischemic cerebral injury (22), this observation may reveal one of the mechanisms by which flunarizine protects the fetal brain from neuronal cell damage.

In the present study there was little or no damage to deeper brain structures. This may be related to minor degrees of the ischemic insult: neuroprotection could not be demonstrated, for example, in diencephalic and brain stem areas. The fact that deeper brain structures were not damaged by the ischemic model used here may constitute an important difference to the clinical situation, since basal ganglia seem to be selectively vulnerable in hypoxic-ischemic encephalopathy (1). However, to mimic a clinical situation asphyxia models are

required, which have the disadvantage that the degree and distribution of neuronal injury varies immensely. Using such models it is, therefore, very difficult to test the neuroprotective properties of different drugs. Since this was the main aim of the present study, we chose to use the ischemic model described.

We observed a more pronounced neuronal cell damage in this study than in a previous one (10), a fact that might be related to differences in methods. We now used a double occlusion of the carotid arteries as described in methods, because double occlusion of the carotid arteries was necessary to account for residual blood flow via various anastomoses between carotid and vertebral arteries. This resulted in a more severe reduction in cerebral blood flow. The differences in the severity of neuronal cell damage between the control group in the present study and that in a study performed by Gunn and co-worker may be explained by strain and vendor differences (7). In fact, the volume of cerebral infarcts, e.g. in adult rats, depends largely on these variables, a fact that is probably due to differences in the cerebrovascular system (23,24).

After cerebral ischemia there was reactive hyperemia in almost all structures of the fetal brain studied. This reactive hyperemia is largely caused by a reduction in vascular tone on the basis of tissue acidosis during and a decrease in blood viscosity after ischemia (25,26). The hyperemic period was followed by a tendency towards hypoperfusion of the brain. Similar changes in cerebral blood flow could be observed in adult animals after ischemia. However, despite extensive research, the biological significance of this phenomenon is not fully understood (27). In the control group, postischemic brain blood flow at 72 h was higher than the preischemic blood flow. This phenomenon has also been described in severely asphyxiated human infants (28). Whether this is related to a loss in CO_2-reactivity and autoregulation of the cerebral vascular bed due to the ischemic insult is unknown.

As discussed previously the decrease in arterial blood pH as well as the increase in mean arterial blood pressure and plasma concentrations of both glucose and lactate are related to the acute experimental animal procedure (10). However, in spite of a few disadvantages of this acute animal model its clear advantage is that total arrest of carotid arterial blood flow could be ensured and controlled visibly.

We conclude that pretreatment by low dose flunarizine (1 mg/kg estimated BW) protects the brain of fetal sheep near term from ischemic injury. We further conclude that this neuroprotective effect is not mediated by drug dependent changes in cerebral blood flow. Since low dose flunarizine does not alter the response of the fetal cardiovascular system to

asphyxia, its clinical use should be reconsidered, particularly in fetuses that are at risk of hypoxic-ischemic encephalopathy.

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Chapter 6

CIRCULATORY RESPONSES TO ACUTE ASPHYXIA ARE NOT AFFECTED BY THE GLUTAMATE ANTAGONIST LUBELUZOLE IN FETAL SHEEP NEAR TERM

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ABSTRACT

Objective: Asphyxia is one of the main causes of perinatal brain damage that can result in psychomotor deficits during later development. Although over the last decade a number of therapeutic strategies have been developed with successful results in animal experiments, various side effects on the cardiovascular system have precluded their clinical application. However, recently lubeluzole, a new glutamate antagonist, was shown to considerably improve clinical outcome in adults suffering from acute ischemic stroke without any safety concerns. In contrast, preliminary experiments in our laboratory have demonstrated transient alterations in heart rate as well as arterial hypertension after intravenous application of this compound in fetal sheep. The aim of the present study was to examine in detail whether lubeluzole affects circulatory responses to acute asphyxia in fetal sheep near term.

Methods: Eleven fetal sheep were chronically instrumented at a mean gestational age of 133 ± 2 (term is at 147 days). The fetuses of the study group (n = 6) received three bolus injections of lubeluzole at 30 min intervals (3 x 0.11 mg/kg estimated body weight), while the remaining received the solvent. Organ blood flows and physiological variables were measured before, during and after arrest of uterine blood flow for 2 min (i.e. at 0, 1, 2, 3, 4, and 30 min).

Results: Before asphyxia distribution of combined ventricular output and physiological variables in fetuses from the control group were in the normal range for chronically prepared fetal sheep near term. During acute asphyxia there was a redistribution of cardiac output towards the central organs accompanied by a pronounced bradycardia and a progressive increase in arterial blood pressure. There were nearly no differences between groups in the time course of physiological and cardiovascular variables measured before, during and after acute intrauterine asphyxia.

Conclusion: From the present study we conclude that lubeluzole does not affect circulatory responses to acute asphyxia in fetal sheep near term.

INTRODUCTION

Asphyxia is one of the main causes of perinatal brain damage that can result in psychomotor deficits during later development (1,2). Over the last decade many therapeutic strategies have been developed to reduce neuronal damage caused by ischemic insults in neonatal and adult animals. Glutamate antagonists in particular have been shown to be highly neuroprotective in various experimental models of cerebral ischemia (3-7). Lubeluzole, a very effective, new glutamate antagonist, considerably improved clinical outcome in patients suffering from acute ischemic stroke (8). Part of its neuroprotective effect may result from inhibition of NO-induced programmed cell death. Thus, lubeluzole decreased DNA fragmentation and annexin-V binding in primary hippocampal neurons (9,10). Furthermore, Ca^{2+} channel-blocking properties of lubeluzole might protect neuronal tissue from ischemic injury as shown by patch clamp techniques in isolated rat dorsal root ganglion cells (11). Drug induced side-effects on the cardiovascular system could be excluded in healthy volunteers except for a significant prolongation of cardiac action potential (12-14). Using lubeluzole as a neuroprotective drug might therefore be of potential interest for many perinatologists. However, our preliminary experiments have shown transient alterations in fetal heart rate as well as arterial hypertension after intravenous application of lubeluzole in sheep near term. Thus, it was thought that any possible neuroprotective effect of this drug might be counteracted by an impairment of fetal circulatory centralisation during acute asphyxia, a crucial mechanism that protects the fetal brain from neuronal injury by increasing cerebral perfusion when oxygen is in short supply. The aim of the present study was therefore to examine in detail whether lubeluzole affects circulatory responses to acute asphyxia in fetal sheep near term. For this purpose we arrested maternal uterine blood flow for 2 min and measured blood flow to fetal organs using the microspheres method.

METHODS

Animal preparation

Eleven fetal sheep were chronically prepared at a mean gestational age of 133 ± 2 days (term is at 147 days). The surgical preparation has been described previously (15). Briefly, all ewes were anesthetized by subarachnoid injection of 8 ml of 0.75% (w/v) bupivacaine at the lower spine, and were operated on under sterile conditions. Polyvinyl catheters were placed into a maternal iliac artery and vein through tibial vessels. The ewe's abdominal wall was opened in the midline and a snare was placed around the descending aorta below the renal artery, which was then used to arrest uterine and ovarian blood flow during the

experiment. Care was taken not to include any nerves. Fetal catheters were inserted and maintained as described previously (15). An additional catheter was inserted into the amniotic cavity. All catheters were filled with heparin (1000 IU/ml), plugged, and passed subcutaneously to the ewe's flank, where they were exteriorized and protected by a pouch sewn to the skin. On the day of surgery and each day thereafter, the ewe received 2 million units of penicillin G (Grünenthal, Germany) and 80 mg of gentamycin sulfate (Merck, Germany), half i.v. and half into the amniotic cavity. The fetuses were allowed to recover for 2 days before being studied. As shown in a variety of previous experiments performed in our laboratory blood gases, cardiovascular variables and plasma catecholamines are within the normal range for chronically prepared fetal sheep near after this time period (16,17).

Experimental protocol

After taking control measurements of the physiological variables, 6 of the 11 fetuses received three bolus injections of lubeluzole at 30 min intervals (3 x 0.11 mg/kg estimated fetal body weight), while the remaining received the solvent. We preferred this application regimen to a single bolus injection of 0.33 mg/kg estimated fetal body weight, since the latter was found to produce cardiovascular side effects. The drugs were donated by Janssen Pharmaceutica, Beerse, Belgium. Sixty minutes later (at 0 min) uterine blood flow was arrested by a single occlusion of the descending aorta for 2 min to induce acute fetal asphyxia. To determine the effects of lubeluzole on the time course of circulatory centralization before, during and after acute asphyxia, blood flow to fetal organs and the distribution of combined ventricular output were measured by injecting six batches of differently labeled isotope microspheres (^{141}Ce, ^{114}In, ^{113}Sn, ^{103}Ru, ^{95}Nb & ^{46}Sc, 16 µm diam., New England Nuclear) into the inferior vena cava (18,19). Depending on the activity, 0.7 to 2.8 million microspheres per batch were applied. Asphyxia was caused by arresting uterine blood flow with the snare. Microspheres were injected at 75 s before asphyxia (control measurement), at 1 and 2 min during arrest of uterine blood flow, and at 1, 2, and 28 min after release of the snare (recovery period). Reference blood samples were withdrawn from both a carotid and a femoral artery at a rate of 2.5 mL/min. Sampling was continuous for 270 s during control, occlusion and the immediate recovery period. Separate samples were taken for 75 s during the 28-min recovery measurement. The volume of blood withdrawn was about 25 mL and was simultaneously replaced by maternal blood maintained at 39 °C in a waterbath. When using the microspheres method to measure blood flow at 1 - 2 min intervals the sampling time for reference blood must be adequate and the number of microspheres still circulating 30 to 40 s after each injection

must be negligible. As shown previously these conditions are fulfilled in the present experimental set up (15).

The experimental protocols including all anesthesiological and surgical procedure were approved by the appropriate institutional review committee and met the guidelines of the responsible governmental agency (Regierungspräsidium Gießen, Germany).

Measurements

Fetal heart rate and arterial blood pressure, amniotic fluid pressure, and maternal arterial blood pressure were recorded on a polygraph (Hellige, Germany) during the experiment. Complete occlusion of the ewe's descending aorta by means of the snare throughout the 2-min study period was confirmed by the fall in maternal arterial blood pressure distal to the obstruction. Before blood flow was measured, a blood sample was taken from the descending aorta and analysed for blood gases, acid-base balance (278 Blood Gas System, Ciba Corning, Frankfurt, Germany), hemoglobin concentration, oxygen saturation (OSM 2 Hemoximeter, Radiometer, Copenhagen, Denmark), glucose and lactate concentrations.

After the experiment a lethal dose of sodium pentobarbitone was given to the ewe and the fetuses were perfused with 300 ml of formalin (15%, w/v, saline). Fetal organs and cotyledons were weighed and placed in vials, which were filled to the same height to reduce variations in geometry. The intestines were separated from the mesentery, opened, and cleared of contents. Paired organs (lungs, kidneys, and adrenals) were counted separately, as were the right and left sides of the cerebrum and of the brainstem regions. No significant preferential streaming of microspheres was found. Specimens of skin and muscle were taken from the hips and shoulders of each side. There were no differences in blood flow between the intact side and the side on which the femoral or brachial arteries had been catheterized.

The applied solid-state semi-conductor germanium (Ge) gamma counter had a high energy resolution of about 3 keV and was connected to a multichannel (2048) pulse height analyzer (ND 62, Nuclear Data Inc., Illinois, USA). The results were normalized with respect to time and sample weight.

Calculations

Blood flow to the various fetal organs were calculated from counts of the injected nuclide recovered in fetal organs or the placenta, from counts in the appropriate reference samples, and from the withdrawal rate of the reference sample (18,19). To determine combined cardiac output absolute blood flows to all fetal organs including the placenta were added together and then divided by fetal body weight. Portal venous blood flow was calculated from the sum of the actual blood flow to each gastro-intestinal organ, including stomach, intestines, mesentery, pancreas, and spleen. The percentage of combined ventricular output distributed to a given organ was calculated from the absolute blood flow to that organ and the combined ventricular output. The vascular resistance was calculated by dividing arterial blood pressure (corrected for amniotic fluid pressure) by blood flow and was expressed in mmHg/ml/min/100g of tissue. Blood flow inferior to 1 ml/min/100g was treated as 1. Umbilical vascular resistance (mmHg/ml/min/kg fetal weight) was calculated by dividing the perfusion pressure of the umbilical circulation (arterial blood pressure minus the estimated umbilical venous blood pressure before (11 mmHg) and during (17 mmHg) reduction in uterine blood flow (20)) by umbilical blood flow.

Statistics

Results are given as means ± SD. The data were analysed for intra- and intergroup differences by two-way multivariate analysis of variance for repeated measures. The Games-Howell-test was used as a post-hoc testing procedure. Statistical analysis was performed by the Super Anova Statistical Package (Abacus, Inc. Ca., USA).

RESULTS

Control group

During the control period combined ventricular output, heart rate, arterial blood pressure, blood gases, pH, glucose, lactate and plasma concentrations of catecholamines were in the normal range for chronically prepared fetal sheep near term (21). Arrest of uterine blood flow for 2 min decreased fetal heart rate, arterial O_2 saturation of hemoglobin, and pH, and increased fetal arterial blood pressure, pCO_2, and plasma concentrations of lactate (Tables 1 and 2).

After 1 min of asphyxia combined ventricular output fell by 51% (Table 2). Whereas the portion distributed to placenta, heart, and brain increased by 57%, 431%, and 87% respectively, that to the adrenals did not change significantly. Within the brain there was a

preferential rise in percent cardiac output distributed to the brainstem. In contrast, blood flow to the choroid plexus decreased (Table 3). After 2 min of asphyxia combined ventricular output was reduced by 57% (Table 2), but the portion distributed towards central organs was raised further (heart 905 %, brain 166%, adrenals 414%).

Table 1. Acid-Base Balance and Blood Gases in Control and Lubeluzole-Treated Term Fetal Sheep.

Groups	Control	Asphyxia 1	Asphyxia 2	Recovery 3	Recovery 4	Recovery 30
pH						
C	7,40 ± 0,04	7,38 ± 0,03	7,30 ± 0,02 f	7,28 ± 0,08 f	7,30 ± 0,05 f	7,33 ± 0,04 e
L	7,41 ± 0,03	7,37 ± 0,03	7,27 ± 0,02 f	7,27 ± 0,04 f	7,29 ± 0,04 f	7,33 ± 0,04 f
Oxygen Saturation (%)						
C	54,0 ± 8,1	14,2 ± 8,7 f	4,8 ± 3,9 f	25,7 ± 11,6 f	43,2 ± 8,9 d	48,8 ± 10,8
L	64,3 ± 5,2	19,1 ± 8,5 f	5,0 ± 1,9 f	31,1 ± 5,5 f	46,2 ± 4,6 f	50,0 ± 8,2 e
pO_2 (mmHg)						
C	21,8 ± 2,7	10,2 ± 2,6 f	5,5 ± 2,2 f	16,9 ± 4,9 d	23,6 ± 3,3	24,3 ± 3,0
L	21,9 ± 1,9	9,0 ± 4,1 f	2,1 ± 1,9 f	15,3 ± 2,2 f	20,0 ± 2,4	19,5 ± 2,6
pCO_2 (mmHg)						
C	47,4 ± 2,6	51,4 ± 2,8	62,8 ± 6,3 f	63,9 ± 9,2 f	57,9 ± 7,4 e	50,0 ± 3,2
L	46,5 ± 2,2	50,9 ± 3,5	64,3 ± 5,3 f	61,3 ± 5,3 f	56,0 ± 4,4 e	48,7 ± 2,5
Hemoglobin (g/dl)						
C	10,8 ± 0,8	11,5 ± 0,9	13,0 ± 1,2 f	12,6 ± 1,1 f	11,7 ± 1,0	10,4 ± 1,2
L	11,6 ± 0,5	12,4 ± 0,4	14,1 ± 0,5 a,f	13,5 ± 0,3 f	12,9 ± 0,5 a,e	11,4 ± 0,5 a
Glucose (mg/ml)						
C	21,4 ± 4,6	21,8 ± 3,8	19,0 ± 2,2	21,0 ± 3,5	25,4 ± 4,3	28,8 ± 5,3 e
L	20,0 ± 3,3	21,5 ± 5,1	18,7 ± 2,6	19,2 ± 4,1	24,3 ± 4,8	22,5 ± 4,5 a
Lactate (mmol/l)						
C	1,64 ± 0,3	1,74 ± 1,6	2,78 ± 0,5 d	3,63 ± 0,7 f	3,69 ± 0,6 f	4,42 ± 0,8 f
L	1,49 ± 0,4	1,92 ± 0,5	2,96 ± 1,0 e	4,03 ± 1,2 f	4,14 ± 1,0 f	4,03 ± 1,5 f

a<0,05, b<0,01, c<0,001 significant between groups; d<0,05, e<0,01, f<0,001 significant versus control within groups
C= control fetuses (n=5), L= treated (lubeluzole) fetuses (n=6)

In the immediate recovery period, i.e. 1 and 2 min after arrest of uterine blood flow had been released, arterial blood pressure was still high (Table 2) and blood flow to the heart and all parts of the brain was increased (Table 3). At 30 min, i.e. after 28 min recovery, these values were not different from control. In all peripheral organs of the upper and lower body segment, including the carcass, skeletal muscle, total gastro-intestinal tract and portal vein, kidneys, spleen, scalp, and body skin, the proportion of combined ventricular output and actual blood flow decreased to very low values during asphyxia, and recovered gradually thereafter (Table 3).

Table 2. Changes of Physiologic Variables and Blood Flow in Control and Lubeluzole-Treated Fetal Sheep Near Term.

Group	Control	Asphyxia 1	Asphyxia 2	Recovery 3	Recovery 4	Recovery 30
Fetal Heart Rate (bpm)						
C	145,4 ± 13,5	93,8 ± 17,4 d	96,9 ± 30,4	159,4 ± 82,5	143,1 ± 36,5	168,0 ± 63,5
L	138,3 ± 25,2	59,2 ± 23,1 f	80,0 ± 23,7 d	124,2 ± 57,2	104,2 ± 15,0	144,2 ± 13,2
Arterial Blood Pressure (mmHg)						
C	47,5 ± 5,3	57,7 ± 6,3 d	71,7 ± 12,7 f	80,2 ± 12,4 f	73,9 ± 7,2 f	48,5 ± 5,1
L	53,2 ± 7,1	55,4 ± 5,9	82,6 ± 5,2 f	87,5 ± 5,9 f	78,3 ± 5,5 f	53,8 ± 4,7
Stroke Volume						
C	3,6 ± 0,3	2,8 ± 0,4	2,7 ± 1,6	2,9 ± 1,4	3,2 ± 0,7	2,9 ± 2,0
L	4,3 ± 0,9	4,6 ± 1,3 a	3,0 ± 0,9	4,8 ± 2,0 a	4,3 ± 0,5	4,5 ± 1,2 a
		Blood Flow (ml/min x kg fetus)				
Cardiac Output						
C	536,6 ± 52,0	265,3 ± 43,8 e	232,6 ± 50,9 f	424,3 ± 140,5	444,5 ± 59,7	485,7 ± 320,3
L	577,1 ± 67,5	279,9 ± 36,1 f	222,9 ± 27,8 f	525,0 ± 87,7	446,4 ± 83,7	637,8 ± 122,0
Placenta						
C	207,1 ± 21,9	167,1 ± 33,2	88,5 ± 68,9 e	179,2 ± 107,7	195,6 ± 70,2	187,6 ± 113,4
L	221,6 ± 21,5	179,7 ± 43,4	84,8 ± 19,7 e	169,2 ± 51,0	158,3 ± 73,3	230,0 ± 112,3
Fetal Body						
C	329,5 ± 42,6	98,2 ± 37,4 f	144,1 ± 38,8 e	245,1 ± 36,8	249,0 ± 41,5	298,1 ± 212,3
L	355,5 ± 67,1	100,2 ± 26,3 f	138,1 ± 20,7 f	355,8 ± 96,9	288,1 ± 51,9	407,8 ± 74,3

a<0,05, b<0,01, c<0,001 significant between groups; d<0,05, e<0,01, f<0,001 significant versus control within groups
C= control fetuses (n=5), L= treated (lubeluzole) fetuses (n=6)

Table 3. Organ Blood Flow in Control and Lubeluzole-Treated Fetal Sheep Near Term

	Blood flow (ml/min/100g)					
Group	**Control**	**Asphyxia 1'**	**Asphyxia 2'**	**Recovery 3'**	**Recovery 4'**	**Recovery 30'**
Heart						
C	215,7 ± 68,5	616,3 ± 293,2 e	884,9 ± 264,0 f	904,5 ± 321,6 f	693,4 ± 313,2 f	418,6 ± 206,4
L	246,2 ± 72,5	458,9 ± 199,3	755,5 ± 92,7 f	670,4 ± 227,3 f	424,1 ± 147,7	427,3 ± 131,2
Brain						
C	205,5 ± 25,9	199,6 ± 53,6	231,0 ± 44,0	361,5 ± 54,8 f	345,2 ± 33,0 f	188,3 ± 47,9
L	184,1 ± 45,9	179,3 ± 22,6	212,0 ± 17,7	360,8 ± 55,2 f	379,0 ± 65,3 f	245,8 ± 79,8
Cerebrum						
C	186,6 ± 27,9	159,0 ± 39,3	182,1 ± 28,6	310,6 ± 54,3 f	296,6 ± 37,6 f	167,2 ± 41,3
L	164,2 ± 35,1	135,4 ± 10,4	162,9 ± 25,5	303,2 ± 41,1 f	314,9 ± 49,6 f	209,2 ± 68,2
Cerebellum						
C	236,7 ± 18,8	269,7 ± 66,3	302,1 ± 58,3	463,0 ± 90,9 f	445,8 ± 40,8 f	240,5 ± 79,1
L	235,5 ± 46,0	243,4 ± 49,7	267,2 ± 50,8	444,7 ± 68,6 f	503,6 ± 82,0 f	317,2 ± 83,0 d
Brainstem						
C	281,5 ± 38,1	385,4 ± 116,6	460,9 ± 111,3 e	593,8 ± 67,0 f	566,9 ± 59,0 f	266,7 ± 66,1
L	228,3 ± 94,6	330,7 ± 76,8	394,2 ± 17,0 e	563,2 ± 99,0 f	593,3 ± 115,4 f	348,1 ± 131,5 d
Choroid plexus						
C	555,7 ± 183,5	78,8 ± 28,3 f	71,9 ± 9,4 f	129,6 ± 54,6 f	121,4 ± 54,7 f	460,0 ± 193,2
L	742,7 ± 276,2	72,5 ± 38,2 f	60,2 ± 16,5 f	196,2 ± 129,6 f	171,7 ± 52,5 f	873,7 ± 427,5
Hippocampus						
C	175,7 ± 41,8	196,8 ± 58,2	238,8 ± 46,1	335,7 ± 44,7 f	313,4 ± 65,0 f	201,2 ± 64,9
L	144,9 ± 37,8	131,7 ± 24,8	180,2 ± 39,0	326,9 ± 44,3 f	305,6 ± 34,2 f	197,8 ± 82,4
Adrenal						
C	240,7 ± 143,3	338,3 ± 280,1	531,6 ± 447,8	545,9 ± 349,7	539,2 ± 282,0	444,4 ± 314,9
L	414,0 ± 144,9	427,4 ± 164,2	558,3 ± 93,2	366,8 ± 175,0	408,0 ± 156,7	436,6 ± 224,0
Kidney						
C	180,1 ± 36,6	30,6 ± 11,3 f	9,9 ± 9,0 f	39,9 ± 20,8 f	69,2 ± 16,3 f	162,7 ± 36,1
L	214,9 ± 27,2	62,0 ± 25,9 f	5,2 ± 3,6 f	49,5 ± 22,9 f	67,8 ± 33,7 f	178,0 ± 81,8
Gastrointestinal tract						
C	83,4 ± 17,9	2,3 ± 1,7 f	6,4 ± 5,6 f	39,5 ± 16,5 e	50,3 ± 6,3 d	82,1 ± 40,4
L	99,7 ± 31,4	4,9 ± 10,0 f	2,5 ± 1,4 f	69,5 ± 29,0 d	46,3 ± 18,7 f	91,0 ± 34,3
Liver, arterial						
C	4,6 ± 4,7	2,1 ± 2,5	7,6 ± 7,7	7,1 ± 6,1	5,3 ± 3,7	4,4 ± 3,4
L	5,2 ± 2,5	0,9 ± 0,7	1,6 ± 0,9	4,5 ± 1,3	3,3 ± 1,5	7,0 ± 5,3
Portal Vein						
C	87,3 ± 18,2	2,3 ± 1,7 f	6,4 ± 5,4 f	39,9 ± 17,5 e	51,7 ± 3,5 d	90,7 ± 41,0
L	109,4 ± 31,6	4,6 ± 9,3 f	2,5 ± 1,4 f	72,0 ± 30,7 e	50,5 ± 18,1 f	97,5 ± 35,9
Spleen						
C	214,8 ± 258,6	0,4 ± 0,4 e	4,3 ± 2,5 d	36,3 ± 49,9 d	67,3 ± 71,9	251,9 ± 118,2
L	273,3 ± 133,5	1,0 ± 1,2 f	2,7 ± 1,6 f	111,2 ± 184,6 d	118,5 ± 89,8 d	201,7 ± 192,4
Scalp						
C	46,7 ± 17,7	26,0 ± 23,0 e	13,3 ± 8,8 f	9,5 ± 4,1 f	12,0 ± 3,3 f	16,2 ± 6,2 f
L	33,0 ± 9,3	19,1 ± 10,2 d	4,2 ± 1,9 f	12,6 ± 5,3 f	12,0 ± 3,1 f	25,0 ± 8,6
Carcass						
C	26,8 ± 3,8	3,5 ± 1,4 f	3,2 ± 0,7 f	9,3 ± 2,6 f	10,9 ± 1,5 f	16,9 ± 5,2 f
L	26,9 ± 3,7	3,5 ± 0,8 f	3,7 ± 0,6 f	14,3 ± 5,0 f	15,6 ± 4,1 f	18,8 ± 3,8 f

a<0,05, b<0,01, c<0,001 significant between groups; d<0,05, e<0,01, f<0,001 significant versus control within groups
C= control fetuses (n=5), L= treated (lubeluzole) fetuses (n=6)

Study group

Fetal heart rate and mean arterial blood pressure were measured 1 min before and 5 min after the three bolus injections of lubeluzole. Application of the drug did not cause any significant changes in these two variables (Table 4). Furthermore, the data from the control and study groups did not differ significantly (Tables 1-3) except for some marginal differences in hemoglobin, stroke volume, percentage cardiac output to the brainstem and thalamus, and vascular resistance of the hippocampus and brainstem (data not shown).

Table 4. Fetal Heart Rate (FHR) and Mean Arterial Blood Pressure (MAP) 1 Minute Before (-1 min) and 5 minutes (+5 min) After Three Bolus Injections of Lubeluzole in Fetal Sheep Near Term

Injection	FHR (bpm)	MAP (mmHg)
1st		
- 1 min	153 ± 14	49 ± 8
+ 5 min	147 ± 14	52 ± 9
2nd		
- 1 min	150 ± 17	50 ± 7
+ 5 min	147 ± 11	53 ± 6
3rd		
- 1 min	144 ± 10	49 ± 8
+ 5 min	138 ± 15	52 ± 5

DISCUSSION

The central finding of the present study is that circulatory responses to acute asphyxia are not impaired by lubeluzole in fetal sheep near term. Before asphyxia, the distribution of combined ventricular output and physiological variables in fetuses from the control group were in the normal range for chronically prepared fetal sheep near term. During acute asphyxia there was a redistribution of cardiac output towards the central organs accompanied by pronounced bradycardia and a progressive increase in arterial blood pressure. After asphyxia circulatory centralization did not resolve quite as rapidly as it had developed, but was almost completely recovered at 30 min after the insult. This pattern of redistribution of cardiac output is very similar to those observed during maternal hypoxemia and umbilical cord occlusion (22,23). Whereas fractions of cardiac output distributed to the brain, heart, and adrenals increase, those to the peripheral organs fall

drastically. However, arresting uterine blood flow has some effects not seen in the other models, for example cardiac output falls markedly. Furthermore, there are differences in actual cerebral vascular resistance and in both blood flow and oxygen delivery to the brain, in that cerebral blood flow does not rise. Thus, oxygen delivery to the cerebrum falls, whereas that to the brainstem is maintained, reflecting a redistribution of brain blood flow in favour of brainstem areas (21). For the present study we preferred this model of fetal asphyxia, since in our laboratory almost all investigations on fetal cardiovascular mechanism have been performed in this experimental set up. This enables us to compare the newly obtained data to the results of our previous experiments.

Although preliminary experiments in our laboratory have shown sporadic alterations in fetal heart rate as well as arterial hypertension after intravenous application of lubeluzole in sheep near term, these observations could not be confirmed in the present study. As shown in Table 4, bolus injections of lubeluzole did not have any significant effects on fetal heart rate and mean arterial blood pressure. In addition, hardly any differences were observed between groups in the time course of physiological and cardiovascular variables measured before, during and after acute intrauterine asphyxia. Furthermore, it should be emphasized that in neither the treated nor the untreated fetuses did cerebral blood flow rise during asphyxia despite an increase in arterial blood pressure, suggesting that during acute asphyxia cerebral autoregulation was maintained and was not affected by lubeluzole. However, one might speculate that effects of lubeluzole on the fetal cardiovascular system could become evident after longer periods of asphyxia. Although the asphyxic insult in the present study lasted only 2 min, its severity is already considerable. Thus, at the end of asphyxia arterial oxygen saturation was nearly zero and combined ventricular output was reduced by 57%. As shown in one of our earlier studies, prolonging the asphyxic period for only two further minutes resulted in death of more than 50% of the fetusus within 10 to 35 min after the arrest of uterine blood flow was released (15). It is therefore obvious that under such experimental conditions pharmacological testing would not seem to be very sensible. To study the effects of lubeluzole on prolonged asphyxic periods an animal model, in which uterine blood flow is gradually reduced, has to be used.

The concentration of lubeluzole used in the present study was within the same range as that applied in previous investigations. In humans the injected dose lay between 2.5 and 25 mg/d, i.e. about 0.036 to 0.36 mg/kg/d (24-26), in rats lubeluzole was applied at a concentration of between 0.62 and 0.8 mg/kg (4,7,27). *In vitro* studies have even shown that lubeluzole has neuroprotective effects down to the pM range (molecular weight: 433,52 g/Mol) (5). As demonstrated in patients with acute ischemic stroke the mean distribution half-life of lubeluzole is 101 min and the mean terminal half-live 27.7 h, if the

drug is applied intravenously at a dose of 15 mg (24). Thus, in the present study the concentration of lubeluzole was within the therapeutic range and possible effects on the fetal cardiovascular system should have become evident during the observation period.

From the present study we conclude that lubeluzole does not affect circulatory responses to acute asphyxia in fetal sheep near term. These results are encouraging enough to justify designing *in vivo* experiments to test the neuroprotective properties of this drug.

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Chapter 7

LUBELUZOLE PRETREATMENT DOES NOT PROVIDE NEURO-PROTECTION AGAINST TRANSIENT GLOBAL CEREBRAL ISCHEMIA IN FETAL SHEEP NEAR TERM

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ABSTRACT

The aim of the present study was to test the neuroprotective effect of the novel benzothiazol compound lubeluzole on neuronal cell damage in fetal sheep arising from global cerebral ischemia. Thirteen fetal sheep were prepared at a mean gestational age of 127 ± 1 d (term is at 147 d). Six fetuses were treated with lubeluzole (0.33 mg/kg estimated body weight) before induction of global cerebral ischemia (-90, -60, and -30 min), while the remainder (n=7) received solvent. Cerebral ischemia was induced by occluding both carotid arteries for 30 min. Cerebral blood flow was measured by injecting radio-labelled microspheres before (-90 min), during (+3 min and +27 min) and after (+40 min, +3 h, and +72 h) cerebral ischemia. Neuronal cell damage was assessed in the cerebrum and deeper brain structures by light microscopy. Values are given as means ± SD. In control fetuses blood flow to the cerebrum was reduced from 100 ± 25 mL·100g^{-1}min^{-1} to less than 20 mL·100g^{-1}min^{-1} during ischemia. Shortly after ischemia hyperperfusion occurred (217 ± 66 mL·100g^{-1}min^{-1}) followed by a tendency towards hypoperfusion (72 ± 17 mL·100g^{-1}min^{-1}) later on (+3 h). Significant differences in blood flow to the various brain structures between the control and study groups could not be observed. Neuronal cell damage was concentrated in the parasagittal regions of the cerebrum. Pre-ischemic application of lubeluzole did not have any effect on the extent of neuronal cell damage. From these results we conclude that pretreatment with lubeluzole fails to protect the brain of fetal sheep near term from injury after transient global cerebral ischemia. However, since the observation period lasted only 3 days, a possible effect of lubeluzole on pathophysiological mechanisms inducing delayed neuronal cell death can not be fully excluded.

INTRODUCTION

Hypoxic-ischemic cerebral damage is an important contributor to perinatal mortality and morbidity including long-term neurologic sequelae in term and preterm fetuses (1). Although over the last decade many therapeutic strategies have been developed in various animal models to reduce neuronal cell damage caused by ischemic insults, their clinical application has often been rejected due to major drug related side-effects (2). Especially after application of calcium antagonists and inhibitors of nitric oxide synthase alterations of fetal cardiovascular control have been observed (3,4). However, the recently developed benzothiazole compound lubeluzole seems to justify further research in this field. The neuroprotective efficiency of this drug has been shown in a variety of *in vitro* as well as *in vivo* experiments (5-10). In clinical safety studies severe drug related side-effects could be excluded (11). Phase III trials have provided different results. Whereas Grotta described a neuroprotective effect of lubeluzole in patients suffering from acute ischemic stroke (12), this finding could not be confirmed in other trials (13,14).

The present knowledge on the safety profile and efficiency of lubeluzole seems to be promising enough to warrant further studies in immature animals. Recently, we were able to show that lubeluzole does not affect fetal circulatory centralisation during acute asphyxia. This mechanism is of major importance, since it protects the fetal brain from neuronal injury by increasing blood flow to the central organs, i.e. brain, heart, adrenals, when oxygen is in short supply (15). The present study was performed to clarify whether lubeluzole protects the brain of fetal sheep near term from neuronal cell damage. Global cerebral ischemia was induced by occluding both carotid arteries for 30 min. To account for drug-related changes in cerebral blood flow that might affect neuronal cell damage we measured cerebral blood flow using the microspheres method.

METHODS

Animal preparation

Thirteen fetal sheep were prepared at a mean gestational age of 127±1 days (term is at 147 days). All ewes were anesthetized by subarachnoid injection of 8 mL of 0.75% (wt/vol) bupivacaine at the lower spine, and were operated under sterile conditions. Polyvinyl catheters were placed in a maternal iliac artery and vein through tibial vessels. The ewe's abdominal wall was opened along the midline and the fetal hindlimbs were exposed through a small uterine incision. Using local anesthesia (prilocaine HCl 1.0%), polyvinyl catheters were inserted via the pedal vein of each hindlimb into the inferior vena cava. The uterine incision was closed and a second uterine incision was performed over the fetal

snout. Head and neck of the fetus were exteriorized. To prevent the fetus from breathing, its head was covered by a water-filled rubber glove. Catheters were inserted into the fetal ascending aorta via both brachial arteries. Furthermore, both fetal common carotid arteries were prepared. Cerebral ischemia was induced by occluding the carotid arteries on both sides simultaneously below the thyroid and above the lingual artery for 30 min as described recently (16,17). Thus, blood flow to the cerebrum via anastomoses between the carotid and vertebral arteries was arrested. After cerebral ischemia, a catheter was inserted into the amniotic cavity, and the second intrauterine incision was closed. All catheters were filled with heparin (1,000 IU/mL), plugged, and passed s.c. to the ewe's flank, where they were exteriorized and protected by a pouch sewn to the skin. On the day of surgery and each day thereafter, the ewe received 2 million units of penicillin G (Grünenthal, Germany) and 80 mg of gentamycin sulfate (Merck, Germany), half i.v. and half into the amniotic cavity.

Experimental Protocol

After insertion of polyvinyl catheters into the inferior vena cava 6 fetuses received three bolus injections of lubeluzole at 30 min intervals (3 x 0.11 mg / kg estimated fetal body weight), while an equal volume (3 x 1.3 mL) of the solvent was administered to the remaining fetuses. The drugs were donated by Janssen Pharmaceutica, Beerse, Belgium. The concentration of lubeluzole used in the present study was within the same range as that applied in previous investigations. In humans the injected dose ranged from 2.5 to 25 mg / d, i.e. about 0.036 to 0.36 mg / kg / d (12,13,18), while in rats lubeluzole was applied at a concentration of between 0.62 and 0.8 mg / kg (9,10,19). *In vitro* studies have even shown that lubeluzole has neuroprotective effects down to the pM range (molecular weight: 433,52 g / Mol) (7). In preliminary experiments we applied lubeluzole to fetal sheep by a single i.v. injection at a concentration of 0.33 mg / kg. This resulted in alterations of fetal heart rate as well as arterial hypotension. We therefore considered the dosage applied in the present study to be at the upper limit of the therapeutic range in fetal sheep near term. A further increase in the administered concentration might have altered fetal cardiovascular control and would therefore have interferred with a possible neuroprotective effect of lubeluzole.

Thirty min after the final injection of lubeluzole cerebral ischemia was induced. To determine the time course of changes in fetal cerebral blood flow six batches of differently isotope-labelled microspheres (^{141}Ce, ^{114}In, ^{113}Sn, ^{103}Ru, ^{95}Nb & ^{46}Sc, 16 µm diam., New England Nuclear) were used. The microspheres, suspended in 10% dextran containing 0.01% Tween 80, were sonicated and checked for size, shape and aggregation. Depending

on the specific activity, 1.2 - 1.8 million microspheres per batch were injected into the inferior vena cava before (-90 min), during (+3 min and +27 min), and after (+40 min, +3 h, and +72 h) 30 min of cerebral ischemia. The number of injected microspheres was large enough to ensure both an adequate number of microspheres per sample and valid blood flow measurements during cerebral ischemia (20,21). Specific calculations revealed that about 400 microspheres were trapped in low flow cerebral areas during ischemia. Thus, for theoretical considerations the blood flow estimates in these areas are within 5 % of the true values (20). During and shortly after injection of the microspheres no significant changes in fetal heart rate or arterial blood pressure were found. Reference blood samples were withdrawn from the brachial artery at a rate of 2.5 $mL \cdot min^{-1}$ for 90 s (16,17). The volume of blood withdrawn was about 22.5 mL and was simultaneously replaced by maternal blood maintained at 39 °C in a water bath.

During and shortly after cerebral ischemia fetal heart rate, ascending aortic and intrauterine pressure were recorded simultaneously. After having injected the fourth batch of microspheres (at +40 min) all catheters were closed and secured as described above, the abdominal wall was closed and the ewe was brought back to the metabolic cage, where the fifth injection (at +3 h) was performed. For technical reasons there were no pressure measurements at this point in time. Before each injection, blood samples were obtained from the brachial artery to measure blood gases, oxygen saturation of hemoglobin, and acid-base balance. At the end of the experiment (at +72 h) the ewe was given a lethal dose of sodium pentobarbitone and saturated potassium chloride intravenously, while the fetus was perfused with 300 mL of formalin (15 %, wt/vol, saline).

The experimental protocols were approved by the appropriate institutional review committee and met the guidelines of the governmental agency responsible.

Measurements

Ascending aortic and intrauterine pressure, and fetal heart rate were recorded on a polygraph (Hellige, Germany). Blood gases and pH were measured in an automatic blood gas analyzer (278 Blood Gas System, Ciba Corning, Frankfurt, Germany), and base excess was calculated. Hb concentration and oxygen saturation of Hb were measured photometrically (OSM 2 Hemoximeter, Radiometer, Copenhagen, Denmark) in duplicate.

Fetal cerebral blood flow and the extent of neuronal cell damage were assessed in identical brain specimens. To determine fetal cerebral blood flow using the microspheres method the fetal brain was removed and fixed in formalin for at least 7 days. Afterwards the

cerebrum was separated from the basal ganglia and divided into four frontal sections (rostral, pericentral, postcentral, occipital) with a thickness of about 1.5 cm. The right and left parts of these four sections were further subdivided into four equal segments (parasagittal 1 and 2, lateral 1 and 2) each weighing 1 - 2 g. In addition to these 32 cerebral specimens, caudate nucleus, thalamus, hippocampus, tegmentum-colliculi-pons, cerebellum and medulla oblongata were separated. These brain structures were placed in vials, which were filled to the same height to reduce variations in geometry during gamma counting.

After cerebral blood flow analysis the specimens of the fetal brain were removed from the counting vials and embedded in paraffin. Coronal subserial sections of 10 µm were obtained and stained with cresylviolet/fuchsin. Every 40th section was mounted to evaluate the extent of neuronal cell damage. Neuronal cell damage was assessed at a magnification of 250x. Neurons with ischemic cell damage were identified according to the criteria of Brown and Brierley (22). Neuronal cell damage in each microscopical visual field was quantified by the following score: 0-5 % damage (score 1), 5-50 % damage (score 2), 50-95 % damage (score 3), 95-99 % damage (score 4), and 100 % damage (score 5). In the present investigation a scoring system was preferred, since evaluation of neuronal cell loss by neuron counts was unfeasible due to the considerable number of histological sections studied. As assessed by the coefficient of variation the intra- and inter-observer reliability of this procedure was 4 and 10 %, respectively (17).

Calculations

Fetal cerebral blood flow was calculated from counts of the injected nuclide recovered in the fetal cerebrum and the appropriate reference sample, and from the withdrawal rate of the reference sample (16,17). The histological score of each cerebral specimen was calculated by averaging the scores of all visual fields analyzed from three sections of that specimen. The number of visual fields evaluated per specimen ranged between 400 and 500 each. The scores from corresponding specimens from the right and left hemisphere were averaged.

Statistics

Values are given as means ± SD. The data were analysed for intra- and intergroup differences by two-way multivariate analysis of variance for repeated measures. The Games-Howell-test was used as a post-hoc testing procedure.

RESULTS

The physiological variables of the control and study groups before, during and after 30 min of global cerebral ischemia are shown in Table 1. Before injection of lubeluzole or carrier no significant differences in these variables were found between the control and study groups. At control the plasma concentrations of both glucose and lactate were slightly above the normal range for chronically prepared fetal sheep (23), but had normalized by the end of the experiments.

Table 1. Physiological Variables, Acid-Base Balance, and Blood Gases Before, During and After Transient Global Cerebral Ischemia in Fetal Sheep Near Term

Group	Control	Ischemia 3′	Ischemia 27′	Recovery 40′	Recovery 3 h	Recovery 72 h
Fetal Heart Rate (beats/min)						
C	166,3 ± 15,1	190,5 ± 17,0	197,8 ± 31,3 d	204,3 ± 33,4 e	n.m.	159,9 ± 9,2
L	170,8 ± 23,4	169,0 ± 27,6	202,4 ± 17,0 d	168,0 ± 20,6 b	n.m.	155,7 ± 19,6
Mean Arterial Pressure (mmHg)						
C	51,5 ± 13,1	67,6 ± 15,9 d	60,8 ± 14,0	55,6 ± 11,4	n.m.	43,3 ± 6,0
L	55,9 ± 12,6	68,3 ± 8,7	64,0 ± 6,5	54,4 ± 4,6	n.m.	35,8 ± 6,4 e
pH						
C	7,35 ± 0,03	7,36 ± 0,03	7,33 ± 0,06	7,32 ± 0,06	7,37 ± 0,03	7,39 ± 0,02 d
L	7,35 ± 0,04	7,35 ± 0,04	7,34 ± 0,05	7,33 ± 0,06	7,36 ± 0,03	7,38 ± 0,02
Oxygen Saturation (%)						
C	67,7 ± 9,0	65,3 ± 14,3	65,9 ± 12,2	65,2 ± 9,28	77,1 ± 4,0	71,4 ± 9,3
L	60,8 ± 9,4	60,9 ± 10,5	62,6 ± 12,5	67,0 ± 15,1	72,7 ± 7,1	61,4 ± 7,3
Oxygen Content (vol%)						
C	9,6 ± 1,5	9,3 ± 2,0	9,4 ± 1,4	9,1 ± 1,4	10,0 ± 1,3	8,5 ± 1,7
L	9,8 ± 1,0	9,8 ± 1,7	9,9 ± 1,8	10,1 ± 1,7	10,9 ± 1,2	8,9 ± 1,4
Base Excess						
C	0,2 ± 1,5	0,2 ± 1,4	-1,5 ± 2,4	-3,1 ± 2,5 e	2,6 ± 1,8 d	3,9 ± 1,3 f
L	1,5 ± 1,4	1,3 ± 1,4	-0,2 ± 1,6	-1,0 ± 1,7 d	1,7 ± 1,4	3,5 ± 2,2 d
Glucose (mg/ml)						
C	56,7 ± 17,8	58,6 ± 13,4	62,7 ± 16,4	58,1 ± 19,0	31,6 ± 11,6 e	18,6 ± 4,2 f
L	54,2 ± 11,7	57,2 ± 16,7	60,7 ± 14,4	56,3 ± 17,2	40,8 ± 15,3	20,8 ± 6,9 f
Lactate (mmol/l)						
C	3,7 ± 0,9	3,6 ± 1,0	4,8 ± 1,7	5,6 ± 1,8 e	4,0 ± 0,9	1,3 ± 0,2 f
L	3,1 ± 1,0	3,2 ± 0,9	4,2 ± 0,9	4,8 ± 1,1 d	3,5 ± 1,1	1,1 ± 0,1 e

C: untreated fetuses (n=7); L: treated fetuses (lubeluzole; n=6). Values are given as means ± SD
Significant differences between groups are indicated by: a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ and within groups vs time point ′control′ by d: $p < 0.05$; e: $p < 0.01$; f: $p < 0.001$
n.m. = not measured

Neuronal cell damage was most pronounced in the parasagittal regions, whereas in the more lateral part of the cortex only minor neuronal damage could be seen (Table 3). Furthermore, cell damage was observed in the cerebellum, hippocampus and caudate nucleus (Table 4). There were almost no significant differences in neuronal cell damage in any parts of the cerebrum or deeper brain structures between treated and untreated fetuses (Tables 3 and 4).

Table 2. Blood Flow to Various Brain Areas Before, During and After Transient Global Cerebral Ischemia in Fetal Sheep Near Term

	Organ Blood Flows (ml/min/100g)				
Group	**Control**	**Ischemia 3´**	**Ischemia 27´**	**Recovery 40´**	**Recovery 3 h**	**Recovery 72 h**
Brain total						
C	141,1 ± 35,7	23,6 ± 11,4 f	34,8 ± 13,5 f	309,5 ± 77,8 f	106,7 ± 25,2	227,0 ± 45,7 f
L	144,3 ± 46,3	15,0 ± 11,0 f	25,8 ± 8,4 f	267,7 ± 68,4 f	102,4 ± 33,3	233,1 ± 66,7 f
Cerebrum						
C	100,2 ± 24,7	15,7 ± 7,8 f	22,3 ± 11,0 f	217,0 ± 65,6 f	72,1 ± 16,6	160,9 ± 34,9 e
L	102,9 ± 34,1	8,6 ± 7,4 f	16,7 ± 6,5 f	198,6 ± 62,2 f	71,6 ± 24,6	163,8 ± 51,2 e
Cerebellum						
C	151,2 ± 64,4	27,1 ± 19,6 e	49,7 ± 22,6 e	413,6 ± 146,8 f	163,8 ± 67,6	261,3 ± 85,6 e
L	156,4 ± 56,7	19,2 ± 16,1 f	38,0 ± 10,3 e	335,7 ± 67,1 a,f	131,4 ± 30,3	261,1 ± 84,2 d
Hippocampus						
C	91,7 ± 18,3	21,9 ± 15,7 f	33,8 ± 16,0 e	233,9 ± 87,0 f	66,9 ± 13,6	162,8 ± 39,2 f
L	101,1 ± 31,6	15,9 ± 13,5 f	27,2 ± 16,5 f	177,7 ± 36,8 b,f	70,8 ± 28,6	168,0 ± 43,1 e
Caudate nucleus						
C	120,8 ± 33,9	33,4 ± 27,2 e	37,7 ± 15,0 e	264,9 ± 89,0 f	94,2 ± 32,0	198,6 ± 90,1 e
L	116,2 ± 32,4	8,9 ± 4,1 f	27,1 ± 9,3 e	217,2 ± 67,8 f	90,2 ± 33,0	194,9 ± 63,2 e
Medulla oblongata						
C	201,4 ± 56,3	61,4 ± 28,2 f	67,8 ± 30,8 e	254,4 ± 145,5	199,7 ± 73,7	354,2 ± 82,8 f
L	196,5 ± 69,0	37,4 ± 21,6 f	51,8 ± 14,6 e	234,6 ± 122,5	147,9 ± 41,0	312,1 ± 79,2 e

C: untreated fetuses (n=7); L: treated fetuses (Lubeluzole; n=6); Values are given as means ± SD.
Significant differences between groups are indicated by:
a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ and within groups vs time point ´control´ by d: $p < 0.05$; e: $p < 0.01$; f: $p < 0.001$

Table 3. Neuronal Cell Damage in the Cerebrum of Fetal Sheep Near Term 72 h after Global Cerebral Ischemia

		Segments			
		Parasagittal		Lateral	
		1	2	1	2
Frontal	C	1,73 ± 0,61	1,49 ± 0,53	1,55 ± 0,69	1,71 ± 0,82
	L	1,72 ± 0,63	1,68 ± 0,67	1,72 ± 0,69	1,75 ± 0,71
Pericentral	C	2,49 ± 0,97	1,93 ± 0,70	1,58 ± 0,89 *	1,16 ± 0,38 **
	L	2,38 ± 1,01	2,10 ± 1,07	1,64 ± 0,59	1,42 ± 0,47 *
Postcentral	C	2,74 ± 1,20	2,34 ± 1,01	1,79 ± 0,71 #	1,16 ± 0,35 ##
	L	2,54 ± 1,11	2,16 ± 0,97	1,77 ± 0,61	1,56 ± 0,61 #
Occipital	C	2,18 ± 1,35	2,33 ± 1,36	2,17 ± 1,24	1,94 ± 1,13
	L	2,06 ± 0,84	2,13 ± 0,85	2,07 ± 0,82	1,88 ± 0,75

C: untreated fetuses (n=7); L: treated fetuses (lubeluzole; n=6) Values are given as means ± SD.
* = P≤0,05; ** = P≤0,01 vs. Parasagittal segment 1 - Perizentral
# = P≤0,05; ## = P≤0,01 vs. Parasagittal segment 1 - Postzentral

Table 4. Neuronal Cell Damage in Various Areas of the Diencephalon, Mesencephalon and Brain Stem in Fetal Sheep Near Term 72 Hours After 30 Min of Global Cerebral Ischemia

Cerebellum	C	1,41 ± 0,42
	L	1,65 ± 0,40
Hippocampus	C	1,71 ± 0,71
	L	2,22 ± 0,57
Caudate nucleus	C	1,10 ± 0,11
	L	1,21 ± 0,15
Medulla	C	1,00 ± 0,00
	L	1,00 ± 0,00

C: untreated fetuses (n=7); L: treated fetuses (lubeluzole; n=6)
Values are given as means ± SD. Significant differences between groups could not be detected.

DISCUSSION

Lubeluzole, the S-isomer of a novel 3,4-difluoro benzothiazole, has been shown to reduce ischemic neuronal cell damage in a variety of *in vitro* as well as *in vivo* studies. The neuroprotective property of lubeluzole may result from various effects on neuronal tissue.

First, lubeluzole has been shown to block ischemia-induced increases in extracellular levels of glutamate and may therefore reduce excitotoxic cell injury (24). Second, lubeluzole inhibits glutamate-stimulated nitric oxide production (25). Nitric oxide combines with superoxide anions to synthesize peroxynitrite, a compound that spontaneously decomposes to form hydroxyl radicals, nitrogen dioxide and NO^{2+}. All these radicals are able to destroy cell membranes and various intracellular structures. Third, lubeluzole has been shown to activate voltage-sensitive Ca^{2+} channels in isolated rat dorsal root ganglion cells thus possibly reducing the tremendous intracellular influx of this ion during an ischemic insult (26). This so-called calcium overload leads to cell damage by activating proteases, lipases and endonucleases (27). Fourth, lubeluzole decreased DNA fragmentation and annexin-V binding in primary hippocampal neurons (28,29). Since these two phenomena are specific markers of apoptosis, lubeluzole might protect neurons from ischemic injury through its inhibitory effects on pathophysiological pathways that trigger the cellular suicide programme. However, these last three lubeluzole-mediated effects have so far only been observed in neuronal tissue *in vitro*. Their *in vivo* significance has still to be confirmed.

Unfortunately, in the present study we were unable to show any neuroprotective effect of lubeluzole on neuronal cell damage in fetal sheep near term after global ischemia. This appears to be inconsistent with various investigations on adult animals (5-10). However, in almost all of these studies models of focal cerebral ischemia were used (5-9). This type of cerebral ischemia is characterized by an ischemic core and a peri-infarct region known as ischemic penumbra (30,31). In this area, brain tissue is perfused at a level between the thresholds of functional impairment and of morphological integrity. Unless cerebral blood flow is rapidly improved in this region within a few hours after the insult, neuronal cell damage is the inevitable result (30,31). As shown in previous studies, glutamate is released in tremendous amounts from the infarct core into the extracellular space after focal ischemia. Increases of up to 80 times above baseline levels have been observed (32). The released glutamate activates the neuronal NO-synthase via calcium influx through NMDA-regulated calcium channels (33,34). In focal ischemia this pathway may be of greater importance for the development of neuronal cell damage than in global ischemia, since in the latter type of ischemia only a moderate and short-lasting increase in glutamate release has been observed (35,36). This may explain in part the differences in neuronal outcome between the present study and previous investigations after treatment of brain injury by lubeluzole. In addition, many other pathophysiological mechanisms such as inhibition of protein synthesis, generation of epileptogenic impulses etc are differently regulated in focal and global ischemia and may therefore alter the neuroprotective efficiency of lubeluzole (37-41).

However, there is one study in adult rats indicating that lubeluzole protects the brain from neuronal cell loss after global ischemia (10). Posttreatment with lubeluzole significantly increased the number of viable neurons in the hippocampus. There could be a number of reasons for the difference in neuronal outcome between this study and our experiments. First, the postischemic pattern of pathophysiological changes in the immature brain may not be the same as in the adult. For example, glutamate release has been reported to be significantly lower in neonates than in adults (42). Although glutamate toxicity has been reported in fetal sheep after cerebral ischemia, this may also affect the induction of NO-synthase via NMDA-receptor regaluted calcium channels. Since the neuroprotective properties of lubeluzole are mediated in part by its effects on the NO-system (25), this may explain the differences in neuronal outcome between the present study and that of Haseldonckx (10). Furthermore, inhibition of the NO-synthase in the fetal sheep had no influence on neuronal cell damage, indicating that NO release may not play a major pathophysiological role in the development of brain injury under these conditions (43). Second, in our study lubeluzole was injected before cerebral ischemia, whereas Haseldonckx and co-workers applied the drug shortly after the insult (10). One might therefore speculate that bolus injection of lubeluzole before ischemia results in an insuffiencient drug supply during the postischemic period. However, as demonstrated in patients with acute ischemic stroke, the mean terminal half-life of this compound in the plasma is 27.7 h (18). Thus, application of lubeluzole one hour before ischemia guarantees postischemic plasma concentrations that are within the therapeutic range. When designing the present study a pretreatment protocol was chosen, since we considered this to be more effective than a posttreatment strategy. Especially under clinical conditions, pretreatment of babies at risk of hypoxic-ischemic encephalopathy by maternal application of various neuroprotective drugs would seem conceivable. Whether an additional application of lubeluzole after cerebral ischemia provides neuroprotection in the present experimental model, will have to be examined in further investigations. Third, the endpoint in our study and that of Haseldonckx and co-workers was different. Whereas neuronal cell damage was evaluated only 3 days after the insult in the present investigation, it was not assessed until 7 days after cerebral ischemia in the latter study. Unfortunately, the sheep model does not allow for longer postischemic observation periods, since this would result in a tremendous increase in the abortion rate due to intrauterine infection and fetal distress. It is conceivable that even several days after the ischemic insult the continued activation of the inducible form of NO-synthase in astrocytes and microglia might result in an increased production of NO (44). One might therefore object that delayed neuronal cell death due to an increased NO production cannot be evaluated using this model (45-47). Since modulation of NO toxicity is a probable target for lubeluzole (25), the negative result of the present study might be due to this phenomenon. This objection cannot be fully excluded. However, as

mentioned above NO toxicity may not play any major part in the development of neuronal cell death in this model, since inhibition of the NO system had no effect on brain injury (43). Furthermore, as shown in a recent study using the MRI-technique, neuroprotective effects of lubeluzole can already be observed within a few hours after ischemia (9). If the main target of lubeluzole is NO toxicity, then a neuroprotective effect should have been observed as early as 3 days after ischemia as demonstrated in a recent study on neonatal rats using an inhibitor of the NO-synthase (48). To exclude very delayed neuroprotective effects of lubeluzole on the neuropathologic outcome in immature animals additional experiments will have to be performed using other ischemic models, e.g. unilateral carotid occlusion in the neonatal rat (49,50). However, it should be taken into account that drug-related side effects of various neuroprotective substances, such as lubeluzole, on the cardiovascular system cannot be monitored in such small animals. We therefore feel that in a first approach the present model was highly appropriate for investigation of the neuroprotective efficacy of lubeluzole.

As already shown for a variety of other drugs (for review: 40), the neuroprotective properties of lubeluzole might depend on the type of ischemia studied and the animal model used. It is therefore conceivable that lubeluzole might protect the fetal brain from ischemic insults in animal models of systemic asphyxia induced by occlusion of the umbilical cord or the uterine arteries. Neuronal loss has been observed after repetitive cord occlusion in the striatum, an area with high glutaminergic input (51). Since lubeluzole is known to inhibit glutamate release during an ischemic insult (24), this drug may be neuroprotective in such an animal model. However, when brain injury is induced by systemic asphyxia the extent and location of neuronal cell loss often vary widely (52-54) and there is a considerable rise in the fetal abortion rate. Since hardly any neuroprotective effect can be demonstrated under such conditions, we preferred the fetal sheep model of global cerebral ischemia caused by occlusion of both carotid arteries. The fact that lubeluzole did not appear to have any neuroprotective effect in the present model cannot be attributed to a lack of glutamate toxicity. As already shown in a previous study the application of MK-801 after global cerebral ischemia caused a significant improvement in neuropathological outcome in fetal sheep (55).

From the present results, we conclude that pretreatment with lubeluzole fails to protect the brain of fetal sheep near term from injury after transient global cerebral ischemia. However, since the observation period lasted only 3 days, a possible effect of lubeluzole on pathophysiological mechanisms inducing delayed neuronal cell death can not be fully excluded.

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Chapter 8

NEUROPROTECTIVE EFFECTS OF MAGNESIUM ON METABOLIC DISTURBANCES IN FETAL HIPPOCAMPAL SLICES AFTER OXYGEN-GLUCOSE DEPRIVATION: MEDIATION BY NITRIC OXIDE SYSTEM

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ABSTRACT

The aim of the present study was to investigate the effects of magnesium on metabolic disturbances in hippocampal slices prepared from fetal guinea pigs after oxygen-glucose deprivation (OGD). Metabolic disturbances were assessed by measuring changes in energy metabolism and protein synthesis. In addition we determined cGMP concentrations in the slices after OGD, as a measure of nitric oxide (NO) production, to clarify whether a possible neuprotective effect of magnesium is mediated in part through the NO-system. Twelve hours after oxygen-glucose deprivation ATP concentration and protein synthesis in the hippocampal slices were significantly reduced depending on the severity of OGD. Increasing magnesium concentration in the incubation medium from 1.3 mM to 3.9 mM 2 h before OGD significantly improved the recovery of ATP and protein synthesis, whereas treatment after OGD was ineffective. cGMP concentrations rose dramatically in hippocampal slices 10 min after OGD indicating a significant increase in NO-production. When the concentration of magnesium in the aCSF was increased 2 h before OGD the rise in tissue levels of cGMP was considerably reduced. Again, treatment after OGD had no effect. From the present study we conclude that increasing magnesium concentration in the aCSF before OGD alleviates metabolic disturbances in hippocampal slices from mature fetal guinea pigs, whereas treatment after OGD has no effect. This neuroprotective property of magnesium may be mediated in part through the inhibition of NO-production shortly after OGD.

INTRODUCTION

Children undergoing hypoxic-ischemic brain damage during birth often suffer from the drastic consequences of this misfortune for the rest of their lives (1). Despite the many far-reaching consequences of this kind of perinatal insult, the range of effective therapeutic measures available is limited (2). In addition, many so-called neuroprotective drugs such as glutamate antagonists cannot be applied during the perinatal period owing to side-effects on the developing brain (3). The retrospective analysis by Nelson and Grether showing that magnesium, a well-known tocolytic agent, also has neuroprotective properties was therefore very encouraging. In fact, the incidence of cerebral palsy in newborns weighing less than 1500 g was significantly lower, if the mother received magnesium before birth (4,5). Unfortunately, a subsequent study of the same research group could not confirm the neuroprotective efficacy of magnesium (6). The authors attributed this to changes in medical practice between the two observation periods. Furthermore, experimental studies with differing perinatal animal models have also yielded inconclusive results with regard to magnesium neuroprotection. Both protective (7-15) and non-protective effects (14,16,17) have been reported with different timing of administration at different stages of development. As recently proposed, the neuroprotective inefficacy of magnesium, especially when applied after cerebral ischemia, may be caused by its hypotensive effects, since magnesium, as a calcium antagonist has vasodilatory properties (14). Furthermore, the pathophysiological mechanisms through which a possible neuroprotective effect of magnesium could be mediated are not well understood. One pathway may be the NO-system. NO is a free radical synthesized by NO-synthase (NOS) in neurons and other cell types in response to rises in levels of intracellular calcium during and after ischemia. During reperfusion, NO and superoxide radicals combine to produce peroxynitrite, leading to the formation of more potent radicals. Destruction of cerebral tissue is the inevitable result (18). Since magnesium has been reported to block cerebral NMDA-receptors which themselves control calcium channels (13), the neuroprotective effect of magnesium may be mediated through an inhibition of NO-production after ischemia brought about by a reduction of neuronal calcium influx.

The aim of the present study was therefore to investigate the effects of magnesium on metabolic disturbances in fetal brain during and after ischemia, while excluding its effects on the cardiovascular system. For this purpose we used the *in vitro* system of oxygen-glucose deprivation (OGD) in hippocampal slices prepared from mature guinea pig fetuses. Metabolic disturbances were assessed by measuring changes in energy metabolism and protein synthesis. In addition we determined cGMP concentrations in the slices after OGD, as a measure of NO-production, to clarify whether a possible neuprotective effect of magnesium is mediated in part through the NO-system (19,20).

METHODS

The present study was performed on guinea pigs at 0.9 gestation (term is at 68 days). The dams were anesthetized with halothane and 34 fetuses were delivered by Caesarean section. The fetal hippocampi were dissected out and cut into 500 µm thick, transverse slices. The tissue slices were transferred to an incubation chamber, containing artificial cerebrospinal fluid (standard-aCSF). To prevent bacterial contamination 10 mg / L each of streptomycin and erythromycin were added to the aCSF. Additionally, aCSF was filtered (pore diameter 0.1 _m) into sterilized containers, and $CuSO_4$ was added to the incubation chamber reservoir. aCSF was pumped through the incubation chamber at a rate of 1 ml / min (21,22). The aCSF was equilibrated with a gas mixture of oxygen and carbon dioxide (95% O_2 / 5% CO_2), and the incubation temperature was held at 37°C. To allow recovery from preparation stress the slices were incubated in standard aCSF for 90 min. The magnesium concentration of the aCSF in the study groups was increased from 1.3 mM to 3.9 mM either at the end of this initial recovery period or immediately after OGD. Ninety minutes after the initial recovery period the glucose concentration of the standard aCSF was lowered from 10 mM to 2 mM for 30 min to accelerate the breakdown of high-energy phosphates in the tissue slices during exposure to OGD (19,21).

The experimental protocol included a 210 min preincubation phase (180 min in 10 mM glucose and 30 min in 2 mM glucose aCSF), an ischemic phase (10 - 40 min) and a recovery phase (12 h starting from the end of OGD). A separate incubation chamber, equilibrated with 95% N_2 / 5% CO_2, was used for the induction of OGD. In contrast to the standard aCSF, the ischemic aCSF contained no glucose or HEPES. HEPES was omitted, because its buffering capacity may influence the fall in pH accompanying OGD. Before the tissue slices were transferred to the anoxic incubation chamber, they were washed in aglycemic aCSF in order to lower the glucose levels in the tissue still further. During OGD the tissue slices were completely submerged in the aCSF. No additional aCSF was pumped through the chamber during this period (flow rate: 0 ml / min). In the postischemic phase the tissue slices were transferred back to standard aCSF (flow rate: 1 ml / min) and equilibrated with carbogen (95% O_2 / 5% CO_2). After a recovery period of 12 h the slices were sampled to measure the tissue concentrations of adenine nucleotides and protein synthesis.

In a second set of experiments we investigated whether a possible neuroprotective effect of magnesium might be mediated in part through the NO-system. Here we determined the tissue concentrations of cGMP 10 min after an OGD-period of between 10 and 40 min using a RIA (NEN, Bad Homburg, Germany). For these measurements tissue slices were frozen in liquid nitrogen and extracted with perchloric acid. As in the first set of

experiments, the magnesium concentration in the incubation medium of the study groups was raised from 1.3 mM to 3.9 mM either 2 h before or immediately after OGD. To confirm that elevated tissue concentrations of cGMP really reflect increased NO-production, we inhibited NO-synthase with the specific blocker N-nitro-L-arginine (L-NNA). L-NNA (100 µM) was added to the incubation medium 30 min prior to, during and 10 min after OGD. By these measures the increase of cGMP tissue concentration 10 min after OGD could be completely suppressed. This observation confirms previous studies showing the same effect (19,20).

To determine the tissue concentrations of adenine nucleotides the slices were frozen in liquid nitrogen. ATP, ADP and AMP were measured by high pressure liquid chromatography (HPLC) after extraction with perchloric acid (21). The adenylate energy charge (AEC), a measure of the relation of energy consumption to energy production, was estimated from the following formula: AEC = ([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP]) (23). The protein content of the tissue slices was measured by the Lowry method (24).

Protein synthesis was assessed from the incorporation rate of ^{14}C-leucine into tissue proteins. After 30 min of incubation in standard aCSF, to which 5 µCi/mL L-[1-^{14}C] leucine (Amersham Buchler, Braunschweig, Germany; specific activity 54 mCi/mMol) had been added, the tissue slices were homogenized in trichloroacetic acid (TCA). The radioactivity of the TCA-precipitated material was then measured by liquid scintillation counting (21,25).

All data are given as means ± SD. The statistical significance of differences between groups was assessed by a two-way analysis of variance (ANOVA), followed by the Scheffé post-hoc test. There were no repeat studies in a single preparation and no data was discarded. The experimental protocols were approved by the appropriate institutional review committee and met the guidelines of the governmental agency responsible.

RESULTS

The concentrations of ATP measured in hippocampal slices from mature guinea pig fetuses are shown in Fig. 1 as percentages of the control values. The ATP tissue levels in the two untreated control groups averaged 20.3 ± 2.3 and 21.2 ± 2.4 µmol / mg protein,

respectively. At the end of the recovery period, ATP concentration in the hippocampal slices was significantly reduced depending on the length of OGD. The tissue content of

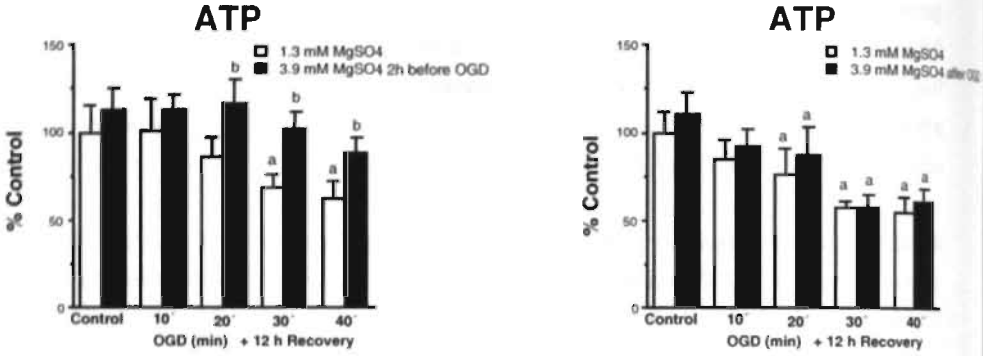

Figure 1. ATP tissue concentration (% of control) in hippocampal slices of mature guinea pig fetuses 12 h after oxygen-glucose deprivation (OGD). OGD lasted between 10 and 40 min. The ATP tissue levels in the two untreated control groups (open bars) averaged 20.3 ± 2.3 and 21.2 ± 2.4 µmol / mg protein, respectively. Magnesium concentration in the aCSF of the study groups was increased from 1.3 mM to 3.9 mM either 2 h before or immediately after OGD. Values are given as means ± SD. Each group comprised 5 slices. Intra- and intergroup differences were measured by a two-way analysis of variance followed by Scheffé-F-test (a: $P < 0.05$ OGD vs. Control; b: $P < 0.05$ $MgSO_4$ vs. Control).

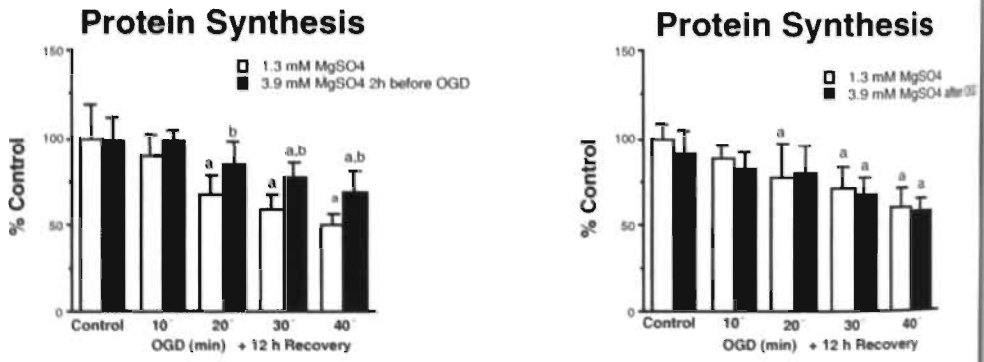

Figure 2. Protein synthesis rate (PSR) (% of control) in hippocampal slices of mature guinea pig fetuses 12 h after oxygen-glucose deprivation (OGD). OGD lasted between 10 and 40 min. PSR in the two untreated control groups (open bars) averaged 124 ± 23 and 125 ± 11 dpm / 30 min / µg protein, respectively. Magnesium concentration in the aCSF of the study groups was increased from 1.3 mM to 3.9 mM either 2 h before or immediately after OGD. Values are given as means ± SD. Each group comprised 5 slices. Intra- and intergroup differences were measured by a two-way analysis of variance followed by Scheffé-F-test (a: $P < 0.05$ OGD vs. Control; b: $P < 0.05$ $MgSO_4$ vs. Control).

adenylates together with the adenylate energy charge in hippocampal slices are given in Table 1. Total adenylate pool in the two untreated control groups averaged 22.4 ± 2.9 and 23.8 ± 2.5 µmol / mg protein and the adenylate energy charge 0.92 ± 0.03 and 0.93 ± 0.02, respectively.

The changes in total adenylate pool after OGD closely resembled those in ATP concentrations (Table 1). Adenylate energy charge scarcely differed at all from control values at the end of the recovery period. Fig. 2 illustrates PSR in hippocampal slices measured as incorporation of ^{14}C-leucine into tissue proteins. PSR in the two untreated control groups averaged 124 ± 23 and 125 ± 11 dpm / 30 min / µg protein, respectively. Like energy metabolism, PSR was severely disturbed by OGD depending on the length of the insult. Increasing magnesium concentration in the aCSF 2 h before OGD significantly improved the recovery of energy metabolism and protein synthesis, whereas treatment after OGD was ineffective.

The cGMP concentrations significantly increased in hippocampal slices 10 min after OGD lasting 10 to 40 min. This rise could be completely suppressed by blocking NO synthase with the specific blocker N-nitro-L-arginine (L-NNA) (Fig. 3). When the magnesium concentration in the aCSF was increased 2 h before OGD the rise in tissue levels of cGMP was considerably reduced. Again, treatment after OGD had no effect (Fig. 4).

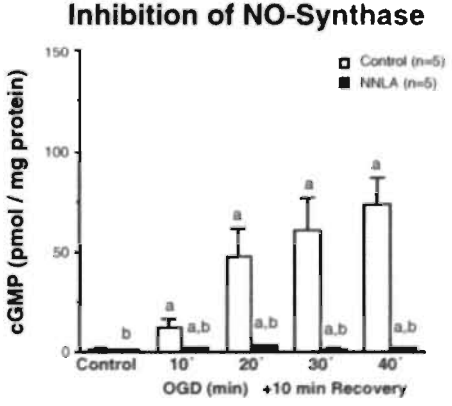

Figure 3 Concentration of cGMP in hippocampal tissue slices after different periods of OGD (10 – 40 min) and a recovery phase of 10 min. NO-synthase was inhibited by incubating slices in 100 µM L-NNA. The cGMP concentration in the slices of the control group (open bar) was 1.47 ± 0.52 pmol / mg protein. Values are given as means ± SD. Each group comprised 5 slices. Intra- and intergroup differences were measured by a two-way analysis of variance followed by Scheffé-F-test (a: $P < 0.05$ OGD vs. Control; b: $P < 0.05$ L-NNA vs. Control).

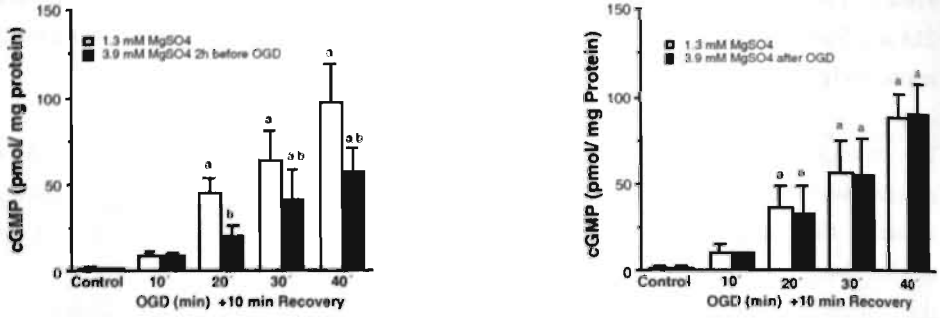

Figure 4. Concentration of cGMP in hippocampal slices of mature guinea pig fetuses 10 min after oxygen-glucose deprivation (OGD). OGD lasted between 10 and 40 min. The cGMP concentration in the slices of the untreated control groups (open bars) averaged 1.53 ± 1.23 and 1.47 ± 0.52 pmol / mg protein, respectively. Magnesium concentration in the aCSF of the study groups was increased from 1.3 mM to 3.9 mM either 2 h before or immediately after OGD. Values are given as means ± SD. Each group comprised 5 slices. Intra- and intergroup differences were measured by a two-way analysis of variance followed by Scheffé-F-test (a: $P < 0.05$ OGD vs. Control; b: $P < 0.05$ $MgSO_4$ vs. Control).

Table 1. Total Adenylate Pool (TAN) and Adenylate Energy Charge (AEC) in Hippocampal Slices 12 h after Oxygen-Glucose Deprivation (OGD).

	Total Adenylate Pool			
Time (min)	$MgSO_4$ 2 h before OGD		$MgSO_4$ after OGD	
	Control	$MgSO_4$	Control	$MgSO_4$
Control	100 ± 17	106 ± 11	100 ± 11	107 ± 10
OGD 10	97 ± 18	106 ± 8	83 ± 10	90 ± 10
OGD 20	84 ± 12	108 ± 13 b	74 ± 13 a	84 ± 15 a
OGD 30	66 ± 9 a	95 ± 8 b	58 ± 4 a	58 ± 6 a
OGD 40	60 ± 9 a	82 ± 7 a,b	56 ± 9 a	62 ± 6 a

	Adenylate Energy Charge			
Time (min)	$MgSO_4$ 2 h before OGD		$MgSO_4$ after OGD	
	Control	$MgSO_4$	Control	$MgSO_4$
Control	100 ± 4	103 ± 1	100 ± 2	101 ± 2
OGD 10	102 ± 1	103 ± 0	102 ± 1	101 ± 1
OGD 20	99 ± 7	104 ± 0	101 ± 2	102 ± 1
OGD 30	101 ± 2	105 ± 0 a,b	99 ± 4	99 ± 2
OGD 40	102 ± 1	104 ± 1 b	99 ± 2	99 ± 3

Values are given as percent of control (mean ± SD). Each group comprised 5 slices. The absolute values of TAN in the two untreated control groups were 22.4 ± 2.9 and 23.8 ± 2.5 µmol / mg protein, and those of AEC were 0.92 ± 0.03 and 0.93 ± 0.02, respectively. Statistically significant differences within and between groups were assessed by ANOVA and the Scheffé post hoc test.
a: $P < 0.05$ (OGD vs control); b: $P < 0.05$ ($MgSO_4$ vs Control)

DISCUSSION

Recently, various retrospective clinical studies have shown that the incidence of cerebral palsy in newborns weighing less than 1500 g can be significantly reduced by administering magnesium to their mothers before birth. The neuroprotective effect was found to be independent of variables such as the administration of tocolytic agents or drugs to accelerate fetal lung development or of any other maternal or fetal risk factors (4,5). Unfortunately, a subsequent study by Grether and co-workers could not confirm the neuroprotective efficacy of magnesium (6). The authors attributed this to changes in medical practice between the two observation periods. A neuroprotective effect of magnesium would be of potential clinical importance, since in contrast to other neuroprotective strategies its application is only very seldom associated with serious complications in either the fetus or the mother (26). A recent study describes an increased paediatric mortality in infants receiving magnesium tocolysis (27). However, few of these children died during the neonatal period and the cause of death could rarely be attributed to the magnesium infusion, thus making a causal link between increased neonatal risk and application of magnesium very unlikely.

A further advantage of using magnesium as a neuroprotective agent would be that the fetus could be treated by application of the drug to the mother, since magnesium crosses the placental as well as the fetal blood brain barrier very easily (28-30). However, experimental studies on application of magnesium in various perinatal models of cerebral hypoxia/ischemia have as yet been inconclusive. While a variety of investigations have shown clear neuroprotective effects of magnesium (7-15), this could not be confirmed by others (14,16,17). Postischemic treatment in particular was seen to be ineffective or even to widen the extent of the resulting damage in the neonatal rat model (Levine-model) (14). As already discussed by Sameshima and co-workers this exacerbation of ischemic damage may arise from magnesium-induced hypotension (14). Using the same animal model Vanucci and Ringel reported almost complete ischemia in the ipsilateral brain hemisphere during 2 h of hypoxia (31,32). Although they did not measure regional blood flow after hypoxia-ischemia, reperfusion to the brain hemisphere could possibly occur during an early stage of recovery. Since magnesium can alter vascular tone by blocking neuromuscular junctions, treatment with magnesium after ischemia may quantitatively change blood flow to brain regions during the reperfusion period and result in more severe brain damage. To overcome this problem we used the *in vitro* system of oxygen-glucose deprivation (OGD) in hippocampal slices prepared from mature guinea pig fetuses: a model that excludes any effect of magnesium on the microcirculation. With this experimental set-up hippocampal slices can be kept metabolically intact for periods of up to 12 h, even when the temperature of the bath is set at 37 °C (19,21).

The neuroprotective effect of magnesium on metabolic disturbances of hippocampal slices after prolonged periods of OGD was investigated by measuring tissue levels of high-energy phosphates and protein synthesis (Fig. 1 and 2). Whereas increasing magnesium concentration in the aCSF 2 h before OGD significantly alleviated metabolic disturbances, treatment immediately after OGD was not effective. Since prolonged inhibition of protein synthesis after an ischemic insult can be viewed as an early marker of neuronal cell damage (33-35), magnesium may also protect the histological integrity of the tissue studied. In fact, protein synthesis has been shown to be reduced both during ischemia and in the early recovery period in vulnerable and non-vulnerable brain areas *in vivo* (36). At the end of the ischemic period, protein synthesis in non-vulnerable regions begins to recover to preischemic levels, while in vulnerable regions, such as the CA 1 sector of the hippocampus, it remains low. The latter phenomenon is then followed by neuronal cell loss (34,35,37,38). As shown in recent studies protein synthesis seems to be involved in the cellular suicide programme known as apoptosis. Apoptotic cell death can be prevented by application of the protein synthesis inhibitor, cycloheximide (39). In the present study protein synthesis was reduced by over 40 % 12 h after OGD. It cannot be excluded that the generation of apoptotic proteins contributes to the residual protein synthesis rate. However, the resulting neuronal cell damage would then be even more severe. Because the extent of apoptotic cell death after cerebral ischemia seems to depend on the severity of the insult (40-42), the proportion of possible apoptotic cells would be greater in slices subjected to shorter periods of OGD.

Increasing the magnesium concentration in the incubation medium before OGD considerably diminished the increase in cGMP tissue levels 10 min after OGD. As shown in this (Fig. 3) and previous studies using specific antagonists, the immediate increase in cGMP tissue levels after OGD is a marker of NO-production in this model (19,20). NO is a free radical synthesized by NO-synthase in neurons and endothelial cells in response to increased levels of intracellular calcium. Beside this endothelial and neuronal form of NO-synthase, another form of the enzyme is found in neutrophil granulocytes and microglia. During and to an even greater extent after ischemia NO as well as large numbers of superoxide radicals are produced by NO synthase, xanthine oxidase and via other pathways in the mitochondria. During reperfusion, NO and superoxide radicals combine to produce peroxynitrite, leading to the formation of more potent radicals. Destruction of cerebral tissue is the inevitable result (18).

The neuronal toxicity of NO after cerebral ischemia was elegantly demonstrated by Hamada and co-workers (43). They applied an inhibitor of the NO-synthase to neonatal rats 1.5 h before an hypoxic-ischemic insult, a procedure that had a highly neuroprotective

effect. In neonatal mice deficient for neuronal nitric oxide synthase (nNOS) similar results were observed following cerebral ischemia, with a reduction in hippocampal and cortical damage compared to the wild type (44). The neuroprotective effect of magnesium application before OGD seems, therefore, to be mediated in part through its inhibitory effects on NO-production. The inhibition of NO-production may be caused by magnesium-blockade of the NMDA-receptor as well as of voltage-dependent calcium channels, hindering the influx of extracellular calcium into the neurons and thus preventing the activation of the NO-synthase (13; for review: 2). In addition, magnesium may exert inhibitory effects of magnesium on NO-synthase. Another mechanism through which magnesium might protect the brain from ischemic injury is through its anti-convulsive properties (45). After an ischemic insult epileptiform activity is often observed in the brain causing an imbalance between blood flow and cell metabolism, which lead to cerebral damage (46). Through its anti-convulsive effects magnesium can diminish epileptiform activity and reduce the extent of possible brain injury. An interesting observation in the present study was that pretreament of hippocampal slices with magnesium did not result in a complete inhibition of cGMP production after OGD. This shows that not all pathophysiological mechanisms causing an activation of NO-synthase are sensitive to magnesium.

In contrast to treatment before OGD, increasing the magnesium concentration in the aCSF after OGD had no impact on cGMP tissue levels. Thus, beside the possible hypotensive effects of magnesium in the postischemic period (14) the neuroprotective inefficacy of post-OGD application may also be attributed to the fact that the NO-production was not inhibited.

After applying L-NNA intravenously to fetal sheep Marks and co-workers observed an increase in ischemic brain injury (47,48). L-NNA is known to block the endothelial form of NO-synthase. This resulted in a reduction in cerebral blood volume after global cerebral ischemia probably through vasoconstriction in the brain. One might argue that in an *in vivo* preparation reduction of NO production through application of magnesium might be harmful, since inhibition of the endothelial isoform of NO-synthase could result in vasoconstriction. However, given the clear vasodilatory properties of magnesium (49) this objection does not seem to be relevant.

From the present study we conclude that increasing magnesium concentration in the aCSF before OGD alleviates metabolic disturbances in hippocampal slices from mature fetal guinea pigs, whereas treatment after OGD has no effect. This neuroprotection may be mediated in part through the inhibition of NO-production shortly after OGD.

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Chapter 9

EFFECTS OF MILD HYPOTHERMIA ON METABOLIC DISTURBANCES IN FETAL HIPPOCAMPAL SLICES AFTER OXYGEN-GLUCOSE DEPRIVATION DEPEND ON DEPTH AND TIME DELAY OF COOLING

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ABSTRACT

Objective: There is a growing body of evidence from animal experiments that mild hypothermia induced during or even after cerebral ischemia may protect the immature brain from neuronal cell damage. However, the exact interrelation between the postischemic time delay and the degree of mild hypothermia by which neuroprotective effects on ischemic insults of different severity can be achieved has not yet been systematically elucidated. To clarify this point we studied the interaction between these variables in a recently modified hippocampal slice model.

Methods: We investigated the recovery of energy metabolism and protein synthesis rate (PSR) in hippocampal slices from mature fetal guinea pigs after 20, 30 or 40 min of oxygen-glucose deprivation (OGD). Hypothermia of varying degrees was induced immediately, 2 h or 4 h after OGD and lasted for 12 h. Prolonged inhibition of PSR after ischemia has been shown to be a particularly sensitive marker of neuronal cell damage.

Results: Hypothermia initiated directly after OGD significantly improved the recovery of energy metabolism and PSR. If there was a time delay of 2 h in the onset of hypothermia, neuroprotection depended on the degree of hypothermia. Reduction of the incubation temperature to 31°C diminished the disturbances of energy metabolism and PSR, whereas lowering the bath temperature to only 34°C was not effective. Inducing hypothermia 4 h after OGD did not have any influence on the recovery of energy metabolism and PSR.

Conclusion: From this study we conclude that the effects of mild hypothermia on metabolic disturbances in hippocampal slices of mature fetal guinea pigs depend on the intervention delay and the degree of cooling. The shorter the postischemic intervention delay and the greater the degree of hypothermia, the better the neuroprotective effect seems to be.

INTRODUCTION

Children undergoing hypoxic-ischemic brain damage during birth often suffer from the drastic consequences of this misfortune for the rest of their lives (1). Despite the many far-reaching consequences of this kind of perinatal insult, the range of effective therapeutic measures available is disturbingly limited (2). However, over the last few years increasing evidence has been obtained from animal experiments that mild hypothermia, i.e. lowering the brain temperature by only a few degree of Celsius, induced during or immediately after cerebral ischemia may protect the immature brain from neuronal cell damage (3-10). Moreover, as shown by recent studies on fetal sheep, hypothermia induced even several hours after cerebral ischemia seems to diminish brain injury (11,12). In contrast to investigations on adult animals (13-18), the exact relation between the postischemic time delay and the degree of mild hypothermia by which neuroprotective effects on ischemic insults of different severity can be achieved has not yet been systematically elucidated in one experimental model in immature animals.

However, if this point were to be clarified in an *in vivo* model of fetal cerebral ischemia various problems would be encountered: First of all a number of experimental variables such as cerebral blood flow, arterial oxygen saturation, blood glucose concentration, catecholamine release etc. have to be kept within very narrow limits, since each of these variables is known to affect neuronal cell loss after an ischemic insult (for review 2). The scatter in the neuronal outcome in a whole animal preparation is therefore usually larger than in an *in vitro* model in which experimental conditions can be easily kept constant or changed over a wide range. When studying a large experimental protocol, with multiple relations between several variables, possible differences between controls and treated groups may remain undetected in an *in vivo* model due to a considerable scatter in the experimental outcome, i.e. extent of brain injury. For this reason we used a recently modified hippocampal slice model (3,19), in which changes in metabolic disturbances during and after oxygen/glucose deprivation have been shown to vary within very narrow limits. As markers for neuronal cell injury we measured energy metabolism and protein synthesis rate (PSR). Prolonged inhibition of PSR after ischemia has been shown to be a particularly sensitive marker of neuronal cell damage (20). To elucidate the relation between the postischemic time delay and the degree of mild hypothermia by which neuroprotective effects on ischemic insults of different severity can be achieved we measured the recovery of energy metabolism and PSR in hippocampal slices from mature fetal guinea pigs after 20 to 40 min of oxygen/glucose deprivation (OGD). Hypothermia of varying degrees was induced immediately, 2 h or 4 h after OGD and lasted for 12 h.

METHODS

Experiments were performed on mature guinea pig fetuses (E 60; 0.9 of gestation). Tissue slices were prepared and incubated as described recently (3,19). For the present study we preferred tissue slices from the hippocampal area, since this region is known to be very sensitive to hypoxic-ischemic insults in comparison with other parts of the brain (20,21).

Guinea pig dams were anesthetized with halothane. The fetuses were delivered by cesarean section and decapitated. Until further preparation the fetal heads were kept in ice-water. The skull was then cut along the midline with scissors and removed with forceps. After removal of the skull the brainstem was divided below the cerebellum. The hemispheres were lifted with a spatula and the cranial nerves and the blood vessels were cut to allow the brain to fall gently into a beaker containing ice-cold artificial cerebrospinal fluid (aCSF) (0°C). This temperature was strictly maintained throughout the slice preparation period, since preliminary experiments have shown that preservation of cellular metabolism is improved under these conditions. Standard aCSF (22) contained (in mM): NaCl, 111; $NaHCO_3$, 26; KCl, 3; KH_2PO_4, 1.4; $CaCl_2$, 1.2; $MgSO_4$, 1.3; glucose, 10 (standard) or 2 (low glucose; in low glucose aCSF NaCl was adjusted to 115 mM); and N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 5. The aCSF was supplemented with amino acids (μM) (22): alanine, 34; arginine, 22; aspartate, 0.13; citrulline, 5.7; cystine, 0.12; histidine, 13; lysine, 27.7; methionine, 3.4; ornithine, 3.8; phenylalanine, 9.1; serine, 28.5; taurine, 8; threonine, 32; tryptophan, 1.5; tyrosine, 8.3; valine, 12.8. To prevent bacterial contamination 10 mg/L each of streptomycin and erythromycin were added to the aCSF. The aCSF was equilibrated with a gas mixture of O_2/CO_2 (95%/ 5%) for at least 60 min prior to the experiments. The pH of the aCSF was adjusted to 7.4 with 1 M NaOH or 1 M HCl. For further preparation the fetal brain was transferred from the beaker onto a cooling plate (0°C) covered with filter paper. The filter paper was moistened with aCSF. The brain was divided into its two halves and the hemispheres were placed with the lateral side down. The brain stem was lifted with a spatula, while the occipital cortex was gently pushed downwards with another spatula until the whole medial and inferior side of the hippocampal formation could be seen. Both ends of the structure were then freed and the hippocampus was rolled upwards and backwards towards the occipital pole by inserting spatulas on both sides under its superior surface. The hippocampal formation now lay with the dentate area down and the alveus up. The hippocampi were then transferred again to chilled standard aCSF until they were cut into slices of 500 μm thickness on a tissue slicer (Rademacher, Cologne, Germany). Before use the tissue slicer had been pre-cooled in a refrigerator. The slices were transferred from the chopper onto a nylon mesh with a painting brush. The nylon mesh was stretched across a plastic ring and immersed in standard aCSF. The incubation took place in a temperature

controlled (37°C) flow-through chamber, gassed with O_2/CO_2 (95%/5%) for 90 min. The flow rate of the aCSF was set to 1 mL/min. Care was taken to ensure that the upper side of the slices was fully exposed to the warm humidified gas mixture, while the underside was lying in aCSF. After the slices had been allowed to recover from the initial preparation stress for 90 min, the flow rate through the chamber was increased to 3 mL/min and standard aCSF with low glucose (2 mM) was pumped through for 30 min. This incubation of slices in low glucose aCSF was introduced to produce a prompt depletion of high energy phosphates during OGD (22).

The experimental protocol consisted of a preincubation period (120 min) and an anoxic period (20, 30 or 40 min OGD) followed by a recovery period with zero time corresponding to the end of the anoxic period (Fig. 1). We decided to vary the duration of OGD, since it is conceivable that the extent of the neuroprotective effect of mild hypothermia may depend on the severity of the insult. Hence, hypothermia may fail to protect tissue slices from metabolic disturbances, if the primary insult is too severe. To induce OGD an additional incubation chamber was set up that was gassed with a mixture of N_2/CO_2 (95%/5%).

Oxygen/glucose-deprived aCSF was prepared in the same manner as standard aCSF except for the addition of HEPES and glucose. HEPES was removed from oxygen/glucose-deprived aCSF, since it provides additional buffering capacity. This could influence the decrease in pH during OGD and alter the tissue response to OGD. Slight changes in osmolarity owing to the removal of glucose and HEPES were compensated by adjusting the NaCl concentration. Before transferring slices to the anoxic chamber, glucose was washed out of the tissue by immersion of the slices in oxygenated, aglycemic aCSF for 2 min. The post-OGD period was initiated by transferring slices to standard aCSF (flow rate 1 mL/min) with O_2/CO_2 (95%/5%). Mild hypothermia was induced immediately, 2 h or 4 h after OGD by lowering the incubation temperature to 34°C or 31°C, respectively. The hypothermic period lasted for 12 h. At the end of the experiments tissue concentrations of adenylates and protein synthesis were determined in slices of the control and intervention groups. Each experiment (normothermia vs. hypothermia) consisted of a normothermic control group and a hypothermic group without OGD. In addition, there were three normothermic and three hypothermic groups of slices that underwent OGD (20 – 40 min) (Fig. 1).

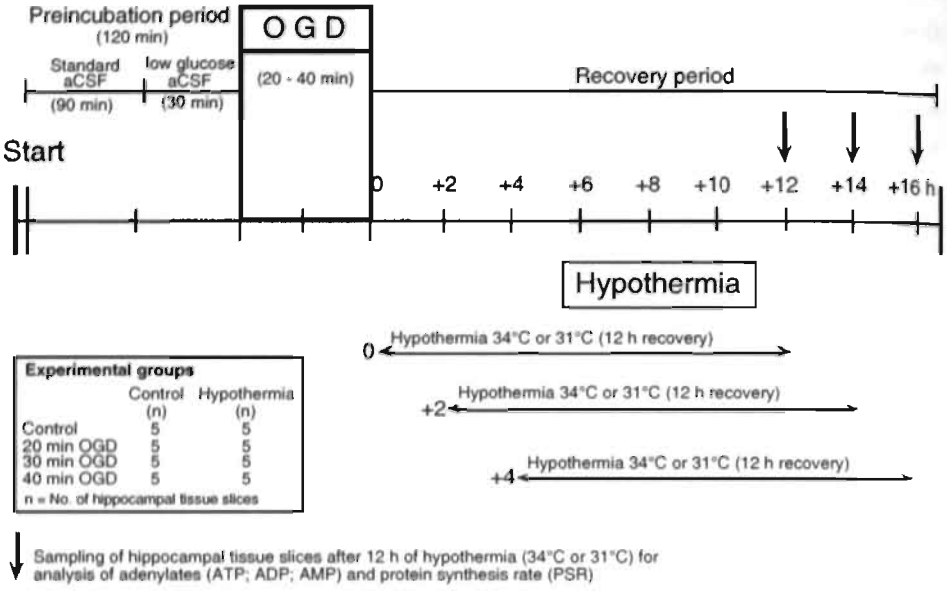

Fig 1. The experimental protocol consisted of a preincubation period (120 min) and an anoxic period (20, 30 or 40 min OGD) followed by a recovery period with zero time corresponding to the end of the anoxic period. To induce OGD, an additional incubation chamber was set up that was gassed with a mixture of N_2/CO_2 (95%/5%). Mild hypothermia was induced immediately, 2 h or 4 h after OGD by lowering the incubation temperature to 34°C or 31°C, respectively. The hypothermic period lasted for 12 h. At the end of the experiments tissue concentrations of adenylates and protein synthesis were determined in slices of the control and intervention groups. Each experiment (normothermia vs. hypothermia) consisted of a normothermic control group and a hypothermic group without OGD. In addition, there were three normothermic and three hypothermic groups of slices that underwent OGD (20 – 40 min).

The concentrations of adenylates in the tissue slices were measured in the supernatant of neutralized tissue homogenates by high-pressure liquid chromatography after extraction with perchloric acid (19). The adenylate energy charge (AEC), a measure of the balance between energy consumption and energy production was calculated as follows (23): AEC = ([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP]); where ATP is adenosine triphosphate; ADP is adenosine diphosphate; and AMP is adenosine monophosphate. The protein content of the tissue slices was measured by the Lowry method (24).

The rate of incorporation of ^{14}C-leucine into tissue proteins was taken as a measure of PSR. For these readings, 30 min before the conclusion of the experiments the tissue slices were incubated in standard-aCSF supplemented with 5 mCi/ml L-(^{14}C)-leucine (Amersham Buchler, Braunschweig, Germany; specific activity: 54 mCi/mMol). The slices remained

there for 30 min and were homogenized in trichloroacetic acid. The radioactivity of the precipitate was measured by liquid scintillation spectrometry after proteins had been dissolved in 1 M NaOH (19). The histological status of the tissue had been investigated in some pilot experiments. Severe deterioration of the tissue could be excluded after an incubation period of up to 24 h.

In contrast to previously described hippocampal slice models, we used an interface technique in which the upper side of the slices was fully exposed to the warm humidified gas mixture while the underside was lying in aCSF. In addition, the slices were kept in a flow-through chamber and the aCSF was supplemented with amino acids. Using this technique, energy metabolism and protein synthesis can be kept constant for up to 24 h as shown in a variety of previous studies (3,19,22). This is especially true of slices from mature fetuses, since they are much less vulnerable to the preparation procedure than those from adult animals (3,19). As shown in a recent study, hippocampal slices from mature fetal guinea pigs are completely depleted of ATP after 20 to 40 min of OGD. Within 24 h a partial recovery in the tissue concentration of ATP can be seen. The extent of the recovery ranges from 40 to 90 % of control levels depending on the duration of OGD (3,19).

Statistics

The values are presented as means ± SD. The data for each experimental group was obtained from 4 to 5 tissue slices. We restricted statistical analysis to each single experiment, which consisted of 8 groups of slices, i.e. a normothermic control group, a hypothermic group without OGD as well as three normothermic and three hypothermic groups of slices that underwent OGD (20–40 min). Significant differences within and between groups were assessed using ANOVA and Scheffé's F-test.

RESULTS

The concentrations of ATP measured in hippocampal slices from mature guinea pig fetuses are shown as % control in Fig 2. The ATP tissue levels in the five untreated control groups averaged 18.2 ± 4.2 µmol/mg protein. At the end of the recovery period, ATP concentration in the hippocampal slices was significantly reduced by up to 50 % depending on the severity of OGD. Moreover, the decrease in ATP concentration was clearly diminished by induction of hypothermia immediately after OGD. The maximum difference (27 %) could be observed between slices that underwent 40 min of OGD and were kept either normothermic or hypothermic at 34°C. If hypothermia was not initiated until 2 h

after OGD, significant alleviation of the fall in ATP tissue levels after OGD could only be observed at an incubation temperature of 31°C (maximum difference 19 %). Inducing hypothermia 4 h after OGD did not have any impact on ATP concentrations in fetal hippocampal slices except for a slight difference in the groups that underwent 20 min of OGD.

The tissue content of adenylates as well as the adenylate energy charge in hippocampal slices are given in Tables 1 and 2. Total adenylate pool and adenylate energy charge in the five untreated control groups averaged 20.9 ± 4.7 µmol/mg protein and 0.91 ± 0.03 respectively. The time course in changes of total adenylate pool after OGD and hypothermia closely resembled that of ATP concentrations (Table 1; Fig. 2). Adenylate energy charge scarcely differed at all from control values at the end of the recovery period (Table 2).

Table 1. Total Adenylate Pool

Onset of Hypothermia (34 °C)				
	Immediately after OGD		**2h after OGD**	
	Control	**Hypothermia**	**Control**	**Hypothermia**
Control	100 ± 15	109 ± 10	100 ± 12	103 ± 5
20 min OGD	76 ± 7	84 ± 7	69 ± 13[a]	81 ± 10[a]
30 min OGD	75 ± 19	80 ± 6[a]	66 ± 7[a]	74 ± 9[a]
40 min OGD	53 ± 8[a]	82 ± 20[a,b]	52 ± 15[a]	48 ± 8[a]

Onset of Hypothermia (31 °C)						
	Immediately after OGD		**2h after OGD**		**4h after OGD**	
	Control	**Hypothermia**	**Control**	**Hypothermia**	**Control**	**Hypothermia**
Control	100 ± 15	109 ± 7	100± 9	102 ± 18	100 ± 16	109 ± 3
20 min OGD	69 ± 11[a]	80 ± 16[a]	71 ± 6[a]	78 ± 8[a]	83 ± 11	68 ± 6[a,b]
30 min OGD	46 ± 8[a]	69 ± 12[a,b]	61 ± 6[a]	78 ± 8[a,b]	50 ± 3[a]	57 ± 4[a]
40 min OGD	44 ± 6[a]	62 ± 16[a,b]	54 ± 7[a]	69 ± 8[a,b]	42 ± 6[a]	48 ± 8[a]

Total adenylate pool Σ(ATP + ADP + AMP) in hippocampal slices from mature guinea pig fetuses 20 - 40 mins after oxygen/glucose deprivation (OGD) and 12 h of hypothermia. In the study groups incubation temperature was reduced to 34°C or 31°C immediately, 2 h or 4 h after OGD. Total adenylate concentration in the five untreated control group was 20.9 ± 4.7 µmol / g. Intra- and intergroup differences were measured by a two-way analysis of variance followed by Scheffé-F-test (a: $P < 0.05$ OGD vs. control; b: $P < 0.05$ hypothermia vs. normothermia).

Table 2. Adenylate Energy Charge

Onset of Hypothermia (34 °C)

	Immediately after OGD		2h after OGD	
	Control	**Hypothermia**	**Control**	**Hypothermia**
Control	100 ± 4	98 ± 2	100 ± 3	103 ± 1
20 min OGD	97 ± 3	98 ± 2	92 ± 11	100 ± 3
30 min OGD	88 ± 12	94 ± 4	97 ± 3	96 ± 4[a]
40 min OGD	89 ± 6	92 ± 8	88 ± 5	92 ± 3[a]

Onset of Hypothermia (31 °C)

	Immediately after OGD		2h after OGD		4h after OGD	
	Control	*Hypothermia*	*Control*	*Hypothermia*	*Control*	*Hypothermia*
Control	100 ± 3	102 ± 3	100 ± 1	101 ± 2	100 ± 1	101 ± 1
20 min OGD	101 ± 3	102 ± 3	103 ± 3	104 ± 1	101 ± 2	97 ± 4
30 min OGD	101 ± 1	102 ± 3	100 ± 2	102 ± 3	99 ± 2	98 ± 2
40 min OGD	97 ± 5	102 ± 4	100 ± 6	102 ± 2	96 ± 2[a]	97 ± 2

The adenylate energy charge (ATP + 0.5 ADP) / Σ(ATP + ADP + AMP) in hippocampal slices from mature guinea pig fetuses 20 - 40 mins after oxygen/glucose deprivation (OGD) and 12 h of hypothermia. In the study groups incubation temperature was reduced to 34°C or 31°C immediately, 2 h or 4 h after OGD. The adenylate energy charge in the five untreated control groups averaged 0.91 ± 0.03. Intra- and intergroup differences were determined by a two-way analysis of variance followed by Scheffé-F-test (a: $P < 0.05$ OGD vs. control; b: $P < 0.05$ hypothermia vs. normothermia).

The PSR in hippocampal slices measured as incorporation of ^{14}C-leucine into tissue proteins is illustrated in fig. 3. PSR in the five untreated control groups averaged 124 ± 47 dpm / 30 min / µg protein. Like energy metabolism, PSR was inhibited by up to 40 % depending on the intensity of the OGD. Hypothermia, initiated at the end of the OGD period, significantly improved the recovery of PSR (maximum difference 25 %). Furthermore, inhibition of PSR could be alleviated, if the temperature of the bath medium was reduced to 31°C 2 h after OGD. The maximum difference (29 %) could be observed between slices that underwent 40 min of OGD. Inducing hypothermia 4 h after OGD had no influence on the recovery of PSR. The impact of hypothermia of 34 °C commencing 4 h after OGD on metabolic disturbances of hippocampal slices has not been studied, since under these conditions no effect could be observed even when the incubation temperature was lowered 2 h after the insult.

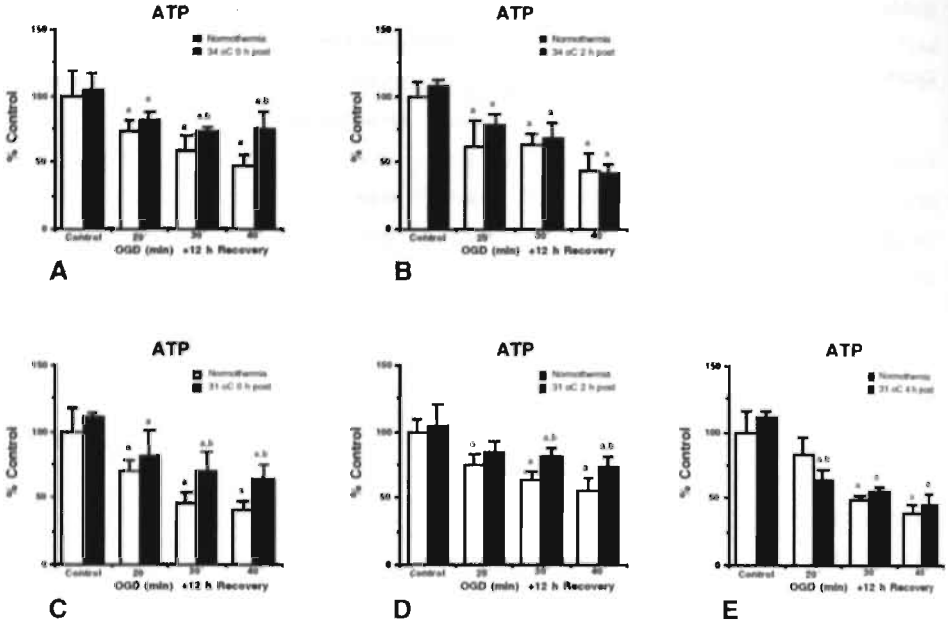

Figure 2. ATP tissue concentration (% of control) in hippocampal slices of mature guinea pig fetuses 20 - 40 mins after oxygen/glucose deprivation (OGD) followed by 12 h of hypothermia. Incubation temperature was reduced from 37°C to 34°C or 31°C immediately [A (34°C); C (31°C)], 2 h [B (34°C); D (31°C)] or 4 h [E (31°C)] after OGD. ATP tissue concentrations in the five untreated control groups averaged 18.2 ± 4.2 µmol / mg protein. Values are given as means ± SD. Intra- and intergroup differences were measured by a two-way analysis of variance followed by Scheffé-F-test (a: $P < 0.05$ OGD vs. Control; b: $P < 0.05$ Hypothermia vs. Normothermia).

DISCUSSION

In recent years, a large number of studies have been performed in fetal as well as in neonatal animals elucidating the neuroprotective effect of mild hypothermia on hypoxic-ischemic insults (3-10). Furthermore, there is also evidence that hypothermia, even when induced several hours after 30 min of cerebral ischemia, protects fetal sheep from brain injury (12). However, the exact relation between the postischemic time delay and the degree of mild postischemic hypothermia at which neuroprotective effects can be achieved on ischemic insults of different severity has not yet been systematically elucidated in one experimental model in immature animals. To clarify this point we studied the interaction between these variables in a recently modified hippocampal slice model (3,19).

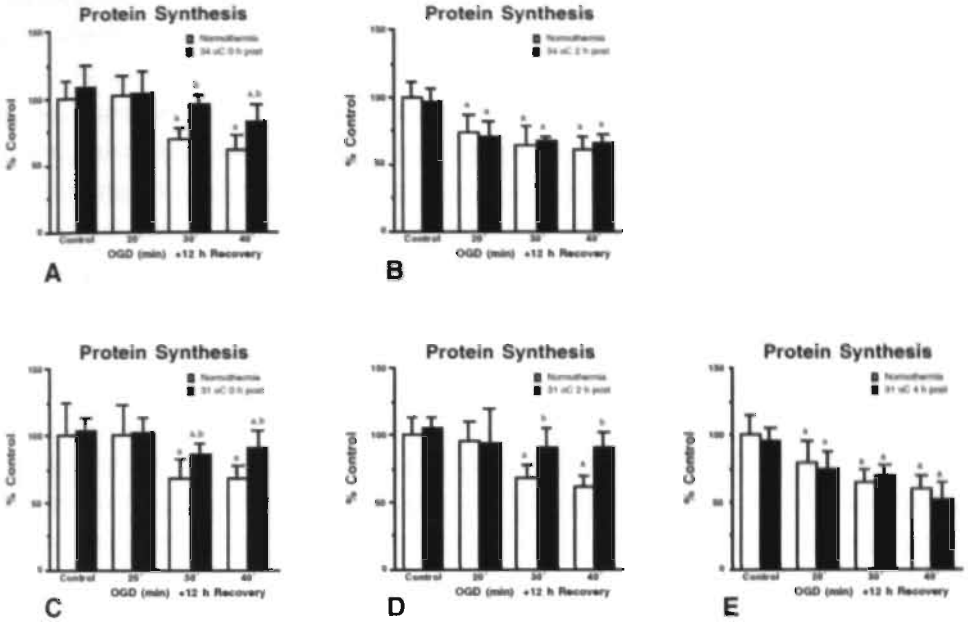

Figure 3. Protein synthesis rate (PSR) (% of control) in hippocampal slices of mature guinea pig fetuses 20 - 40 mins after oxygen/glucose deprivation (OGD) followed by 12 h of hypothermia. Incubation temperature was reduced from 37°C to 34°C or 31°C immediately [A (34°C); C (31°C)], 2 h [B (34°C); D (31°C)] or 4 h [E (31°C)] after OGD. PSR in the five untreated control groups averaged 124 ± 47 dpm / 30 min / _g protein. Values are given as means ± SD. Intra- and intergroup differences were measured by a two-way analysis of variance followed by Scheffé-F-test (a: $P < 0.05$ OGD vs. Control; b: $P < 0.05$ Hypothermia vs. Normothermia).

Hypothermia initiated directly after OGD significantly improved the recovery of energy metabolism and PSR. If there was a time delay of 2 h in the onset of hypothermia, neuroprotection depended on the degree of hypothermia. Whereas reduction of the incubation temperature to 31°C diminished the disturbances in energy metabolism and PSR, lowering the bath temperature to 34°C was found to be ineffective. The intervention delay of 2 h is quite consistent with a recently published *in vivo* study on fetal sheep (11), where the extradural temperature was reduced to 32.4 ± 0.8°C by placing a cooling coil over the fetal scalp connected to a pump in a cooled water bath. After 5 d of recovery there was a dramatic reduction in brain injury. In the present study, if hypothermia was first initiated 4 h after OGD, a neuroprotective effect was no longer observed. These results contradict a previous publication on fetal sheep illustrating a reduction in neuronal damage

due to delayed hypothermia induced 5.5 h after cerebral ischemia (12). However, this could be accounted for by the different experimental models used in the two studies. Hypothermia induced 8.5 h after cerebral ischemia has recently been shown to be ineffective in preventing brain injury in fetal sheep (25). Although these studies on fetal sheep have elucidated the time limit up to which the induction of hypothermia may be neuroprotective, they did not investigate whether the extent of the intervention delay is influenced by the degree of cooling. Such a relation was demonstrated in the present study, where the effects of mild hypothermia on metabolic disturbances in fetal hippocampal slices were shown to depend on both the intervention delay and the degree of cooling. The longer the postischemic intervention delay and the lower the degree of hypothermia, the weaker the neuroprotective effect seems to be. This finding is consistent with investigations in adult animals demonstrating a close relationship between the onset of postischemic hypothermia and neuronal cell loss (18). Thus, in adult gerbils hypothermia of 32°C initiated 1 h after ischemia and continued for 24 h provided substantial protection in the CA1 sector of the hippocampus. Although treatment delayed by 4 h also reduced hippocampal injury, the effect was significantly less than that of hypothermia initiated at 1 h (15,16). In these studies the neuroprotective effect of delayed hypothermia also depended on the depth of cooling (32°C vs. 34°C) (15,16,18).

From the results of the present study it is difficult to establish whether metabolic disturbances are affected by interaction between the severity of the ischemic insult and the length of intervention delay or the degree of cooling. Because hypothermia induced immediately after OGD improved the recovery of ATP concentrations in slices that had undergone 30 to 40 min of OGD, but not in those subjected to 20 min of OGD, one may speculate that the neuroprotective effect of hypothermia is more pronounced after longer OGD-periods. However, this effect could not be observed in experiments investigating the recovery of PSR after OGD, where this variable returned almost to control values after 20 min of OGD. Furthermore, no significant differences in the recovery of ATP or of PSR could be detected between the two groups of slices that had undergone 30 and 40 min of OGD, respectively.

One might speculate that inducing hypothermia after a long time delay after the primary insult may reverse its neuroprotective effect. In fact, we found that when hypothermia was started 4 h after OGD, ATP tissue concentrations in hippocampal slices that had undergone 20 min of OGD were significantly higher under normothermic conditions. However, at present there is no evidence in the literature to suggest that extending the postischemic time delay of hypothermia has any deleterious effect on cerebral tissue. To the best of our

knowledge the side-effects of hypothermia seem to be determined chiefly by the degree and duration of the treatment (18).

The mechanisms by which mild hypothermia alleviates postischemic inhibition of protein synthesis are not fully understood. After ischemia, PSR is severely depressed throughout the brain but recovers in cerebral areas where the insult does not lead to cell death (26,27). It also returns to control values in vulnerable regions, when the development of cell injury is blocked by therapeutic intervention (28). The inhibition of protein synthesis is caused by a blocking of the initiation step (29,30) through increased phosphorylation of the eukaryotic initiation factor eIF-2α (31). In eukaryotic cells this phosphorylation reaction is catalysed by the RNA doublestrand-activated protein kinase (PKR) (32). Besides viral infections, one important way to activate PKR is by disturbing endoplasmic reticulum (ER) calcium homeostasis (33,34). It has been concluded, therefore, that a disturbance of ER calcium homeostasis may contribute to the postischemic suppression of protein synthesis and to the pathological processes producing ischemic cell injury, a view which is corroborated by the results of several studies (for review see: 35). Whether the neuroprotective effect of mild hypothermia arises from an alleviation of a postischemic disturbance of ER calcium homeostasis remains to be established in future experiments.

The alleviation of disturbances in energy metabolism after OGD in slices from the study group may be a consequence of the well-known inhibitory effect of hypothermia on cerebral metabolism (36,37), an effect helping to maintain intracellular energy stores (38-42). Furthermore, as shown in a recent study in neonatal rats, secondary energy failure after a hypoxic-ischemic insult can be diminished by mild hypothermia (43). Thus, hypothermia of 24 h duration induced immediately after ischemia reduced the secondary decline of PCr/Pi ratio from 0.48 ± 0.22 to 1.07 ± 0.19. On the other hand, the neuroprotective effect of mild hypothermia is not thought to result from a delay in the depletion of high energy phosphate during OGD. This was shown in a previous study on hippocampal slices from mature fetal guinea pigs (3). Whether alterations in polyamine metabolism interfere with the neuroprotective effect of mild hypothermia on metabolic disturbances after OGD remains to be established in further studies (44,45).

From the present study we conclude that hypothermia induced after OGD diminishes disturbances in energy metabolism and PSR in hippocampal slices from mature fetal guinea pigs. The extent of this effect depends on the intervention delay and the degree of cooling. The shorter the postischemic intervention delay and the greater the degree of hypothermia, the better the neuroprotective effect seems to be.

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Chapter 10

SUMMARY, CONCLUSIONS, AND FUTURE PERSPECTIVES

Although perinatal brain injury is a major contributor to perinatal morbidity and mortality, present clinical strategies are not capable of reducing the incidence of perinatal hypoxic-ischemic brain damage significantly. However, a wealth of experimental studies has emerged describing the pathophysiological mechanisms that are implicated in perinatal brain injury (*Chapter 2*). These involve the acute breakdown of cerebral energy metabolism leading to the release of excitatory amino acids such as glutamate and aspartate. Glutamate binds to postsynaptically located glutamate receptors that regulate calcium channels. The resulting calcium influx activates proteases, lipases and endonucleases which in turn destroy the cellular skeleton. The acute lack of cellular energy arising during ischemia induces almost complete inhibition of cerebral protein biosynthesis. Once the ischemic period is over, protein biosynthesis returns to preischemic levels in non-vulnerable regions of the brain, while in more vulnerable areas it remains inhibited. The inhibition of protein synthesis therefore appears to be an early indicator of subsequent neuronal cell death. A second wave of neuronal cell damage occurs during the reperfusion phase. This cell damage is thought to be caused by the postischemic release of oxygen radicals, synthesis of nitric oxide, inflammatory reactions and an imbalance between the excitatory and inhibitory neurotransmitter systems. Part of the secondary neuronal cell damage may be caused by induction of a kind of cellular suicide programme known as apoptosis. Recent studies have shown that inflammatory reactions not only exacerbate secondary neuronal damage after cerebral ischemia, but may also injure the immature brain directly. This damage seems to be brought about by the cardiovascular effects of endotoxins leading to cerebral hypoperfusion and by activation of apoptotic pathways in oligodendrocyte progenitors through the release of TNF-α.

Since a significant proportion of neuronal cell damage is brought about by pathophysiological processes that first begin several hours or even days after an ischemic insult, the setting up of a therapeutic window would be feasible. Knowledge of these pathophysiological mechanisms has enabled scientists to develop new therapeutic strategies with successful results in animal experiments. The potential of such therapies was subject of the studies presented in this thesis, particularly the promising effects of the application of the calcium-antagonist flunarizine, the administration of magnesium and postischemic induction of mild cerebral hypothermia.

Flunarizine

Since flunarizine, a class IV calcium antagonist, is a potent vasodilator, the neuroprotective properties of this drug may be counteracted by its cardiovascular side-effects. To clarify this point we subjected mature fetal sheep to acute intrauterine asphyxia (*Chapter 4*). Fetuses from the study group received flunarizine i.v. before asphyxia. During acute asphyxia there was a redistribution of cardiac output towards the central organs accompanied by a pronounced bradycardia and a progressive increase in arterial blood pressure. Fetuses of the study group had higher plasma catecholamine levels than the control group. After asphyxia, circulatory centralization did not resolve quite as rapidly as it had developed, but was almost completely recovered at 30 min after the insult. There were hardly any differences between treated animals and controls in the time course of physiological and cardiovascular variables measured before, during and after acute intrauterine asphyxia.

Given the key significance of the so-called calcium overload for the development of neuronal cell death we tested the neuroprotective effect of flunarizine, an antagonist of voltage-dependent calcium channels in a fetal sheep model (*Chapter 5*). Ischemic brain injury was caused by occlusion of both carotid arteries for 30 min. One hour before the insult we applied the calcium-antagonist flunarizine at a concentration of 1 mg/kg fetal body weight i.v. This regimen reduced neuronal cell damage significantly, especially in the parasagittal cortex (Chapter 5). We conclude that pretreatment by low dose flunarizine protects the brain of fetal sheep near term from ischemic injury. We further conclude that this neuroprotective effect is not mediated by drug dependent changes in cerebral blood flow. Since low dose flunarizine does not alter the response of the fetal cardiovascular system to asphyxia, its clinical use should be reconsidered, particularly in fetuses that are at risk of hypoxic-ischemic encephalopathy.

Lubeluzole

During the last decade several *in vitro* and *in vivo* studies have shown that the benzothiazole compound lubeluzole is able to protect the brain from hypoxic-ischemic injury. In clinical studies the results were controversial. Protective and non-protective effects were seen in adult patients suffering from acute ischemic stroke. However, the knowledge on the safety profile and efficiency of lubeluzole seemed to be promising enough to warrant further studies in immature animals. We firstly studied the effects of lubeluzole on the fetal cardiovascular system (*Chapter 6*). Lubeluzole did not affect fetal circulatory centralisation during acute asphyxia in term fetal sheep. This mechanism is of major importance, since it protects the fetal brain from neuronal injury by increasing blood

flow to the central organs, i.e. brain, heart, adrenals, when oxygen is in short supply. Because of these promising results, we further evaluated the neuroprotective properties of lubeluzole in fetal sheep. Unfortunately, we were unable to show any cerebroprotective effect of the drug on neuronal cell damage induced by transient global cerebral ischemia. This appears to be inconsistent with investigations in adult human beings (for detailed discussion see *Chapter 7*). However, in almost all of these studies models of focal instead of global cerebral ischemia were used. This type of cerebral ischemia is characterized by an ischemic core and a peri-infarct region known as ischemic penumbra. In this area, brain tissue is perfused at a level between the thresholds of functional impairment and of morphological integrity. Unless cerebral blood flow is rapidly improved in this region within a few hours after the insult, neuronal cell damage is the inevitable result. As shown previously, glutamate is released in tremendous amounts from the infarct core into the extracellular space after focal ischemia. The released glutamate activates the neuronal NO-synthase via calcium influx through NMDA-regulated calcium channels. In focal ischemia this pathway may be of greater importance for the development of neuronal cell damage than in global ischemia, since in the latter type of ischemia only a moderate and short-lasting increase in glutamate release has been observed. This may explain in part the differences in neuronal outcome between the present study and previous investigations after treatment of brain injury by lubeluzole.

Magnesium

In the clinical setting, $MgSO_4$ has been used widely used in obstetric practice for over 60 years. Its indications include suppression of preterm labor and management of pregnancyinduced hypertension. A retrospective epidemiological study by Nelson and Grether (1995) suggested that premature fetuses whose mothers received $MgSO_4$ for the treatment of preeclampsia or as a tocolytic agent are less likely to develop cerebral palsy compared to a gestational age-matched group of fetuses not exposed to the drug. This difference was statistically highly significant. Almost identical results were obtained in a retrospective study carried out by Schendel and co-workers (1996).

The neuroprotective efficacy of magnesium has been attributed to a variety of effects of this molecule on pathophysiological mechanisms during and after cerebral ischemia, i.e. vasodilation, inhibition of the NMDA-receptor, anti-convulsive properties. Furthermore, magnesium reduces the ischemia induced release of NO by blocking the NO-synthase (*Chapter 8*). NO is a free radical synthesized by NO-synthase in neurones and other cell types in response to rises in levels of intracellular calcium during and after ischemia. During reperfusion, NO and superoxide radicals combine to produce peroxynitrite, leading

to the formation of more potent radicals. Destruction of cerebral tissue is the inevitable result. Since magnesium has been reported to block cerebral NMDA-receptors which themselves control calcium channels, the neuroprotective effect of magnesium may be mediated through an inhibition of NO-production after ischemia brought about by a reduction of neuronal calcium influx. On the strength of these results, several clinical studies have been conducted to test the effect of magnesium on the incidence of cerebral palsy in preterm fetuses. Recently, Mittendorf and co-workers (2002) reported a significant association between tocolytic $MgSO_4$ exposure and total pediatric mortality rates from the MagNET trial. Children who had cord blood ionized magnesium levels above the measured cohort median level had significantly more adverse events – i.e. periventricular leukomalacia, intraventricular hemorrhage, cerebral palsy, and death – than occurred in those infants who had levels below the median. Thus, they found that combined adverse outcomes were associated with magnesium exposure in a dose-related fashion. Therefore, future studies have to be conducted to test the putative neuroprotective benefits of antenatal $MgSO_4$ in the setting of premature labor.

Mild hypothermia

The induction of mild hypothermia has raised interesting possibilities for neuroprotection in cerebral ischemia. Over the last few years, the effect of mild hypothermia in protecting the brain from ischemically induced damage has been examined. Experimental studies on adult animals have shown that lowering of the brain temperature by 3-4°C during global cerebral ischemia reduces neuronal cell damage dramatically. Furthermore, treated animals were found to perform better than controls in subsequent learning and behavioural tests. In the present study, we evaluated the exact interrelation between the postischemic time delay and the degree of mild hypothermia by which neuroprotective effects on ischemic insults of different severity can be achieved (*Chapter 9*). Therefore, we used the well described model of oxygen-glucose deprivation (OGD) in hippocampal tissue slices derived from mature fetal guinea pigs. Hypothermia initiated directly after OGD significantly improved the recovery of energy metabolism and protein synthesis. If there was a time delay of 2 h before the onset of hypothermia, neuroprotection depended on the degree of hypothermia. Reduction of the incubation temperature to 31°C diminished the disturbances of energy metabolism and protein synthesis, whereas lowering the bath temperature to only 34°C was not effective. Inducing hypothermia 4 h after OGD did not have any influence on the recovery of energy metabolism and protein synthesis. These results tie in with studies performed in fetal sheep. Based on these results, many authors now consider the induction of hypothermia during and particularly after a hypoxic-ischemic insult to be an effective therapeutic strategy. In fact, recent clinical safety studies have demonstrated that induction

of mild cerebral hypothermia in newborn infants after perinatal asphyxia has no harmful side-effects and improves outcome in infants who suffered from moderate to severe encephalopathy (Gunn et al. 1998; Battin et al. 2001).

FUTURE PERSPECTIVES ON PERINATAL NEUROPROTECTION

Significant advances have been made over the past few years concerning the cellular and molecular events underlying hypoxic-ischemic brain injury. There are several new neuroprotective therapies available that have shown promise in animal and adult studies. Additional controlled, prospective trials are warranted, which hopefully will be designed and conducted within the next few years. The following paragraphs will briefly provide some new strategies which are currently under investigation (Table 3).

Combination therapy

The deleterious cascades that ensue following ischemia are many. It, therefore, stands to reason that an approach to neuroprotection that targets a single mechanism of action (e.g., either calcium, glutamate or free radicals) is unlikely to be of maximal benefit. Consequently, some investigators have looked to combination treatments to try to achieve a beneficial synergistic or additive effect. Combination therapy may include, initially, a neuroprotective agent that preferably acts at multiple sites in the ischemic cascades (e.g., magnesium) to protect the brain from subsequent injury. This may be followed by by prevention of reperfusion injury, and finally we might administer treatment to improve plasticity in the developing brain (e.g., growth factors, gene therapy, or stem cells).

Creatine

Mathews and co workers (1998) reported, that creatine protects the brain against malonate-induced increases in hydroxyl radical production and 3-nitropropionic-caused rise in 3-nitrotyrosine concentration, a marker of peroxynitrite-mediated oxidative injury. In fetal and neonatal animals creatine supplementation alleviates inhibition of protein synthesis and reduces hypoxic-ischemic brain injury (Berger et al. in press). In vitro it was found, that the glutamate uptake into synaptic vesicles is stimulated by phosphocreatine even in the absence of added ATP. At a glutamate concentration of 50 μM, maximal phosphocreatine-stimulated glutamate uptake was significantly higher than that maximally stimulated by ATP (Xu et al., 1996). A mechanism by which creatine supplementation

might protect the immature brain from ischemic injury could therefore be the activation of glutamate uptake into synaptic vesicles. Since creatine crosses the placental barrier and has been reported to accumulate in the brain of adults after oral supplementation (Dechent et al., 1999), further studies should be performed to test the neuroprotective efficacy of creatine on hypoxic-ischemic perinatal brain damage.

Table 3. New Therapies for Hypoxic-Ischemic Encephalopathy

Hypothermia, brain only
$MgSO_4$
Oxygen-free radical inhibitors, e.g. allopurinol
Calcium channel blockers, e.g. flunarizine
Inhibitors of nitric oxide production
Monoganglioside GM
NMDA receptor antagonists, e.g. MK-801
Creatine
Phenobarbital
Melatonin
Cannabidiol
Nerve growth factor (NGF)
Glial cell line-derived neurotrophic factor (GDNF)
CO_2 control
Glucocorticosteroids ?
Gene therapy ?
Stem cells ?
Combination of above

Selective Nitric Oxide Synthase Inhibition by 2-Iminobiotin

Recently, Peeters-Scholte and coworkers reported about the neuroprotective properties of selective NOS inhibition in the newborn piglet model of perinatal hypoxia-ischemia (Peeters-Scholte, 2002). They investigated the effects of NOS inhibition by 2-iminobiotin on histological outcome and clinical parameters, such as cerebral oxygenation, hemodynamics, and electrocortical brain activity. The drug provides selective inhibition of neuronal (nNOS) and inducible (iNOS), but not endothelial (eNOS) nitric oxide synthase. Intravenous application of 2-iminobiotin preserved cerebral oxygenation and hemodynamics, and electrocortical brain activity. Moreover, 2-iminobiotin treatment improved cerebral energy state, reduced the amount of vasogenic edema, and decreased the apoptosis-related neuronal cell death. These promising neuroprotective effects of 2-iminobiotin treatment after hypoxia-ischemia remain to be confirmed in the human.

Melatonin

Another interesting molecule under investigation is melatonin, a secretory product of the vertebrate pineal gland. Melatonin was recently shown to possess free radical scavenging activity, especially in the brain (for review: Reiter et al. 2001). This high efficacy may relate to the ease with which melatonin crosses the blood-brain barrier and to the fact that melatonin levels in the cerebrospinal fluid are orders of magnitude higher than in the blood. Furthermore, melatonin seems to lack significant toxicity and preliminary evidence in humans suggests that it may be protective against neurodegenerative disorders.

Cannabidiol

The non-psychoactive marijuana constituent, cannabidiol, can prevent both hypoxia-ischemia induced glutamate neurotoxicity and oxygen radical induced cell death (for review: Hampson et al. 2000). Tetrahydrocannabinol the psychoactive constituent of Cannabis, also blocks neurotoxicity with a potency similar to that of cannabidiol. Using a glutamate neuronal toxicity model cannabidiol was demonstrated to be significantly more protective than either of the antioxidant vitamins, ß-tocopherol or ascorbate. These properties of cannabinoids suggest they may have a therapeutic role as neuroprotectants, and the particular properties of cannabidiol make it a good candidate for such development.

Glial cell line-derived neurotrophic factor

Glial cell line-derived neurotrophic factor (GDNF), a protein in transforming growth factor-ß (TGF-ß) superfamily, was initially considered to be a specific trophic factor and neuroprotective agent for dopaminergic neurons (for review: Wang et al. 2002). Recent studies indicate that GDNF can protect the cerebral hemispheres from damage induced by middle cerebral arterial ligation. These effects are mediated through specific GDNF receptor alpha-1 (GFR1). Furthermore, it was shown that hypoxia-ischemia induced nitric oxide synthase (NOS) activity can be attenuated by GDNF.

Gene Therapy

Recently, it is becoming clear that some of genes induced during cerebral ischemia may actually serve to rescue the cell from death. However, the injured cell may not be capable of expressing protein at high enough levels to be protective. One interesting arena of such intervention is the use of viral vectors to deliver potentially neuroprotective genes at high levels (for review: Yenari et al. 2001). Recent scientific advances in the area of stroke and

neurodegeneration have led to the discovery of specific cellular events that occur during necrosis and apoptosis. It is now possible to therapeutically target these events with the hope of rescuing brain cells from death. Several studies have shown that cerebral ischemia alters gene expression and that some of the induced genes may serve a protective or damaging role. Among the many genes that have been identified to participate in ischemia, those possessing neuroprotective properties may be candidates for gene therapy.

Final Comment

Neuroprotection of the developing brain can have an enormous impact on the quality of life of an individual and on public health. In the absence of appropriate neuroprotection, severe injury of a fetal or infant brain will result in lifelong disability. Neuroprotection of the immature brain is feasible, since short-term neuroprotection during critical periods of brain development can have life long effects in preserving normal neurological function. However, identification of the critical periods for neuroprotection against various insults remains an area in need of continued intensive study.

For the future, it is important to know what we don't know.

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Hoofdstuk 10

SAMENVATTING, CONCLUSSIE EN TOEKOMSTPERSPECTIEF

Perinatale hersenbeschadiging is een belangrijke oorzaak van perinatale morbiditeit en mortaliteit. Met de huidige klinische detectiemethoden is het echter niet mogelijk de incidentie van perinatale hersenbeschadiging ten gevolge van hypoxie en/of ischaemie daadwerkelijk te beïnvloeden. Er zijn echter wel vele experimentele studies uitgevoerd die de pathofysiologische processen analyseren welke ten grondslag liggen aan perinatale hersenbeschadiging. Dit betreft onder meer de acute stilstand van de energievoorziening van de hersenen leidende tot het vrijkomen van prikkelende aminozuren zoals glutamaat en aspartaat. Glutamaat bindt zich aan postsynaptische glutamaat receptoren welke de calcium kanalen reguleren. De hieruit resulterende calcium influx activeert proteases, lipases en endonucleases welke vervolgens het cellulaire skelet vernielen. Het acute tekort aan cellulaire energie dat optreedt tijdens ischaemie veroorzaakt een nagenoeg algehele remming van de cerebrale eiwit aanmaak. Wanneer de ischaemische periode voorbij is, herstelt de eiwitsynthese zich tot het pre-ischaemische niveau in de minder kwetsbare gebieden van de hersenen terwijl deze productie op een lager niveau blijft in de meer kwetsbare gebieden.

Dientengevolge lijkt de verminderde eiwitproductie een vroege indicator te zijn voor de daaropvolgende dood van de zenuwcellen. Een tweede fase van zenuwcel beschadiging treedt op tijdens de reperfusie fase. Men veronderstelt dat deze celbeschadiging wordt veroorzaakt door het vrijkomen van zuurstof radicalen, de synthese van NO, ontstekingsreacties en een uit balans raken van stimulerende en remmende neurotransmitter systemen na de ischaemie. Een deel van deze secundair optredende neurogene schade wordt mogelijk veroorzaakt door het op gang komen van een cellulaire suïcideproces, apoptose genaamd. Recente studies lieten zien dat na cerebrale ischaemie ontstekingsreacties niet alleen secundaire neurogene schade bevorderen maar ook de immature hersenen direct beschadigen. Deze beschadiging lijkt te worden veroorzaakt door de cardiovasculaire effecten van endotoxinen waardoor cerebrale hypoperfusie ontstaat en door het activeren van apoptotische processen in voorlopers van de oligodendrocyten ten gevolge van het vrijkomen van TNF-α.

Daar een belangrijk deel van de zenuwcelbeschadiging ontstaat door pathofysiologische processen, die pas uren of zelfs dagen na het ischaemisch insult beginnen, is het kunnen vaststellen van de juiste therapeutische periode van groot belang. De kennis van deze pathofysiologische mechanismen heeft het wel al mogelijk gemaakt nieuwe therapeutische strategieën te ontwikkelen met goede resultaten in dierexperimenten. De mogelijke effectiviteit van zulke therapieën was het onderwerp van de studies gepresenteerd in dit

proefschrift in het bijzonder de veelbelovende effecten van het toepassen van de calcium-antagonist flunarizine, het toedienen van magnesium en het aanwenden van milde cerebrale hypothermie in de post ischaemische periode.

Flunarizine

Daar flunarizine, een klasse IV calcium antagonist, een krachtige vaatverwijder is, kunnen de beschermende eigenschappen van deze stof op het centrale zenuwstelsel geheel of gedeeltelijk teniet worden gedaan door de cardiovasculaire neveneffecten. Om dit probleem te analyseren onderwierpen we mature foetale schapen aan acute intrauteriene asphyxie (*Hoofdstuk 4*). Foetale lammeren uit de studiegroep kregen flunarizine intraveneus toegediend vóór het begin van de asphyxie. Tijdens acute asphyxie was er een herverdeling van de cardiac output naar de centrale organen, samen met een uitgesproken bradycardie en een toenemende arteriële bloeddruk. Foetale lammeren in de studiegroep hadden hogere plasma catecholamine spiegels dan in de controlegroep. Na de asphyxie, normaliseerde de centralisatie van de circulatie niet zo snel als deze was ontstaan: het herstel duurde ongeveer 30 minuten. Tussen de dieren in studie en de controlegroep bestonden nauwelijks verschillen in het tijdsverloop van de geregistreerde fysiologische en cardiovasculaire parameters voor, tijdens en na acute intrauteriene asphyxie.

Gezien de sleutelrol van de overmaat aan Ca-influx voor het ontstaan van celdood in het centrale zenuwstelsel werd de beschermende invloed van flunarizine, een Ca-influx antagonist, op het hersenweefsel van het foetale lam getest (*Hoofdstuk 5*). Hieruit werd de conclusie getrokken dat voorbehandeling met een lage dosis flunarizine de hersenen van het bijna à terme foetale lam beschermt tegen ischaemische schade. Tevens werd de conclusie getrokken dat deze bescherming niet verloopt via flunarizine afhankelijke veranderingen in cerebrale bloeddoorstroming. Daar een lage dosering flunarizine de reactie van het cardiovasculaire systeem op asphyxie niet verandert zou klinisch gebruik heroverwogen moeten worden, vooral bij foetús welke risico lopen op hypoxische-ischaemische hersenbeschadiging.

Lubeluzol

In vitro en in vivo studies uit de laatste tien jaar toonden aan dat het benzothiazol derivaat lubeluzol in staat is de hersenen te beschermen tegen hypoxische-ischaemische schade. In klinische studies waren de resultaten tegenstrijdig. Beschermende en niet beschermende effecten werden waargenomen bij volwassen patiënten met acuut herseninfarct. Echter het veiligheidsprofiel en effectiviteit van lubeluzol leek veelbelovend genoeg om verdere

studies bij immature dieren te rechtvaardigen. Eerst werd het effect van lubeluzol op het foetale cardiovasculaire systeem onderzocht (*Hoofdstuk 6*). Lubeluzol beïnvloedde de centralisatie in de foetale circulatie niet tijdens acute asphyxie in voldragen foetale lammeren. Dit mechanisme is van groot belang daar het de foetale hersenen beschermt tegen celdood schade door de bloeddoorstroming naar de centrale organen (zoals hersenen, hart, bijnieren) te verhogen wanneer er zuurstoftekort dreigt. Op grond van deze veelbelovende resultaten werden voorts de beschermende effecten van lubeluzol op de hersenen van het foetale schaap onderzocht. Helaas kon geen enkel beschermend effect van deze stof op de schade in het centrale zenuwstelsel, geïnduceerd door kortdurende algehele cerebrale ischaemie, worden gevonden. Dit lijkt in strijd met de bevindingen bij volwassenen (zie voor gedetailleerde overwegingen *Hoofdstuk 7*).

Echter in bijna al deze studies werd focale in plaats van globale cerebrale ischaemie gebruikt. Dit type cerebrale ischaemie wordt gekenmerkt door een ischaemische kern en een gebied hieromheen, ischaemische penumbra genoemd. In dit gebied wordt het hersenweefsel doorstroomd op een niveau gelegen tussen de grens van functionele beperkingen en/of morphologische integriteit. Tenzij de hersendoorbloeding binnen een paar uur na het insult snel verbetert in dit gebied, is celbeschadiging het onvermijdbare resultaat. Zoals voorheen aangetoond, wordt glutamaat in grote hoeveelheid geproduceerd centraal in het infarct en getransporteerd naar de extracellulaire ruimte. Het vrijgekomen glutamaat activeert de productie van NO in de hersenen door middel van Ca^{2+}-influx via de NMDA-gereguleerde calcium kanalen. Bij focale ischaemie is dit mechanisme waarschijnlijk belangrijker voor het ontstaan van neurogene celbeschadiging dan bij globale ischaemie, daar bij globale ischaemie slechts een lichte en kortdurende stijging in de glutamaat productie wordt gezien. Dit zou gedeeltelijk het verschil in uitkomst tussen de huidige studie en voorafgaand onderzoek na behandeling van hersenbeschadiging met lubeluzol, kunnen verklaren.

Magnesium

In de klinische obstetrie wordt magnesium sulfaat reeds meer dan zestig jaar gebruikt voor de indicaties premature weeënactiviteit en de behandeling van pre-eclampsie. Nelson en Grether (1995) stelden op grond van een retrospectieve epidemiologische studie vast, dat in de groep prematuur geboren kinderen wiens moeders waren behandeld met magnesium sulfaat, hetzij wegens pre-eclampsie hetzij wegens premature weeënactiviteit, minder frequent ischaemische encephalopathie voorkwam dan in een voor de zwangerschapsduur gematchte groep kinderen waarvan de moeders niet met dit middel waren behandeld. Dit

verschil was statistisch zeer significant. Vrijwel dezelfde resultaten werden vastgesteld in een retrospectieve studie uitgevoerd door Schendel et al. (1996).

Deze beschermende eigenschap van magnesium voor zenuwcellen wordt toegeschreven aan verschillende effecten van dit molecuul op de pathofysiologische mechanismen tijdens en na cerebrale ischaemie zoals vaatverwijding, remming van de NMDA-receptor, anti-convulsieve eigenschappen. Verder reduceert magnesium de door ischaemie geïnduceerde NO-productie door het blokkeren van de NO-synthase (*Hoofdstuk 8*). NO is een vrije radicaal gesynthetiseerd door NO-synthase in neuronen en andere celsoorten als reactie op de toename van intracellulair calcium tijdens en na ischaemie. Tijdens reperfusie produceren NO en vrije zuurstof radicalen samen peroxynitriet, hetgeen leidt tot de vorming van krachtiger radicalen, welke het hersenweefsel irreversibel beschadigen. Daar van magnesium is beschreven dat het cerebrale NMDA-receptoren kan blokkeren, die de calcium kanalen controleren, zou de beschermende werking van magnesium voor de hersencellen kunnen berusten op de remming van NO-productie na ischaemie door een reductie van Ca-influx in de neuronen. Gebaseerd op de deze veelbelovende bevindingen zijn verschillende klinische studies uitgevoerd om het effect van magnesium op de incidentie van ischaemische encephalopathie na te gaan bij de premature foetus.

Recent rapporteerde Mittendorf et al. (2002) een significante associatie tussen $MgSO_4$ toegediend als weeënremmer en de totale kindersterfte in de MagNet studie. Kinderen die navelstreng bloedspiegels van geïoniseerd magnesium hadden boven de vastgestelde mediaan van het bestudeerde cohort vertoonden significant meer negatieve gevolgen, onder meer PVL, IVH, CP, sterfte, dan kinderen waarbij deze concentratie zich onder de mediaan bevond. Dus zij vonden dat gecombineerde negatieve uitkomsten geassocieerd waren met het blootgesteld worden aan magnesium in een dosisafhankelijke relatie. Dientengevolge moeten nieuwe studies worden uitgevoerd met $MgSO_4$ om de veronderstelde beschermende werking op de hersenen van het voor de geboorte toedienen van deze stof als weeënremmer bij partus prematurus, nader te onderzoeken.

Milde hypothermie

De beschermende werking van milde hypothermie op de hersenen is de laatste jaren onderzocht en het toepassen hiervan biedt interessante mogelijkheden tot neuroprotectie. Experimentele studies bij volwassen dieren lieten zien dat verlaging van de hersentemperatuur met 3-4°C, tijdens globale cerebrale ischaemie, de neurogene schade sterk beperkt. Daarenboven presteerden de behandelde dieren nadien beter dan de controledieren in leer- en gedragtesten. In deze studie onderzochten we de precieze relatie

tussen het postischaemische tijdsverloop en de mate van milde hypothermie waarbij de beschermende werking kon worden bereikt bij ischaemische insulten van verschillende ernst (*Hoofdstuk 9*). Hiertoe maakten we gebruik van weefselcoupes uit de hippocampus van à terme foetal cavia's die werden blootgesteld aan een tekort aan zuurstof en glucose (het OGD model).

Hypothermie, toegepast direct na OGD, verbeterde het herstel van het energiemetabolisme en eiwitsynthese significant. Bij een interval van twee uur tussen noxe en hypothermie was de beschermende werking afhankelijk van de mate van hypothermie. Het terugbrengen van de incubatietemperatuur tot 31°C verminderde de verstoringen in het energiemetabolisme en de eiwitsynthese, terwijl het verlagen van de temperatuur tot maar 34°C niet effectief bleek. Het toepassen van hypothermie 4 uur na OGD had geen enkele invloed op het herstel van het energiemetabolisme en eiwitproductie. Deze resultaten zijn in overeenstemming met studies uitgevoerd bij foetale schapen. Gebaseerd op deze resultaten, beschouwen vele onderzoekers het toepassen van hypothermie, tijdens en vooral na een hypoxisch ischaemisch insult, als een effectieve therapeutische benadering. Recente klinische veiligheidsstudies toonden aan dat, milde cerebrale hypothermie bij asphyctische pasgeborenen, geen schadelijke bijwerkingen veroorzaakte en de uitkomst bij kinderen met matige tot ernstige encephalopathie verbeterde (Gunn et al. 1998; Bathin et al. 2001).

DE TOEKOMST VAN PERINATALE NEUROPROTECTIE

Gedurende de laatste jaren is er belangrijke voortgang geboekt in het ontrafelen van de cellulaire en moleculaire processen die ten grondslag liggen aan hypoxische-ischaemische hersenbeschadiging. Er zijn verschillende nieuwe neuroprotectieve therapieën beschikbaar gekomen die zowel in dierexperimenten als bij volwassenen veelbelovend zijn. Echter prospectieve gecontroleerde studies zijn noodzakelijk. De volgende paragrafen beschrijven kort enkele nieuwe strategieën welke momenteel onderwerp van studie zijn (Tabel 3).

Combinatie therapie

Na ischaemie doen zich vele schadelijke processen voor die met elkaar zijn verweven. Het is daarom zeer onwaarschijnlijk dat goede neuroprotectie bewerkstelligd kan worden via één enkel werkingsmechanisme (bijvoorbeeld of calcium, glutamaat of vrije radicalen). Dientengevolge hebben sommige onderzoekers gezocht naar combinatie therapieën om

synergische effecten te bereiken. Combinatie therapie kan initieel een neuroprotectieve stof betreffen, die bij voorkeur werkt op meerdere aangrijpingspunten in de keten van processen die op gang komen bij ischaemie (bijvoorbeeld magnesium). De volgende stap is het voorkomen van reperfusieschade, en tenslotte kan het toedienen van stoffen overwogen worden welke de plasticiteit van de zich ontwikkelende hersenen verbetert (bijvoorbeeld groeifactoren, gentherapie, stamcellen).

Tabel 3 Nieuwe Therapieën Bij Hypoxische-Ischaemische Encephalopathie

Hypothermie van de hersenen alleen
$MgSO_4$
Remmers van vrije zuurstofradicalen bijvoorbeeld allopurinol
Calcium kanaal blokkers bijvoorbeel flunarizine
Remmers van NO productie
Monoganglioside GM
NMDA receptor antagonisten, bijvoorbeeld MK-801
Creatine
Phenobarbital
Melatonine
Cannabidiol
Nerve growth factor (NGF)
Glial cell line derived neurotrophic factor (GDNF)
CO_2 controle
Glucocorticoiden ?
Gen therapie ?
Stamcellen ?
Combinatie van bovenstaande

Creatine

Mathews et al. (1998) rapporteerden dat creatine de hersenen beschermd tegen de door maloneaat veroorzaakte toename van hydroxyl radicalen, en tegen de door 3-nitropropion veroorzaakte stijging van 3-nitrotyrosine, een marker van peroxynitriet gestuurde oxydatieve beschadiging. Bij foetale en neonatale dieren vermindert creatine de remming van eiwitsynthese en vermindert daarmee de mate van hypoxische ischaemische hersenbeschadiging (Berger et al., in druk). In vitro werd vastgesteld dat de glutamaat opname in de synaptische blaasjes wordt gestimuleerd door fosfocreatine zelfs in afwezigheid van toegevoegd ATP. Bij een glutamaat concentratie van 50μM, was de maximale fosfocreatine gestimuleerde glutamaat opname significant hoger dan die welke maximaal gestimuleerd kon worden met ATP (Xu et al. 1996). Een mogelijk mechanisme waardoor creatine toediening de immature hersenen zou kunnen beschermen tegen

ischaemische schade, zou het activeren van de glutamaat opname in de synaptische blaasjes kunnen zijn. Daar creatine de placenta passeert en bekend is dat het zich ophoopt in de hersenen van volwassenen (Dechent et al. 1999), zijn verdere studies welke de neuroprotectieve werking van creatine op hypoxische-ischaemische perinatale hersenschade bestuderen voor de hand liggend.

Selectieve Nitric Oxide Synthase Inhibitie door 2-Iminobiotine

In een recente publicatie van Peeters-Scholte et al. (2002) werden de neuroprotectieve eigenschappen van selectieve NOS inhibitie na perinatale hypoxie-ischemie in een neonataal biggenmodel beschreven. Zij onderzochten de effecten van NOS inhibitie door 2-iminobiotine op hersenweefsel en klinische parameters zoals cerebrale oxygenatie, hemodynamiek en electrische hersenactiviteit. 2-Iminobiotine veroorzaakte selectieve inhibitie van het neuronale (nNOS) en induceerbare (iNOS) NO synthase maar niet van het endotheliale (eNOS) enzym. Intraveneuze toediening van 2-iminobiotine hield de cerebrale oxygenatie, hemodynamiek en electrische hersenactiviteit in stand. Bovendien werd de energie status van de hersenen door 2-iminobiotine behandeling verbeterd, verminderde de hoeveelheid vasogeen oedeem en nam de kwantiteit van door apoptose geïnduceerde celdood af. Of deze veelbelovende neuroprotectieve eigenschappen van 2-iminobiotine na een episode van hypoxie-ischemie ook bij de mens effectief zijn, dient in verder onderzoek bevestigd te worden.

Melatonine

Een ander interessante stof in dit verband is melatonine, een stof geproduceerd bij vertebraten door de glandula pinealis. Van melatonine werd recent bekend dat het vooral in de hersenen vrije radicalen opruimt (voor overzicht zie Reiter et al. 2001). De grote efficiëntie waarmee dit gebeurt kan te danken zijn aan de gemakkelijke passage van melatonine door de bloed-hersen barrière alsmede aan het feit dat de melatonine concentratie in de cerebrospinale vloeistof zeer veel hoger is dan de concentratie in het bloed. Daarenboven lijkt melatonine niet giftig te zijn en voorlopige gegevens bij mensen suggereren dat het kan beschermen tegen neuro-degeneratieve ziekten.

Cannabidiol

Het niet psychoactieve marihuana bestanddeel, cannabidiol, kan zowel de door hypoxie-ischaemie geïnduceerde glutamaat neurotoxiciteit als de door vrije zuurstof radicalen geïnduceerde celdood voorkomen (voor overzicht zie Hampson et al. 2000). Tetrahydro-

cannabinol, het psychoactieve bestanddeel van cannabis, blokkeert neurotoxiciteit eveneens in dezelfde mate als cannabidiol. In een glutamaat neurotoxisch model werkte cannabidiol significant beter beschermend dan de antioxydatieve vitamines β-tocopherol of ascorbinezuur. Deze eigenschappen van cannabinoiden wekken de suggestie dat ze een therapeutische rol kunnen spelen bij neuroprotectie en de specifieke eigenschappen van cannabidiol maken het voor dit doel een goede kandidaat.

Glial cell line derived neurotrophic factor (GDNF)

GDNF, een eiwit uit de transforming growth factor-β (TGF-β) super familie, werd aanvankelijk beschouwd als een specifieke trophische factor en als een beschermende stof voor dopaminerge neuronen (voor overzicht zie Wang et al. 2002). Recente studies laten zien dat GDNF de cerebrale hemisferen kan beschermen tegen schade geïnduceerd door het afklemmen van de a. cerebri media. Deze effecten verlopen via de specifieke GDNF receptor alpha-1 (GFR1). Voorts kon worden aangetoond dat door hypoxie-ischaemie geïnduceerde NO-synthase (NOS) activiteit kan worden onderdrukt door GDNF.

Gen therapie

Recent is het duidelijk geworden dat het tot expressie komen van bepaalde genen tijdens cerebrale ischaemie tot doel heeft het voorkomen van celdood. Echter de beschadigde cel zou niet in staat kunnen zijn voldoende eiwit te produceren om de beschermende werking te kunnen waarborgen. Een interessant onderzoeksgebied in dit opzicht is het gebruik van virale vectoren om mogelijke neuroprotectieve genen tot expressie te laten komen (voor overzicht zie Yenari et al. 2001). Recente wetenschappelijke resultaten op het terrein van stroke en neurodegeneratie hebben geleid tot de ontdekking van specifieke cellulaire processen die optreden tijdens necrose en/of apoptose. Het is nu mogelijk deze specifieke cellulaire processen te beïnvloeden met als doel de dood van hersencellen te voorkomen. Uit verschillende studies blijkt dat cerebrale ischaemie de genexpressie verandert en dat sommige van de tot expressie gebrachte genen een beschermende of een beschadigende rol hebben. Onder de vele genen die een rol spelen bij ischaemie zijn die welke neuroprotectieve eigenschappen hebben kandidaten voor gen therapie.

SLOTCONCLUSIE

Bescherming van de zich ontwikkelende hersenen bij dreigende schade kan van grote betekenis zijn op de kwaliteit van leven van een individu en op de maatschappij als geheel. Zolang de mogelijkheid van daadwerkelijke bescherming echter ontbreekt zal ernstige beschadiging van de foetale of neonatale hersenen uitmonden in levenslange invaliditeit. Indien het mogelijk wordt de exacte intervallen tussen het hypoxische-ischaemische insult en de mogelijke adequate therapie waarbinnen deze moet worden ingesteld, te definiëren dan lijkt bescherming van de hersenen haalbaar.

Voor de toekomst is het belangrijk te weten wat we niet weten.

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Perinatal brain damage is a major cause of subsequent neurological disability in both premature and term infants. Although multiple causes, e.g., hypoxia-ischemia, hemorrhage, infection, metabolic derangement, are recognized, hypoxic-ischemic disease predominates. During the past decade, a wealth of experimental studies has emerged describing the pathophysiological mechanisms that are implicated in perinatal brain injury. Knowledge of these pathways has enabled scientists to develop new therapeutic strategies which have been shown to be neuroprotective in perinatal animal and adult studies. The potential of such therapies in perinatal medicine is subject of the present book, particularly the promising effects of postischemic induction of mild cerebral hypothermia, the application of calcium channel blockers and the administration of magnesium.

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