Hyperglycemia and Focal Brain Ischemia

Clinical and Experimental Studies

NASIM FARROKHNIA
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Abstract

Diabetes is a major risk factor for ischemic stroke and is associated with increased mortality. Additionally, hyperglycemia, a common complication in acute stroke, is associated with poor outcome.

In order to identify the correlation between blood glucose and early mortality, multiple logistic regression analyses were used and odds ratios calculated in a retrospective study of 447 stroke patients. Eighty-one patients (18%) had diabetes. The odds ratios for 30-day case-fatality and blood glucose were 1.9 and 1.6 in diabetic and non-diabetic patients respectively. Optimal blood glucose concentrations in respective group were 10.3 and 6.3 mmol/L, as determined by receiver operator characteristic (ROC) curves.

Cerebral ischemia triggers different signaling pathways including mitogen-activated protein kinases (MAPK) which regulate fundamental cell functions. In an experimental rat model of combined hyperglycemia and transient middle cerebral artery occlusion (MCAO), the activation pattern of one such MAPK, extracellular signal-regulated kinase (ERK) was studied along with infarct volumes and neurological function. Hyperglycemia resulted in markedly increased ERK activation and approximately three-fold increase of infarcts compared with controls.

Based on the increased ERK activation, further experiments were conducted to limit the hyperglycemic-ischemic damage by interfering with ERK and supposedly related mechanisms. Consequently, rats were given U0126 (inhibiting ERK activation), PBN (anti-oxidative), PP2 (inhibiting src-family kinases), or vehicle. PBN reduced infarcts and improved neurological function compared with controls while no statistically significant effects were observed for U0126 or PP2. However, when the dose was doubled, U0126 significantly reduced infarcts and improved neurological function after 1 day in hyperglycemic rats. Post-ischemic ERK activation was completely inhibited by U0126 as demonstrated with Western immunoblotting. The findings suggest that ERK is an important mediator of hyperglycemic-ischemic brain injury and possible target for future interventions.

Keywords: cerebrovascular disorders, diabetes mellitus, hyperglycemia, infarction, middle cerebral artery, ischemia, mitogen-activated protein kinases, mortality, rats, reactive oxygen species, reperfusion, signal transduction, therapeutics

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“You cannot hope to build a better world without improving the individuals. To that end, each of us must work for our own improvement and, at the same time, share a general responsibility for all humanity, our particular duty being to aid those to whom we think we can be most useful.”

Marie Skłodowska Curie
Nobel Prize winner 1903 and 1911
List of papers


*Paper I and II are printed with permission from the publishers.*
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Abbreviations

AA   Arachidonic acid
ABG  Admission blood glucose
AD   Alzheimer’s disease
AVM  Arteriovenous malformations
CBF  Cerebral blood flow
CT   Computed tomography
DMSO Dimethyl sulfoxide
DWI  Diffusion-weighted imaging
ER   Endoplasmic reticulum
ERK  Extracellular-signal regulated kinase
FFA  Free fatty acids
FITC Fluorescein isothiocyanate
HRP  Horseradish peroxidase
ICH  Intracerebral hemorrhage
JNK  c-jun-N-terminal kinase
MAPK Mitogen-activated protein kinase
MBG  Mean blood glucose
MCAO Middle cerebral artery occlusion
MRA  Magnetic resonance angiography
MRI  Magnetic resonance imaging
OGD  Oxygen glucose deprivation
PBN  Alpha-phenyl-N-tert-butynitrone
PD   Parkinson’s disease
pH   Extracellular pH
pH_i Intracellular pH
PKC  Protein kinase C
PP2  4-amino-5(4-chlorophenyl)-7-(t-butyl)pyrazol[3,4-d]pyrimidine
PVDF Polyvinylidene fluoride
ROC  Receiver operator characteristic curve
ROS  Reactive oxygen species
RTK  Receptor tyrosine kinases
SAH  Subarachnoid hemorrhage
SAPK Stress-activated protein kinases
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sp-D rat Sprague-Dawley rat
TGI  Transient global ischemia
TIA  Transient ischemic attack
TMCAO Transient middle cerebral artery occlusion
U0126 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
VEGF Vascular endothelial growth factor
WHO World health organization
Introduction

Clinical aspects of stroke
Definition
Stroke is the third common cause of death in Sweden (Socialstyrelsen 2003) and the care of stroke patients is a large public health issue. The approximate number of stroke events in Sweden is 30000 annually (Socialstyrelsen 2001). The age at the time of stroke onset is on an average 75.7 years, 73.4 for men and 78 for women (Riks-Stroke 2004). Age is the risk factor most closely correlated with stroke, and the incidence of stroke doubles with each decade between the ages of 45 and 85 years (Asplund et al. 1998). The proportion of men and women is almost equal among stroke patients (49.9% and 50.1%) (Riks-Stroke 2004).

According to the World Health Organisation (WHO), stroke is defined as rapidly developing clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than vascular (Aho et al. 1980). Episodes with durations less than 24 hours, transient ischemic attacks (TIAs), are consequently not included in this definition. However, the annual stroke risk following TIA is increased by 3-5% per year (Nguyen-Huynh et al. 2005).

Diagnostic tools
Almost all (97.9%) stroke patients in Sweden undergo computed tomography scan (CT) examinations of the brain (Riks-Stroke 2004). The proportion of deceased stroke patients who undergo autopsy is low, about 5%, 4% in women and 6% in men (Riks-Stroke 2004). This slight difference may be explained by the higher mean age of the women.

For intracerebral hemorrhage, the sensitivity of CT is nearly 100%. For subarachnoid hemorrhage (SAH), the sensitivity of CT approaches 95%. In those examined within 3 hours of stroke, CT signs of ischemia can be detected in up to 20% of the patients. The earliest CT sign of developing infarction is a hyperdensity (increased attenuation) within
the cerebral artery supplying the area corresponding to the neurological deficit. This hyperdensity represents an acute intramural thrombus (clot). Decreased attenuation on CT develops later, usually after 60 minutes or more (Gacs et al. 1983; Leys et al. 1992). Magnetic resonance imaging (MRI) is more sensitive, but currently not a widespread emergency imaging modality for patients with acute ischemic stroke. In contrast to CT, MRI examines pathophysiology as well as anatomy (Biola et al. 2005; Chandromohan et al. 2005). MRI perfusion imaging and diffusion-weighted imaging (DWI) can be performed acutely. Early signs of infarctions that are not detectable in CT can be seen as areas of abnormalities by DWI within minutes following stroke onset. Magnetic resonance angiography (MRA) is possible to obtain more rapidly than conventional cerebral angiography, and MRA allows identification of intracranial occlusions potentially treatable with thrombolytic therapy. Finally, MRI provides valuable information in the differential diagnosis of ischemic stroke and can be necessary in the ethiologic diagnosis of hemorrhages (Socialstyrelsen 2002).

Ischemic stroke
Ischemic stroke is the most common type of stroke in Sweden and accounts for about 85% of all stroke events (Socialstyrelsen 1996). Ischemic strokes are mainly attributed to cardioembolism, atherosclerotic cerebrovascular disease, or non-atherosclerotic cerebral vasculopathies (figure 1). Hypercoagulable etiologies of stroke and metabolic strokes comprise a very small fraction of ischemic strokes (Ionta et al. 2005). Cardiac embolism accounts for 12% to 35% of strokes but these figures may reflect an underestimation. The most common cardiac sources are: atrial fibrillation (25% of all patients according to Riks-Stroke 2003), mitral or aortic valve disease, prosthetic hearth valves, infective endocarditis, cardiomyopathies, dyskinetic myocardial segments (after myocardial infarctions), and ventricular aneurysms. A presumed atherosclerotic etiology is found in nearly 50% of the patients with ischemic stroke. Atherosclerosis primarily affecting larger (extracranial and intracranial) vessels is responsible for about 25% of all ischemic strokes (Socialstyrelsen 2002). About 20% of ischemic strokes are lacunar infarcts caused by local atherosclerotic occlusions of small vessels in the deeper regions of the brain. These infarcts are principally small, defined as less than 15 mm in diameter (Socialstyrelsen 2002). The major risk factors for atherosclerotic ischemic stroke are hypertension, smoking (15% of all patients according to Riks-Stroke 2003), obesity and/or lack of exercise, diabetes, and dyslipidemia. However, dyslipidemia seems to correlate more strongly with coronary artery disease than with stroke.
Hemorrhagic stroke
Hemorrhagic stroke comprises intracerebral hemorrhages (ICH, 10%) and subarachnoid hemorrhages (SAH, 5%) (Socialstyrelsen 1996). In young patients, ICH occurs spontaneously in four common settings: (1) ruptured arteriovenous malformations (AVMs), (2) ruptured intracranial aneurysms, (3) arterial hypertension, and (4) abuse of sympathomimetic drugs (Toffol et al. 1987). SAH most often results from
rupture of an intracranial aneurysm or AVM (Biller et al. 1987). Women are slightly over-represented (3:2) in (aneurysmal) SAH (Locksley et al. 1966), but age-adjusted rates of ICH are about 50% higher in men than in women.

Prognosis

Early mortality after ischemic stroke
The highest mortality rate for patients with cerebral infarction is seen within the first 30 days, with case-fatality rates ranging from 8% to 20% (Wilterdink et al. 1992). During this early period, death is most likely due to the stroke itself (“malignant brain edema”) or cardiopulmonary complications (Howard et al. 1989). In many countries, early stroke mortality rates have been declining (Garraway et al. 1983; Modan et al. 1992). In Söderhamn, Sweden, during the period 1975 to 1990, stroke incidence and short-term case-fatality was stable over time. On the other hand, long-term survival after stroke has continued to improve (Terent 2003). The predominant cause of death among Swedish stroke patients in the 1970s and 1980s, was cerebrovascular disease (Terent 2004).

Late mortality after ischemic stroke
It is unclear to what extent post-stroke mortality is influenced by (1) the initial stroke severity, (2) the recurrence of stroke, or (3) other associated co-morbidities such as cardiovascular diseases. Improved control and treatment of “modifiable” stroke risk factors could have a significant impact on late mortality, although the therapeutic potential in the acute stage is uncertain. In Sweden, the age-related stroke mortality has declined during the last 25 years, primarily due to declined acute mortality (Socialstyrelsen 2002).

Medical management of stroke
Accurate diagnosis of the type and cause of stroke is fundamental for successful treatment (Table 1). Management of patients with ischemic stroke differs considerably from that of patients with hemorrhagic stroke. According to the Swedish national guidelines for stroke care (Socialstyrelsen 2002), the recommended acute management of stroke patients is as followed:

At the emergency department:
- Assessment and stabilization of airways, breathing and circulation
- Obtaining the history from the patient and observers
• Physical examination of all organ systems with focus on vascular signs
• Measurements of blood pressure (BP) and body temperature (BT)
• Neurological examination (according to NIH Stroke Scale)
• Blood samples: hematological and renal analyses, S-electrolytes, B-glucose
• Electrocardiography (ECG)
• Cranial CT scan

At the ward:
• Continued measurements of BP, BT, B-glucose
• Complementary blood chemistry tests for example S-cholesterol
• Urine analysis
• Examination and evaluation by a multidisciplinary stroke rehabilitation team

And at special medical or neurological indications:
• Extracranial ultrasonography
• Echocardiography (including transesophageal)
• MRI and/or MRA
• Lumbar puncture (if suspected SAH and negative CT)

Although the goals of treatment vary, many therapies are complementary (Fulgham et al. 2004). Patients with cerebrovascular disease often receive several interventions. Some treatment modalities (for example antihypertensive drugs or anticoagulants) are aimed at preventing recurrent stroke or other vascular events. Others (for example thrombolytic therapy or surgery) directed towards the treatment of an acutely ill stroke patient, are aimed at limiting or reversing the neurological consequences of the vascular event. Additional, acutely prescribed treatments, such as antibiotics and anticonvulsants, may be given to avoid or treat serious medical or neurological complications. Finally, education and rehabilitation are important to speed recovery and help the patient to return to their usual activities. The care of stroke patients is best handled in a specialized facility, a stroke unit, that is devoted to stroke management (Langhorne et al. 1993; Glader et al. 2001).

Some special aspects on acute stroke management

Hypertension
Hypertension is the most important modifiable cerebrovascular risk factor, confirmed by both previous epidemiological data and by more recent intervention trials of primary (HOT, Syst-Eur, LiFE) and sec-
ondary (PROGRESS) prevention of stroke in hypertensive patients (Pinto et al. 2004). Acute elevation of the blood pressure is most often transient (Broderick et al. 1993). Although treatment is recommended (Socialstyrelsen 2002) when systolic blood pressure exceeds 220 mmHg or diastolic blood pressure exceeds 120 to 140 mmHg, the rational for treatment is largely theoretical. With such severe elevation in blood pressure, vasoconstriction may be excessive and ischemia may worsen. Alternatively, vasoconstriction may fail and cerebral edema thereby may be potentiated. If antihypertensive therapy becomes necessary, agents that have a very rapid onset and a predictable response are preferred.

**Infections**

Infection is identified as one of the most important risk factors in cerebral atherosclerosis (Diaz et al. 2004). The role of Chlamydia pneumoniae is still controversial while influenza vaccination is a simple and effective preventive measure against stroke. Pneumonia (Yoshikawa 1989) accounts for 15% to 25% of the deaths following stroke. The majority are bacterial infections secondary to aspiration. The pneumonia is likely to be community acquired in patients with radiographically detectable infiltrates within the first 24 hours. In order to detect pneumonia early, body temperature should be measured frequently. Antibiotic therapy is administered until a specific etiology diagnosis is established and antibiotic sensitivities determined. Urinary tract infection (Yoshikawa 1989) also presents a management dilemma and is present in up to 40% of patients dying from stroke. Patients are often confused and incontinent. The use of catheters should be avoided, if possible, and limited to the first days of the hospitalization. Antibiotic therapy should be reserved to patients with symptomatic infections.

**Elevated body temperature**

Stroke patients frequently have elevations in temperature. Fever (body temperature >37.5°C) negatively influences outcome after stroke (Kammersgaard et al. 2002) and is shown to be independently related to initial stroke severity, infarct size, mortality and neurological outcome in survivors (Reith et al. 1996). However, some studies claim that body temperature is unaffected at stroke onset but increases in the early hours after onset, in relation to the severity of the stroke (Boysen et al. 2001). However, for each degree Celsius change in body temperature, glucose utilization in most regions of the central nervous system (CNS) is shown to be altered between 5% and 10% (McCulloch et al. 1982). For patients with ischemic stroke and fever, with or without infection, measures should be taken to lower the temperature into the
normal range (Socialstyrelsen 2002). For alert patients, oral administra-
tion of paracetamol is recommended. Severe or prolonged fever (if not
secondary to infections or deep venous thrombosis, DVT) reflecting a
severe cerebral damage and more often a large hemorrhage, is accom-
panied with massive neurological symptoms and impaired conscious-
ness.

Elevated blood glucose
Elevated blood glucose is a common phenomenon in acute stroke. 
Plasma glucose >6.7 mmol/L is reported in 20-43% of patients admi-
itted to hospital (Warlow et al. 2003). However, hyperglycemia, defined
as a blood glucose concentration >6.0 mmol/L, can be observed in two
thirds of all patients with ischemic stroke (Scott et al. 1999). Accord-
ing to Swedish, European and American guidelines, glucose-containing
infusions are discouraged in the first few days, but the evidence for this
recommendation is vague. An ongoing intervention trial, the United
Kingdom Glucose Insulin in Stroke Trial (GIST) is aimed to answer
the question whether lowering and maintaining blood glucose concen-
trations after stroke can reduce early mortality (Scott et al. 1999; Gray
et al. 2004). Preliminary results of a pilot study including 53 patients
has shown a significant reduction of blood pressure in glucose, potas-
sium, and insulin treated patients, while 4-week mortality was 28%
and 32% among treated and control patients respectively (Scott et al.
1999; Scott et al. 2001). Another interventional study in 24 diabetic
patients with acute cerebral infarction has confirmed the feasibility and
relative safety of treatment with i.v. insulin as (Bruno et al. 2004).
Elevated blood glucose is associated with worse stroke outcome (table
1) and may reflect diabetic comorbidity as well as increased stroke
severity. On the other hand, hypoglycemia may aggravate brain dys-
function (Sahay et al. 2001). Impaired glucose tolerance i.e. relative
insulin deficiency or insulin resistance has been found in a majority of
patients with recent myocardial infarction (Norhammar et al. 2002)
and is found in about 50% of the stroke patients as well (Kernan et al.
2002; Kernan et al. 2003; Kernan et al. 2005). Reactive hyperglyce-
mia, presumably due to major stress response, is associated with worse
prognosis in non-diabetic patients with pre-stroke normoglycemia
(Lindsberg et al. 2004), based on the correlation between high cortisol
and blood glucose concentrations (Murros et al. 1993). On the other
hand, hyperglycemia has been associated with worse stroke outcome
but no such an association was seen with stroke severity. Furthermore,
there was no correlation between plasma norepinephrine, epinephrine,
and glucose concentration in these patients (van Kooten et al. 1993).
Table 1. Prevalence and outcome of hyperglycemia in clinical studies (in chronological order) of ischemic (I) and hemorrhagic (H) stroke in diabetic (D) and non-diabetic (ND) patients. The definition of hyperglycemia ranges from >6 to >10 mmol/l (fasting or random blood glucose) in the different studies.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Prevalence</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Dora et al. 2004)</td>
<td>ND, I</td>
<td>- (Not available)</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td></td>
</tr>
<tr>
<td>(Bhalla et al. 2002)</td>
<td>All together</td>
<td>22-35%</td>
</tr>
<tr>
<td>(Els et al. 2002)</td>
<td>ND, I</td>
<td>45%</td>
</tr>
<tr>
<td>(Szczudlik et al. 2001)</td>
<td>ND, I</td>
<td>36%</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td>[All patients]</td>
</tr>
<tr>
<td>(Bruno et al. 1999)</td>
<td>All together</td>
<td>- (Not available)</td>
</tr>
<tr>
<td>(Weir et al. 1997)</td>
<td>All together</td>
<td>- (Not available)</td>
</tr>
<tr>
<td>(Sacco et al. 1994)</td>
<td>ND+D, I</td>
<td>39% (D)</td>
</tr>
<tr>
<td>(Toni et al. 1994)</td>
<td>ND, I</td>
<td>49%</td>
</tr>
<tr>
<td>(de Falco et al. 1993)</td>
<td>ND, I</td>
<td>52%</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td>[All patients]</td>
</tr>
<tr>
<td>(van Kooten et al. 1993)</td>
<td>ND, I+H</td>
<td>35% (of all patients)</td>
</tr>
<tr>
<td></td>
<td>D, I+H</td>
<td></td>
</tr>
<tr>
<td>(Kiers et al. 1992)</td>
<td>ND, I</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td>[All patients]</td>
</tr>
<tr>
<td></td>
<td>D, H</td>
<td>[All patients]</td>
</tr>
<tr>
<td>(Matchar et al. 1992)</td>
<td>ND+D, I</td>
<td>53% (D)</td>
</tr>
<tr>
<td>(Toni et al. 1992)</td>
<td>ND, I</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td>[All patients]</td>
</tr>
<tr>
<td>(Cazzato et al. 1991)</td>
<td>ND, I</td>
<td>63%</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td>-</td>
</tr>
<tr>
<td>(Lee et al. 1991)</td>
<td>ND+D, I</td>
<td>83% (D), 30%</td>
</tr>
<tr>
<td></td>
<td>ND+D, H</td>
<td>(ND)</td>
</tr>
<tr>
<td>(Tuhrim et al. 1991)</td>
<td>ND+D, H</td>
<td>- (Not available)</td>
</tr>
<tr>
<td>(Kushner et al. 1990)</td>
<td>ND, I</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td>-</td>
</tr>
<tr>
<td>(Woo et al. 1990)</td>
<td>ND, I</td>
<td>8%</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ND, H</td>
<td>28%</td>
</tr>
<tr>
<td>(Adams et al. 1988)</td>
<td>All together</td>
<td>52%</td>
</tr>
<tr>
<td>(Woo et al. 1988)</td>
<td>All together</td>
<td>- (Not available)</td>
</tr>
<tr>
<td>(Gray et al. 1987)</td>
<td>ND, I</td>
<td>28%</td>
</tr>
<tr>
<td>(Candelise et al. 1985)</td>
<td>ND, I</td>
<td>19%</td>
</tr>
<tr>
<td></td>
<td>ND, H</td>
<td>13%</td>
</tr>
<tr>
<td>(Pulsinelli et al. 1983)</td>
<td>ND, I</td>
<td>45%</td>
</tr>
<tr>
<td></td>
<td>D, I</td>
<td>-</td>
</tr>
<tr>
<td>(Melamed 1976)</td>
<td>ND, H</td>
<td>28%</td>
</tr>
</tbody>
</table>
In summary, numerous clinical studies link hyperglycemia to worse stroke outcome (Capes et al. 2001; Bruno et al. 2004). However, it is controversial whether hyperglycemia, independently of other prognostic factors, worsens the brain damage or simply reflects stroke severity per se.

Diabetes and stroke

Diabetic patients have an increased susceptibility to atherosclerosis and have an increased prevalence of atherogenic risk factors: hypertension, obesity, and abnormal blood lipids in particular. Case-control studies of stroke patients and prospective epidemiological surveys have confirmed an independent relationship with diabetes. Diabetes is associated with a two- to fourfold increase in the risk of ischemic stroke (Abbott et al. 1987). Diabetic patients suffer more often from ischemic than hemorrhagic stroke (Roehmholdt et al. 1983). Ischemic stroke in diabetic patients is associated with a poorer outcome and increased mortality (Olsson et al. 1990); the 28-day case- fatality rate after acute stroke is shown to be higher in diabetic than non-diabetic patients (Asplund et al. 1980; Jorgensen et al. 1994; Stegmayr et al. 1995). In observational studies, one third of patients with acute stroke have diabetes (Olsson et al. 1990; Jorgensen et al. 1994). According to the latest report (2003) from Riks-Stroke (The National Stroke Register in Sweden) over 20% of all stroke patients have diabetes.

Diabetes can affect the central nervous system in several ways (Sahay et al. 2001). The damage may be due to involvement of the large and small cerebral blood vessels by more severe atherosclerosis, but also due to metabolic derangement in the brain tissue by prolonged hypoglycemia, anoxia or ketoacidosis. Other emergencies that occur in diabetic patients are convulsive disorders in the setting of hypo- and hyperglycemia, coma, cranial neuropathies and acute proximal muscle weakness. Hyperglycemia at the time of stroke seems to be an important risk factor for a poor outcome and the use of MRI methods to delineate stroke topography and pathophysiology appear helpful to describe the effects of hyperglycemia (Baird et al. 2002). An aggressive treatment of hypertension and dyslipidemia is shown to decrease the risk of stroke in diabetic patients, while the importance of a stable diabetic control for stroke prevention is still unproven (Mankovsky et al. 2004).
Experimental stroke

Studies in small-animal models of cerebral ischemia play a central role both in investigations of the pathophysiology of ischemic brain injury and in preclinical studies of potentially neuroprotective drugs. Larger animal species, such as rabbits and primates, are of several aspects less desirable and serviceable. From the ecological as well as the animal-rights perspectives, the use of rodents is preferable. Furthermore, small-animal models have the advantage of lower maintenance and supply expenses. Inbreeding of small animals assures relative homogeneity within strains. Finally, with respect to both cerebrovascular anatomy and physiology, neuronal/glial cellular biology, and molecular biology, there are strong homologies between rodents and higher animals (Graham et al. 2004). During the last few decades, the development of less invasive methods of focal ischemia, the availability of transgenic mice, and the applications of imaging strategies such as MRI have contributed to the firmer establishment of the small-animal models (Takizawa S et al. 1991; Graham et al. 2004).

Rat models of cerebral ischemia

The rat models of cerebral ischemia can mainly be divided into global and focal. Global ischemia is always transient (TGI). The rat models most commonly used are the “two-vessel” occlusion (bilateral carotid artery occlusions) and the “four-vessel” occlusion (bilateral carotid artery occlusions with bilateral vertebral artery electrocoagulation). The “two-vessel” occlusion model of forebrain ischemia is designed to produce an abrupt onset of ischemia and an acute reestablishment of recirculation at the end of the insult (Smith et al. 1984). This model is suitable for chronic survival studies. The “four-vessel” occlusion model produces a high-grade transient ischemia and is applicable to either awake, freely moving or anesthetized rats (Pulsinelli et al. 1979). However, the latter model suffers from poor reproducibility. Moreover, ischemic periods longer than 30 minutes may lead to complicating seizures.

TGI affects widespread brain areas and typically alters the neurons within special brain regions (selective neuronal necrosis). By contrast, focal ischemia (either with or without reperfusion) leads to a histopathology characterized chiefly by focal pan-necrosis or infarction (Kuroiwa et al. 1994).
Focal cerebral ischemia in the rat

Middle cerebral artery occlusion (MCAO)

Rat models of middle cerebral artery occlusion (MCAO) have been widely used in recent years because of their similarity to the clinical condition of thromboembolic stroke (Garcia 1984; Hossmann 1991). A number of serviceable models of MCAO have been developed, and can basically be divided into (1) surgical access to the MCA through a craniotomy (Tamura et al. 1981), or (2) vascular access to the MCA through the carotid vessels and the circle of Willis (Koizumi et al. 1986; Longa et al. 1989). Numerous studies of potentially neuroprotective pharmacological agents have utilized these models.

MCAO via surgical exposure, introduced by Tamura (Tamura et al. 1981), requires a subtemporal craniotomy. Both the cortex and the caudoputamen can be infarcted dependent on the level of the MCAO (proximal-distal). The MCAO may be temporary or permanent dependent on the exact method of occlusion using for instance a minianeurysm clip (Morikawa et al. 1992), or a reversible snare ligature (Shigeno et al. 1985).

The method used in our laboratory (Lennmyr et al. 1998) is a modified MCAO by intraluminal suture, first described by Koizumi et al (Koizumi et al. 1986) and Zea-Longa et al (Longa et al. 1989). The procedure consists of inserting a monofilament e.g. a 3-0 nylon suture into the internal carotid artery (ICA) and advancing it cranially to the circle of Willis in Sprague-Dawly (Sp-D) rats to block MCA flow. The suture is introduced retrogradely through the stump of the external carotid artery (ECA), which branches together with the pterygopalatine artery (PPA) are interrupted. The heat blunted tip of the suture which passes beyond the origin of MCA effectively occludes collateral circulation from the anterior communicating arteries.

Cerebral blood flow (CBF) in the center of the MCA territory in the Zea-Longa method is shown to be reduced to about 1/3 (33 ml/100g/min) (Laing et al. 1993). Among normoventriculoplasty strains, mean infarct volume is somewhat larger and more consistent in Sp-D rats (Duverger et al. 1988). Periods of MCAO of 2 to 3 hours yields extensive neocortical and basal ganglionic infarction comparable in size to that produced by permanent MCAO (Memezawa et al. 1992). Moreover, spontaneous hyperthermia (>39°C) is shown to occur in ischemia lasting longer than 90 minutes (Li et al. 1999). Shorter periods of MCAO (90-120 minutes) lead to necrotic neurons in the cerebral cortex, whose number remain relatively stable over a 7-day survival period. Zhu et al have shown that with each 20 min of MCAO during the first two hours of ischemia, infarct size increases (Zhu et al. 1995). Furthermore, this process is extremely sensitive to systemic hypoten-
sion: for example, 120 min of MCAO at a mean arterial blood pressure of 80 mmHg leads to the same damage as only 80 min of MCAO at a pressure of 60 mmHg. Thus, rigorous control of physiological variables is needed to assure the model consistency. Infarct volume, particularly in models of temporary MCAO, is critically dependent on brain temperature (Morikawa et al. 1992). Mild to moderate hypothermia instituted in the first hours of MCAO significantly reduces the volume of brain infarction (Morikawa et al. 1992).

The morphological changes after focal cerebral ischemia

As in global cerebral ischemia, the cerebral cortex in focal ischemia may show both selective neuronal necrosis and infarction. In 1977, the threshold concept of critical levels of hypoxia was extended to cerebral ischemia by Astrup and coworkers (Astrup et al. 1977). They established the precisely defined threshold of CBF needed to support synaptic transmission (at the upper flow range) and ion homeostasis (at the lower). Brain regions with flow-rates lying between these two thresholds were termed *penumbra*. In other words, the penumbra is characterized as an ischemic condition that causes functional suppression without structural damage (Astrup et al. 1981). Thus the penumbra represents tissue at risk, carrying a potential of functional and morphological recovery. Therefore, treatment strategies in experimental stroke can be regarded as attempts to salvage the ischemic penumbra. In the clinical context, the same concept applies to early thrombolytic therapy. However, the predominant symptoms in focal brain ischemia are due not to selective neuronal necrosis but to functional suppression due to hypoperfusion. While the peri-infarct tissue exhibits some potential for effective treatment of ischemic stroke, currently available therapies cannot prevent subsequent progression to necrosis. Pan-necrosis in the setting of cerebral ischemia, results in total removal of all tissue elements of the neuropil except for blood vessels which are often spared (Garcia et al. 1971). Basal ganglia e.g. *Caudate nucleus* is affected by focal ischemia resulting from MCAO, if occlusion occurs proximal to the origin of the lenticulostriate arteries (Bederson et al. 1986). When MCAO occurs more distally, flow to the basal ganglia is preserved and purely cortical ischemia results. Chronic neuronal atrophy, and shrinkage of the surrounding neuropil, can be observed in the *thalamus, hippocampus*, and *cerebellum* after focal ischemia. The thalamus, if not directly damaged by focal ischemia, can undergo transsynaptic atrophy (diaschisis) when ischemia destroys large cortical regions (Nagasawa et al. 1990).
Glucose metabolism in MCAO in the rat

Nedergaard et al used multiple-label autoradiography to correlate changes in CBF with cerebral glucose metabolism at three time points during ischemia (Nedergaard et al. 1986). In the center of the ischemia where the CBF is less than 10% of control, there is no sign of glucose utilization, either early or after 20 hours of ischemia. On the contrary, in the first few minutes of ischemia, extensive areas of enhanced glucose utilization occur in both cortex and striatum surrounding the core of the ischemia, ranging from 160% to 180% of that in the contralateral hemisphere. The hyper-metabolic areas in the cortex have blood flows of 20% to 30% of those in homologous areas on the contralateral side, suggesting that this enhancement of glucose metabolism is caused by anaerobic glycolysis precipitated by relative hypoxia. The rim of hypermetabolism correlates spatially with the zone of sporadic neuronal injury that surrounds the sharply bounded infarct area consequent to MCAO. Hyperglycemia significantly eliminates this hypermetabolism (Nedergaard et al. 1988). The absence of increased glucose utilization in hyperglycemia can probably be attributed to reduced ion transport and higher threshold for spreading depression, which may be responsible for the enhancement of glucose utilization in normoglycemia (Nedergaard et al. 1988).

During recirculation following MCAO, regional glucose metabolism varies. In the filament model, blood flow in the ischemic core returns to the control level within 3 hours of reperfusion, whereas glucose metabolism remains at the same level as during ischemia (approximately 40% to 45% of control). In the periphery of the MCA-territory, where blood flow remains decreased at the ischemic level (35%-50%), glucose utilization increases to 160% of control, which hypothetically may be a result of edema in the ischemic core (Nagasawa et al. 1990).

Focal brain ischemia and pH changes

Tissue average measurements of intracellular pH (pH) during focal ischemia are as low as 6.3. Loss of cellular bicarbonate during acidosis from ischemia may be a primary cause of brain injury. The molecular mechanisms by which acidosis is harmful remain unclear. A free radical mechanism has been proposed to be dependent on a bicarbonate-required sequestration of iron. In an excessively acidic environment, free iron can catalyze the generation of free radicals with subsequent injury of membranes by lipid peroxidation (Siesjö et al. 1985). It seems likely that acidosis is a primary (or the sole) precipitant of brain cell death from ischemia under extreme conditions. Reasonably, many individual physiologic variables taken to extremes can be lethal and in
case of acidosis, less severe brain acid-base changes may modulate ischemic brain at a sublethal way. For example, experiments in tissue cultures (Giffard et al. 1990) and acute brain slices (Tombaugh et al. 1990; Tombaugh 1994) show that mild interstitial acidosis may be protective against hypoxia and excitotoxicity (glutamate exposure), by inhibition of calcium influx from extra- to intracellular spaces. However, the situation in vivo seems radically different since the extracellular calcium content is limited and the total tissue calcium content during ischemia is not altered for 1-2 days (Kristian et al. 1996). Possibly, the multitude of effects pH can exert on brain function, and its response to injury, reflects the diversity of pH changes that occur within specific types of brain cells and the interstitial space. Experiments with graded increase in plasma glucose have revealed linear relationship between tissue lactate content and delta pH, which varies from <0.4 to >1.4 units irrespective of preischemic CO₂ tension (Katsura et al. 1992). Measurements of extracellular pH (pHᵢ) have shown a threshold for seizure incidence and density of neuronal death at values of 6.40-6.45, corresponding to pH 6.2-6.3 (Li et al. 1995).

Hyperglycemia leads to a further decrease in intra-ischemic pH level compared with normoglycemia, since measurements of pHᵢ and pHᵣ demonstrate an accentuated reduction during transient ischemia in hyperglycemic animals (Smith et al. 1986; Chopp et al. 1988). This phenomenon may provide a possible explanation for the hyperglycemic aggravation of ischemic brain damage. The measured values are pHᵢ 6.6-6.8 in normoglycemia compared to 6.2-6.4 in moderate hyperglycemia (>12 mmol/l) with a small additional acidic shift following recirculation (Li et al. 1995). As pointed out above, pHᵢ and pHᵣ during ischemia vary almost linearly with the tissue lactate concentration (Katsura et al. 1992), the latter being a direct function of the preischemic plasma glucose concentration. Tissue damage is exaggerated over a very narrow range of plasma glucose concentrations and pHᵢ values (Li et al. 1997). However, it is unlikely that the hyperglycemia-accelerated injury in the reperfusion phase is due to lactic acid buildup since pHᵢ and pHᵣ usually returns to normal level after 15-30 min of recirculation in both normo- and hyperglycemic animals (Smith et al. 1986).

Brain ischemia and mitogen-activated protein kinases (MAPK)

The transmission of extracellular signals into their intracellular targets is mediated by a network of interacting proteins that regulate a large number of cellular processes. One such group of signal transduction
proteins is the MAPK, which regulates cell proliferation, differentiation, and development (Seger et al. 1995; Peyssonnaux et al. 2001). Following ischemia many factors are released, including growth factors, cytokines, glutamates, and free radicals, all of which have been shown to stimulate MAPK pathways (Irving et al. 2002).

The first MAPK to be characterized was the extracellular signal-regulated protein kinase (ERK), which typically responds to growth factor stimulation (Boulton et al. 1990; Segal et al. 1996). Subsequently other MAPKs have been identified, including JNK and p38 (Kyriakis et al. 1990; Han et al. 1994). The latter have also been named stress-activated protein kinases (SAPK), due to the responsiveness to stress stimuli such as UV-light and osmotic stress (Paul et al. 1997). The MAPK pathway, here exemplified with ERK, can be schematically divided into membranous and cytoplasmic phases. The first phase occurs in close proximity of the growth factor receptors and involves the activation of a small GTP binding protein ras, which is followed by the sequential stimulation of several cytoplasmic protein kinases, MAP3K (Raf-1), MAPKK (MEK 1/2), MAPK (ERK 1/2) (Segal et al. 1996), resulting in phosphorylation of a conserved threonine-X-tyrosine activation motif, figure 2.

MAPK interacts with a number of intracellular pathways, including the Src family kinases. The Src kinases regulate tyrosine phosphorylation, and the member Src has been linked to VEGF-mediated increase in vascular permeability, supposedly mediating ischemia-induced brain damage (Paul et al. 2001). Experimental data from rodents suggest that Src inhibitors (PP1, PP2) as well as Src -/- knockout (mice) can reduce brain injury after focal ischemia (Paul et al. 2001; Lennmyr et al. 2004). Moreover, studies in neuronal cell-cultures suggest that Src kinases, via NMDA receptor signaling, may contribute to an ERK-dependent injury mechanism in brain ischemia (Crossthwaite et al. 2004).
The stress-activated protein kinases (SAPK)

JNK and p38 are the most common SAPKs which are activated in response to stress stimuli and are associated with apoptosis, survival, proliferation, and differentiation depending on the cell type studied (Herdegen et al. 1998; Alessandrini et al. 1999; Irving et al. 2000; Wu et al. 2000). Three JNK isoforms have been detected in the mammalian system; JNK1 and JNK2 are expressed in all tissues, and JNK3 is predominantly found in the CNS (Martin et al. 1996). JNK activity in the brain is partly regulated by the scaffolding protein JIP-1 (JNK-interacting protein 1), which inhibits the phosphorylation of JNK and its translocation to the nucleus (Dickens et al. 1997). A number of studies have investigated the activation of JNK and c-jun in brain ischemia. However, the profile of activation appears to differ between experimental studies. Increased JNK activation is reported after global and transient focal ischemia in the rat (Herdegen et al. 1998; Hu et al. 2000). In the latter model, early and sustained JNK activity is found within the ischemic lesion (Hayashi et al. 2000; Wu et al. 2000), which may suggest a role in the mechanisms underlying ischemia-induced cell death. Further supporting a role of JNK in cell death, hydrogen peroxide has been shown to increase JNK activity and inhibition of JNK by alpha-synuclein (most probably mediated through increased expression of JIP-1) is protective (Hashimoto et al. 2002).

Alterations in gene expression and enzyme activity induced by cellular stress such as oxidative stress are mediated by the interplay of multiple signaling pathways, among which the SAPKs are the central mediators. Recently, the usage of specific JNK inhibitors has provided evidence that JNK activation is also involved in free-radical mediated cell death. However, studies are mainly conducted in neurodegenerative diseases, rheumatoid arthritis, and asthma models of inflammation (Waetzig et al. 2005). In transient focal brain ischemia, specific JNK inhibition is reported to reduce infarct volume and decrease the number of apoptotic cells present in the infarct region by inactivating with the mitochondrial apoptosis-signaling pathway (Gao et al. 2005). Another recent study describes the neuroprotective effect of specific JNK inhibition in transient global ischemia in the rat (Guan et al. 2005).
The extracellular signal-regulated kinase (ERK)

Post-mortem studies of human brain tissue indicate that there may be an association between ERK activation and central nervous system (CNS) disease. Aberrant neuronal presence of phospho-ERK (and other MAPKs) is noted in Alzheimer’s disease (AD), Parkinson’s disease (PD), and other Lewy body diseases (Zhu et al. 2002; Zhu et al. 2002). After fatal ischemic stroke (1-44 days), ERK phosphorylation has been described along with tyrosine phosphorylation and VEGF expression, mainly in the cortical infarct border zones (Slevin et al. 2000).

Phospho-ERK can phosphorylate substrate in different subcellular compartments, since ERK is continuously shuttled between cytoplasm and nucleus. Phospho-ERK localization depends on cytoplasmic or nuclear anchors, including cytoplasmic MEK, and specific ERK phosphatases as well as nuclear phosphatases (Colucci-D’Amato et al. 2003). Experimental evidence demonstrates that the active ERK subcellular localization can affect the biological function of the cell differently. ERK signaling is necessary for NGF-stimulated differentiation and survival in PC12 cells (Cowley et al. 1994). Paradoxically, EGF and insulin, which act as mitogens for PC12 cells, also activate ERK. The different duration of ERK activation, which correlates with different compartmentalization, is a key difference between differentiating agents (e.g. NGF) and mitogens. Prolonged, NGF-induced activation of ERK causes a long-lasting nuclear translocation whereas short, EGF-induced activation of ERK is localized to the cytoplasm (Colucci-D’Amato et al. 2003). While data from several laboratories reveal nuclear localization of active ERK in physiological functions, this property of ERK may not strictly apply to injured neurons. After injury induced by cerebral ischemia where cellular integrity is compromised, the degree of cellular regulation of kinase activity may be reduced or lost, thus leading to uncontrolled signaling in response to stimulation. For example, the ischemic breakdown of the cytoskeleton may lead to the loss of spatial separation of kinases and also to the disruption of the scaffolding proteins. Thus, the consequence of kinase activation after injury may thus be dependent on the immediate intracellular environment, the cell type, the number of kinase pathways activated, and the duration of kinase activation (Irving et al. 2002).

The activation of ERK and other MAPKs in cerebral ischemia, which was first reported one decade ago (Hu et al. 1994), has since been observed in various models of focal and global ischemia. However, the kinetics, duration and regional distribution of phospho-ERK differs in various models (Irving et al. 2002). In transient MCAO in the rat, phospho-ERK persists for up to 24 h (Irving et al. 2000) and
72 h (Lennmyr et al. 2002) within penumbral neurons and glia and is mainly localized to the cytoplasm, perikarya or neuropil. In contrast, chronically activated ERK is retained in the nuclei of damaged neurons from various brain regions following hypoxia/ischemia in neonatal rats (Wang et al. 2003) and in neuronal cell cultures subjected to glutamate-induced oxidative stress (Stanciu et al. 2002). In neurodegenerative diseases mentioned above (PD and AD and Lewy body dementias) ERK is localized within cytoplasmic granules (Zhu et al. 2002; Zhu et al. 2004). The observation of transiently increased phospho-ERK in the ischemic regions before cell death in both focal and global models of ischemia suggests involvement of ERK activation in ischemia-induced cell-death.

Neuroprotective effects of ERK inhibition
The specific MEK-inhibitors PD98059 and U0126 (figure 3) have allowed investigating the role of ERK in ischemia-induced cell death. PD98059 blocks the phosphorylation of MEK1 but can not effectively inhibit the activity of MEK1 once it is phosphorylated (Alessi et al. 1995). U0126 blocks the enzymatic activity of MEK 1/2 and thereby ERK activation (DeSilva et al. 1998; Favata et al. 1998) and thereby is more potent. Treatment of cell cultures with PD98059 or U0126 have been shown to reduce damage in response to various cytotoxic stimuli such as NMDA-mediated glutamate excitotoxicity, seizure activity, hypoxia, and oxygen glucose deprivation (OGD) (Bading et al. 1991; Murray et al. 1998; Runden et al. 1998; Satoh et al. 2000; Stanciu et al. 2000; Namura et al. 2001; Skaper et al. 2001). However, it is important to take into consideration that in cultured cell-lines and enriched primary neuron cultures, direct effects of pharmacological inhibitors of ERK activation can not be established, as contributions of glial cells derived cytokines or vascular effects of the inhibitors are not present.
In vivo, experimental data show that intravenous administration of U0126 can reduce the infarct volume after transient focal cerebral ischemia in the rat (Namura et al. 2001). These findings have been corroborated by other groups and in other experimental models (Wang et al. 2003; Wang et al. 2004). Although the MEK inhibitor studies offer evidence supporting a detrimental role for ERK signaling in acute brain injuries, other studies indicate that ERK promotes functional recovery following mild trauma (Dash et al. 2002). Differences in outcome resulting from MEK-inhibition may depend on the nature and severity of injury and even on drug dosing regimens and or the cell type expressing activated ERK (e.g. neurons contra glia) (Chu et al. 2004).

Ischemia and reactive oxygen species (ROS)
It is technically difficult to measure free radicals directly. Consequently, much of the evidence of the effects of ROS rely on indirect
observations. More direct evidence for participation of ROS in neuronal injury is derived from studies on specific free radical-mediated damage such as lipid peroxidation (Watson et al. 1984), or hydroxyl radical production (Globus et al. 1995) in the ischemic penumbra. These results point out ischemia, followed by reperfusion, as the major factor facilitating peroxidative free-radical chain process in brain. There are also results emphasizing the potential interaction between ROS and excitatory amino acids (Hammer et al. 1993; Globus et al. 1995), since high extracellular glutamate levels coincide with generation of hydroxyl radicals in the penumbral cortex (Morimoto et al. 1996). Although the majority of tissue damage occurs within the first few hours following cerebral ischemia (Dereski et al. 1993), penumbral tissue may continue to deteriorate later (Heiss et al. 1992; Dereski et al. 1993). Ischemia leads to a continued release of free fatty acids (FFA), which has been demonstrated to be due to calcium influx (Kunievsky et al. 1992). Later in an ischemic episode, the release of arachidonic acids (AA) increases, and is maintained during reperfusion. In sensitive areas such as the pyramidal cell layer in hippocampus, AA levels remains significantly high until cell death is observed (Kunievsky et al. 1992). Due to metabolism of AA to eicosanoids, a prolonged increase in AA promotes free radical production, which has deleterious effects on neuronal cell membranes. In addition, AA is shown to modulate kinase activity (Bramham et al. 1994) and increase Ras activity (Valitutti et al. 1991).

ERK has been shown to have a neuroprotective effect following oxidative stress (Guyton et al. 1996). In contrast, other experimental findings show that ERK inhibition reduces or blocks cytotoxicity following brain injuries (such as seizures, cerebral ischemia and trauma) (Murray et al. 1998; Alessandrini et al. 1999; Sugino et al. 2000; Mori et al. 2002). ERK is also activated by ROS (Sato et al. 2000) and its inhibition reduces ROS-induced cell death (Seo et al. 2001). As in the case of ischemia, oxidative ERK activation may result in survival or death, depending on different experimental conditions, such as duration of ischemia and animal species (Colucci-D’Amato et al. 2003). In addition, it is interesting to note that many model systems that suggest ERK in a detrimental role are associated with a delayed, sustained phase of ERK activation (Zhu et al. 2002). In the case of neurodegenerative diseases, phospho-ERK is found both in the substantia nigra of PD patients and in brain extracts from AD patients, but not in control individuals. The evidence linking chronic ERK activation to neurodegeneration are derived from studies on neuronal cell death induced by glutamate (Simonian et al. 1996) and neuronal cell cultures exposed to NMDA-mediated excitotoxicity (Stanciu et al. 2000). Furthermore, the SAPK pathways activated by oxidative stress, have been attributed
a role in chronic neurodegenerative diseases such as AD, where JNK has been found activated and redistributed from nuclei to cytoplasm (Zhu et al. 2004). In that study, dysfunctional mitochondria were pointed out as one of the major sources of oxidative stress by releasing excess levels of free radicals.

Redox regulation has been demonstrated at multiple steps of the classic receptor-regulated activation of ERK, including the inactivating phosphatases (figure 2) (Kamata et al. 1999). Generation of ROS leads to the activation of protein tyrosine kinases followed by the stimulation of downstream signaling systems including different MAPK (Jope et al. 2000; Yoshizumi et al. 2000). On the other hand, reductants generally suppress the upstream signaling cascade resulting in the suppression of related transcription factors. Not only are the cellular signaling pathways subjected to redox regulation, but also the signaling systems regulate the cellular redox state. When cells are activated by extracellular stimuli, they produce ROS, which in turn stimulate other cellular signaling pathways, indicating that ROS acts as a second messenger. Thus, there is evidence of cross-talking between the cellular signaling system and the cellular redox state.

Interestingly, from the cell signaling perspective described above, experimental evidence has been presented that Src kinases may serve as intermediate steps between ROS and MAPK activation in vitro (Jope et al. 2000; Yoshizumi et al. 2000).

Neuroprotection and brain ischemia

Numerous studies of the pathology and pathophysiology of ischemic brain injury are the main contributors to the concept of neuroprotection. Oxygen and glucose deprivation to neuronal cells elicits a series of pathological cascades that lead to neuronal death. Some of these numerous pathways documented in ischemic damage are believed to play critical roles, especially in the penumbra e.g.: (1) excessive activation of glutamate receptors, (2) intracellular accumulation of calcium ions, and (3) excessive production of free radicals (Cheng et al. 2004). During the last two decades, neuroprotective agents designed to block these cascades have been investigated in animal models of cerebral ischemia. Anti-oxidative treatment, as exemplified by the spin trap agent alpha-phenyl-N-tert-butyl nitronate (PBN) (figure 4), has proved effective in experimental stroke models (Folbergrova et al. 1995; Lancelot et al. 1997; Yang et al. 2000; Zausinger et al. 2000; Li et al. 2001). However, the literature offers sparse or no information on the efficacy of PBN in hyperglycemic-ischemic injury.
Another nitrone-based free-radical trapping agent, NXY-059, has been proven neuroprotective by improving the neurological outcome and reducing the infarct size when administered after the ischemia onset (Marshall et al. 2001; Zhao et al. 2001; Green et al. 2003). The safety of NXY-059 is proven in stroke patients (Lees et al. 2003) and clinical phase III trials have been performed.

**Hyperglycemia and MAPK in brain ischemia**

The MAPK have mainly been investigated in normoglycemic experimental stroke. In rat glomeruli, mesangial cells, and brain tissue (Bhardwaj et al. 1999) hyperglycemia is able to induce phosphorylation of ERK, through activation of PKC and PLA₂ (Haneda et al. 1997). In an experimental study of transient forebrain ischemia, pre-ischemic hyperglycemia has been shown to increase the activation of ERK and phospho-ERK has been found in areas of hyperglycemia-augmented damage (Li et al. 2001). In the same study, phosphorylation of ERK in the dentate gyrus was weaker in hyperglycemia at 30
minutes but stronger at 1 and 3 hours of reperfusion. This may reflect a biphasic effect of hyperglycemia on cell death by initially promoting mitochondrial respiration and preserving mitochondrial energetic status in the early reperfusion stage (Hillered et al. 1985; Wagner et al. 1986) but later accelerating cell death through other pathways. JNK and p38 have been investigated in the same model of transient global ischemia. However, they do not appear to be over-activated in the same manner (He et al. 2003).

Transient ischemia leads to production of ROS (Zini et al. 1992; Sen et al. 1993), the quantity of which is likely to be substantially increased by hyperglycemia in the ischemic brain (Li et al. 1999; Bonnefont-Rousselot 2002). This supports the hypothesis that ROS and associated mechanisms, including MAPKs/ERK, may constitute a major injury modality in hyperglycemic-ischemic brain injury. However, the evidence on the effect of hyperglycemia on the interacting intracellular pathways is sparse.

Hyperglycemic ischemic brain injury
Several studies in animals as well as in humans have shown that hyperglycemia, during both the ischemic and the recovery period, can accentuate neuronal damage (Myers et al. 1977; Siemkowicz et al. 1980; Pulsinelli et al. 1983). However, the results concerning the role of hyperglycemia in experimental models of both global and focal ischemia are conflicting. In case of transient ischemia, the detrimental effect of pre-ischemic hyperglycemia on the final damage is relatively uncontroversial (Myers et al. 1977; Gjedde et al. 1978; Ginsberg et al. 1980; Pulsinelli et al. 1982; Voll et al. 1991; Yip et al. 1991; Li et al. 1994; Cherian et al. 1997; Li et al. 2001). In case of permanent ischemia, hyperglycemia is shown to result in both larger (Nedergaard et al. 1987) and smaller infarct size (Ginsberg et al. 1987; Prado et al. 1988). Here it can be argued that if the ischemia is severe enough and prolonged enough to yield maximal infarct size, it is not likely that hyperglycemia can further aggravate damage unless it exaggerates edema or compromises collateral flow to such an extent that intracranial pressure increases. In permanent MCAO, hyperglycemia may therefore not be expected to influence the final infarct size. If hyperglycemia decreases infarct size, the increased glucose supply must allow additional ATP to be formed without enhancing acidosis or other negative factors to a detrimental level (Folbergrova et al. 1992).

Anaerobic metabolism, lactic acidosis and free radical production have been proposed to explain hyperglycemic-ischemic brain injury (Siesjö et al. 1996). The pathogenesis of this explanatory model includes: (1) damage to cell membranes and their way of handling cal-
cium ions, (2) impaired protein synthesis and (3) initiated programmed cell death (Siesjö et al. 1996). However, hyperglycemia can also exaggerate accumulation of extracellular glutamate release, brain edema formation, blood-brain barrier disruption, and the tendency for hemorrhagic transformation. Furthermore, repeated waves of spreading depression can lead to necrosis of the penumbral tissue (Kagansky et al. 2001). Evidence exists that hyperglycemia and enhanced intracellular acidosis prevents recovery of mitochondrial function after ischemia periods longer than 30 minutes (Hillered et al. 1984). This suggests that acidosis can trigger rapidly developing mitochondrial failure, possibly by mechanisms related to increased free radical formation (Siesjö et al. 1996).

Compared to normoglycemic ischemic brain injury, hyperglycemia augments edema development due to swelling of endothelial cells, dendrites, glial cells and ER (Kalimo et al. 1981; Inamura et al. 1987; Inamura et al. 1988). Moreover, hyperglycemia fastens transformation of selective neuronal damage to pan-necrosis, and shortens the maturation time of the brain injury. Severe hyperglycemia leads to post-ischemic (about 18-36 hours after ischemia) seizures (Cherian et al. 1997; Alessandrini et al. 1999). Hyperglycemia enhances the damage to vulnerable areas such as the hippocampal CA1 sector, caudoputamen and neocortex, and leads to damage of areas such as hippocampal CA3, cingulate cortex, ventroposterior nucleus of thalamus, and substantia nigra pars reticulata that normally survive short periods of ischemia in normoglycemic condition (Inamura et al. 1987; Inamura et al. 1988; Bramham et al. 1994).
Aims

Paper I

1. To investigate the relationship between the blood glucose concentrations and early stroke mortality in diabetic and non-diabetic stroke patients.

2. To identify optimal blood glucose concentrations in diabetic and non-diabetic stroke patients in routine care.

Paper II

3. To validate the selected model of hyperglycemia-aggravated focal ischemic brain injury in the rat.

4. To investigate the impact of hyperglycemia on cerebral ERK and JNK activation in focal ischemia-reperfusion injury.

Paper III

5. To investigate the therapeutic potential of intervention directed against ERK, Src and ROS-associated mechanisms in hyperglycemic-ischemic brain injury.

Paper IV

6. To investigate the effects of the specific MEK inhibitor U0126 on transient focal ischemic brain injury in both normo- and hyperglycemia.
Methods

Paper I

In order to investigate the correlation between blood glucose concentrations and early stroke mortality, we designed a retrospective study of hospitalized patients. The medical records of all patients discharged from the University Hospital in Uppsala with a diagnosis of acute stroke (ICD 10, I60-I64) in the period January 1- December 31, 1998 were reviewed. Only the first occasion of hospital stay was included in this study (first-event strokes).

According to existing routines, blood glucose was to be measured five and three times daily in diabetic and non-diabetic patients respectively. During hospital stay, extra doses of short-acting insulin were given as required to 45% of the diabetic stroke patients. No insulin therapy was given to non-diabetic patients. The total number of blood glucose measurements was 1 839 and 1 074 among diabetic and non-diabetic patients respectively.

The metabolic control of each patient was defined as the mean blood glucose concentration (MBG). MBG was calculated according to the formula: mean blood glucose value for day 1 + mean blood glucose value for day 2 etc./number of days that blood glucose was measured in that specific patient. Mean body temperature (MBT) was calculated by the same formula as was used for MBG. The mean values were based on rectal temperatures recorded twice daily in the wards.

Case-fatality rates at 30 days were studied in relation to blood glucose on admission (ABG) and the mean value during hospital stay (MBG). Other possibly predictive factors considered in the analyses were: age, sex, stroke subtype (ischemic or hemorrhagic), level of consciousness (as a marker of initial stroke severity), body temperature, blood pressure and infection. Simple and multiple logistic regression analyses were used to estimate odds ratios for 30-day case-fatality.

By estimating the areas under the receiver operating characteristic (ROC) curves, we compared the predictive value of blood glucose concentrations on 30-day case-fatality. The ROC curve is a plot of the sensitivity of a test versus its false-positive rate for all possible cut points (Obuchowski 2003). The optimal cut-off level for the predi-
tion of early mortality was identified for diabetic and non-diabetic patients separately.

**Paper II, III, and IV**

Experimental model of transient focal brain ischemia

Focal cerebral ischemia was induced in male Sp-D rats using the filament technique of middle cerebral artery occlusion (MCAO) as described by Longa et al. (Longa et al. 1989). The left carotid region was exposed through a midline neck incision, and the external carotid artery (ECA) was divided after dividing the small ECA branches. The pterygopalatine artery (PPA) was temporary ligated to facilitate passage of a 3/0 suture with a rounded tip. The tail artery was catheterized for invasive blood pressure and blood samples. Peri-operatively, physiological data (below) were registered and electroencephalogram (EEG) recorded to confirm ischemia over the left MCA territory (Williams et al. 2001). In paper III and IV, a PE-50 catheter was inserted into the left femoral vein for the i.v. access of the drugs.

Table 2. Experimental design of paper II, III, and IV. In all studies, 90 minutes of MCAO was used for cerebral ischemia.

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<th>Groups</th>
<th>Survival</th>
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<td>Neurological outcome</td>
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<td>Mechanisms</td>
<td>0.5 h and 4.5 h&lt;sup&gt;↑&lt;/sup&gt; of reperfusion</td>
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<td>Western immunoblotting&lt;sup&gt;↑&lt;/sup&gt;</td>
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<sup>(Bederson et al. 1986)</sup>
<sup>↑Indicating muscular strength and proprioception (Johansson et al. 1996; Roos et al. 2003)</sup>
<sup>TTC=triphenyl-tetrazolium-chloride</sup>
<sup>↑Paper II only</sup>
<sup>↑Paper II and IV</sup>

In paper II, the rats were randomised to glucose or control solution, and hyperglycemia was induced by an IP injection of glucose (2g/kg (Yip et al. 1991)) given as a 15% solution and 60 minutes prior to MCAO.

In paper III, i.v. infusion was started 60 minutes after the onset of brain ischemia and continued for a total time of 60 minutes. The animals were given either (1) 1% DMSO in 0.9% NaCl (physiological
saline), (2) N-tert-Butyl-alpha-phenylnitrone, PBN (100 mg kg⁻¹), (3) Src inhibitor PP2 (10µg kg⁻¹), or (4) the specific MEK-inhibitor (upstream of ERK) U0126 (200 µg kg⁻¹). The administration of the drug was randomized and blinded to the operator until the volumetric analyses were completed.

In paper IV, U0126 was given in a dosage of 400 µg/kg and the vehicle in an equal volume of 1% DMSO in 0.9% NaCl. The compounds were given as 60-min intravenous infusions starting either 30 minutes prior to the MCAO or after 60 min of MCAO.

Immunohistochemistry
Perfusion-fixated, paraffin embedded coronal sections cut into 5 µm slices were used. Hematoxylin-eosin was used for light microscopy and immunofluorescence was performed according to standard protocols. The primary antibodies used were mouse monoclonals directed against phospho-ERK and phospho-JNK. FITC-conjugated rabbit anti-mouse was used as secondary antibodies and propidium iodide was used as counter staining.

Western immunoblotting
The brains were frozen in liquid nitrogen and kept in −70°C before preparation of lysates. SDS-PAGE was performed under reducing conditions. The proteins were then transferred to a PVDF-membrane and Western immunoblotting was performed with mouse monoclonal anti-phospho-ERK or anti-phospho-JNK antibodies. HRP-conjugated rabbit anti-mouse was used as secondary antibody and the blots were developed using ECL (enhanced chemiluminescence).
Results and discussion

Paper I

The study included 447 patients. Diabetes was previously diagnosed in 81 (18.1%) patients. The MBG values were 3.5 mmol/l higher in diabetic than non-diabetic patients.

Simple logistic regression analyses of 30-day case-fatality identified the highest odds-ratios for mean blood glucose (MBG), decreased level of consciousness, age and mean body temperature in both diabetic and non-diabetic patients. These risk factors that were most strongly related to mortality were then included in the multiple logistic regression analyses. The strongest relationships were found between MBG, level of consciousness and early mortality in both groups. Depressed consciousness, as well as infarct size and the severity of the neurological deficit have previously been reported as clinical predictors of early outcome and age is shown as the risk factor most closely correlated to stroke (Socialstyrelsen 2002). Furthermore, both elevated blood glucose and elevated body temperature have been shown to have a negative impact on stroke outcome (Boysen et al. 2001). However, very few (if any) of previous studies have included all these risk factors in their analyses. Our study confirms the close association between elevated blood glucose and early stroke mortality.

Several mechanisms may contribute to worse outcome in hyperglycemic stroke. For example, animal studies have shown that increased blood glucose impairs the metabolic state in the ischemic penumbra (Lindsberg et al. 2004). Two recent MRI-studies including both non-diabetic and diabetic patients have shown that post-stroke hyperglycemia is associated with expanding brain infarctions (Baird et al. 2002; Parsons et al. 2002; Baird et al. 2003). In one of these studies, subcutaneous glucose concentrations were measured continuously for 72 hours and glucose concentrations ≥7 mmol/l predicted infarct expansion. On the other hand, neither an admission blood glucose ≥8 mmol/l nor an elevated HbA1c were associated with progression of the lesion volume (Parsons et al. 2002). Thus, blood glucose concentration after stroke seems to be of greater importance than blood glucose concentration on admission, both in these MRI-studies and in our relatively large clinical study.
In our analyses MBG seems to be a considerably stronger predictor of early stroke mortality than ABG in both diabetic and non-diabetic patients and despite stroke subtype. According to the ROC-curves and assuming the equal importance of sensitivity and specificity, the most optimal concentrations of MBG for predicting 30-day case-fatality was 10.3 mmol/l in diabetic and 6.3 mmol/l in non-diabetic patients. The reason for this discrepancy is obscure. One can speculate whether this is a random phenomenon or if the brain tissue of diabetic patients is adapted to higher blood glucose concentrations. Another possible explanation may be that small (lacunar) infarctions are the typical type of stroke in diabetic patients (Ghika et al. 1989). Similarly, a meta-analysis of 26 clinical studies (Capes et al. 2001), estimated the pooled relative risk of 30-day stroke mortality associated with hyperglycemia to be higher in non-diabetic than diabetic patients (3.07 (95% CI-2.50-3.79) compared to 1.30 (95% CI-0.49-3.43)).

In this context, it is of interest to notice that the target values for insulin therapy after stroke differs between guidelines. According to the recommendations issued by the European Stroke Initiative (EUSI), blood glucose concentrations above 10 mmol/l should be treated, while the American Stroke Association recommends that concentrations above 16 mmol/l (300 mg/dl) are to be treated (Lindsberg et al. 2004).

It remains to be determined whether lowering and maintaining of blood glucose concentrations after stroke can reduce early mortality. This question can only be answered by randomized trials similar to those performed in myocardial infarction and intensive care (Malmberg et al. 1995; van den Bergh et al. 2001). Data from the United Kingdom Glucose Insulin in Stroke Trial (GIST) show that the concept of glucose-insulin infusion is relatively safe in non-diabetic stroke patients (Gray et al. 2004). In view of our current results, it seems relevant and important to design and conduct randomized trials also for diabetic stroke patients with as well.

Paper II

The model of injecting intraperitoneal glucose bolus resulted in moderate hyperglycemia (generally <15 mmol/l), as expected from the description of this particular subgroup in an earlier characterization of the model (Yip et al. 1991). Other physiological parameters were stable and similar in the two groups, except for significantly lower base excess after 60 min in the glucose-treated group. This difference in base excess was found along with significantly higher blood glucose concentrations. However, the blood lactate concentrations of some of
these rats were measured and appeared to be within normal ranges and no acidotic pH-shift could be observed in these rats.

Volumetric analysis revealed significantly larger infarct volumes in the hyperglycemic group (193 ± 74 mm$^3$ vs. 61 ± 43 mm$^3$; p<0.01, figure 5) which is consistent with previous findings in other models of cerebral ischemia (Voll et al. 1991; Yip et al. 1991).

![Figure 5](image)

*Figure 5.* Volumetric analysis of infarct size (left), which is significantly larger in glucose than control rats (n=7 in each group) and performance on the inclined plane (right), where the average of three maximal angles was recorded prior to middle cerebral artery occlusion (MCAO) and after 1 day of survival. The glucose group managed significantly worse than controls both in terms of absolute performance and the relative difference between the two recordings. Significance marks on the inclined plane (*/**) refers to absolute and relative differences respectively, *=p<0.05, **=p<0.01.

Neurological testing included the Bederson scoring (Bederson et al. 1986) and the inclined plane test (figure 5) (Johansson et al. 1996; Roos et al. 2003), which has previously been proved useful in other experimental models of brain ischemia. The performances of the two groups in these tests were consistent with the results of the volumetric analysis supporting the finding of aggravation of the ischemic brain damage by hyperglycemia.

In addition, infarct size expectedly correlated both to blood glucose and to the relative difference in the performance of the animals on the inclined plane (figure 6). This supports the relevance of “inclined plane test” in evaluating the neurological injury in the present MCAO model.
Immunolabeling revealed that hyperglycemic rats had widespread cortical ERK activation on the lesion side, and to a certain extent also in the hippocampus-dentate gyrus nerve fibers on the ipsilateral side. In controls, ERK activation was present in the infarct region, but generally sparse in comparison with hyperglycemia. Western blot analysis showed consistent findings.

As pointed out in a recent review on the role of ERK in oxidative stress (Chu et al. 2004), the outcome of ERK activation depends, among other factors, on cell type and subcellular localization. In the present work, we did not study these questions specifically; however, we were able to identify activated ERK in both the neuronal cytoplasm and the nuclei. Whether ERK is present in other cell-types in the brain is not contradicted by our data, but this was on the other hand not apparent.

Our results resemble the data reported in a model of transient forebrain ischemia. In that study, the changes were seen in the hippocampus-dentate gyrus, which is considered the most vulnerable regions in that model (Li et al. 2001). In the present material, some changes were seen in the hippocampus-dentate gyrus as well, although these regions belong to another vascular territory than MCA. According to our observations, ERK activation was mainly seen in the MCA territory, anatomically corresponding to the primary olfactory cortex. These phospho-ERK positive regions coincided with regions identified as infarcted in the volumetric analyses, indicating that the combination of hyperglycemia and ischemia-reperfusion is a strong triggering factor for ERK activation in the brain. Regarding the data from normoglycemic models, it is imaginable that the increased damage seen in hyperglycemic-
ischemic brain injury is partly mediated by ERK, although its exact pathogenesis is unclear.

It is well known that hyperglycemia has a deleterious effect in stroke patients by accelerating ischemic brain injury (Kagansky et al. 2001). However, interventional studies are needed for proving the beneficial effect of strict glycemic control. Recent thrombolysis data identify the harmful effect of hyperglycemia, especially when recanalization is successful early after ischemia (Alvarez-Sabin et al. 2004; Ribo et al. 2005). Conventional insulin therapy may be inappropriate in the narrow time window of a few hours, which offers an opening for alternatives to insulin therapy. Consequently, ERK should be considered as such a target for future interventional studies in hyperglycemic-ischemic brain injury.

JNK activation was present in deep cortical regions throughout both hemispheres of hyperglycemic as well as normoglycemic rats. Western blot analysis revealed increased activation in the contralateral hemisphere in the hyperglycemic rats. It is uncertain whether this represents an ipsilateral decrease or a contralateral (non-ischemic side) increase. Possibly, a deactivation of JNK might be mediated by excessive activation of survival pathways such as ERK (Zablocka et al. 2003). In fact the neuroprotective effects of insulin have recently been attributed Akt/PI3K dependent negative regulation of JNK (Hui et al. 2005). Whether this is the case in the present model remains to be shown. Nevertheless, the present data do not support a role for JNK in hyperglycemic-ischemic brain injury, which is consistent with previous observations (He et al. 2003). However, it should be pointed out the data so far can not be regarded as conclusive and further functional studies may be indicated.

Paper III

Thirty-three animals were randomized to either treatment with PBN, PP2, and U0126 or control (1% DMSO in 0.9% NaCl) and evaluated with lesion size measurement and neurological assessment (table 3).

PBN significantly reduced infarct volumes approximately 70% compared with controls. PBN rats also performed significantly better in neurological tests. A tendency towards infarct size reduction was seen also in the PP2 and U0126 groups.
Table 3. Number of rats in different treatment groups in paper III. The perioperative complication rate varied between the groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Excluded</th>
<th>Included</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>Post-operative death with SAH</td>
</tr>
<tr>
<td>PBN</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>SAH on post-mortem exam (n=2), post-op death with and without SAH (n=2)</td>
</tr>
<tr>
<td>PP2</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>U0126</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>Post-operative death without SAH</td>
</tr>
<tr>
<td>All</td>
<td>33</td>
<td>7</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

The mechanism behind the effect of PBN in the present model is unknown. PBN is primarily recognized as a spin trap agent interfering with ROS toxicity, which could be a plausible explanation with respect to the increased oxidative stress in hyperglycemia-ischemia (Bonnefont-Rousselot 2002). Furthermore, PBN has been suggested to suppress: (1) ROS production in mitochondria, (2) reactive nitrogen species (RNS) formation in neuroinflammation, and (3) to interact with signal transduction processes (Floyd 1999) including MAPK (Tsuji et al. 2000). There is also evidence of a protective effect of PBN against excitotoxicity (Lancelot et al. 1997).

Ischemia is accompanied by mitochondrial dysfunction, as assessed by measurements of mitochondrial respiratory activities (Perez-Pinzon 2004). Particularly after ischemia of longer duration, reperfusion may be accompanied by secondary mitochondrial failure. Experimental data suggest that stabilization of the mitochondrial membrane is neuroprotective (Siesjö et al. 1999). Oxidative stress leads to collapse of the mitochondrial membrane potential, to ATP hydrolysis, to enhanced production of reactive oxygen species (ROS), and finally to cell death. Thus, prevention of the secondary mitochondrial failure during early circulation could be a possible mechanism behind the observed effects of PBN.

PP2 and U0126 did not prove effective in the present experimental design in paper III. There was a tendency towards reduced infarct size in these groups, an observation that should be interpreted with precaution since these groups also carried the highest peri-operative mortality. The intention with U0126 was to attenuate the ERK hyperactivation observed in paper II. The neuroprotective effect of specific ERK inhibition had been shown in a normoglycemic ischemia model, from which the dosage of U0126 was copied (Alessandrini et al. 1999; Namura et al. 2001). The aim with PP2 was to block src-family kinases, however, no intravenous dosage could be extracted from the literature. Previously, neuroprotective effects have been described for this type of inhibitor at 1500 g/kg intraperitoneally (Paul et al. 2001; Lennmyr et
al. 2004). Therefore, we made an estimation of 100 g/kg, which was adjusted to 10 g/kg due to toxicity with the higher dosage in preliminary experiments.

In summary, PBN appeared highly neuroprotective, while no conclusion could be made whether ERK or SFK inhibition is beneficial in hyperglycemic-ischemic brain injury. Therefore, additional experiments with adjusted dosage and evaluation of drug effect were warranted and conducted.

**Paper IV**

The findings by Namura et al in normoglycemic stroke rats suggests that pretreatment as well as early administration of U0126 ameliorates the ischemic damage (Namura et al. 2001). To cover that aspect, the therapeutic potential of high dose U0126 was examined. In paper III, 200 g/kg of U0126 was not effective in reducing the brain damage, although reported neuroprotective in earlier studies (Namura et al. 2001). Considering the strong ERK hyperactivation seen in paper II, the hypothesis of a stochiometric problem was formed. In line with this, an experiment was designed where the timing of administration was varied and carried out in both hyperglycemic and normoglycemic rats. The rats were assigned a regime with two serial 60 minutes-infusions containing either U0126 (U) or intravenous control vehicle (C). The first infusion was started 30 minutes prior to MCAO and the second 30 minutes prior to reperfusion (i.e. after 60 minutes of MCAO) (table 4).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>i.p injection (MCAO -60 min)</th>
<th>1st i.v. infusion (MCAO -30 min)</th>
<th>2nd i.v. infusion (MCAO +60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC</td>
<td>7</td>
<td>Glucose</td>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td>HCU</td>
<td>7</td>
<td>Glucose</td>
<td>Vehicle</td>
<td>U0126</td>
</tr>
<tr>
<td>HUC</td>
<td>7</td>
<td>Glucose</td>
<td>U0126</td>
<td>Vehicle</td>
</tr>
<tr>
<td>NCC</td>
<td>7</td>
<td>Control</td>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td>NCU</td>
<td>7</td>
<td>Control</td>
<td>Vehicle</td>
<td>U0126</td>
</tr>
</tbody>
</table>

TTC staining revealed significantly smaller infarct volumes in the normoglycemic groups compared to hyperglycemic controls (NCC and NCU vs. HCC; p<0.001) (figure 7). These infarct volumes are similar to those of paper II, which corroborates the stability of the ischemia model used. U0126-treatment in hyperglycemic groups significantly
reduced infarct volumes compared with controls (HCU and HUC vs. HCC; p<0.01). To our knowledge, the previous experience of MEK inhibition in MCAO is limited to normoglycemia (Alessandrin et al. 1999; Namura et al. 2001), which makes this the first report on hyperglycemia.

![Infarct size](image)

*Figure 7.* The volumetric analyses of infarct sizes (N=7 in each group), which are significantly smaller in U0126-treated groups (HCU and HUC) compared with control. The normoglycemic groups showed the smallest infarct volumes.

The lack of statistically significant reduction of infarct volumes in normoglycemic animals in the present study may represent a beta error since the study was not powered to exclude such a difference between groups. Thus, the present data do not contradict previous findings on the neuroprotective effects of U0126 in NG-ischemia (Namura et al. 2001).

On the inclined plane, U0126 significantly improved the performance in hyperglycemic rats compared with untreated controls (figure 8). The difference between the two normoglycemic groups was not statistically significant. The hyperglycemic rats treated with U0126 during ischemia-reperfusion had significantly lower scores than hyperglycemic controls. In contrast, the lower scores of rats treated with U0126 prior to ischemia were not statistically significant. In line with paper II, both normoglycemic groups had better neurological scores than the hyperglycemic controls. In concordance with the volumetric
analysis the difference between the two normoglycemic groups was not statistically significant.

![Graph](image)

**Figure 8.** The performance on the inclined plane (left), where the average of three maximal angles was recorded prior to MCAO and after 1 day of survival. The two hyperglycemic groups treated with U0126 (HCU and HUC), as well as both normoglycemic groups (NCC and NCU), managed significantly better than hyperglycemic, untreated controls (HCC). The neurological scoring (right), where the medians are indicated, ranging from (0) normal, (1) forelimb flexion, (2) decreased resistance to lateral push or (3) circling. The HCU, NCC, and NCU groups scored significantly better than controls (HCC, p<0.05).

Western immunoblotting (phospho-ERK) revealed a double-band consistent with the expected size of 44/42 kDa. A strong band signal in cortical samples from the occluded hemisphere of hyperglycemic rats, along with weak signals in corresponding samples from U0126 treated animals, confirmed the effective inhibition of ERK by U0126.

Considering the results from paper IV, it appeared beneficial to use a higher dose of U0126 compared to paper III. In summary, this study demonstrates the novel finding of neuroprotective effect of specific MEK-inhibitor U0126 on hyperglycemic focal brain injury.
Concluding remarks

This doctoral thesis spans over clinical observations and animal experiments on stroke and hyperglycemia. In paper I, we identified a correlation between early stroke mortality and mean blood glucose concentrations during hospital stay in both diabetic and non-diabetic patients. In paper II, we showed that glucose administration leads to increased infarct size along with ERK hyperactivation. In paper III, we were able to demonstrate that alpha-phenyl-N-tert-butylnitrone (PBN) reduced the hyperglycemic-ischemic brain injury, and in paper IV, we showed that U0126 reduced the hyperglycemic-ischemic brain injury along with inhibited ERK activation.

Hyperglycemia might, as commonly assumed, aggravate ischemic brain damage by further decreasing intra-ischemic pH levels. However, this mechanism has mainly been associated with higher blood glucose concentrations than observed in the present model. Therefore, it is not evident that intracellular changes such as the observed ERK activation in paper II depend on acid-base shifts. Irrespective of the presence of acidosis, enhanced production of reactive oxygen species (ROS) occur in hyperglycemia, and might represent a damaging component itself, or by providing a link to the observed ERK activation. The finding in paper III that PBN ameliorates the hyperglycemic ischemic injury indicates a role of ROS as pathogenic mediator. However, the mechanisms, including the effects on the redox state, need to be addressed further. There is growing evidence of a connection between ROS and ERK (Chu et al. 2004), and taken together, the findings in paper II-IV are consistent with such a mechanism. There are several possible levels of interaction between ROS and ERK including both upstream activation and defective downstream deactivation. Outlining this pathway will be an interesting challenge for future studies.

It is possible that research never will fully clarify the exact mechanisms of ROS, ERK and other mediators in hyperglycemic-ischemic brain injury. Nevertheless, one could argue that, from an empirical point of view, the neuroprotective effects of substances such as PBN and U0126 render them a possible role in the development of future therapies. Hopefully, mechanistic and empirical approaches will complement each other in the search for useful tools in treating hyperglycemic-ischemic brain injury.
Conclusions

- Blood glucose concentrations are strongly and independently correlated to early stroke mortality in diabetic as well as non-diabetic patients.

- The optimal blood glucose concentration seems to be lower in non-diabetic than diabetic stroke patients.

- Hyperglycemic rats develop larger infarct volumes in the experimental model of transient focal ischemia (MCAO).

- Consistent with the larger brain injury, hyperglycemic rats also show worsened neurological outcome 1 day after MCAO.

- ERK is hyper-activated in the brains of hyperglycemic rats with transient MCAO.

- PBN reduces hyperglycemic-ischemic brain injury in transient MCAO in the rat.

- High dose U0126 eliminates ERK activation in the post-ischemic brain in the rat.

- U0126 reduces infarct volume and improves neurological function.
Allmän bakgrund


Förhöjt blodsocker (hyperglykemi) förekommer hos upp till 2/3 av strokepatienterna, och kan förklaras av ökad halt av stresshormoner och därmed spegla slaganfallets svårighetsgrad. Många patienter med hyperglykemi kan dessutom ha en försämrad sockertolerans eller insulinkänslighet och löper viss risk för att i framtiden utveckla diabetes. Huruvida hyperglykemi är farlig för den hjärnvävnad som är drabbad av akut nedsatt blodcirkulation (ischemi), är noggrant studerat och forskningen har bedrivits såväl kliniskt som experimentellt. Hos de flesta patientgrupper som undersöks har ett samband funnits mellan hyperglykemi och sämre prognos. I djurmodeller däremot är utfällen varierande, men hyperglykemi förefaller leda till större hjärnskada om infarktområdet får blodcirkulationen tillbaka.

Idag saknas vetenskapligt grundade råd för behandling av högt blodsocker hos strokepatienter. Europeiska strokeallskapet rekommenderar att blodsocker över 10 mmol/l ska behandlas medan American Heart Association sätter gränsen vid drygt 16 mmol/l. Två undersökningar av socker-insulin-dropp hos patienter med akut hjärtinfarkt respektive patienter i intensivvård, har resulterat i minskad dödlighet hos de behandlade patienterna. En liknande undersökning pågår bland strokepatienter i Storbritannien. Preliminära data bekräftar att droppbehandlingen är relativt enkel och säker att utföra.

Delarbete I

Denna doktorsavhandlings delarbete I är en tillbakkblickande (retrospektiv) studie av strokepatienter vårdade på Akademiska sjukhuset

Experimentell bakgrund

Experimenten i denna doktorsavhandling designades med utgångspunkt i resultaten från delarbete I samt data om cellernas signalvägar. Målet var att undersöka det förhöjda blodsockrets skademekanismer. Signalproteinet ERK reglerar cellens basala funktioner såsom tillväxt, utveckling och överlevnad. ERK som tidigare visats skadligt vid experimentell stroke, ökar i aktivitet vid kombinationen hyperglykemi och global hjärnischemi. I den experimentella modellen i delarbeten II, III och IV, framkallas stroke med hjälp av en nylontäta (filament) som förs in via vänster sidans halsblodkärl hos rätta. Filamentet täpper till avgången för arteria cerebri media, motsvarande det vanligaste strokeområdet hos människor, och avlägsnas efter 90 minuter vilket häver cirkulationsstoppet (ischemin) och hjärnan återfår blodflödet (reperfusion). Ett dygn efter strokeen genomgår djuren neurologisk testning, varefter de avlivas och hjärnan fixeras, färgas och snittas för volymbestämning av infarkterna.

Delarbete II

I delarbete II jämfördes två grupper av rättor, varav den ena fick hyperglykemi medelst en sockerinjektion i buken och den andra saltlösning före induktionen av stroke. Den hyperglykemiska gruppen visade sig ha cirka tre gånger större hjärninfarkter än gruppen med normalt blodsocker. Dessutom visade färgning med antikroppar mot ERK i tunna hjärnnsnitt och efter proteinelektrofores en överaktivering av proteinet i infarktområdena. Slutsatsen av detta arbete är att kombinationen av högt blodsocker och fokal ischemi i hjärnan hos rätta leder till en överaktivering av ERK samtidigt som hjärnskadans storlek ökar.
Delarbete III

I delarbete III testades behandling riktad mot ERK (delarbete II) och två möjliga associerade skadesteg, nämligen fria radikaler och vissa tyrosinkinaser. Således gavs olika substanser som modulerar nämnda signalvägar till råttor med högt blodsocker. Fyra grupper, inklusive kontroll, fick droppbehandling kring tiden för återställandet av blodflödet. Hos råttor behandlade med PBN (mot fria radikaler) återfanns minska
de hjärninfarkter med ca 70 % samt signifikant bättre neurologisk funktion jämfört med kontroll. Grupperna behandlade med U0126 (mot ERK-aktivering) och PP2 (mot tyrosinkinaser tillhörande src-familjen) visade tendenser till minskade hjärninfarkter, men dessa fynd blev inte statistiskt signifikanta. Slutsatsen av delarbete 3 är att PBN eliminerar den av högt blodsocker förvärrade skadan beskriven i delarbete II.

Delarbete IV

Med stöd av tendensen till minskade hjärninfarkter av U0126- i delarbete III samt den uttalade ERK-aktivering i delarbete II, uppställdes hypotesen att skadan skulle kunna minska med en högre dos av U0126 än i delarbete III. Således gavs U0126 i en högre dos samt vid två olika tidpunkter till råttor med högt blodsocker. Som ytterligare kontroller gavs dessutom U0126 eller kontrolllösning till råttor med normalt blodsocker. Den hämmande effekten på ERK-aktiveringen visades med antikroppsanalys av vävnadsprover som i arbete II. U0126 resulterade i mindre hjärninfarkter samt bättre neurologisk funktion jämfört med kontrolllösning hos hyperglykemiska råttor. De minsta hjärninfarkterna fanns hos råttor med normalt blodsocker, där även en tendens till minskad skada kunde ses efter U0126.

Sammanfattningsvis förefaller ERK ha en mer framträdande roll vid kombinationen hyperglykemi och ischemi än bara vid ischemi i hjärn-
an.
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