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Antti Nurmi

The Role of Nuclear Factor kappa-B in Models of Adult and Neonatal Cerebral Ischemia: The Effects of Pyrrolidine Dithiocarbamate

Doctoral dissertation

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Series editors: Professor Karl Åkerman
Department of Neurobiology
A.I.Virtanen Institute for Molecular Sciences
University of Kuopio

Research Director Jarmo Wahlfors
Department of Biotechnology and Molecular Medicine
A.I.Virtanen Institute for Molecular Sciences
University of Kuopio

Author's address: Department of Neurobiology
A.I.Virtanen Institute for Molecular Sciences
University of Kuopio
P.O.Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 162 790
Fax +358 17 163 030

Supervisors: Professor Jari Koistinaho, M.D., PhD.
Department of Neurobiology
A.I.Virtanen Institute for Molecular Sciences
University of Kuopio

Dr. Nina Vartiainen, Ph.D.
Cerebricon Ltd
Kuopio, Finland

Dr Juha Yrjänheikki, Ph.D.
Cerebricon Ltd
Kuopio, Finland

Reviewers: Docent Jyrki Kukkonen, Ph.D.
Department of Neuroscience
University of Uppsala
Sweden

Docent Jouni Sirviö, Ph.D.
Orion Pharma
Finland

Opponent: Professor Dan Lindholm, M.D., D.M.Sc.
Minerva Foundation Institute for Medical Research
Biomedicum Helsinki
Finland

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ABSTRACT

Several pathophysiological mechanisms are involved in cerbral ischemia. Therapeutic interventions targeting delayed injury mechanisms have been promising in experimental ischemia models. Yet no clinically relevant treatments have been established besides intravenous thrombolysis. Several transcription factors, including nuclear factor kappa-B (NF-κB), play a role in cerebral ischemia. Because NF-κB was recently found to promote ischemic neuronal death, the potential of genetic or pharmacological inhibition of NF-κB to provide protection in brain ischemia models was investigated.

In permanent ischemia model genetic deletion of p50 subunit of NF-κB or treatment with pyrrolidine dithiocarbamate (PDTC) provided neuroprotection. This protection was strain and species independent. In models of transient focal ischemia, global forebrain ischemia and neonatal hypoxia/ischemia PDTC reduced the infarction with a wide therapeutic time window. In addition, PDTC provided protection in *in vitro* models of excitotoxicity and hypoxia/reoxygenation.

Human brain samples showed increased immunoreactivity and nuclear translocation of p65 subunit of NF-κB. Permanent ischemia model involved increased transcriptional activity of NF-κB, which was inhibited by PDTC. In models of transient focal cerebral ischemia and neonatal hypoxia/ischemia increased neuronal immunoreactivity and nuclear translocation of NF-κB p50 and p65 subunits were observed. Increased neuronal immunoreactivity and nuclear localization of p50 subunit was observed also *in vitro* models of neuronal injury. PDTC treatment effectively inhibited nuclear translocation of p50 subunit *in vitro* models.

Ischemia induced expression of inflammatory molecules tumor necrosis factor -α (TNF-α), interleukin -1β (IL-1β) and cyclooxygenase-2 (COX-2) 24 hours after the onset of brain ischemia. In PDTC treated animals the expression of all inflammatory markers studied were reduced. In neonatal brain elevated level of apoptotic marker, cleaved caspase-3, was transiently reduced in PDTC treated animals. Production of reactive oxygen species was reduced by PDTC *in vitro*.

To summarize, the results suggest that genetic and pharmacologic inhibition of NF-κB provides protection against various *in vivo* and *in vitro* models of brain ischemia. The protective effect of PDTC is associated with reduced transcriptional activity of NF-κB and inhibition of NF-κB regulated genes. In addition, PDTC has antiapoptotic and antioxidant properties. These results suggest that NF-κB may be a relevant therapeutic target in stroke treatment strategies.

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To beloved memory of Eila Nurmi
1921-1999

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In Kuopio, December 2004

Antti Nurmi

ABBREVIATIONS

Aβ	amyloid beta
ABI	atherothrombotic brain infarction
ACA	anterior carotid artery
ACPD	1-aminocyclopentane-1,3,-dicarboxylic acid
AD	Alzheimer's disease
AIDS	acquired immunodeficiency syndrome
AIF	apoptosis inducing factor
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazole proprionic acid
Apaf-1	apoptotic protease activating factor 1
APP	amyloid precursor protein
AP-1	activating protein -1
ATF	activating transcription factor protein
ATP	adenosine triphosphate
BBB	blood brain barrier
BCAO	bilateral carotid artery occlusion
bFGF	basic fibroblast growth factor
CA	cornu ammonis
CAD	caspase activated deoxiribonuclease
CBF	cerebral blood flow
CCA	common carotid artery
CINC	cytokine-induced neutrophil chemoattractant
CNS	central nervous system
COX-2	cyclooxygenase -2
CREB	cyclic adenosine monophosphate response element binding protein
DAB	diamino benzidine
DAG	diacylglycerol
DCF	2', 7'-dichlorofluorescein diacetate
DDTC	diethyl dithiocarbamate
Div	divisions
ECA	external carotid artery
EEG	electro enkephalography
ELAM	endothelium leukocyte adhesion molecule
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
EndoG	endonuclease G
FADD	Fas associated death domain
Gadd45β	Growth arrest and DNA damage-inducible protein
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte/macrophage colony stimulating factor

HIV	human immunodeficiency virus
HIF-1α	hypoxia inducible factor -1 alpha
H/I	hypoxia/ischemia
IAP	inhibitor of apoptosis
ICA	internal carotid artery
ICAM	intercellular cell adhesion molecule
ICE	inteleukin -1 converting enzyme
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL-1	interleukin -1
IL-1R	interleukin -1 receptor
IL-1ra	interleukin -1 receptor antagonist
IL-6	intereukin -6
IL-8	interleukin -8
IL-10	interleukin -10
iNOS	inducible nitric oxide synthase
IP_3	inositol 1,4,5-trisphosphate
JNK	c-Jun NH_2-terminal kinase
KA	kainic acid
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein -1
MEKK	mitogen-activated protein kinase/ERK kinase kinase -1
mGluR	metabotrobic glutamate receptor
MIP-1α	macrophage inflammatory protein - 1 alpha
MK-801	dizocilpine maleate
MMP	matrix metalloproteinase
MnSOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
NAC	N- acetyl cysteine
NADPH	nicotineamide adenine dinucleotide phosphate
NF-κB	nuclear factor κB
NiDAB	nickel enhanced diamino bentzidine
NIK	NF-κB inducing kinase
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NSAID	non-steroidal anti-inflammatory drug
pO_2	partial oxygen pressure
pCO_2	partial carbon dioxide pressure
PARP	poly (ADP-ribose) polymerase
PAF	platelet activating factor
PCA	posterior carotid artery
PCR	polymerase chain reaction
PD	Parkinson's disease

PDTC	pyrrolidine dithiocarbamate
PLC	phospholipase C
PMCAO	permanent middle cerebral artery occlusion
PMNL	polymorphonuclear leukocyte
Pro-DTC	proline dithiocarbamate
p50	50 kDa subunit of NF-κB
p65	65 kDa subunit of NF-κB
RANTES	regulated on activation, normal T expressed and secreted
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
SAPK	stress activated protein kinase
SDS-PAGE	sodium dodecyl sulfate polyacryl amide gel electrophoresis
SHR	spontaneously hypertensive rat
SN	substantia nigra
STAT	signal transducers and activators of transcription
TACE	TNF alpha converting enzyme
TGF-β	transforming growth factor beta
TIA	transient ischemic attack
TMCAO	transient middle cerebral artery occlusion
TNF-α	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TRADD	TNF receptor associated death domain
TTC	2,3,5 triphenyl tetrazolium chloride
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
VACC	voltage activated calcium channel
VCAM	vascular cell adhesion molecule

LIST OF ORIGINAL PAPERS

This thesis is based on the following scientific papers that are referred to by their corresponding Roman numerals:

I) Nurmi A, Lindsberg PJ, Koistinaho M, Zhang W, Juettler E, Karjalainen-Lindsberg ML, Weih F, Frank N, Schwaninger M, Koistinaho J.(2004) Nuclear factor-kappaB contributes to infarction after permanent focal ischemia. Stroke. 2004 Apr;35 (4): 987-91.

II) Nurmi A, Vartiainen N, Pihlaja R, Goldsteins G, Yrjänheikki J and Koistinaho J (2004) Pyrrolidine dithiocarbamate inhibits translocation of nuclear factor kappa-B in neurons and protects against brain ischemia with a wide therapeutic time window. Journal of Neurochemistry (in press).

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1 INTRODUCTION

Stroke is the third leading cause of death after cancer and heart disease. It is estimated that in the United States every 45 seconds someone has a stroke, and on the average, every 3 minutes someone dies of stroke (AHA, 2003). Stroke is also a major cause of serious and long term disability. In 1999, 1.1 million Americans reported difficulty with functional limitations and with activities of normal daily living resulting from stroke (CDC/NCHS, 1999). In Europe, the situation is not less dramatic since more than one million strokes per year occur in a population ofover 500 million (Brainin et al., 2000). Moreover, more than 10 000 individuals per year suffer from stroke in Finland. As strokes are considered a significant cause of death especially in elderly populations, hypoxic ischemia is also a common cause of damage to the fetal and neonatal brain. Neonatal strokes occur in approximately 1 in 4,000 to 1 in 10,000 newborns, and more than 80% of the cases involve the vascular territory supplied by the middle cerebral artery (Ashwal and Pearce, 2001).

In addition to its life-threatening properties, stroke is also a major cause of disability in the elderly and often requires long-term institutionalization. The economic burden of stroke to modern society is therefore overwhelming, since direct and indirect cost of stroke in the U.S. is estimated at more than $53.6 billion dollars in 2004 (AHA, 2003). At the moment no effective stroke therapy exists beyond thrombolysis, which is safe and effective for only a limited population of stroke patients. Therefore, the importance of long term rehabilitation and physiotherapy *after* stroke are more pronounced in modern stroke treatment (Pomeroy and Tallis, 2000).

Stroke is caused by disrupted blood flow to the major arteries of brain. Even a short lasting obstruction of cerebral arteries or hypoperfusion of the brain may cause

quickly irreversible brain damage. However, brain damage may further evolve for a relatively long period after acute insult. The concept of "penumbra", a salvageable tissue surrounding the center of the irreversibly damaged brain tissue in experimental settings has fueled extensive research aimed to establish a mechanism or mechanisms behind ischemic brain pathology and to find potential treatments for stroke. Indeed, several targets for neuroprotection including excitatory amino acids, calcium overload, enzymes, free radicals, gene expression, apoptosis and inflammation have been explored (Barone and Feuerstein, 1999; Dirnagl et al., 1999). Experimental therapies aimed at the very acute phase after ischemic insults have been found sometimes to be highly neuroprotective, but may be of no clinical relevance (Heiss et al., 1999), since the majority of stroke patients arrive at emergency units too late. Importantly, experimental and clinical evidence also point out to delayed mechanisms contributing to brain injury that can be potentially interfered by therapy.

Inflammatory response after stroke has been established in experimental and clinical settings. Inflammation is mediated by a concerted production of inflammatory molecules, such as cytokines (interleukin -1 (IL-1), tumor necrosis factor alpha (TNF-α), chemokines, adhesion molecules, and enzymes (cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), and matrix metalloproteinase (MMP)) that regulate the infiltration of peripheral leukocytes to brain parenchyma and contribute to brain damage (Barone and Feuerstein, 1999; del Zoppo et al., 2000). Inflammation affects also directly brain cells, namely neurons, glial cells and brain vasculature. Importantly, anti-inflammatory therapies have been established to have beneficial effects especially in experimental stroke.

Apoptosis, also called programmed cell death, has been suggested to contribute cell death after stroke (Love, 2003). Especially after mild ischemic insults, brain cells

adopt apoptotic characteristics instead of passive necrotic cell death (Du et al., 1996). This active process of cell death involves also activation of several biochemical cascades that may be overlapping with mechanisms seen in inflammatory processes. Currently, experimental manipulations targeted to regulate the expression of molecules involved in both inflammation and apoptosis hold potential as targets for therapy in cerebrovascular diseases (del Zoppo et al., 2000; Love, 2003). For example, genetic disruption of pro-inflammatory or pro-apoptotic genes has been shown to have beneficial effect in stroke models (Boutin et al., 2001; Connolly et al., 1996; Friedlander et al., 1997; Nogawa et al., 1998; Schielke et al., 1998).

At the important level of gene expression are transcription factors. These are protein molecules responsible for binding to specific sites in the nuclear DNA and regulate the expression of their target genes. Recently, widely expressed transcription factor nuclear factor κB (NF-κB) has been suggested to play a role in various brain disease models. Depending of the experimental paradigm, NF-κB activity has been reported to either enhance neuronal death or promote their survival (Grilli et al., 1996b; Lezoualc'h et al., 1998; O'Neill and Kaltschmidt, 1997; Pennypacker et al., 2001; Schneider et al., 1999). Originally, NF-κB was found to regulate inflammation in the peripheral leukocytes (Sen and Baltimore, 1986), but soon after its discovery it was found to be expressed virtually in all tissues, including brain. Importantly, NF-κB has been shown to regulate the expression of several genes involved in inflammation and apoptosis. Moreover, NF-κB was also recently suggested to play a detrimental role in brain ischemia, since genetic deletion of the p50 subunit of NF-κB complex reduced the vulnerability of brain tissue to focal cerebral ischemia in mice (Schneider et al., 1999). Application of antioxidants and proteosome inhibitors has been found to be

neuroprotective and protection in these studies has been often associated with reduction of NF-κB activity in experimental stroke models.

Since, the role of NF-κB in stroke is not well established we sought to investigate if inhibition of NF-κB with genetic or pharmacologial tools is beneficial in stroke models. Of special interest was to study the effects of NF-κB inhibition with dithiocarbamate derivatives on inflammatory and apoptotic mechanisms that previously have been shown to contribute to brain damage in experimental stroke models. Dithiocarbamates have been reported to have antioxidant, anti-inflammatory and anti-apoptotic properties and they are widely used inhibitors of NF-κB. Since dithiocarbamates have been reported to have also minor clinical utilities the relevance of dithiocarbamates as a therapy in brain ischemia models was explored.

2 REVIEW OF THE LITERATURE

2.1 Cerebral ischemia (Clinical stroke)

Cerebral ischemia is characterized as a cessation of blood flow resulting in insufficient oxygen and glucose delivery to the brain tissue and cells (Sharp, 1998). There are several superficially defined subgroups and types of brain ischemia characterizing a broad spectrum of ischemic conditions in clinical and experimental situations. Clinically, eighty-percent of the stroke cases are ischemic resulting either from atherothrombotic brain infarctions (ABI) or emboli of the cerebral vasculature (AHA, 2003; Wolf, 1998). Eighty-eight percent of all strokes are ischemic whereas 12 % comprise of intracerebral and subarachnoidal hemorrhage. Additionally, global ischemia, an important type of ischemic brain insult resulting from the collapse of systemic circulation after cardiac arrest, leads to transitory hypoperfusion of the brain and brain damage at various brain areas (Horn and Schlote, 1992). Finally, perinatal asphyxia can cause cerebral hypoxic/ischemic injury, which results in severe neurological sequelae and death (Johnston et al., 2001).

In nearly all of the above described conditions, brain ischemia causes characteristic destruction and malfunction of brain cells or areas leading to sensorimotor and cognitive impairments both in human individuals and in experimental animals. The effects of cerebral ischemia can be temporary or permanent, depending on how quickly blood flow is restored, how many brain cells are damaged, and how quickly treatment is administered. In the worst cases, cerebral ischemia leads to death.

Table 1. Ischemia types in clinical stroke cases

Ischemic stroke	Cerebral thrombosis Cerebral embolism
Intracerebral hemorrhage	Cerebral hemorrhage Subarachnoid hemorrhage
Transient ischemic attack (TIA)	Cerebral embolism
Perinatal hypoxic ischemia (HI)	Hypoxia/Asphyxia
Cardiac arrest	Hypoperfusion/ Hypotension

2.2 Ischemic changes in the brain

Ischemic conditions challenge the brain tissue in several ways. Depending of the time course of ischemic cascade, cellular and molecular events can roughly be divided to acute and delayed cascades, which will contribute to the final outcome (Figure 1). However, some elements are characteristics to both cascades and therefore also temporally and spatially overlapping. Cell death by *necrosis* is an acute, irreversible passive response of the brain tissue to ischemia whereas *apoptosis* develops during a longer time window after the insult and involves complex and active processes leading ultimately towards cell suicide and which has been often referred as "programmed cell death". A dynamic process, *inflammation*, contributes to both early brain injury but also to the delayed cascades of brain injury after stroke. However, not only has inflammation been recognized as a detrimental response following stroke, but also as a course of action which is involved in the recovery process of the brain following ischemic insult (Barone and Feuerstein, 1999). Altogether, all of these

cascades comprise a vast array of functional and morphological events that take place in the brain immediately or even months after the ischemic insult.

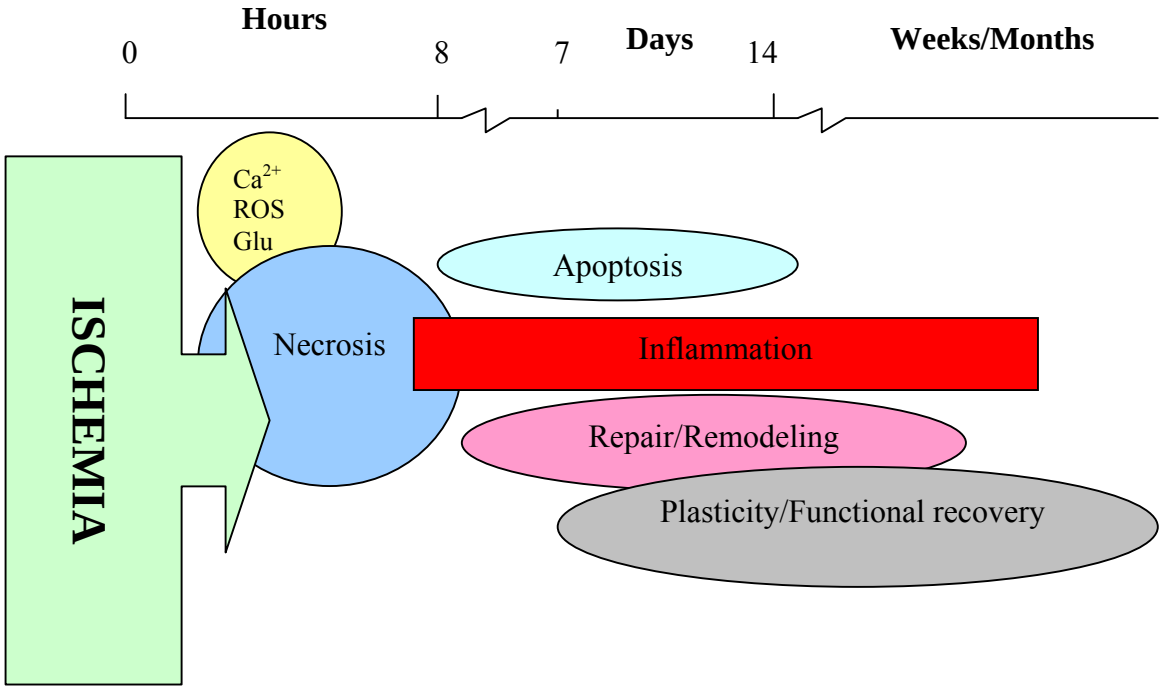


Figure 1. Schematic illustration of the temporal profile of the cascades contributing to brain damage in brain ischemia. An important cascade, inflammation, is involved throughout the evolution of ischemic injury, participating to the exacerbation of the ischemic damage in the early phases, but later also promoting tissue repair and recovery of function in the brain (Modified from Barone and Feuerstein, 1999).

2.2.1 Mechanisms underlying the acute brain damage

The brain is about two percent of the total human body mass but consumes 15 percent of the energy generated in the body. Most of this energy is used by neurons to maintain ionic gradients that are important for conductivity and normal synaptic function. During brain ischemia, energy production from glucose and oxygen in the mitochondria is compromised leading to a fall in ATP or phosphocreatine levels. Since brain tissue mainly relies on oxidative energy production, anaerobic energy

production that ensues from ischemia leads quickly to production of lactic acid and fall in pH. Increase in lactic acid content and increased amount in H^+ attracts water and causes cerebral edema, even though most of the cell edema is thought to develop by a leakage of extracellular Na^+ inside the cells and due to a compromised pumping activity of ATP driven Na^+/K^+ ATPase (Siesjö, 1988a; Siesjö, 1988b; Symon, 1980). The resulting edema can affect perfusion in the brain negatively and lead to increased intracranial pressure, vascular compression and herniation. Importantly, brain edema is clinically one of the most important determinants of whether a patient survives the first few hours after stroke (Dirnagl et al., 1999)

In rodent models of focal cerebral ischemia blood flow at the artery occlusion territory or in the *ischemic core* is diminished to about 5-10% from its normal flow rate (Duverger and MacKenzie, 1988; Nedergaard et al., 1986; Tamura et al., 1981b). In the ischemic core, cell death occurs within minutes after initiation of the ischemia due to homeostatic failure, depolarization of neurons and glia, and loss of synaptic transmission. As a result, somatodendritic as well presynaptic voltage-dependent Ca^{2+} channels become activated leading to the release of excitatory amino acids into the extracellular space. Simultaneously, the energy consuming processes such as reuptake of excitatory amino acids are compromised, which leads to accumulation of excitatory transmitters, especially glutamate, in the extracellular space (Dirnagl et al., 1999; Martin et al., 1997). This region of the brain, which is often referred as *ischemic core*, cannot be saved by any pharmacological interventions. However, the area surrounding the ischemic core, often referred as a *penumbra*, is also suffering from reduced blood flow. In the penumbra region, blood flow is 20%-60% of its normal values and is at very high risk of tissue damage (Back et al., 1995; Ginsberg and Pulsinelli, 1994). In the penumbra region, normal electrical neuronal functions are interrupted but the ATP

levels are maintained at normal or near-normal levels. The neuronal dysfunction is manifested by decreased voltage leading to abnormal EEG and failure to detect sensory evoked potentials even though ionic gradients and membrane pumps are generally maintained and functional. As a result, cells composing the penumbra are at this point viable but at very high risk to die. Ultimately, if cerebral blood flow (CBF) is not restored to adequate levels, selective gray matter necrosis will occur.

Restoration of cerebral blood flow after ischemia does not guarantee adequate perfusion and glucose metabolism in brain tissue. Pulsinelli and colleagues (1982b) found that after 30-minute forebrain ischemia, CBF remained below normal for 24 hours after the insult. Also, post-ischemic glucose utilization in the forebrain, except in the hippocampus, was depressed below control values and either remained low (neocortex, striatum) or gradually rose to normal (white matter) by 48 hours after the insult.

Acute breakdown in energy metabolism results in disturbed protein metabolism and has been observed in response to decreased cerebral blood flow in animal experiments. For example, it has been shown that in the rodent brain, protein synthesis decreases almost linearly in response to even slight reductions in CBF (Hossmann, 1993; Nedergaard et al., 1988). Using a primate global ischemia model, Kleihues and colleagues (1975) found that protein synthesis reduced significantly in the brain during ischemia and early post-ischemic phase. Interestingly, cerebral polysome profiles remained unaltered during the ischemic period in the primate brain, but a marked disaggregation of polyribosomes with a concomitant increase in free ribosomal subunits occurred early after the onset of recirculation (Kleihues et al., 1975). Recovered recirculation in the primate brain did not restore functional protein synthesis. Kleihues and colleagues (1975) found that elongation and termination, but

not initiation stages of protein synthesis were functional after the insult. In the fetal brain, protein synthesis was found to recover faster than adult brain from hypoxic/ischemic insults suggesting developmental differences in response to hypoxic/ischemic insults (Berger et al., 1996). It should be noted, however, that hypoxic or ischemic conditions do not inhibit all protein synthesis in the brain since during an immediate reperfusion period increased production of several transcription factors and heat shock protein can be observed (Ikeda et al., 2000). Interestingly, inhibitors of protein synthesis, cycloheximide (Goto et al., 1990) and anisomycin (Shigeno et al., 1990) have been found to protect neurons from ischemia, suggesting the involvement of newly synthesized 'killer proteins'.

2.2.1.1 Necrosis

Necrosis means mortification of tissue. It is a passive pathological event arising from the spontaneous insults such as trauma or ischemia. Microscopically it is characterized by cell, organelle and mitochondrial swelling or dilatation, cytoplasmic vacuolation, breaking of cell membranes, disintegration of organelles, and a final cell bursting (Jellinger, 2003; Kerr et al., 1972; Wyllie et al., 1980).

Defects in membrane permeability and ion transport proteins as well as impairments in oxidative phosphorylation and depletion of high-energy phosphates are early, causal mechanisms for cellular necrosis. The mitochondria undergo a complex sequence of changes that involves contraction or condensation of the inner membrane and dissipation of matrical granules, inner membrane swelling and cristaeolysis, formation of flocculent aggregates, and then disintegration. Evolution of

mitochondrial abnormalities has been demonstrated in various forms of cellular necrosis (Martin et al., 1998).

In necrotic cells, ribosomes are dispersed from the rough endoplasmic reticulum and polyribosomes disassociate, resulting in many monomeric ribosomes that are found "free" in the cytoplasm, causing the cytoplasmic matrix to appear dense and granular. Also, cisterns of the endoplasmic reticulum and Golgi apparatus have been found to dilate, fragment, and vesiculate, and the plasma membrane can undergo a process called blebbing (Kerr, 1995). Because cellular necrosis results in the liberation of antigenically active denatured intracellular debris, it is accompanied by an inflammatory response, which includes leukocytic infiltration, tissue edema, and ultimately a gross change in the overall histology of the focus of tissue damage due to the formation of a "scar."

Du and colleagues (1996) have reported that 90-min transient focal ischemia in rats causes substantial cortical infarction within 6 h which in their model fully developed 1 day after the insult. However, in permanent focal ischemia of the rat and mouse, anti-inflammatory compounds may reduce the infarction by 20-30 % even when administered 24 hours after the onset of stroke, indicating that the evolution of infarct may last for several days (Iadecola et al., 1995b; Nagayama et al., 1999). Du and coworkers found also in their study that the majority of the cell death in severe ischemia was necrotic, whereas in a mild (30-min) transient focal ischemia characteristics of delayed and apoptotic cell death were more evident (Du et al., 1996). Excitotoxicity causes necrosis of neurons in less than 24 h (Ferrer et al., 1995; Portera-Cailliau et al., 1997a; Portera-Cailliau et al., 1997b). In addition, global cerebral ischemia causes acute necrosis of principal striatal neurons in 24 h (Martin et al., 1997; Petito and Pulsinelli, 1984b), but delayed degeneration of hippocampal CA1

neurons 2–4 days later (Ito et al., 1975; Kirino, 1982; Petito and Pulsinelli, 1984a; Pulsinelli et al., 1982a). In the hippocampus, this neuronal death has been called post-ischemic "delayed neuronal death" coined by Kirino (1982), and cannot be directly categorized as necrotic death.

2.2.1.2 Excitotoxicity

Excitotoxicity is a phenomenon in which prolonged activation of excitatory amino acid receptors leads to cell death. The excessive release of glutamate activates glutamate receptors, which can be divided functionally to two subgroups: ionotropic and metabotropic receptors. Ionotropic receptors are directly coupled to ion channels whereas metabotropic glutamate receptors are coupled to more complex intermediary compounds, such as G-proteins and phospholipase C (PLC) which modulate intracellular second messengers, such as inositol-1,4,5-trisphosphate (IP_3), calcium and cyclic nucleotides (Matute et al., 2002; Nicotera and Lipton, 1999). The directly coupled ionotropic receptors can be further divided to three subtypes named by their selective chemical agonists: NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-isoxazolepropionate), and kainate.

Metabotropic glutamate receptors (mGluRs) are classified according to their amino acid sequence homology (reviewed by Conn and Pin, 1997). Class I receptors are positively coupled to PLC and thereby regulate Ca^{2+} release from IP_3-sensitive internal stores and via diacylglycerol (DAG) and IP_3 (Masu et al., 1991). Class II and III mGluRs are, on the other hand, negatively coupled to adenylyl cyclase regulating decreasing the level of cAMP (Tanabe et al., 1993). Metabotropic glutamate receptors are found pre- and post synaptically and they may modulate the toxicity of

ionotropic glutamate receptors, for example during excitotoxicity (Nicoletti et al., 1996; Yu et al., 1997). However, direct stimulation of mGluRs with the selective agonist 1-aminocyclopentane-1,3,-dicarboxylic acid (ACPD) does not result in neurotoxicity (Durkin et al., 1996).

AMPA receptors belong to the class of ionotropic glutamate receptors. They are as widespread in the CNS as NMDA receptors, have fast gating kinetics, and are involved in the generation of the fast component of excitatory postsynaptic potentials. Structurally, AMPA receptors are composed of subunits termed GluR1-GluR4 (Hollmann et al., 1991). All subunits have characteristics of a 900 amino acid long polypeptide chain and are subject to alternative splicing (Sommer et al., 1990). This alternative splicing allows different expression profiles of different AMPA receptors in mature and developing brain. Also, splicing directed channel modifications may also explain the functional differences and cell specific distribution of different AMPA receptors. Although most neuronal AMPA receptors show small Ca^{2+} permeability upon glutamate stimulation (Brorson et al., 1992; Jonas et al., 1994; Moudy et al., 1994), they may modulate Ca^{2+} influx due to AMPA receptor mediated Na^{+}-dependent depolarization and the subsequent opening of voltage activated calcium channels (VACCs) leading to release of Mg^{2+} blockade and NMDA receptor activation (Carriedo et al., 1995; Lu et al., 1996).

NMDA ionotropic receptors have an essential role in many functions of CNS, and have been suggested to be involved in glutamate mediated processes such as memory acquisition, cognitive processes and learning (Mori and Mishina, 1995). These receptors are also crucial for many forms of excitotoxicity, where excessive release of synaptic glutamate and inability of neurons to respond properly, leads to neuronal death (Lipton and Rosenberg, 1994). NMDA receptors are heteromeric structures

composed of two subunit types, NMDAR (NR) 1 subunit and one of four NR2 subunits (NR2A-NR2D) (Sucher et al., 1996). The NMDA receptor channels have fundamental differences compared to AMPA and kainate receptor channels, which is related to their physiological role. NMDA receptors have a high single channel conductance, high Ca^{2+}/Na^{+} permeability ratio, a voltage dependent Mg^{2+} block and high affinity to glutamate (Collinridge and Watkins, 1994).

The least studied glutamate receptor is the ionotropic kainate (KA) receptor. Kainate receptors are difficult to distinguish from AMPA receptors pharmacologically since selective agonists and antagonists are not available (Bleakman, 1999). Therefore, kainate/AMPA receptors are often referred together as non-NMDA receptors. Also, even though AMPA and KA receptors share a considerable amount of sequence homology, immunoprecipitation studies do not support the hypothesis that KA and AMPA receptors are composed of the same subunits (Puchalski et al., 1994). Indeed, molecular cloning has revealed that kainate receptors are composed of high affinity KA1 and KA2 subunits and low affinity GluR5, GluR6 and GluR7 subunits, which may form functional ion channels (Hollmann and Heinemann, 1994).

Several brain insults elicit a pronounced release of glutamate characteristic to excitotoxicity (Benveniste et al., 1984; Jorgensen and Diemer, 1982; Shimada et al., 1990). Reductions in blood flow and strong membrane depolarizations, for example in cerebral ischemia, increase the open probability of voltage- dependent ion channels and activation of glutamate receptors in neurons. Excessive glutamate release from the presynaptic membrane leads to excessive accumulation of postsynaptic intracellular calcium. Post-synaptically, NMDA receptors are mainly responsible for the influx of Ca^{2+}. Also, some types of AMPA and KA receptors may also contribute to Ca^{2+} flux, since their coupled ion channels are, as mentioned above, partially

permeable to calcium. Excessive neurotransmitter release leading to Ca^{2+} overload triggers cascades that activate several potentially detrimental enzymes, lead to DNA fragmentation, proteolysis and lipolysis (Dirnagl et al., 1999). As an example, Ca^{2+} overload leads to activation of phospholipase A which produces free fatty acids including arachidonic acid and platelet-activating factor. Arachidonic acid inhibits glutamate reuptake from the synaptic cleft, which in turn leads to further activation of glutamate receptors, Ca^{2+} accumulation, and further arachidonic acid formation (Katsuki and Okuda, 1995). Instead, platelet-activating factor (PAF) increases the glutamate release from the presynaptic membrane (Bazan et al., 1995). Arachidonic acid is also a substrate for ischemia inducible enzyme cyclooxygenase -2 (COX-2) which plays a significant role in the development of delayed ischemic brain damage and brain inflammation (Adams et al., 1996; Miettinen et al., 1997; Planas et al., 1995). Other neurotoxic cascades triggered by excitotoxicity include depolarization of mitochondria, calcium overload of mitochondria, and production of reactive oxygen species (ROS) (Nicotera and Lipton, 1999).

Inhibition or blockade of glutamate receptors has been extensively studied as a treatment for brain ischemia. In cat the selective NMDA receptor antagonist, MK-801, significantly reduced the ischemic infarct size, supporting the important role of NMDA receptors in mediating brain tissue injury (Ozyurt et al., 1988; Park et al., 1988a). Similar reports have been obtained from rats even if MK-801 is given early after the insult (Park et al., 1988b). Also, several AMPA receptor antagonists have been found to provide protection against ischemic insults (Graham et al., 1996; Kawasaki-Yatsugi et al., 1998; Shimizu-Sasamata et al., 1998). Moreover, voltage gated ion channel antagonists, such as phenytoin, carbamazepine and lamotrigine for Na^{+} channels (Rataud et al., 1994) and nicarpidine (Kittaka et al., 1997) for Ca^{2+}

channels have been found protective in focal ischemia experiments as well as brain lesion models induced by electric convulsions. However, even though therapeutic approaches aimed against excitotoxicicty have been shown to protect in experimental brain ischemia models, the narrow therapeutic time-window, together with severe side effects eliminate the use of glutamate receptor antagonists in humans (Dirnagl et al., 1999).

2.2.1.3 Reactive oxygen species (ROS)

Oxygen-free radicals have been suggested to be involved in the pathogenesis of cerebral ischemia and reperfusion injury (Barone and Feuerstein, 1999). At reperfusion after ischemic insult, several molecular events such as phospholipase activation, lipid peroxidation and dysfunction of the mitochondrial respiratory chain have been shown to lead to production of free radicals and oxidative stress (Chan, 1994).

Oxidative stress is defined as an increase in intracellular ROS such H_2O_2, superoxide anion (O_2^-), or hydroxyl radical ($^{\cdot}OH$). Among oxygen-free radicals, superoxide (O_2^-) is directly toxic to neurons (Patel et al., 1996). Importantly, oxygen-free radicals may cause oxidation of proteins and DNA damage, both of which are hallmarks of ischemic tissue. In addition, there are several lines of evidence suggesting that superoxide (O_2^-) contributes indirectly to tissue damage by enhancing vasogenic edema and blood brain barrier (BBB) disruption after brain ischemia. The role of superoxide (O_2^-) in compromised BBB is supported by the fact that endothelial cells are the cellular constituents of blood brain barrier (BBB), but also the major source of superoxide (O_2^-) production (Terada et al., 1991). This way endothelial cells

damage themselves in a self-inflicting manner. Moreover, reperfusion-induced oxidative stress (ROS) increases phagocytic activity of infiltrating peripheral leukocytes (neutrophils and macrophages) (Benjelloun et al., 1999; Mabuchi et al., 2000) and resident brain microglial cells after ischemia (Kreutzberg, 1996).

Oxidative stress and especially ROS are important activators of certain transcription factors, which control the expression of several stress related genes. Activating protein -1 (AP-1) and nuclear factor kappa-B (NF-κB) are among the most well described (Lavrovsky et al., 2000). In the case of NF-κB, increase in intracellular ROS and H_2O_2 are suggested to be the most potent activators of NF-κB (Bowie and O'Neill, 2000). Macromolecules such as interleukin-1 (IL-1) and tumor necrosis factor –α (TNF-α) are target genes of activated NF-κB, but by themselves may also activate NF-κB via their cell surface receptors and subsequent intracellular signaling pathways. Importantly, these cytokines play an important role in the pathogenesis of brain ischemia and post ischemic inflammation in the brain (see chapter 2.3.3.1). Ultimately, early oxidative stress and ROS production predisposes delayed mechanisms of brain injury such as disruption of BBB, vasogenic edema, gene expression and inflammation, but also form a link between acute and delayed mechanisms contributing to brain injury.

2.2.2 Delayed mechanisms contributing to brain damage

Although a fall in energy homeostasis contributes to acute necrotic cell death during the early phases of ischemia, delayed and selective neuronal death also occurs. This is supported by the notion that restoration of cerebral blood flow rapidly normalizes the ionic disturbances and ATP levels in the brain but delayed cell death cannot be

prevented. The classic example of the delayed effects of ischemia is the selective neuronal cell death in the vulnerable CA1 area of the hippocampus, which occurs several days after the ischemic insult (Kirino, 1982). It has been suggested that transient energy depletion and loss of ionic homeostasis triggers the delayed or secondary damaging cellular events that eventually will kill vulnerable cells.

2.2.2.1 Apoptosis

Apoptosis is an active, energy consuming process of self-destruction in which unnecessary or damaged cells are eliminated. It can be also considered as an intrinsic suicidal program of the cell triggered by a wide array of stimuli. Therefore, apoptosis is physiologically an important way to maintain homeostasis of the organism and apoptotic machinery is evolutionarily conserved (Jellinger, 2003; Reed, 2000).

Cell death by apoptosis is carried out by several facilitating receptors or factors. These include apoptosis inducing or death receptors (e.g. Apo-1/Fas, Apaf-1), apoptosis initiating factors (AIFs), members of the Bcl-2 family proteins (not the Bcl-2 itself) and cysteine proteases of the caspase/calpain family (Reed, 2000; Yuan and Yankner, 2000). However, as mentioned previously multiple pathways can lead to apoptosis. These apoptotic pathways and mechanisms can be roughly divided to caspase -dependent and caspase -independent mechanisms (Fig. 2).

Caspases have been widely recognized as the key apoptotic molecules (Hengartner, 2000; Leist and Jäättela, 2001). Caspases are cysteine containing enzymes having a pentapeptide motif Gln-Ala-Cys-X-Gly, where X is Arg, Gln or Gly (Earnshaw et al., 1999). This family of proteases is synthetized as zymogens and various apoptotic upstream signals mediate maturation of these precursors to mature proteases. Upon activation these proteases are cleaved to small (10kD) and large (20kD) subunits from

pro-caspase to yield active enzymes, which consists of two small and two large subunits as heterotetramers (Thornberry and Lazebnik, 1998). Caspases can be divided into two categories: initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10 and -14), which are activated by oligomerization-induced autoprocessing, while effector caspases (caspase-3, -6,-7) are activated by initiator caspases or other proteases. Two major pathways have been recognized according to their initiator caspase: death receptor mediated apoptosis involving caspase-8 and mitochondrial pathway where various signals can trigger the release of harmful proteins by mitochondria, especially cytochrome c into the cytoplasm which further promotes aggregation caspase-9, Apaf-1 and cytochrome c to form the apoptosome. The apoptosome instead cleaves and activates procaspase-3 into active caspase-3, the key effector caspase involved in caspase-mediated apoptosis (Antonsson, 2001; Wang, 2001).

Although caspase inhibition has been shown to prevent apoptosis, cell death with apoptotic morphology still occurs suggesting caspase-independent apoptosis (Carmody and Cotter, 2000; Lorenzo et al., 1999). Cell death by caspase-independent mechanisms has been shown to involve activation of other proteases such as calpain (Squier et al., 1994), proteasome (Hirsch et al., 1998) and serine proteases (Hughes et al., 1998), apoptosis inducing factor (AIF) (Susin et al., 1999) endonuclease G (EndoG) (Li et al., 2001) or some times even Bax mediated apoptosis without caspase activation (Jurgensmeier et al., 1998).

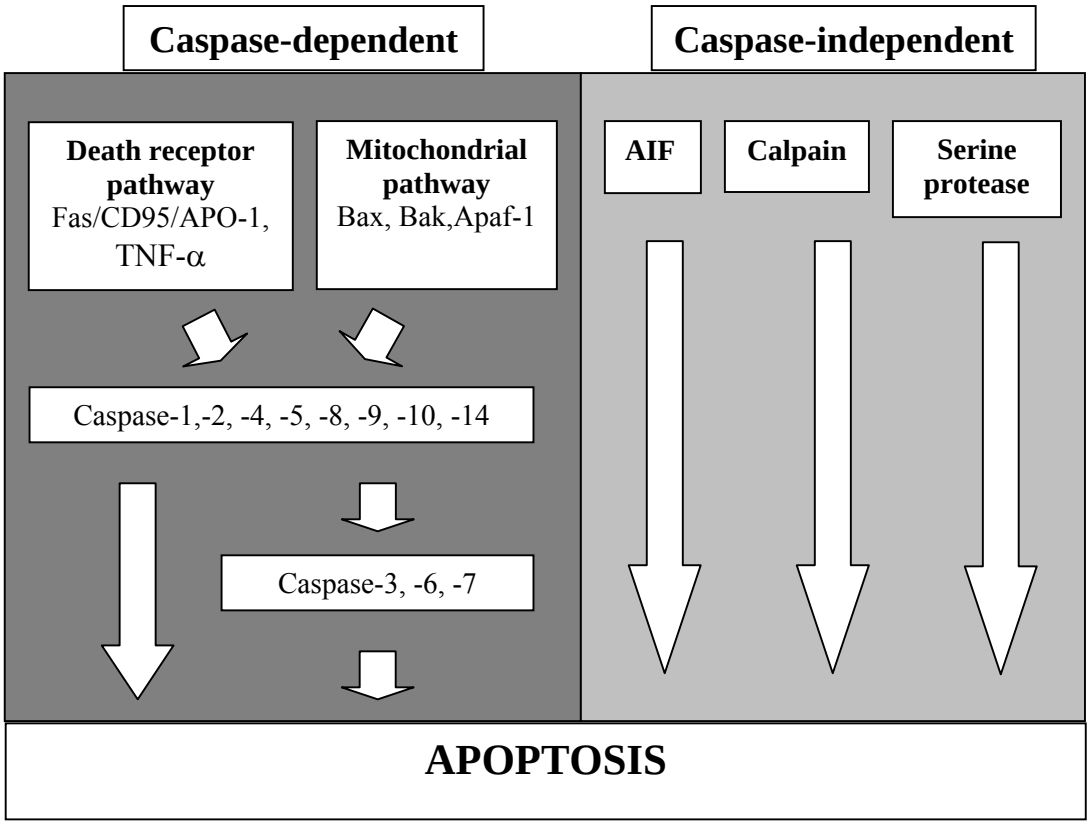


Figure 2. Caspase dependent and independent pathways leading to apoptosis. See text for references.

Neurons and other brain cells share the same basic apoptosis machinery with all other cell types. However, there are some differences in the combinations of these factors in the nervous system. Also, whether apoptosis is a physiological (i.e. developmentally regulated) or pathological event, the mechanisms by which apoptosis is triggered can be different. Several lines of evidence have suggested the involvement of apoptosis in cerebral ischemia, although acute cell death in ischemia has been traditionally considered necrotic. Apoptotic neurons are predominantly located within the surrounding tissue (penumbra), not in the center of the ischemic core. Indeed, many experimental studies of transient cerebral ischemia and cerebral hypoxia/ischemia have demonstrated that neurons in the border zone of infarcts, scattered neurons in the cerebral cortex and striatum, and in the vulnerable CA1 area of the hippocampus

become susceptible to TUNEL-staning, (Charriaut-Marlangue et al., 1996; Cheng et al., 1998; Clemens et al., 1997b; Endres et al., 1998; Gillardon et al., 1997), a commonly used indicator of apoptosis.

Ischemia induced apoptotic cell death has been suggested to involve activation of caspases, which has been supported by several studies (Reviewed by Love, 2003). Caspase-3 activity and its cleavage from its precursor has been detected in adult rats subjected to cerebral ischemia (Namura et al., 1998) as well as in neonatal hypoxia/ischemia model (Arvin et al., 2002; Han et al., 2000). Caspase-3 is a well known effector caspase and its inhibition has been found to prevent caspase activated deoxiribonuclease activity (CAD) and subsequent DNA cleavage (Luo Y, 2002). Moreover, inhibitors of caspase activity have been found neuroprotective in transient and permanent focal ischemia models in adult animals (Hara et al., 1997a; Hara et al., 1997b; Krupinski et al., 2000; Loddick and Rothwell, 1996; Schulz et al., 1998) as well as neonates (Cheng et al., 1998). In focal ischemia, caspase cleavage products and TUNEL staining have been found to colocalize in neurons starting very early 1-2 hours after severe and 9-12 hours later after mild ischemia (Fink et al., 1998; Schulz et al., 1998). Also, in global ischemia models, expression of caspase-3 mRNA have been found to be up-regulated (Ni et al., 1998). Importantly, there are also indications of increased neuronal procaspase-3 levels in clinical atherothrombotic strokes, although this increase is not associated with activated caspase -3 or cleavage of poly (ADP-ribose) polymerase (PARP) (Love et al., 2000a). However, clinical cases of cardiac arrest show activated caspase-3 and cleavage product of PARP in cortical neurons as well as macrophages and microglia during 6 to 9 days after the insult (Love et al., 2000b)

In addition to caspase-3, several other caspases have been found to be activated or upregulated in response to ischemic insults. For example, caspase-1 or interleukin-1 converting enzyme (ICE) mRNA have been shown to be up-regulated in response to ischemic insults (Bhat et al., 1996; Yrjänheikki et al., 1999). Importanly, increased expression of caspase-1 substrate pro-interleukin-1β (pro-IL-1β) and its product IL-1β have been widely established in ischemia models (See 2.2.3.1 Cytokines). The important role of caspase-1 in ischemic cell death is supported by the protective effects of dominant negative mutation of caspase-1 in mice after ischemia (Friedlander et al., 1997). Alternatively, mice with knock out caspase-1 show reduced brain damage after ischemic insults (Schielke et al., 1998)

Apoptosis can occur also independent of caspase activation. Ischemic and excitotoxic insults have been reported to involve caspase-independent apoptotic mechanisms (Cao et al., 2001; Zhan et al., 2001). Most of these mechanisms are yet to be established in brain disease models, although their existence has been shown in several other experimental settings.

2.2.2.2 Inflammation

Inflammation is a defense reaction caused by tissue damage or injury. The primary objective of inflammation is to localize and eliminate the irritant and repair the surrounding tissue. For the survival of the host, inflammation is a necessary and beneficial process. Since the time of ancient Rome, physicians have known the four basic signs of inflammation: rednesss, warmth, swelling, and tenderness ("rubor, calor, tumor, et dolor"). The acute inflammatory response involves three major stages: first, dilation of capillaries to increase blood flow; second, microvascular structural

changes and escape of plasma proteins from the blood stream; and third, leukocyte transmigration through endothelium and accumulation at the site of injury. Inflammation is mediated by a complex array of mechanism involving both cellular and molecular components. Cells of the immune system, mainly white blood cells (granulocytes, monocytes and leukocytes) compose the cellular components of immune response whereas molecular components such as cytokines, chemokines, adhesion molecules and inflammatory enzymes act as mediators and regulators of function of the cellular components.

As shown in previous chapters, excitotoxicity and apoptosis interact and share many features. The same applies to inflammation where inflammatory responses are mediated by signaling pathways involved also in excitotoxic and apoptotic cell death. For example, apoptotic FasL has been shown to upregulate the expression of tumor necrosis factor -α (TNF-α) via regulation of NF-κB in monocytes/macrophages (Lu et al., 2002). The effects of newly synthetized TNF-α on monocytes/macrophages itself are then mediated via binding to TNF-receptors (TNFR1 and TNFR2) leading to further activation of nuclear factor -κB (NF-κB) and possibly promoting inflammatory gene expression. Alternatively, TNF-receptor binding has been shown to promote apoptosis through mechanisms involving TNF receptor associated death domain (TRADD), Fas associated death domain (FADD) and caspase-8 ultimately leading to activation of effector caspases and caspase-dependent apoptosis (reviewed by Hu, 2003). In a study of excitotoxicity in cerebellar neurons, exposure to glutamate leads to activation of calpain and caspase-3, mediators involved in caspase-independent and -dependent apoptosis, respectively (Schölzke et al., 2003). Importantly, excitotoxicity-induced calpain activity in this study was found to induce also the activation of NF-κB in cerebellar neurons. Also, glutamate excitotoxicity -

induced activation of NF-κB has been found to correlate with neuronal cell death in cerebellar granule cells and in hippocampal slices (Grilli et al., 1996b). Moreover, Grilli and colleagues (1996a) found that both inflammatory IL-1β and excitotoxic glutamate activated NF-κB and induced expression of amyloid precursor protein (APP), another NF-κB regulated gene.

2.3 Brain ischemia and inflammation

2.3.1 Inflammation in the brain

There is abundant evidence that an acute inflammatory reaction contributes to the development of delayed brain damage. In brain, post ischemic inflammation is mediated by infiltrating peripheral leukocytes (see chapter 2.3.2.1 and 2.3.2.3) and production of inflammatory mediators such as cytokines (IL-1, IL-6, TNF-α), chemokines (MCP-1, RANTES), adhesion molecules (integrins, ICAMs) and inflammatory enzymes (iNOS, COX-2, MMPs) (Barone and Feuerstein, 1999). Indeed, anti-inflammatory compounds or genetic manipulations targeted to inflammatory genes and their products have been shown to have protective effects (Chamorro and Planas, 2004). Cytokine and adhesion molecule production combined with recruitment of leukocytes to ischemic zone occurs early in brain ischemia and underlie the transition from early ischemic to late or delayed inflammatory injury (Fig. 3).

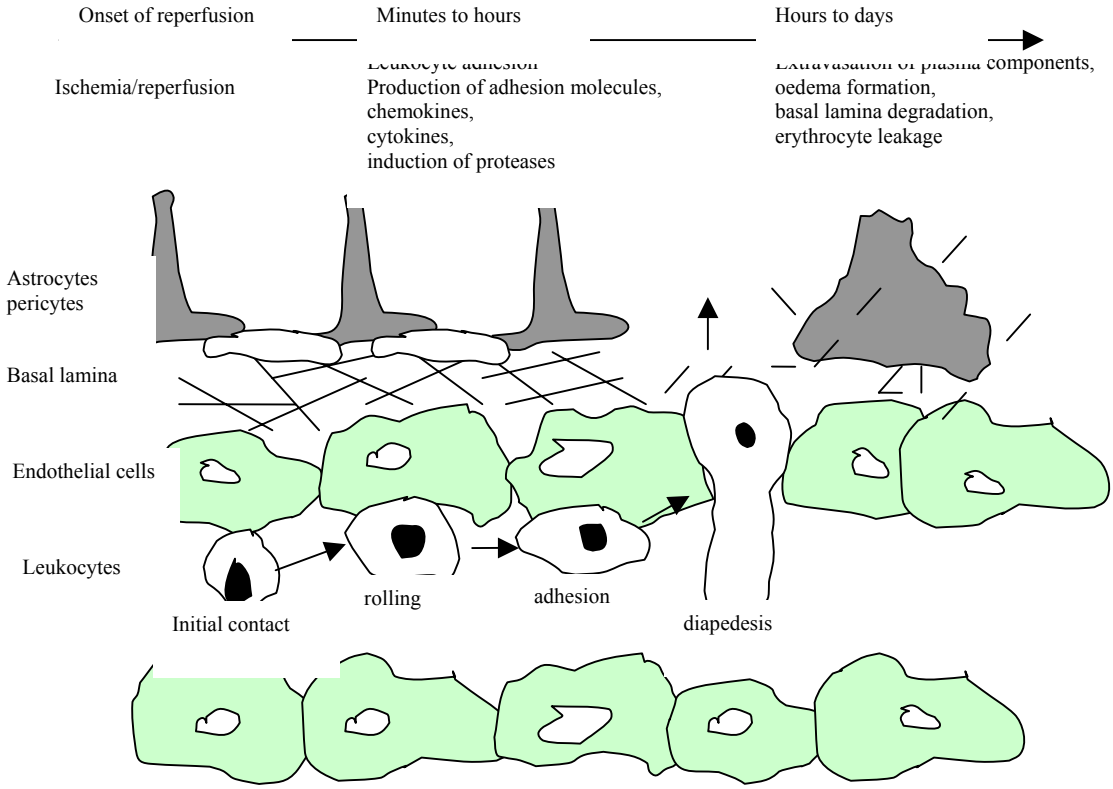


Figure 3. The process of leukocyte recruitment and blood-brain-barrier disruption in brain ischemia. Leukocyte recruitment can be divided into a cascade of steps: initial contact, rolling, firm adhesion and diapedesis or transmigration. In addition, dysfunction of the blood-brain barrier, the primary barrier in capillary and post- venule endothelium, results in extravasation of plasma components oedema formation. Moreover, loss of basal lamina, specialized extracellular matrix that connects endothelial cells to adjacent cell layer (astrocytes, pericytes), results in erythrocyte leakage and hemorrhagic complications (based on reviews by del Zoppo and Mabuchi, 2003; Petty and Wettstein, 2001).

2.3.2 Cellular components inflammation in the brain

2.3.2.1 Peripheral leukocytes

Leukocyte infiltration into brain infarction is a widely documented histopathological event in experimental studies, but also routinely observed in patients suffering from stroke. Leukocytes are considered to be key mediators of the host response in reperfusion injury in the brain (Kochanek and Hallenbeck, 1992). Leukocytes are the

main cellular components of the immune system and are produced by two lineages of hematopoietic cells: myeloid lineage and lymphoid lineage. Bone marrow derived myeloid lineage gives rise to granulocytes, which can be further divided to three lineages of cells: neurophils, eosinophils and basophils. Neutrophils are considered the most important cell type against acute infection and they are able to phagocytose infectious material (Roitt et al., 1998). Importantly, granulocytes are also the first hematogenous cells that appear in the brain in response to ischemia. In MCAO model, granulocytes have been shown to accumulate to cerebral vessels as early as 30 minutes after the onset of insult, prior the infiltration to infarction core and penumbra (Clark et al., 1993; Garcia et al., 1994; Kochanek and Hallenbeck, 1992). The highest number of intravascular neutrophils was found at 12 hours after occlusion, whereas the number of granulocytes peaked in ischemic parenchyma 24 hours after occlusion (Garcia et al., 1994). On day 7 after the insult, only few granulocytes were seen.

In addition to granulocytes, cells of the monocyte/macrophage lineage derived from the myeloid lineage as well are the most abundant hematogenous cells that enter the brain after the focal cerebral ischemia (Garcia et al., 1994). Garcia and colleagues (1994) found that circulating monocytes were detected within the capillaries/venules of the ischemic area after 4 to 6 hours. Also, in a study by Schroeter and colleagues (1994), ED1 immunoreactive macrophages were found to infiltrate the core of the infarct starting on day 1 which persisted for 14 days. At day 3, the entire infarct area was covered by macropahages (Schroeter et al., 1994). During days 5 to 15 after PMCAO in spontaneously hypertensive rats (SHR), Clark and colleagues (1993) found that both the infarct and the ipsilateral hemisphere decreased in size, which correlated well with the presence of abundant macrophages and cavitation of the lesion along its medial border. By the end of the 4th week after the insult, tissue debris

was almost completely cleared (Clark et al., 1993). These observations provided evidence about the important role of macrophages in phagocytic activity in ischemic brain tissue.

The body of evidence suggests that leukocytes may play a key role leading to secondary brain damage after ischemia. First of all, there was a considerable correlation between polymorphic neutrophil (PMN) accumulation in the ischemic zone and the expansion of cerebral damage in human brain (Akopov et al., 1996). Secondly, it has been shown experimentally that neutropenia has a beneficial effect to the reperfusion injury after ischemia (Heinel et al., 1994; Matsuo et al., 1994a; Vasthare et al., 1990). Thirdly, genetic manipulations or treatments that prevent PMN recruitment and adhesion to microvessel endothelia are protective against cerebral ischemia (Connolly et al., 1996; Hartl et al., 1996; Matsuo et al., 1994b).

After re-canalization, recruitment of leukocytes may lead to an obstruction of microvessels in a phenomenon called “no-reflow”, which further hinders recirculation of blood in the ischemic zone (del Zoppo, 1991). Moreover, the same leukocytes produce also proteolytic enzymes, reactive oxygen species (ROS), and other inflammatory mediators that may contribute to additional damage (Barone and Feuerstein, 1999). For example, inducible nitric oxide synthase (iNOS) has been shown to be active and to produce toxic nitric oxide (NO) in infiltrating neutrophils (Forster et al., 1999; Iadecola et al., 1995a). Other free radical producing mechanisms in neutrophils include NADPH oxidase (Walder et al., 1997). Neutrophils oxidize NADPH to generate superoxide and thus are important potential free radical donors during and after focal ischemia. Different proteins, including receptors, ionic channels, transporters or components of transduction pathways are substrates of

oxidation by ROS and other free radicals (see chapter 2.2.1.2.2). Therefore, infiltrating neutrophils promote tissue injury via production of free radicals.

2.3.2.2 Microglia

Microglia are the resident macrophage cell population and represent the primary immunocompetent cells within CNS. Microglia comprise 10-20% of total glial cell population in the CNS and act in concert with astrocytes and neurons to maintain normal brain homeostasis (Hansson and Ronnback, 2003). They respond to several transmitters and have a capacity to release regulatory molecules involved in inflammation, such as cytokines (Hanisch, 2002). Moreover, together with astrocytes, they release trophic factors such as nerve growth factor (NGF) and basic fibroblast growth factor (bFGF).

Microglia are involved in several pathological situations. They react on invasions by infectious agents and tumors but also participate in removing cellular debris after traumatic or ischemic injury. Moreover, microglia are currently considered as “a hot topic” in pathological neurodegenerative diseases involving neuroinflammation, such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis (ALS) (Wood, 2003).

In the normal mature brain microglia adopt a typical ramified “resting” morphology with variable number of highly branching processes. Upon stimulation, microglia transform into the ameboid shape, characteristic for activated microglia (Tikka et al., 2001; Tikka and Koistinaho, 2001; Tikka et al., 2002). In focal cerebral ischemia, microglial activation occurs very rapidly within hours after the onset of ischemia and persists for weeks (Lehrmann et al., 1997; Yrjänheikki et al., 1999). For example, in TMCAO models, increased immunoreactivity for phosphotyrosine or isolectin

(markers for microglia) can be detected as early as 3-6 hours after the onset of ischemia (Korematsu et al., 1994; Mabuchi et al., 2000). Moreover, activated microglia with ameboid shape and retracted processes are clearly seen 24 hours after the ischemia. Activation is seen not only in the core of the infarct but also in the adjacent surviving tissue, suggesting a role for microglia in tissue damage expansion in cerebral ischemia. Even though it is still under debate about what is the contribution of microglia in the development of ischemic brain damage, there are clear indications that microglial activation *precedes* rather than is a consequence of neuronal damage and this activation indicates inflammatory reaction in the brain.

Several inflammatory mediators are produced by microglia in ischemic insults. Classical inflammatory mediators produced by microglia are cytokines such as IL-1α, IL-1β, IL-6 and TNF-α (Hanisch, 2002). Moreover, lipid derivatives such as arachidonic acid and prostaglandins as well as free radicals such as super oxide and nitric oxide are produced. Free radicals or ROS produced by brain microglia promote abnormal phagocytic activity of microglia leading to further damage of ischemic tissue (Kreutzberg, 1996). However, as mentioned in chapter 2.2.1.2.2, ROS produced by microglia can damage neurons also directly. Enzymes catalyzing the production of several pro-inflammatory mediators are also produced in microglia. These include, for example, COX-2, MMP- family enzymes, caspase-1 (ICE), and iNOS (Bhat et al., 1996; Gottschall et al., 1995; Nakashima et al., 1995; Sairanen et al., 1998).

Microglia have been shown to produce also beneficial and anti-inflammatory mediators that may protect from the ischemic insults. These mediators include trophic factors like transforming growth factor -β (TGF-β) and cytokines such as interleukin-10 (IL-10) and antiapoptosis protein Bcl-2 (Lehrmann et al., 1998; Mesples et al., 2003; Urabe et al., 1998). Moreover, phagocytosis of cellular debris from degenerated

neurons have been shown to support wound healing and peripheral neuronal regrowth (Zeev-Brann et al., 1998), and secretion of beneficial molecules can support neuronal recovery (Faber-Elman et al., 1996).

2.3.2.3 Vascular endothelium and blood-brain barrier

Loss of vascular integrity is a widely described phenomenon in cerebral ischemia. Occlusion in a major cerebral artery causes the deprivation of blood delivery in the downstream microvasculature, which in turn results in the activation of coagulation factors, increased capillary density and disruption of the extracellular matrix (Petty and Wettstein, 2001).

There are anatomical and functional barriers that maintain vascular integrity as well as transport and transmigration of circulating blood cells. Blood-brain barrier (BBB) enables fluid retention within plasma space and prevents cellular extravasation during normal conditions. Blood brain barrier is composed of endothelium, which is the primary barrier in brain capillaries and in post-capillary venules. Beneath the bed of endothelium is the basal lamina that contributes to the extravasation of blood cells, but which also connects endothelium to cell layers beneath. Basal lamina is comprised of type IV collagen, laminin, fibronectin, various proteoglycans, and heparan sulphates (Scherrmann, 2002). Moreover, maintenance and integrity of BBB is thought to be under regulatory control of perivascular astrocytes, the third component of the functional BBB (Petty and Wettstein, 2001; Tran et al., 1998)

Several factors and mechanisms affect to the integrity of BBB. Ischemia, inflammation and inflammatory mediators (cytokines, adhesion molecules), free radicals and proteases (matrix metalloproteinases, plasminogen activators, thrombin) have been suggested to increase BBB permeability and subsequent vascular oedema

in several experimental settings (Ayata and Ropper, 2002). For example, 3 -hour transient middle cerebral artery occlusion (TMCAO) followed by 3 -hour reperfusion period in rats has been shown to cause a significant opening of the BBB (Yang and Betz, 1994). Moreover, the role of specific Zn^{2+} and Ca^{2+} dependent MMPs have been extensively studied in stroke and neuroinflammation (Rosenberg, 2002; Rosenberg and Mun-Bryce, 2004). These enzymes are capable of degrading components of basal lamina and extracellular matrix. Brain cells express both constitutive and inducible MMPs in response to cellular stress. MMPs are tightly regulated to avoid unwanted proteolysis. Therefore, MMPs are secreted as inactive enzymes and require activation by other proteases and free radicals.

Gelatinase B (MMP-9), one of the enzymes belonging to the matrix metalloproteinase (MMP) family, was markedly increased in rats at 12 and 24 h after PMCAO. This increased coincided with secondary vasogenic edema, which was maximal 1-2 days after a stroke. The time of appearance of gelatinase B suggested therefore a role in secondary tissue damage and vasogenic oedema (Rosenberg et al., 1996). Also, in another study in mice permanent ischemia, Gasche and coworkers (1999) found increased pro-MMP-9 expression already 2 hours after the onset of ichemia. Importantly, the expression of activated MMP-9 was apparent 4 hours after the onset of PMCAO, which coincided with BBB disruption detected as Evan's blue extravasation. Another important member of the MMP family, pro-MMP-2 (gelatinase A) expression was found to increase only 24 hours after PMCAO. In TMCAO model in rats, MMP-2 (gelatinase A) expression was found to correlate during the initial opening of BBB, whereas peak MMP-9 expression coincided with maximal opening of the BBB 48 hours after the insult (Rosenberg et al., 1998). However, Fujimura and coworkers (1999) found that MMP-9 activity was rapidly

increased in TMCAO model in mice and correlated with early BBB opening. These differences in MMP expression and odema formation may reflect the differences between permanent and transient ischemia models or differences between species and need to be studied further. However, intravenous treatment with anti-MMP-9 antibody have been shown to result in nearly 30% reduction in brain infarct volume, suggesting that anti-MMP therapies may be a beneficial approach to stroke therapies (Romanic et al., 1998).

In summary, dysfunctional BBB and brain edema appear to play an important role in the development of brain damage in animal models of brain ischemia. Moreover, clinical manifestations of ischemic brain oedema as a result of impaired BBB function is one of the most important causes of clinical deterioration and death within the first 2-5 days after stroke (Davalos et al., 1999).

2.3.2.4 Astrocytes

Astrocytes are the predominant glial cell population in the CNS. They provide metabolically important nutrients to high-energy consuming neurons, maintain the ionic balance in the extracellular space and remove waste metabolites as well as excitatory transmitters (Tsacopoulos and Magistretti, 1996). Astrocytes provide also physical support to the surrounding neurons, vasculature and meninges. In this regard, intraparenchymal capillaries are surrounded by astrocytic processes, called end-feet, which support the maintenance of blood-brain barrier (BBB) (Janzer, 1993; Janzer and Raff, 1987) and may participate in regulation of capillary microcirculation (Sporbert et al., 1999).

Role of astrocytes in ischemic brain insults is not clear. They exist in two typical forms: stellar-fibrillary astrocytes and protoplasmatic astrocytes. In the normal brain, the majority of stellar-fibrillary astrocytes are localized to white matter and can be detected by using anti-bodies against glial fibrillary acidic protein (GFAP), whereas protoplasmatic astrocytes are localized to grey matter but cannot be visualized the same way since these cells are GFAP -negative (Bignami et al., 1972). However, early astroglial activation can be seen within hours after ischemic, traumatic or infectious insult (Raivich et al., 1999). This is manifested by increased GFAP- positive immunoreactivity plaque -like cells, which are gradually superseded by cells with stellar morphology. Chen and coworkers (1993) found increased astroglial proliferation as evidenced by GFAP immunoreactivity in the rat brain 24 hours after transient focal ischemia. Immunoreactivity was most prominent in the perifocal areas (penumbra), whereas a dramatic decrease in GFAP immunoreactivity was seen in the ischemic core (Chen et al., 1993). Increased GFAP immunoreactivity has also been observed in the CA1 subfield of stratum radiatum as late as 6 days after the insult in a global ischemia model in gerbils (Yrjänheikki et al., 1998).

2.3.2.5 Neurons

There are reports showing that neurons themselves can produce inflammatory mediators and enzymes that may contribute to tissue injury progression. For example, Nagayama et al. (1998) showed that COX-2 is produced in neurons after ischemia. In addition to COX-2, neurons are able to produce cytokines such IL-6, IL-1 and TNF-α (Murphy et al., 1999; Tchelingerian et al., 1996; Tchelingerian et al., 1993; Yang et al., 2004)

Recently, Kaltschmidt et al. (2002) found that COX-2 is a target gene for transcription factor NF-κB in neurons. Since NF-κB is activated and COX-2 is expressed in neurons after ischemia (see section 2.4.2 and section 2.3.2.3.2), this may lead to the exacerbation of neuronal damage in a self-inflicted manner.

2.3.3 Molecular components of brain inflammation

It has become increasingly evident that inflammatory response is mediated by the induction of a wide array of deleterious proteins, which are up-regulated in response to brain ischemia.

2.3.3.1 Cytokines

Cytokines are a naturally occurring group of low-molecular-weight glycoproteins that act as intercellular messengers and participate in intercellular communication. In the periphery, cytokines are produced by activated cells of the immune system, namely macrophages, monocytes and lymphocytes, but also by endothelial cells, fibroblasts, platelets, and many other cell types (Pantoni et al., 1998). These hormone-like polypeptides regulate cell function during development, maintain homeostasis in mature organisms and during responses to infections, inflammatory, autoimmune, traumatic, and ischemic injury (Allan and Rothwell, 2001; Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995; Szelenyi, 2001; Wang and Shuaib, 2002).

Neurotoxic and neuroprotective mechanisms are closely related to the balance between pro-inflammatory and anti-inflammatory cytokines in several brain diseases. The process of neurodegeneration is closely associated with the shift of cytokine balance towards the side of pro-inflammatory cytokines like IL-1 and TNF-α

regardless of where they are produced (CNS or periphery). In contrast, production of anti-inflammatory cytokines in the CNS maintain homeostasis and protect brain tissue by inhibiting inflammatory response (Rothwell, 1999; Szelenyi, 2001). Two major cytokines, interleukin -1β (IL-1β) and tumor necrosis factor -α (TNF-α), are the classic pro-inflammatory cytokines exacerbating the cerebral ischemic injury, whereas the anti-inflammatory molecules such as interleukin-10 and the naturally occurring IL-1 receptor antagonist (IL-1ra) appear to have neuroprotective properties (Allan and Rothwell, 2001). Interleukin -1β (IL-1β) and tumor necrosis factor -α (TNF-α) will be reviewed further due to their essential role in cerebral ischemia models.

2.3.3.1.1 Interleukin -1β (IL-1β)

The interleukin -1 family exists in three major forms; IL-1α, IL-1β and IL-1ra. All forms have a molecular weight of 17-22 kD and about 25% of amino acid sequence homology (Dinarello, 1988; Dinarello, 1991). The IL-1 proteins are products of separate genes which are formed as precursors, and are enzymatically cleaved to the mature form by specific cellular proteases. Mature IL-1α usually remains membrane associated whereas IL-1β is secreted (Rothwell, 1999). Interleukin -1 converting enzyme is required for pro-IL-1β to be cleaved to a mature IL-1β (Cerretti et al., 1992). IL-1 receptor antagonist (IL-1ra) acts as an inhibitor of IL-1(α,β) activity in the periphery (Dinarello, 1996) and in the brain (Rothwell, 1999) and has been used also as a useful pharmacological tool to study IL-1 functions. Other components of the IL-1 family have also been identified in the central nervous system. The three members of the IL-1 family share the same receptors: IL-1 receptor-1 and -2 (IL-1RI and RII). IL-1R1 transduce intracellular signal when bound to IL-1(α,β,ra), whereas

IL-1RII does not. Currently, it is thought that IL-1RII serves probably as a decoy by blocking IL-1 activity (Dinarello, 1996).

2.3.3.1.2 Interleukin -1β and brain ischemia

The expression of IL-1β in the brain is dramatically increased in response to clinical stroke or animal models of stroke. Despite several lines of evidence supporting the role of IL-1β in the pathogenesis of cerebral ischemia, its functional role has not been fully elucidated. In a transient global ischemia model, IL-1β mRNA levels are elevated in 15 minutes but also elevated IL-1β protein levels are seen already 3-6 hours after the ischemia (Minami et al., 1992; Saito et al., 1996). Also, in TMCAO model in SHR, levels of IL-1β mRNA were elevated already 1h hour after the onset of reperfusion (Wang et al., 1994). Correspondingly, immunohistochemical data from a mouse TMCAO model suggested that IL-1β protein levels are also increased within 2 hours after the insult and remained elevated for 24 hours (Buttini et al., 1994). In a PMCAO model IL-1β mRNA transcription was found to increase, but later than in reperfusion models, reaching the peak of transcription at 10-12 hours (Hill et al., 1999; Liu et al., 1993) and peak protein levels at 24 hours (Davies et al., 1999; Hillhouse et al., 1998) after the onset of ischemia. Also, excitotoxins such as kainic acid or selective NMDA receptor agonists have been found to induce rapid expression of IL-1β mRNA and protein in the brain, suggesting close interplay between excitotoxic and inflammatory processes. Importantly, distribution and cell source of IL-1β protein induced by NMDA receptor activation in rat brain is similar to responses which follow cerebral ischemia or brain trauma (Pearson et al., 1999; Rothwell, 1999).

The main source of the IL-1β after brain ischemia is considered to be microglial, but also endothelial cells, astrocytes and neurons as well as invading monocytes/macrophages from the peripheral circulation have been reported to express IL-1β in rodents (Buttini et al., 1994; Davies et al., 1999; Sairanen et al., 1997; Saito et al., 1996; Zhang et al., 1998).

Despite of the evidence showing significant role for the IL-1 –family genes in brain pathology, knock out and transgenic animal models targeted to IL-1 family genes or related regulatory genes have also shed complexity to the functional significance of IL-1β in brain ischemia. For example, mice deficient of both IL-1α and IL-1β genes showed significantly reduced ischemic damage against 30 minute transient cerebral ischemia, whereas mice deficient only IL-1α or IL-1β failed to influence brain damage suggesting that chronic deletion of either IL-1α or IL-1β results in compensatory mechanisms in the IL-1 system (Boutin et al., 2001).

Systemic or intracerebroventricular application of IL-1ra provides protection against both permanent or transient MCAO (Betz et al., 1995; Garcia et al., 1995; Loddick and Rothwell, 1996; Martin et al., 1994; Relton and Rothwell, 1992). Since IL-1ra blocks all known actions of both IL-1α and IL-1β, the results described above do not distinguish the relative importance of each of these proteins. There is, however, evidence suggesting that IL-1β is the primary mediator of neurodegeneration. IL-1β has been shown to be the predominant form of IL-1 induced by brain insults. For example, intracerebroventricular injection of IL-1β has been shown to exacerbate ischemic brain damage, brain edema, necrosis and neutrophil infiltration (Yamasaki et al., 1995). Finally, administration of neutralizing antibody against IL-1β was found to inhibit damage caused by transient focal cerebral ischemia suggesting a central role

for IL-1β in brain ischemia (Yamasaki et al., 1995; Yamasaki et al., 1994; Yamasaki et al., 1992).

2.3.3.1.3 Tumor necrosis factor -α (TNF-α)

Tumor necrosis factor -α is a pleiotropic cytokine that is rapidly upregulated in the CNS following brain injury, including trauma and ischemia, but also in neurodegenerative diseases like Parkinson's disease (Mogi et al., 1994). Human TNF-α is produced as membrane-bound polypeptide precursor (26 kDa) and undergoes proteolytic cleavage to yield the soluble form (17 kDa) which forms the active soluble trimeric polypeptide. TNF-α is a member of a family of peptide signaling molecules that exert their activity by interacting with target receptors (Tracey and Cerami, 1993a; Tracey and Cerami, 1993b). TNF-α exerts its biological activity by interacting with two high-affinity receptors: TNFR1 (p55) and TNFR2 (p75). Cytosolic portions of both receptors upon TNF-α binding recruit multiple intracellular adapter proteins. For example, when TNF-α receptor associated death domain (TRADD) is activated via TNFR1, this leads to signal transduction through Fas associated death domain (FADD) eventually activating some caspase proteases. In addition, TNF-α receptors may interact with TNF-α receptor associated factors (TRAFs) like TRAF1 and TRAF2, which can in turn lead to nuclear factor -κB (NF-κB) (see chapter 2.4.1), a transcription factor regulating the expression of various pro- and antiinflammatory genes important to the physiological function of the immune system (Shohami et al., 1999). Also, several kinases are activated by TNF-α of which mitogen-activated protein kinases (MAPKs) are the most extensively studied. Two subfamilies, the c-Jun NH_2-terminal kinases (JNKs) and p38 kinases, also known as stress activated protein

kinases (SAPKs), are activated by TNF-α. Activation of JNKs and p38 kinases may lead to activation of transcription factor AP-1 complex, another important regulator of inflammation and inflammatory gene expression (Herlaar and Brown, 1999).

2.3.3.1.4 TNF-α and brain ischemia

Evidence is accumulating for a detrimental role for TNF-α in brain ischemia. Tumor necrosis factor-α is up-regulated in the post mortem brain tissue of patients with acute brain infarction (Tomimoto et al., 1996). Also, cerebrospinal fluid (Vila et al., 2000) and serum (Carlstedt et al., 1997; Vila et al., 2000) TNF-α levels are elevated in patients with acute stroke. In one study there was also correlation between raised plasma TNF-α levels and early neurological deterioration and poor functional outcome (Castellanos et al., 2002). From the experimental point of view, induction of TNF-α mRNA and protein occurs in the ischemic brain after permanent (Buttini et al., 1996) and transient MCAO (Wang et al., 1994) in rats. It has been shown experimentally that inhibition of TNF-α activity by using soluble TNF-receptor 1 reduces the ischemic brain damage rats, whereas it is exacerbated by intracerebroventricular administration of TNF-α (Barone et al., 1997). Moreover, intracerebroventricular administration of neutralizing monoclonal antibodies against TNF-α protected ischemic mouse brain and reduced the expression of intercellular adhesion molecule -1 (ICAM-1) in vessels of the ischemic area (Yang et al., 1998).

Tumor necrosis factor -α is a mediator of several cellular and molecular mechanisms potentially meaningful to the development of ischemic damage. For example, TNF -α promotes activation and proliferation of microglia and astroglia.

Tumor necrosis factor -α molecules are also secreted by glial cells but also by neurons, and may therefore act in a autocrine manner on these cell types (Barna et al., 1990; Gong et al., 1998; Merrill, 1991). Endothelial cell permeability and cell adhesion molecule expression are also regulated by TNF-α. This is manifested by increased leukocyte accumulation in blood vessels at the site of TNF-α injection (Liu et al., 1994). Increased blood-brain barrier permeability as well as pial artery constriction as a response to TNF-α have also been reported (Kim et al., 1992; Megyeri et al., 1992). Liberation of membrane bound, inactive precursor of TNF-α is mediated by TNF-α converting enzyme (TACE or ADAM 17), and inhibition of TACE activity by selective inhibitor, DPH-067517, was recently found to reduce ischemic brain damage by 40-50%. Ultimately, observations suggest that TNF-α plays a crucial role in brain inflammation by activating neutrophils, increasing leukocyte-endothelial cell adhesion molecule expression and increasing leukocyte adherence to blood vessels and leukocyte infiltration into the brain.

TNF-α may have also neuroprotective properties since genetically generated TNF-receptor deficient mice show increased vulnerability to epileptic seizures and ischemic injury (Bruce et al., 1996). Moreover, TNF-α can protect cultured embryonic rat hippocampal, septal, and cortical neurons against glucose induced injury and excitatory amino acid toxicity (Cheng et al., 1994). Importantly, preconditioning treatment with TNF-α has been shown to protect brain from ischemic insults (Nawashiro et al., 1997).

2.3.3.2 Chemokines and adhesion molecules

As cytokines such as IL-1β and TNF-α play a major role in promoting inflammation and adhesion between endothelial cells and leukocytes, they are poor attractants for polymorphonuclear leukocytes and monocytes to ischemic parenchyma; other chemoattractant cytokines (called chemokines), such as CINC (cytokine-induced neutrophil chemoattractant), MCP-1 (monocyte chemoattractant protein -1) and MIP-1α (macrophage inflammatory protein -1α) are believed to be specifically involved in guiding leukocytes toward the ischemic area (Pantoni et al., 1998).

Chemokines are low-molecular-weight polypeptides that bind to specific receptors formed by seven transmembrane domains and, in turn, activate G-proteins and subsequently intracellular kinases (Baggiolini et al., 1997). Chemokines mainly attract neutrophils and monocytes/macrophages; attraction is dependent of the structural differences in the location of four cysteine residues in chemokine molecules (Furie and Randolph, 1995). Other chemokines such as interleukin -8 (IL-8) and RANTES (Regulated on Activation Normal T Expressed and Secreted) are also involved in leukocyte chemotaxis, cellular activation, inflammation and gliosis after brain ischemia (Barone and Feuerstein, 1999).

Expression of adhesion molecules that promote leukocyte binding to endothelium is a well-described phenomenon in several animal models of brain ischemia. Binding to these adhesion molecules is the primordial step in the leukocyte entry into the brain tissue. Increased mRNA expression of adhesion molecules usually occurs within a few hours after the onset of ischemia in the brain in transient ischemia models, but also in permanent ischemia models without reperfusion (Wang and Feuerstein, 1995). For example, increased P-selectin expression is seen already during MCAO, whereas

intercellular adhesion molecule -1 (ICAM-1) is increased within 1-4 hours after the onset of reperfusion (Okada et al., 1994). In addition, endothelium-leukocyte adhesion molecule -1 (ELAM-1 or E-selectin) mRNA expression has been found to increase in rat ischemic cortex already 12 hours after the ischemia and but also persisting for 2 days suggesting that E-selectin also promotes leukocyte adhesion to endothelium and leukocyte infiltration to the ischemic brain (Wang et al., 1995).

A detrimental role for adhesion molecules in the cerebral ischemia has been suggested. This suggestion is supported by the studies carried out in ICAM –1 knock out (-/-) mice, which exhibited reduced brain infarction volume in TMCAO model when compared to ICAM –1 wild type (+/+) controls (Connolly et al., 1996). In addition, cerebral blood flow to the infarcted hemisphere was 3.1-fold greater in ICAM-1 -/- mice compared with ICAM-1 +/+ controls, suggesting an important role for ICAM-1 in the genesis of postischemic cerebral “no-reflow”. The importance of ICAM –1 was recently highlighted by the findings where murine antibodies against ICAM –1 (Enlimomab) were found to reduce neuronal damage and neuronal deficits in animal models of cerebral ischemia (Bowes et al., 1995; Clark et al., 1991; Zhang et al., 1994). The results were so promising that they gave a rationale to evaluate Enlimomab in human patients. Unfortunately, the trial failed due to adverse side effects and increased mortality in patients treated with Enlimonmab (Enlimomab Acute Stroke Trial 2001).

2.3.3.3 Inflammatory enzymes

Cerebral ischemia induces the expression of multiple molecules and proteins, which can be detected at very early stages after stroke. As reviewed earlier, these molecules

are composed of inflammatory cytokines, chemokines and adhesion molecules. However, there are also well-characterized enzymes, such as nitric oxide synthases (especially iNOS), cyclooxygenases (especially COX-2), and matrix metalloproteinases (MMPs; see chapter 2.3.2.3.) that have also been shown to be upregulated in response to ischemia and to possibly contribute to the ischemic brain damage. The role of cyclooxygenase -2 (COX-2) in ischemic stroke will be reviewed further in this thesis.

2.3.3.3.1 Cyclooxygenase –2 (COX-2)

Cyclooxygenases participate in normal physiological processes such as normal renal maintenance, vascular function, wound healing, bone development glomerular filtration by mediating the production of prostanoids. They are, especially the inducible form COX-2, involved in several pathophysiological conditions such as arthritis, asthma, atherosclerosis, hypotension, shock, and acute inflammation (Herschman, 1994). Also, the expression of cyclooxygenases (COXs) has recently emerged as an important determinant of the cytotoxicity associated with cerebral ischemia and in post-ischemic inflammation (Govoni et al., 2001; Nogawa et al., 1998; Nogawa et al., 1997; Planas et al., 1995; Tomimoto et al., 2000).

Cyclooxygenases, also known as prostaglandin H synthases, are the rate limiting enzymes for prostaglandin and thromboxane biosynthesis. Arachidonic acid, a substrate for COXs, can be metabolized to prostanoids by cyclooxygenase (COX; prostaglandin G/H_2 synthase) after being first released from membrane fatty acids by the action of phospholipase A_2. There are two major isoforms of COX identified so far: COX -1 and COX -2. Cyclooxygenase -1 is constitutively expressed in a variety

of cells. In contrast, COX -2 is the product of an immediate early response gene and induced by variety of stimuli, such as cytokines and growth factors. This inducible COX -2 is linked to inflammatory cell types and is believed to be the target enzyme for the anti-inflammatory activity of non-steroidal anti-inflammatory drugs (NSAIDs) (Seibert et al., 1994; Smith et al., 1996).

2.3.3.3.2 COX-2 in brain ischemia

The expression of COX -2 has been demonstrated to be induced in the brain in response to transient MCAO (Miettinen et al., 1997; Nogawa et al., 1997; Planas et al., 1995), transient global ischemia (Koistinaho et al., 1999; Ohtsuki et al., 1996) and permanent MCAO (Nagayama et al., 1999). Ischemia induced COX-2 expression has been suggested to contribute to production of superoxide and proinflammatory prostanoids from arachidonic acid (Miettinen et al., 1997; Nogawa et al., 1997). However, recently Manabe and coworkers (2004) found that genetic or pharmacologic inhibition COX-2 results in protection against NMDA –induced toxicity independent of free radical production. These results suggest that COX-2 prostanoids rather than free radicals are behind COX-2 dependent neurotoxicity. However, the exact role of COX-2 in neuropathology is unclear and future studies need to address its contribution.

In experimental cerebral ischemia studies, the major site of COX-2 induction has been shown to be in neurons and endothelial cells. However, Sairanen and coworkers have shown from autopsy material from stroke patients that COX-2 protein is also induced in microglia in acute cases (Sairanen et al., 1998). Furthermore, microglia has

been shown express COX-2 also in patients suffering from chronic cerebral ischemia (Tomimoto et al., 2000).

Selective inhibition of COX-2 with SC58236 (Govoni et al., 2001) and NS398 (Nogawa et al., 1998; Nogawa et al., 1997; Sugimoto and Iadecola, 2003) has been applied in several experimental ischemia models. The beneficial effect of these inhibitors is that they inhibit specifically COX-2 enzymatic activity without interfering with transcription, translation or enzymatic activity of other COX genes (Gierse et al., 1996; Kurumbail et al., 1996). In addition, administration of NS398 has been found to have neuroptotective effects if the treatment is started as late as 6 and 18 hours after the onset of brain ischemia in rats and mice, respectively (Nogawa et al., 1997; Sugimoto and Iadecola, 2003).

2.4 Transcription factors as regulators of brain damage

Several transcription factors and their activity have been implicated in various acute brain insults, including stroke. These transcription factors regulate expression of a vast array of genes which may enhance cell death or promote survival. For example, immeadiate early genes such as Fos and Jun family members are up-regulated in response to ischemia (An et al., 1993; Hsu et al., 1993). Importantly, these proteins are components of transcription factor AP-1, which has been shown to activate and mediate the expression of several injury related genes. Other injury inducible transcription factors in the brain include hypoxia inducible factor -1 alpha (HIF-1α) (Pichiule et al., 2003), cAMP responsive element binding protein (CREB) (Sugiura et al., 2004; Zhu et al., 2004), activating transcription factor proteins (ATFs) (Morooka et al., 1995; Zaman et al., 1999), signal transducers and activators of transcription

(STATs) (Takagi et al., 2002), and as will be reviewed further, nuclear factor kappa B (NF-κB).

2.4.1 Transcription factor nuclear factor kappa B (NF-κB)

Activation of transcription factor nuclear factor kappa B (NF-κB) has been implicated in a vast array of normal physiological and developmental, but also in pathological processes. It is a ubiquitous transcription factor composed of a complex of proteins, which are critical regulators of a variety of responses, especially in inflammation. Indeed, NF-κB was originally identified from the B-cells of the immune system being a nuclear factor that bound to the site in the immunoglobulin κ enhancer (Sen and Baltimore, 1986). Since then, nuclear factor kappa B (NF-κB) has been shown to have a central role in immunological processes and have apparent involvement in several other diseases as well (Baldwin, 1996).

2.4.1.1 Molecular properties of NF-κB

Nuclear factor κB (NF-κB) is a heterogenous collection of dimer proteins, composed of various combinations of members of the NF-κB/Rel family. There are five Rel family proteins identified so far: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), c-Rel, RelA (p65) and RelB (Ghosh et al., 1998; Karin and Ben-Neriah, 2000; Siebenlist et al., 1994). All of these proteins share a highly conserved 300-amino-acid Rel homology region, which is responsible for the dimerization, DNA binding, and interaction with the inhibitory IκB proteins.

However, p50-p65/RelA-complex was the first form of NF-κB to be identified and found in most cell types (Siebenlist et al., 1994), including the cells of the mammalian CNS (Gabriel et al., 1999; Schneider et al., 1999). As a consequence, p50-p65/RelA complex is the most often used term to describe NF-κB.

2.4.1.1.1 Activation of NF-κB

Transcription factor NF-κB has interesting qualities that have attracted several researchers: its unusual and rapid activation and regulation and wide range of genes that it controls. NF-κB has been found to respond to a broad range of stimuli and conditions, including inflammatory cytokines such as IL-1 and TNF-α, growth factors, adhesion molecules, cell surface receptors, extracellular stress as diverse as UV-light and H_2O_2 and intracellular oxidative stress (Bowie and O'Neill, 2000; Pahl, 1999) (Figure 4).

Nuclear factor kappa B, in its inactive state, is a cytoplasmic multiprotein complex that is associated to inhibitory complex IκB. Upon stimulation, inhibitory subunit IκB is dissociated from the complex by phosphorylation leading ultimately to the nuclear translocation of active NF-κB (DiDonato et al., 1995). In turn, inhibitory subunit IκB is ubiquitinated and degraded by the proteosome. In the nucleus, NF-κB binds to a specific DNA motif and regulates transcription of target genes containing NF-κB consensus sequences (GGG GAC TTT CCC) in their promoter region (Figure 4) (Siebenlist et al., 1994).

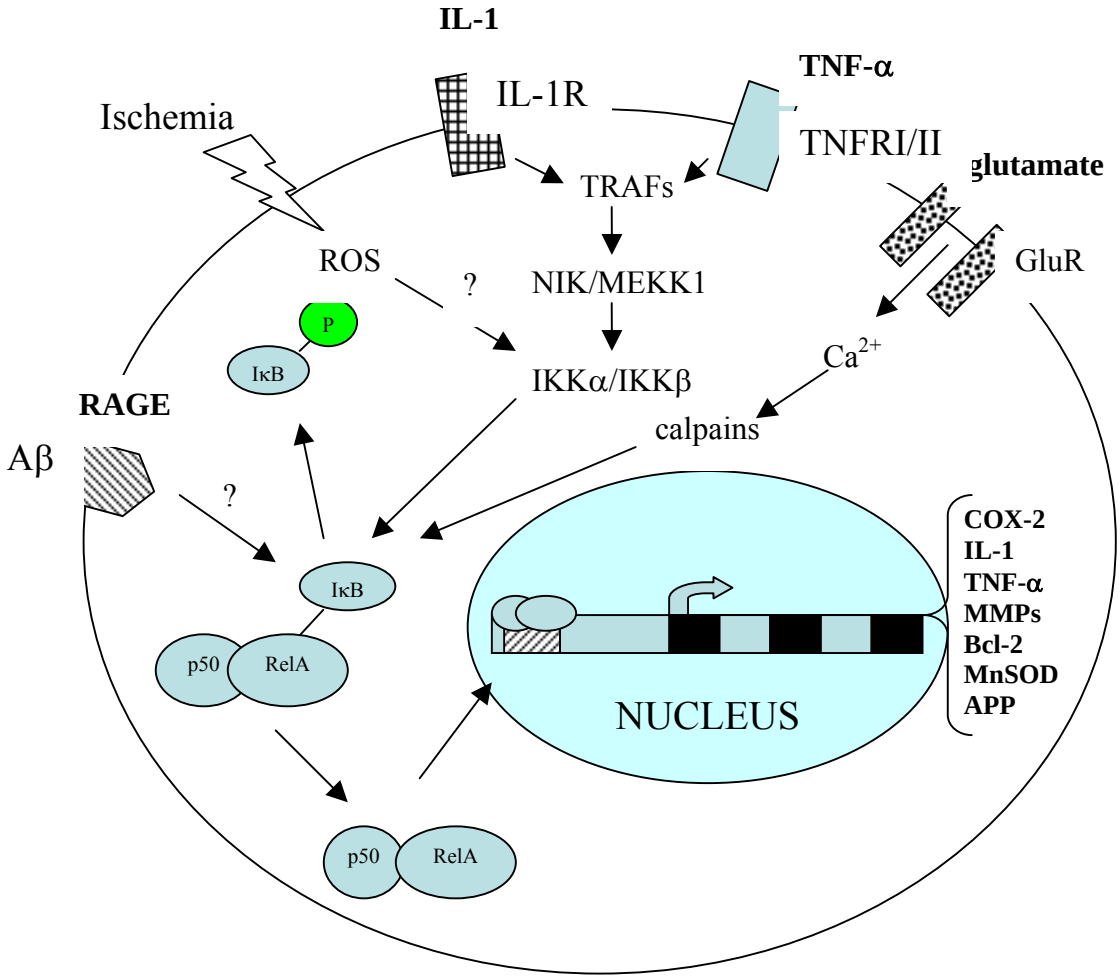


Figure 4. Activating signals responsible for NF-κB activation in brain pathology. Simplified presentation of a few, but important pathways leading to activation of NF-κB. Upon various stimuli activity of the IKK complex leads to phosphorylation and dissociation of inhibitory IκB molecule from the NF-κB complex. Dissociation of the inhibitory IκB releases the NF-κB complex (p50/RelA), which translocates to nucleus and binds to a consensus sequence in their promoter region (Moynagh et al., 1993; Nadjar et al., 2003; Schreck et al., 1992a; Schölzke et al., 2003; Yan et al., 1996).

Inhibitory IκB proteins (inhibitor of NF-κB) are non-covalently linked to NF-κB proteins in the cytosol and maintain NF-κB in the inactive state (Ghosh et al., 1998; Karin, 1999a). Mammalian IκB proteins are composed of several IκB family members: IκBα, IκBβ, IκBγ, IκBε, precursor proteins p100 and p105, IκBR, and Bcl-3 (Gilmore and Morin, 1993; Li and Nabel, 1997). The N-terminal region of IκB

proteins contain both phosphorylation and ubiquitination sites, both vital for NF-κB activation and subsequent IκB protein degradation by the proteosome (Karin and Ben-Neriah, 2000). IκBα and IκBβ are primarily responsible for regulation of NF-κB activity since they interact with the potent transctivators p65 and c-Rel. IκBα and IκBβ exhibit similar inhibitory properties, but they respond to different stimuli. For example, almost all inducers of NF-κB target IκBα causing rapid and transient activation of NF-κB. A few inducers (lipopolysacharide (LPS) and IL-1), however, lead to degradation of IκBβ, which is reportedly slower, phosphorylation dependent and a more sustained process (DiDonato et al., 1996; Thompson et al., 1995).

Key steps in phosphorylation of IκBs are the activities of protein kinases called IκB- kinases (IKKs), which are known to be responsive to a number of potent NF-κB activators. Indeed, the extent to which IKKs are activated seems to dictate the extent of IκB degradation and subsequent NF-κB activation (DiDonato et al., 1997; Karin, 1999a; Karin, 1999b; Mercurio et al., 1997). Three different IKK –kinases have been described; IKKα and IKKβ are functional kinases (Zandi et al., 1997), whereas the third, IKKγ, is suggested to link IKK- complex to upstream activators (Rothwarf et al., 1998). IKKs phosphorylate IκBα and IκBβ at different sites (Ser-32 and Ser-36 of IκBα and Ser-19 and Ser-23 of IκBβ) (Zandi et al., 1998; Zandi and Karin, 1999).

Upstream of IKKα/β is a complex and yet not fully revealed array of signalling pathways that regulate the activation of NF-κB. In the case of interleukin 1 (IL-1) and TNF-α, binding of these proinflammatory cytokines to their receptors in the cell membrane leads to activation of TRAF proteins: TRAF 6 for IL-1 receptor and TRAF 2 for TNF-receptor I/II. The subsequent step in this pathway is the activation of NIK

(NF-κB inducing kinase) and MEKK1 leading ultimately to the activation of IκB-kinases, IKKα and IKKβ (Karin, 1999a; Karin, 1999b).

2.4.1.1.2 Inhibition of NF-κB activity

Negative regulation of NF-κB activity is very complex, and a variety of mechanisms are involved in both termination of NF-κB activation and its down-regulation in response to specific signals (Karin and Ben-Neriah, 2000). The critical physiological inhibitory step is considered to involve the binding of newly synthetized inhibitory IκBs to NF-κB in the nucleus, thereby preventing NF-κB activity (Arenzana-Seisdedos et al., 1995). Also, the role of different combinations of Rel family proteins have also been suggested to regulate NF-κB mediated expression of its target genes. For example, it has been found that IL-10 induced translocation and DNA binding of Rel family homodimers composed of p50 subunits (p50/p50) strongly inhibited NF-κB mediated transcriptional activity, suggesting that homodimers of the Rel family may act as repressors of inflammatory gene transcription (Driessler et al., 2004). There are also reports showing that molecules involved in inflammation can regulate NF-κB activity. For example, nitric oxide (NO) has been shown to inhibit NF-κB activity in endothelial cells, suggesting an anti-inflammatory effect for NO (Zeiher et al., 1995). Interestingly, since NF-κB regulates the transcription of the iNOS gene, a well documented enzyme involved in several disease models such as rodent focal cerebral ischemia (Iadecola et al., 1995a; Iadecola et al., 1995b; Samdani et al., 1997), production of NO may have a negative feed back to inhibit its own production through the inhibition of NF-κB transcriptional activity.

Several pharmacological compounds have been reported to inhibit NF-κB activity. For example, glucocorticoids (Ray and Prefontaine, 1994), nonsteroidal anti-inflammatory drugs (NSAIDS) such as salicylates (Yin et al., 1998) and other immunosupressants such as methotrexate (Majumdar and Aggarwal, 2001) inhibit NF-κB activity. Recently, a fluorinated derivative of acetylsalicylic acid, triflusal posttreatment was found to inhibit glial NF-κB, downregulate the glial response, and was neuroprotective in an excitotoxic injury model in postnatal brain (Acarin et al., 2001).

In addition, several antioxidants such as N-acetyl cysteine (Zafarullah et al., 2003), vitamin C (Carcamo et al., 2002), as well as several naturally derived products are potent inhibitors of NF-κB (Bremner and Heinrich, 2002).

2.4.1.2 Gene expression regulated by NF-κB

Several inflammatory and apoptotic genes are up-regulated in response to tissue injury and inflammation. Interestingly, anti-apoptotic and anti-inflammatory genes are also simultaneously regulated in the same conditions within the same tissue. NF-κB has been suggested to regulate a wide array of these target genes (Table 2), since many of them have been shown to have regulatory binding sites for NF-κB in their promoter region.

Table 2. NF-κB responsive genes in mammalian cells

Gene	Reference
Injury and inflammation	
Tumor necrosis factor -α (TNF-α)	(Shakhov et al., 1990)
Interleukins (IL-1, -2, -6, -8, -10, -12)	(Hiscott et al., 1993; Kunsch and Rosen, 1993; Lai et al., 1995; Libermann and Baltimore, 1990; Mori and Prager, 1996; Murphy et al., 1995; Smith et al., 1994; Xu et al., 2002b)
COX-2	(Kaltschmidt et al., 2002; Yamamoto et al., 1995)
iNOS	(Morris et al., 2003)
MMPs (MMP-1, -3, -9)	(Bond et al., 2001; Bond et al., 1998; Yan et al., 2004)
APP	(Song and Lahiri, 1998)
RAGE	(Li and Schmidt, 1997)
GM-CSF	(Schreck and Baeuerle, 1990)
MCP-1	(Ueda et al., 1994)
ICAM-1	(van de Stolpe et al., 1994)
VCAM-1	(Iademarco et al., 1992)
E-selectin	(Schindler and Baichwal, 1994)
MHC proteins	(Israel et al., 1989a; Israel et al., 1989b; Johnson and Pober, 1994)
RANTES	(Moriuchi et al., 1997)
Cell survival and tolerance	
MnSOD	(Das et al., 1995)
Bcl-2	(Catz and Johnson, 2001)
Bcl-X_L	(Chen et al., 1999)
Gadd45β	(De Smaele et al., 2001)
IAPs	(Stehlik et al., 1998a; Stehlik et al., 1998b)

2.4.2 Nuclear factor kappa B in the brain and in brain disease models

2.4.2.1 Normal physiological functions

Several physiological conditions have been shown to modulate NF-κB. For example, NF-κB has been suggested to be involved in the development of the nervous system (Cauley and Verma, 1994; Schmidt-Ullrich et al., 1996) as well as memory formation (Freudenthal and Romano, 2000). Also, a constitutively active form of NF-κB can be seen in mediating effects of the neurotransmitter glutamate during brain development (Guerrini et al., 1995; Kaltschmidt et al., 1995).

2.4.2.2 Neurodegenerative diseases

Immunohistochemical analysis of the post-mortem brain tissue of Alzheimer's disease patients showed strong NF-κB immunoreactivity in the center of primitive plaques and in cholinergic neurons and astroglia surrounding early plaque stages (Boissiere et al., 1997; Kaltschmidt et al., 1997). Also, activation of NF-κB has been observed in dopaminergic neurons of the substantia nigra (SN) in PD patients, with a more than 70-fold increased nuclear translocation compared to controls (Hunot et al., 1997). Since there seems to be association between disease pathology and activity of NF-κB, several studies have attempted to clarify the role of NF-κB in experimental models of these neurodegenerative diseases. Despite these efforts, the role of NF-κB as "a friend or foe" in neurodegenerative diseases is still unclear and highly controversial.

2.4.2.3 Brain ischemia

There is an increasing body of evidence showing activation of transcription factor NF-κB in brain ischemia models (Clemens et al., 1997a; Clemens et al., 1997b; Gabriel et al., 1999; Salminen et al., 1995; Schneider et al., 1999; Seegers et al., 2000; Stephenson et al., 2000). NF-κB activation has been detected within hours after the ischemic insult and found to sustain over days independent of the study (global vs. focal ischemia). Based on recent reports most prominent NF-κB activation after brain ischemia is seen in neurons (Clemens et al., 1997a; Clemens et al., 1997b; Schneider et al., 1999; Stephenson et al., 2000), although glial cells are also probably involved . For example, in a study by Stephenson et al. (2000) both p50 and p65 subunits of NF-κB complex were found to localize mainly to cortical and striatal neurons in the ischemic brain. Moreover, nuclear preparations from the ischemic brain showed increased binding of NF-κB complex to consensus sequence early after insult. Importantly, Schneider et al. (1999) showed that genetic disruption of inducible NF-κB activity in p50 knock out mice was able to prevent neuronal cell death significantly, supporting a direct cell death promoting role for NF-κB in transient cerebral ischemia model. However, causality between NF-κB activity and neuronal cell death have not been established well enough in brain disease models (Mattson and Camandola, 2001).

2.4.2.4. NF-κB as a therapeutic target in brain ischemia models

Several compounds that have been shown to have neuroprotective effects in brain ischemia models have also been shown to inhibit NF-κB activity. For example, recombinant adenoviral expression of dominant negative IκBα has been shown to protect brain from cerebral ischemic injury (Xu et al., 2002a). Also, NF-κB oligodeoxynucleotide decoys have been shown to attenuate neuronal damage after global brain ischemia (Ueno et al., 2001). Antioxidants such as N-acetylcysteine (NAC) have been found to inhibit NF-κB and provide protection against focal cerebral ischemia (Carroll et al., 1998). Also, Shen et al. (2003) showed that antioxidants attenuate reperfusion injury also in global brain ischemia through inhibiting NF-κB activity. Recently, proteosome inhibitors were found highly protective and the protective effect was associated to NF-κB inhibition, reduced expression of inflammatory molecules and inhibited leukocyte recruitment (Berti et al., 2003; Williams et al., 2004; Williams et al., 2003).

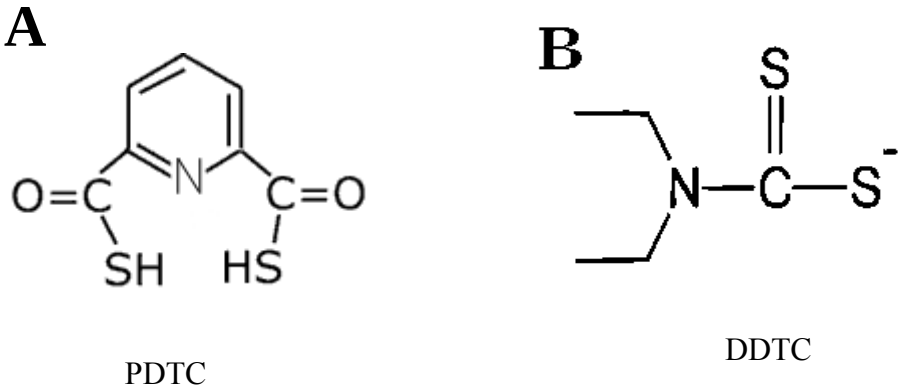
Whether the neuroprotective effect in the above-mentioned studies is a result of direct modulation of NF-κB activity in neurons or glia or just a consequence of other mechanisms affecting NF-κB activity is not clear. However, these studies suggest an important role for NF-κB in these experimental models regulating the development of brain damage as well as inflammation after brain ischemia.

2.5 Dithiocarbamates as therapeutic agents in disease models

2.5.1 Overview

Dithiocarbamates are thiol-containing molecules defined by the possession of an $(R_1)(R_2)N\text{-}C(S)\text{-}S\text{-}R_3$ functional group that have a diversity of applications, both experimentally as pharmacological agents in molecular and cell biology, and commercially as an important class of agricultural pesticides with additional minor clinical utilities (Bowie and O'Neill, 2000; Reisinger et al., 1990; Schreck et al., 1992b; Spruit et al., 1978).

Pyrrolidine dithiocarbamate (PDTC) has been considered a stable dithiocarbamate in physiological surroundings compared to other experimentally used dithiocarbamate analogues such as diethyl dithiocarbamate (DDTC) (Topping and Jones, 1988). Also, a stable dithiocarbamate derivative proline dithiocarbamate (Pro-DTC) possess similar properties than DDTC and PDTC, but is less lipophilic and does not cross the blood brain barrier (Frank et al., 1995).



PDTC DDTC

Figure 5. Dithiocarbamate derivatives. A) pyrrolidine dihiocarbamate (PDTC) B) diethyldithiocarbamate (DDTC).

2.5.2 Dithiocarbamate analogues as therapeutics

At the moment, wide clinical use dithiocarbamates is limited to disulfiram (Antabus), which is used to induce alcohol aversion therapy through the inhibition of aldehyde dehydrogenase. Minor clinical uses of dithiocarbamates include treatments against heavy metal poisoning (Shinobu et al., 1983a; Shinobu et al., 1983b). However, several experimental models have revealed that dithiocarbamates may have additional properties which could be applied in the context of diseases. For example, dithiocarbamates have been reported to exhibit antioxidant, metal-ion chelating and anti-apoptosis activities in various experimental systems (Nobel et al., 1997a; Nobel et al., 1997b). They have also anti-inflammatory and anti-carcinogenic properties (Gopalaswamy et al., 1998; Kovacich et al., 1999) Also, dithiocarbamates have been proposed for the treatment of a number of diseases including atherosclerosis (Moellering et al., 1999) and diabetic retinopathy (Yoshida et al., 1999). Moreover, a clinical trial using dithiocarbamates to inhibit HIV progression was found successful where dithiocarbamate treatment delayed the disease progression to AIDS significantly (Reisinger et al., 1990)

2.5.3 Dithiocarbamates as inhibitors of NF-κB

Dithiocarbamates are widely used inhibitors of NF-κB (Bowie and O'Neill, 2000). Their NF-κB inhibiting properties have been exploited in various experimental settings. Many of these models have focused on the relationship of NF-κB to inflammation, ischemia/reperfusion or cancer. In several experimental models of

systemic shock or inflammation, dithiocarbamates have showed anti-inflammatory properties (Boyle et al., 1998; Cuzzocrea et al., 2002; Frode-Saleh and Calixto, 2000; Kovacich et al., 1999; Rangan et al., 1999) and improved survival (Muller et al., 2000). Also, dithiocarbamates have been shown to be protective in various organ and tissue models of ischemia/reperfusion injury (Kis et al., 2003; Lille et al., 2001; Long et al., 2003), even though there are exceptions (Hill et al., 2001). Also, the beneficial effect of NF-κB inhibition in cancer has been studied with dithiocarbamates as NF-κB inhibitor (Santos-Silva et al., 2001; Sumitomo et al., 1999)

Recently, a dithiocarbamate analogue, pyrrolidine dithiocarbamate (PDTC), was found to inhibit NF-κB activity by inhibiting IκB -ubiquitine ligase activity independently of its antioxidative functions (Hayakawa et al., 2003). Ubiquitinylation of IκB is one of the key elements regulating degradation of inhibitory IκB proteins and therefore NF-κB activity. Interestingly, PDTC has been shown to hold also pro-oxidative properties in some settings by increasing the amount of oxidized glutathione, which also results in NF-κB inhibition (Brennan and O'Neill, 1996; Mihm et al., 1995; Nobel et al., 1995). Therefore, the exact mechanisms how dithiocarbamates inhibit NF-κB is unknown.

2.6 Experimental models to study cerebral ischemia

2.6.1 Overview

The literature describes a vast amount of different approaches to study cerebral ischemia. These experimental models are designed to describe the cellular and molecular events that take place in the brain after ischemic insults.

Primary cultures, co-cultures, cell lines and tissue cultures are widely used methods to study ischemia at cellular level. These *in vitro* models are relatively simple but powerful tools to study elements in brain ischemia in a highly controlled environment. On the other extreme are the animal models, which mainly exploit mammals such as rodents, canines and primates. The most widely used rodent cerebral ischemia models will be reviewed further.

2.6.1.1 Animal models for cerebral ischemia

An animal model for stroke is a living experimental system that contains most of the necessary elements: neurons, glia, brain vasculature and the whole complex physiology of the animal. These elements are needed to ensure reproducibility of ischemic insults and to make correct and statistically valid conclusions (Ginsberg, 1996). The four most widely used animal models of cerebral ischemia are transient or permanent focal ischemia, global ischemia, and hypoxic ischemia in neonates (Lipton, 1999).

2.6.1.1.1 Transient focal cerebral ischemia

Originally described by Koizumi (1986), transient middle cerebral artery occlusion (TMCAO) in rodents has been used routinely to model transient focal cerebral ischemia. The essential feature of this model is the temporary reduction of local blood flow in a defined area of the brain. The TMCAO model has received wide popularity, since it is a simple technique and it produces consistent MCA occlusion and re-canalization without craniectomy. In this model, a (nylon) thread is inserted to the

internal carotid artery via common carotid artery (CCA) or external carotid artery (ECA) (Longa et al., 1989) and advanced so that MCA, anterior cerebral artery (ACA) and posterior cerebral artery (PCA) are blocked. In this model, the severity of the insult is controlled by varying the occlusion time. Another essential feature of this model is that ischemic damage, rather paradoxically, progresses for days and even weeks after the restoration of the blood flow. This phenomenon called "reperfusion injury" is well characterized and characteristic for the model.

2.6.1.1.2 Permanent focal cerebral ischemia

In the permanent focal cerebral ischemia model, MCA is occluded permanently unilaterally. The original model of MCA occlusion was developed by Tamura and colleagues (1981a). In this rodent model, the coronoid process of mandible and zygoma is removed and a hole is opened lateral to the foramen ovale. The exposed MCA is permanently electrocoaculated via opening in the dura. This procedure permanently prevents blood flow from MCA territory of the brain and produces an ischemic lesion.

There are several modifications for permanent MCA occlusion model, for example, the model described by (Welsh et al., 1987) applies the occlusion of the MCA via burr hole made through the temporoparietal bone, which produces relatively consistent ischemic lesions to the cortical areas of mice brains (Koistinaho et al., 2002a; Koistinaho et al., 2002b). However, in the distal occlusion model consistent infarcts are difficult to produce in rats (Bederson et al., 1986), without temporarily (60 min) occluding common carotid arteries (CCAs) bilaterally (Chen et al., 1986). Importantly, in this model no reperfusion or blood reflow occurs to the core of the

infarct after the occlusion of the MCA, which is the case in the transient focal ischemia model. A permanent model of ischemia may also be of clinical significance since it has been reported that a considerable rate of the human stroke cases are not associated with reperfusion during the first 24 hours after clinical stroke (Lindsberg and Kaste, 2003).

2.6.1.1.3 Global forebrain ischemia

Global brain ischemia is induced by cardiac arrest, which results in cessation of systemic blood circulation, hypotension and hypoperfusion of the brain. This animal model has been applied in several mammals such as cats (Cervantes et al., 2002; Hamberg et al., 1993), rats (Pulsinelli and Brierley, 1979), and gerbils (Kirino, 1982; Levine and Payan, 1966) as well as in primates (Myers and Yamaguchi, 1977). There are several ways to produce global cerebral ischemia, but the most common methods employ the occlusion of common cerebral arteries and vertebral arteries for 5-30 minutes. Gerbils have been acknowledged to have ideal vasculature for the global forebrain ischemia studies. Due to absence of anastomosis between vertebral and internal carotid arteries, global forebrain ischemia is produced simply by occluding common carotid arteries bilaterally (BCAO) (Levine and Payan, 1966). This method produces widely used and highly reproducible model to study delayed neuronal cell death in the hippocampus (Kirino, 1982). Delayed neuronal cell death occurs selectively in highly vulnerable brain regions such as neocortical layers (layers 3, 5 and 6), dorsomedial striatum and, perhaps most importantly, in CA1 region of the hippocampus (Kirino, 1982; Kirino and Sano, 1984a; Kirino and Sano, 1984b; Kirino et al., 1984). Other global ischemia models employ occlusion of CCAs bilaterally

combined with hypotension (Smith et al., 1984), increase of intracranial pressure (Ljunggren et al., 1974) , or controlled asphyxiation (Hendrickx et al., 1984).

2.6.1.1.4 Neonatal hypoxia/ischemia model

Occluding carotid artery unilaterally combined with timed exposure to hypoxia produces classical animal model of hypoxia/ischemia. Since the original description by Levine (1960) this model has been applied successfully in neonates (Vannucci, 1990), which are more resistant to prolonged hypoxia than adults (Rice et al., 1981). Hypoxic/ischemic insult in neonate rodents produces lesions affecting cortex, sub-cortical striatum and hippocampus ipsilateral to carotid ligation (Aden et al., 2002; Arvin et al., 2002). A fundamental process believed to be responsible for hypoxic-ischemic damage to neurons is excitotoxicity (Choi and Rothman, 1990; Johnston, 2001), but also apoptotic mechanisms have been suggested (Cheng et al., 1998; Gill et al., 2002; Hu et al., 2000). However, brain injury in this model evolves over hours to days after the neurotoxic cascade that is triggered by hypoxia/ischemia.

3 AIMS OF THE STUDY

Transcription factor NF-κB has been proposed to contribute to neuronal cell death and survival. Furthermore, NF-κB regulates the expression of several inflammatory and apoptotic factors, which are known to be induced in ischemic brain insults. This motivated us to study the contribution of NF-κB in neuronal cell death and its role in the expression of inflammatory and apoptotic factors in brain ischemia models. A particular emphasis of this thesis was to study the effects of the established inhibitor NF-κB and antioxidant, pyrrolidine dithiocarbamate (PDTC) in ischemic brain disease models. The aims of this study were:

1) To explore if transcription factor NF-κB is activated in ischemic brain disease models of adult and neonatal brain

2) To study the effect of PDTC on NF-κB activation and transcriptional activity in ischemic brain insults

3) To investigate the effects of PDTC on the expression of inflammatory and apoptotic markers in the post-ischemic brain

4) To study if genetic inhibition of NF-κB or treatment with pyrrolidine dithiocarbamate provides protection against ischemic brain insults in various *in vivo* and *in vitro* models

4 MATERIALS AND METHODS

4.1 Animal models

All animal studies were conducted according to National Institute of Health (USA) guidelines and approved by the Ethical Committee of National Laboratory Animal Center, University of Kuopio, Finland and by the guidelines of care and use of animals at the University of Heidelberg, Germany.

4.1.1 Transient focal cerebral ischemia (II)

Male, Wistar (n=60) rats weighing 230-280 g (National Animal Center, University of Kuopio, Finland) and spontaneously hypertensive rats (n= 63) weighing 220-290 g (M&B A/S, Denmark) were housed in randomly divided treatment and time-point groups of 5 animals/cage with ad libitum access to food and water. Transient focal cerebral ischemia was produced by occluding the middle cerebral artery (MCA) according to Koizumi et al. (1986) with modifications. Briefly, the rats were anesthetized with 5% halothane in 70% N_2O and 30% O_2. During anesthesia, concentration of the halothane was lowered and maintained at 0.7-1.1%. To avoid changes in body temperature, animals were monitored and rectal temperature was maintained at ~37 $^{\circ}$C with a heating pad during the operation. A midline incision was made and right common carotid artery (CCA) was exposed. External carotid artery (ECA) was ligated distal from the carotid bifurcation point. Side-branches of the ECA were ligated or/and electrocoaculated prior to insertion of 0.25 mm ∅ nylon monofilament thread to internal carotid artery (ICA) via an opening in the ECA. The

filament was inserted 22-24 mm into the internal carotid artery until light resistance was felt. After 90 minutes of ischemia the filament was removed to restore MCA blood flow. Animals were allowed to recover in a cage and sacrificed when needed for experiments.

4.1.2 Permanent focal cerebral ischemia (I)

Male (mean body wt 28.2 ± 3.3 g, n=12) and female (mean body wt 23.3 ± 1.2 g, n=12) p50 knock out mice were used for studying the role of NF-κB in permanent focal cerebral ischemia. Mice having a targeted disruption of p50 subunit are viable, show no abnormalities during development and have only specific defects in immune responses (Sha et al., 1995). Male (mean body wt 27.5 ± 2.7 g, n=9) and female (mean body wt 22.4 ± 1.8 g, n=9) 129/SV X C57BL/6 mice (strain 100 903; Jackson Laboratories, Bar Harbor, Maine) were used as a control mice in p50 knock out mice experiments, which have been described previously (Barone et al. 1993, Connolly et al. 1996, Schneider et al. 1999). Male, Balb/c mice (n=69) weighing 19-26g were used to study the neuroprotective effect of PDTC -treatment after permanent model of ischemia and physiological effects of PDTC-treatment and immunohistochemistry. Permanent focal ischemia was produced by directly occluding the MCA using a method originally described by Tamura et al. (1981) with modifications. Briefly, in p50 knock out mice studies, mice were anesthetized with i.p injection of 2.5% tribromoethanol (Fluka) (given 0.015 ml/g body wt) in saline. In studies with Balb/c mice, 5% halothane in 70% N_2O and 30% O_2 was used for induction of anesthesia and 0.8 - 1.2% halothane for maintenance. In anesthetized animals, MCA was exposed as described by Welsh et al. (1987). The left temporoparietal region of the head was

shaved and the skull was exposed by a midline incision between orbit and ear. A burr hole (diameter ~ 2 mm) was drilled in the temporal bone above the MCA with concomitant cooling of the area with saline. Skull material and dura were carefully removed from the burr hole area prior to permanent electrocauterization of the MCA. The burr hole was filled with porous material (Spongostan, Johnsson & Johnsson, New Brunswick, New Jersey, USA), wounds were sutured and mice were allowed to recover from anesthesia. To asses neuroprotection in permanent focal ischemia model, mice were sacrificed 72 hours after ischemia. Mice body temperature was kept at 36-37.5 °C throughout the operations.

4.1.3 Global forebrain ischemia (II)

Male Mongolian gerbils (n= 18, M&B A/S, Ry, Denmark) weighing 50-67 g were subjected to 6-min bilateral carotid artery occlusion under halothane anesthesia (Yrjänheikki et al., 1998) to induced global brain ischemia, a model of brain damage induced by cardiac arrest. Five percent halothane in 70% N_2O and 30% O_2 was used for induction and 1.5% halothane was used for maintenance. During surgery, the body temperature was monitored and maintained at 37 °C. A midline incision was made and both carotid arteries exposed and miniature aneurysm clips were attached to occlude both carotid arteries for 6 minutes. During the ischemic period, anesthesia was disconnected. After 6 min of ischemia, clips were removed and gerbils were sutured prior to the return to housing cages for 7 days before the loss of CA1 neurons was assessed.

4.1.4 Neonatal cerebral hypoxia/ischemia (III)

Post natal (P7) Wistar rat pups were subjected to hypoxia/ischemia by the method described originally by Rice et al. (1981) with modifications. Briefly, rat pups were subjected to unilateral right common carotid artery ligation under halothane anesthesia (induction 5%, maintenance 1% in 30% O_2/70% N_2O), after which pups were returned to their damn for 2 hour recovering period prior to hypoxic period. After a recovery period, pups were placed to a hypoxic (8% O_2 in balance N_2) chamber immersed in normothermic (37 °C) water bath for 2.5 hours. After the hypoxic period, pups were again returned to their damn. Control animals (P8 and P14) used in a biochemical analysis were subjected to carotid artery ligation without hypoxic period. During surgery animal body temperatures were maintained at 37 °C with a heating blanket.

4.2 Drug treatments (I-III)

Pyrrolidine dithiocarbamate ammonium salt (PDTC) was obtained from Sigma (Germany) and was dissolved in saline (pH 7.4). Proline dithiocarbamate (Pro-DTC) was a generous gift from Dr Norbert Frank at DKFZ (Division of toxicology and cancer risk factors, Cancer Research Center) in Heidelberg, Germany. Treatments were given as follows: pre-treatment group in transient focal ischemia model rats received PDTC 50 mg/kg body wt i.p. 12 hours prior to the onset of ischemia, the next immediately after the onset of ischemia, and the last 12 hours later. In transient focal ischemia model, rat post-treatment groups received PDTC either 100 or 200 mg/kg

body wt i.p. either 2 or 6 hours after the onset of ischemia and again 12 hours later. In permanent focal cerebral ischemia model, mice received PDTC either 100 or 200 mg/kg i.p. 2 hours after the onset of ischemia and again 12 hours later. In neonatal model of hypoxia/ischemia rat pups received a single injection of PDTC 50 mg/kg i.p. 30 minutes after the hypoxic period (normoxia). Animals that served as controls or shams received vehicle solution (0.9% NaCl, pH 7.4) in equivalent amounts as PDTC -treated animals in all models of this study.

4.3 Assessment of ischemic damage (I-III)

In adult rats and mice, determination of the infarct volume was done with 2,3,5-triphenyltetrazolium chloride (TTC) staining according to Bedersen et al. (1986). In transient focal cerebral ischemia model, rats (Wistar) in the pre-treatment groups were sacrificed 24 hours after the onset of ischemia. In post-treatment groups (Wistar & SHR), rats were sacrificed 72 hours after the onset of ischemia. In permanent focal cerebral ischemia model, mice were sacrificed 72 hours after the onset of ischemia. Mice were anesthetized with 2.5% tribromoethanol or with a 50:50 mixture of fentanyl-fluanisone (Hypnorm, Janssen Pharmaceutica) and midazolam (Dormicum, Roche) prior to decapitation. Rats were anesthetized with an overdose of pentobarbital and decapitated. The brains were quickly removed and placed on tissue slicer matrix immersed in ice-cold saline. One-mm coronal sections were cut starting rostrally and slices were incubated in TTC- solution for 20 minutes at 37 °C. Stained slices were scanned on a flatbed scanner and analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Total infarct volume was obtained by

integrating infarcted area of 1-mm brain slices and corrected for brain edema (Swanson et al., 1990).

In cerebral hypoxia/ischemia (H/I) model in neonate rats, determination of stroke volume was done by T_2 -weighed magnetic resonance imaging (MRI) 7 days after the insult and performed at 9.4 T (vertical Oxford magnet (Oxford Instruments PLC, Abingdon, UK) interfaced to a s.m.i.s. console (s.m.i.s., Surrey, UK)) using a 30 mm surface coil. During the procedure, neonatal rats were anesthetized using 1% halothane (carrier gas 70% N_2O and 30% O_2) and a stack of 18 T_2-weighted coronal images (echo time 58 ms, repetition time 2.5 s, two averages/image, slice thickness 1 mm, field-of-view 19.2 mm^2, resolution 256 x 128) were positioned according to pilot images to cover the whole brain. Lesion volumes were estimated slice-by slice by drawing a region-of-interest around the area of hyperintensity in the images. Total infarct volumes were obtained by integrating brain edema corrected infarcted areas of 1 mm brain slices (Swanson et al., 1990).

4.4 Physiological parameters (I, II)

Arterial blood gases (pO_2, pCO_2), pH and blood glucose were determined to follow physiological responses to ischemia and PDTC –treatment during the first 24 hours after the onset of ischemia. Sham -operated and ischemic spontaneously hypertensive rats and Balb/c mice were sedated with ongoing halothane as in ischemia experiments. A midline incision was made and right CCA exposed and cannulated to withdraw small (50-100µl) blood samples for analysis with an ABL-5 blood gas analyzer (Radiometer Inc. Copenhagen, Denmark) and EuroFlash™ blood glucose analyzer (LifeScan, Johnsson & Johnsson AB, Sollentuna, Sveden). Cerebral blood flow (CBF)

was measured from the MCA territory via a small burrhole in the skull with a laser Doppler probe (OxyFlo, Oxford Optronix, Oxford, UK) and analyzed with PowerLab System software (A. D. Instruments, Castle Hill, Australia). In all experiments, CBF was allowed to stabilize 15 minutes prior to measurements (baseline) and monitoring was continued 45 minutes after drug application. CBF data is presented as relative changes from the baseline.

4.5 *In vitro* models of ischemia (I-III)

4.5.1 Primary hippocampal neuronal cultures (III)

Hippocampal neuronal cells were prepared from C57Bl/6 mouse embryos (E18) (National Laboratory Animal Center, University of Kuopio). The tissue was suspended on Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FBS and the cells were plated on poly-DL-ornithine-coated 48-well plate, 2.0 x 10^5 cells/well. On day 2 *in vitro*, the medium was changed into fresh Neurobasal medium (with penicillin-streptomycin and 2% B27 supplement) where 10 μM araC and 25 μM glutamate were added. On days 4 and 8 *in vitro*, medium was changed to fresh Neurobasal. The cultures were used for experiments on day 10 *in vitro*.

4.5.2 ROS measurement after hypoxia-reoxygenation in hippocampal neurons (II)

For studies of ROS production, the medium was changed into Hepes buffer solution (25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$ and 15 mM glucose, pH 7.4).The cells were treated first with 5, 20 or 100 μM PDTC and then exposed to hypoxia for 6 h (hypoxia chamber in 37 °C). After 6 h hypoxia, 10 μM 2', 7'-dichlorofluorescein diacetate (DCF) was loaded with fresh buffer and the

cells were incubated for 20 min. Finally, fresh medium without DCF was changed. After 40, 60, 180 and 300 min the fluorescence intensity was quantified using Storm fluoroimager.

4.5.3 Cell death measurement after hypoxia-reoxygenation in hippocampal neurons (III)

The hippocampal cells were treated with PDTC (20 or 100 μM) and exposed to hypoxia for 9 h by placing the plates into a humidified chamber (modular incubator chamber, Billups Rothenberg, Del Mar, CA) and flushing for 10 minutes with 95% N_2 and 5% CO_2 to achieve a low-oxygen environment. The sealed chamber was placed into a 37 °C incubator. After the hypoxia period, the culture plates were placed back into a normoxic incubator for the time desired. The control plates were kept in normoxic conditions for a corresponding time. Cell death was determined after 20 h reoxygenation using lactate dehydrogenase (LDH) assay with the Sigma Kinetic LDH Kit. Briefly, a 100 μl aliquot was pipetted to a microtiter plate and mixed with 100 μl of LDH reagent. The change of absorbance per minute was determined using a Multiskan plate reader (Labsystems, Helsinki, Finland).

4.5.4 Primary cortical co-cultures (II)

A co-culture system of separable glial and neuronal cell layers was used as previously described (Chen et al., 2001b) with modifications. This model was chosen because the presence of glial cells may influence neuronal injury both *in vivo* and *in vitro*. Cortical glial cells were prepared from newborn NIH/S mice (National Institute of Health, Kuopio). The tissue was suspended in Minimal Essential Medium (MEM)

supplemented with 2 mM glutamine, 10 μg/ml gentamicin and 10% FBS-HI, and were plated on 75 cm^2 flasks. The confluent cultures were trypsinized after 15 div and plated on collagen-coated circles of dialysis membranes. The cells were used after 8 div. Cortical neurons (>99% pure neuronal cultures) were prepared from 15-day old mouse embryos (NIH/S). The tissue was suspended on MEM supplemented with 2 g/L glucose, 2 mM L-glutamine, 10 μg/mL gentamicin and 10% FBS-HI, and the cells were plated on poly-L-lysine –coated 24-well plates, 0.5 x 10^6 cells/well. After 24 h, the confluent glial cells on dialysis membranes were placed on top of the neurons, the cells facing each other. The cultures were used for experiments on day 8 *in vitro*.

4.5.5 NMDA toxicity in primary cortical co-cultures (II)

The cultures were exposed to 200 μM N-Methyl-D-Aspartate (NMDA) for 5 min. PDTC at 10 μM was introduced to the cells 30 min before the NMDA insult, or was added to the fresh medium changed to the cultured after the 5-min NMDA exposure. MK-801 (10 μM), an NMDA receptor antagonist, was used as a positive control. NMDA at 300 μM for 24 h was used as control for total neuronal death. The cell death was determined using lactate dehydrogenase (LDH) assay. Twenty-four hours after the NMDA exposure, the medium was collected, prepared cell-free by centrifugation, and the release of LDH was measured immediately using a Sigma Kinetic LDH kit. Briefly, a 100-μl aliquot was pipetted to microtiter plate and mixed with 100 μL of LDH reagent. Results from at least three separate experiments were combined.

4.5.6 NMDA toxicity in primary mouse cortical culture (I)

Cortices were prepared from 17-day-old mouse embryos (BALB/c) as previously described (Koponen et al. 2003). On day 12, 10 μmol/l PDTC or Pro-DTCwas added in the culture medium 30 minutes before the cultures were exposed to 50 μmol/l *N*-methyl-D-aspartate (NMDA) for 5 minutes. Twenty-four hours later the medium was collected and prepared cell-free by centrifugation, and the release of LDH was measured immediately with a Sigma Kinetic LDH kit and ELISA reader. The culture experiments were repeated 3 times.

4.6 Cytokine measurements (II)

Tissue samples from ischemic penumbras and contralateral cortices were homogenized (4 × vol/wt) in ice-cold phosphate buffered saline (0.1 mM) containing a commercial protease inhibitor cocktail (Complete^{TM}, Roche, Germany). Tissue samples were homogenized with a pellet pestle motor (Kontes, USA) for 25 seconds and then centrifuged for 15 minutes at 13000 rpm at +4 °C. Resulting supernatants were removed, aliquoted, and stored at -80 °C until used for IL-1β and TNF-α measurements. Diluted supernatants (1:3 - 1:7) were used to determine IL-1β and TNF-α concentrations (pg/mg wet tissue weight) in the ischemic brain using commercial 96-well enzyme linked immuno-sorbent assay (ELISA) plates (Quantikine® M, R&D Systems Inc., USA). Cytokine levels were determined from supernatants as duplicates with the microplate reader set to 450 nm with appropriate correction wavelengths. All procedures and steps were done as instructed by the manufacturer of the assay.

4.7 Nuclear protein isolation and electrophoretic mobility shift assay (EMSA) (II)

Nuclear protein extracts were isolated from ischemic penumbra and contralateral cortex of the PDTC –treated and non-treated animals at different timepoints using the method of Dignam (1983) with modifications. Briefly, tissue samples in a hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1.0 mM DTT, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 0.2 mM PMSF) were first homogenized with a pellet pestle motor (Kontes, USA) and incubated on ice for 15 minutes. The resulting homogenate was centrifuged for 10 minutes at 4000 rpm at +4^{o}C. Then, supernatant was removed and the pellet was resuspended to both low-salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 1 mg/ml aprotinin, 1mg/ml leupeptin, 0.2 mM PMSF, pH 7.9) and high-salt buffer (the same as the low-salt buffer except 1.2 M KCl) and incubated for 30 minutes to release nuclear proteins. The mixture was centrifuged at 13000 rpm for 30 minutes at +4^{o}C, and the resulting supernatant containing nuclear proteins were collected and used for EMSA. A consensus oligonucleotide for NF-κB E3291 (Promega) was labeled with γ–[^{32}P]ATP (Amersham) using a T4 polynucleotide kinase (Promega). The probe was purified using Pharmacia Microspin columns (Pharmacia Biotech). The binding reactions were conducted by incubating 5 μg of nuclear proteins in reaction solution containing 5 × binding buffer (100 mM HEPES pH 7.9, 7.6 mM $MgCl_2$, 5 mM EDTA, 50% glycerol), 1.0 mM DTT, 0.1 M NaCl, and poly-dIdC 1.5 μg/μl (Pharmacia Biotech) for 20 minutes. Separation of bound and free probe was conducted by 6% non-denaturing polyacrylamide gel electrophoresis. Gel run was performed at room temp in 5 × Tris-glycine-EDTA buffer (pH 8.5). A phosphoscreen (Molecular Dynamics, Sunnyvale, CA, USA) exposed to bound probes on the gel was

visualized with a STORM™ fluoroimager (Molecular Dynamics) and analyzed with ImageQuant software (Molecular Dynamics).

4.8 SDS-PAGE and Western Blotting (II, III)

Rat brain tissue samples were homogenized in SDS-PAGE Laemli sample buffer and incubated at 100 °C for 10 min. Ten μg of sample protein was loaded per lane. Electrophoresis was carried out in 10% SDS-PAGE gel, in MiniProtean (Bio-Rad) apparatus according to the manufacturer's instructions. Separated proteins were transfered onto Hybond-P membrane (Amersham-Pharmacia Biotech) in MiniTransBlot (Bio-Rad) wet blotting apparatus according manufacturers instructions.

COX-2 was detected using rabbit polyclonal antibodies (Cayman) in 1:1000 dilution. Cleaved caspase-3 was detected using polyclonal antibodies (Pharmingen) in 1:1000 dilution. Secondary HRP-labeled anti rabbit IgG antibodies (Amersham-Pharmacia Biotech) were taken in 1:2000 dilution and HRP label detected using ECL Plus kit (Amersham-Pharmacia Biotech). Membranes were directly scanned on STORM (Molecular Dynamics) fluoroimager, and detected bands quantified using ImageQuant software.

4.9 Immunohistochemistry (I-III)

Immunohistochemistry was done from both transient focal cerebral ischemia model (Wistar) and permanent focal cerebral ischemia model (Balb/c) at 6 and 24 hours after the onset of ischemia. Animals were anesthetized with pentobarbital (60 mg/kg)

(Wistar) or with a mixture of fentanyl-fluanisone and midazolam (Balb/c) and then transcardially perfused first with ice-cold phosphate buffered saline followed by 4% paraformaldehyde. Fifty- micrometer –thick floating sections were prepared with a Leica VT 1000M –vibratome. Sections were incubated in primary antibodies in appropriate dilutions for 48-72 hours at 4 °C. For transient focal ischemia model, sections from Wistar rats were incubated with goat polyclonal anti-p50 (1:500, Santa Cruz, La Jolla, CA, USA), rabbit polyclonal anti-p65/RelA (1:500, Santa Cruz, La Jolla, CA, USA), rabbit polyclonal anti-COX-2 (1:300, Cayman; Ann Arbor, MI, USA), mouse anti-CD11b (OX-42) (1:200, Serotec; Oxford, UK) antibodies. For permanent focal ischemia model, sections from Balb/c mice were incubated with goat polyclonal anti-p50 (1:500, Santa Cruz, La Jolla, CA, USA), rabbit polyclonal anti-p65/RelA (1:500, Santa Cruz, CA, USA), and rabbit polyclonal anti-COX-2 (1:300, Cayman; Ann Arbor, MI, USA) antibodies.

For hypoxia/ischemia model, sections from neonatal (P7-P8) Wistar rats were incubated with goat polyclonal anti-p50 antibody (1:500; Santa Cruz, La Jolla, CA, USA), monoclonal anti-MAP-2 antibody (Chemicon, Temecula, CA, USA) and with rabbit polyclonal antibody against cleaved caspase-3 (Pharmingen, San Diego, CA). After incubation with primary antibodies, sections were reacted with an appropriate biotinylated antibody (diluted 1:200) and avidin-biotin complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 2 hours as recommended by the manufacturer. Immunoreactivity was visualized with H_2O_2 and chromogens diaminobenzidine (DAB) or nickel -enhanced diaminobenzidine (NiDAB). Sections were coverslipped and examined with a Nikon Eclipse E400 microscope equipped with a digital camera (Nikon Coolpix 4500).

For human post mortem samples, tissue blocks were formalin-fixed and embedded in paraffin blocks until dissected. After cutting the tissue on 5 μm thin sections onto Superfrost -glass slides, the sections were deparaffinized and subjected to antigen-retrieval by three 5 min periods of microwave irradiation in citrate-buffer at pH 6. Following routine procedures to remove endogenous peroxidase reactivity with methanol combined with hydrogen peroxide (30 %) and blocking with normal serum, the sections were incubated with a mouse monoclonal antibody against the p65 subunit of the human NF-kB heterodimer (Chemicon Int., Temecula, CA, USA) with a dilution of 1:200. A Vectastain avidin-biotin kit including a secondary anti-mouse IgG (Vector, Burlingame, CA, USA) was used according to the manufacturer's intructions before application of the chromogen diaminobenzidine (Sigma Chemical, St. Louis, MO, USA). To visualize cell nuclei, the sections were briefly dipped in Meyer's Hemalum and then mounted with Permount. Light microscopy was performed with Nikon Eclipse E600 and photographed with Nikon Coolpix 995 digital camera.

4.10 κB5- reporter gene assay (I)

Transgenic mice containing a β-globin reporter under transcriptional control of three binding sites for NF-κB were used. Mice were reanesthetized and perfused with Ringer's solution 24 h after MCAO. The ischemic and the corresponding contralateral cortices were quickly dissected and frozen on dry ice. Tissue was stored at -80 ˚C. RNA was extracted with the RNA pure kit (Peqlab, Erlangen, Germany) according the manufacturer's instructions. Ten μg RNA was transcribed with MMLV reverse transcriptase and random hexamers. For PCR the following primers were used: β-

globin transgene 5', AGC TGC ATG TGG ATC CTG AGA; β–globin transgene 3',GAT AGG CAG CCT GCA CTG GT; cyclophilin 5', AGG TCC TGG CAT CTT GTC CAT; cyclophilin 3', GAA CCG TTT GTG TTT GGT CCA. The taqman probe was labeled with 6-FAM and had the following sequence: CTG GTC TGT GTG CTG GCC CAT CAC T. PCR was perfomed according to the following protocol: 10 min 95 °C, 15 sec 95 °C, and 1 min 60 °C (40 cycles). Amplification was quantified with the Gene Amp 5700 sequence detector and the Taqman kit (Perkin Elmer, Weiterstadt, Germany). The quantification of the β-globin transgene PCR was normalized to the quantification of the cyclophilin PCR in individual samples.

5 RESULTS

5.1 Genetic deletion of p50 subunit of NF-κB or PDTC protects brain *in vivo*

In mouse model of PMCAO, genetic deletion of p50 subunit (p50-/-) resulted in significantly smaller cortical infarcts compared to wild type mice three days after the insult (I, Fig 2). PDTC treatment provided protection against permanent ischemia in Balb/c and C57B6 x SV129 (p50+/+) mouse strains, as well as in spontaneously hypertensive rats (not shown) using the same model (I, Fig. 2B and Fig. 3). However, PDTC did not provide any additional protection in p50-/- mice.

In experiments applying 90 minute transient focal ischemia (TMCAO) model PDTC treatment started 12 hours before the onset of insult provided a significant protection when evaluated 24 hours after the insult (II, Fig 1A). In PDTC treated groups both cortical and subcortical (striatum) areas were protected against ischemia if compared to vehicle treated animals (II, Fig 1A). Protection against TMCAO was obtained if PDTC treatment was given 2 or 6 hours after the onset of ischemia (i.e 4.5 hours reperfusion) (II, Fig. 1C-D). In addition, similar experiments showed that PDTC treatment provided protection also in spontaneously hypertensive rats (II, Fig. 2A-B) in which TMCAO model produces large infarcts and the efficacy of protective treatments is poor compared to normotensive strains (Relton et al., 2001).

In experiments modeling cardiac arrest in Mongolian gerbils, vulnerable CA1 hippocampal neurons were protected against BCAO by PDTC treatment as evaluated 6 days after the insult by cresyl violet staining (II, Fig 3A-D).

In the neonatal model of hypoxia/ischemia, PDTC treatment protected immature rat brain resulting in significantly smaller brain lesions 7 days after the insult as

evidenced by T_2-weighed MRI (III, Fig 1A-B). The results from the *in vivo* studies are summarized in table 3.

Table 3. The effect of dithiocarbamates in animal models of stroke.

Treatment protocol	Strain	Dose (mg/kg)	End point	Effect
PMCAO model				
Post 2h + 14h	p50 +/+ *	200	72h	- 40 %
Post 2h + 14h	p50 -/- *	200	72h	No effect
Post 2h + 14h	Balb/c	100	72h	No effect
Post 2h + 14h	Balb/c	200	72h	- 33 %
Post 2h + 14h	Balb/c	200†	72h	No effect
Post 2h + 14h	SHR	200	72h	- 28 %
TMCAO model				
Pre 12h + 0 + Post 12h	Wistar	50	24h	- 58 %
Post 2h + 12h	Wistar	200	72h	- 52 %
Post 6h + 12h	Wistar	200	72h	- 48 %
Post 2h + every 12h	Wistar	50	72h	No effect
Post 2h + 10h	SHR	100	72h	- 15 %
Post 2h + 10h	SHR	200	72h	- 20 %
BCAO model				
Post 2h	Mongolian gerbil	200	6 days	- 50 %
Neonatal H/I model				
Post 2h	Wistar	50	7 days	- 59 %

* background strain C57B6 x SV129
† Proline dithiocarbmate (Pro-DTC)

5.2 Neuroprotective effects of dithiocarbamates *in vitro*

Three different *in vitro* models were applied to study the effect of PDTC in NMDA toxicity and hypoxia/reoxygenation. LDH release from the culture medium was used as a marker of cell death. The results are summarized in table 4. In a model of pure cortical neurons (I), pre-treatment with 10 μM PDTC and Pro-DTC reduced LDH release 24 hours after 5 min exposure to 50 μM NMDA 31.8 % and 29.2 %,

respectively. A co-culture model of glia and neurons (Chen et al., 2001b) was also used to study NMDA excitotoxicity *in vitro* (II, Figure 8). In this model, 5-min exposure to 200 μM NMDA produced a significant increase in LDH release 24 hours after exposure, which was reduced by 59 % and 71 % when administered 30 min prior or 5 min after the NMDA exposure, respectively. Primary hippocampal neurons exposed to 9 h of hypoxia followed by 20 h of reoxygenation induced a 5 -fold increase in LDH-release, which was inhibited 79 % by 20 μM PDTC (III, Fig. 3).

Table 4. The effect of PDTC *in vitro* brain ischemia models.

Injury model	Cell type	Culture type	Treatment	Effect (LDH release)
NMDA toxicity 50 μM	Cortical neurons	Primary culture, monolayer	PDTC 10μM, Pro-DTC 10μM	-29 % - 32 %
NMDA toxicity 200 μM	Astrocytes and neurons	Co-culture, Separable layers	PDTC 10μM	-59 to -71%
Hypoxia/ Reoxygenation (9h / 20h)	Hippocampal neurons	Primary culture, monolayer	PDTC 20μM	-79%

5.3 Cerebral ischemia induces NF-κB binding, nuclear p50/p65 immunoreactivity and NF-κB driven gene expression which all are inhibited by PDTC treatment

Increased binding of NF-κB proteins to their radiolabeled consensus sequence was seen in brain samples prepared from ischemic penumbras 6 and 24 h after the onset of TMCAO model in rats (II, Fig. 4A). This increased binding was effectively inhibited in PDTC treated ischemic animals, especially at the 24 h time point. In addition, immunohistochemical preparations from rats subjected to TMCAO revealed increased p65 immunoreactivity located in the ischemic penumbra 24 h after the insult, which was markedly reduced in rats treated with PDTC (II, Fig. 4B-D). In the TMCAO

model, p65 immunoreactivity colocalized with COX-2 immunopositive cells with neuronal morphology (II, Fig. 6A-C). Correspondingly, a similar pattern of reduced p50 immunoreactivity was seen in PDTC treated neonatal rats 6 h after the insult in neonatal model of hypoxia/ischemia (III, Fig. 2A-F). In this model some of the p50 immunoreactivity was colocalized with MAP-2 immunoreactivity, a neuronal marker. In the PMCAO model, transgenic mice expressing chimeric human β-globin gene under exclusive control of NF-κB showed significant induction (260%) of NF-κB driven gene expression in the ischemic cortex compared to non-affected cortex 24 h after the insult (I, Fig 2A). Importantly, PDTC treatment almost completely prevented the ischemia induced increase in β-globin gene expression.

In vitro, hypoxia-reoxygenation induced nuclear localization of p50 subunit of NF-κB in hippocampal neurons, which was effectively inhibited by PDTC (III, Fig. 3). Also, exposure of cortical neurons to NMDA increased nuclear localization of p50 immunoreactivity, which was effectively inhibited by PDTC (II, Fig. 8). Finally, Western blot experiments revealed that NMDA exposure caused a significant reduction of cytosolic p50 protein in cultured neurons, but not in astrocytes 10 min after NMDA exposure. The change in cytosolic p50 protein neurons was prevented by PDTC treatment.

5.4 Stroke induces p65 immunoreactivity in human post mortem tissues

In human post mortem samples collected at autopsy from patients who had died after stroke, increased p65 immunoreactivity was seen during the first 1-2 days after insult. In addition, p65 immunoreactivity in cortical samples surrounding the infarct was

localized to nuclei of neurons (I, Fig. 1). In contrast, no detectable p65 immunoreactivity was seen in patients who had died of a non-cerebral cause.

5.5 PDTC inhibits the expression of inflammatory mediators after brain ischemia

The expression of two inflammatory cytokines, TNF-α and IL-1β, was studied from rat ischemic brain samples with the ELISA method. Twelve hours after TMCAO, a significantly increased expression of both TNF-α and IL-1β protein was detected in tissue samples prepared from areas surrounding the ischemic core (II, Fig. 5A-B). No significant change in cytokine expression was seen in PDTC treated rats at this time-point. However, increase of TNF-α and IL-1β protein in brain samples was effectively inhibited in PDTC (200 mg/kg) treated rats 24 hours after ischemia (II, Fig. 5A-B).

We also studied the expression of cyclooxygenase -2 (COX-2) in rats and mice after brain ischemia. In TMCAO model, Western blot experiments revealed increased expression of COX-2 protein in the ischemic brain samples 24 hours after the insult (II, Fig. 5C). In PDTC treated rats the amount of COX-2 protein was reduced by 50%. Immunohistochemical analysis from the ischemic brains supported Western blot data, since a clear COX-2 immunoreactivity was seen in brain sections (II, Fig. 5E) but not in sham operated rats (II, Fig. 5D). Importantly, COX-2 immunoreactivity was reduced in ischemic brains of PDTC treated rats (II, Fig. 5F). In the PMCAO model in mice increased COX-2 expression was also seen in the brain areas surrounding ischemic core (I, Fig. 4A). Again, in PDTC treated mice a clear reduction in COX-2 immunoreactivity in the brain was seen (I, Fig 4B).

5.6 PDTC attenuates hypoxia/ischemia induced elevation of cleaved caspase-3 level in neonatal brain

In neonatal rats, hypoxia/ischemia induced a significant increase in cleaved caspase-3 level as evidenced by Western blot experiments and immunohistochemical data (III, Fig 5A-E). Twenty-four hours after H/I insult, a significant increase in cleaved caspase-3 level was seen in the ipsilateral hemisphere of neonatal rat when compared to control samples. In PDTC (50 mg/kg) treated rat pups, no significant increase in cleaved caspase-3 level was seen at 24 hours after H/I insult. Seven days after the insult, cleaved caspase-3 level was still significantly increased, but was not affected by PDTC treatment.

5.7 PDTC reduces the production of reactive oxygen species (ROS) in neurons and in blood neutrophils

Exposure of neurons to 200 μM NMDA *in vitro* did not increase the production of ROS (II, Fig. 8F), and was not affected by PDTC. Instead, when cortical neurons were exposed to artificial pro-oxidant (haemin) increased ROS production in cortical neurons was observed, but which was effectively inhibited by PDTC. Neurons exposed to hypoxia-reoxygenation induced a clear induction of ROS production, which was inhibited by PDTC in a concentration dependent manner. Moreover, blood neutrophils, prepared from adult rats, showed a robust production of ROS upon phorbol myristate acid (PMA) stimulation. However, ROS production was, again, effectively inhibited by PDTC treatment.

6 DISCUSSION

This study was designed to clarify the role of NF-κB in ischemic insults of adult and immature brain. The effects of NF-κB inhibition in the ischemic brain were evaluated, with emphasis on the pharmacological inhibitor of NF-κB and antioxidant, pyrrolidine dithiocarbamate (PDTC). This study aimed at providing further knowledge about the role of NF-κB in neuronal cell death after ischemic insult in several animal models and in several animal strains. Moreover, we designed cell culture experiments to provide supportive information in relation to the animal experiments. A very important goal was to provide relevant information about the functional role of NF-κB in brain ischemia, mainly by following the expression of NF-κB driven gene expression in the transgenic animal model and by studying the expression of several ischemia inducible markers, which have been shown to be regulated by NF-κB in other systems. Finally, some elements involved in apoptosis were also studied in this context.

6.1 NF-κB is induced in ischemic insults of adult and neonatal rodent brain and in human clinical stroke

An important observation of this study was that NF-κB was activated in neurons of adult and immature brain after experimental ischemic insult as well as *in vitro* models of excitotoxicity and hypoxia/reoxygenation. Clinical observations from human autopsy material presented here suggest that NF-κB is activated also in neurons in clinical cases of stroke.

Our studies with transient focal ischemia models in adult animals are in agreement with previous studies, where NF-κB binding to its consensus sequence in ischemic tissue samples and nuclear immunoreactivity of p50/p65 subunits in neurons have been observed after *in vivo* ischemia (Schneider et al., 1999; Stephenson et al., 2000) or *in vitro* excitotoxicity (Schölzke et al., 2003). Moreover, we extended knowledge about NF-κB in ischemic brain insults as we showed for the first time an increased immunoreactivity of p50 NF-κB subunit in neonatal brain after hypoxia/ischemia.

We did not study the expression of subunits, immunoreactivity or binding activity NF-κB beyond 24 hours, but this has been previously demonstrated in transient focal ischemia studies (Clemens et al., 1997a; Clemens et al., 1997b; Gabriel et al., 1999; Salminen et al., 1995; Schneider et al., 1999; Stephenson et al., 2000). Salminen and coworkers (1995) first reported a delayed induction of NF-κB in brain tissue 5 days after transient focal cerebral ischemia, even though the source of NF-κB activity was not resolved. Gabriel and coworkers (1999) found that 24 hours after transient focal ischemia p65 immunoreactivity was increased in Hsp70 positive neurons. In the same study Gabriel and coworkers (1999) found that from day 4 to day 7, p65 immunoreactivity was mainly changed from neurons to astrocytes and microglia. Clemens and coworkers (1997a) found that neuronal NF-κB immunoreactivity in the hippocampus was induced already 6 hours after a 4- vessel occlusion, but which also persisted three days until apoptotic degeneration occurred in these neurons. Importantly, similar observations were obtained by Schneider and coworkers (1999) in mice where prominent p65 immunorectivity was seen in the apoptotic neurons of the penumbra area 3 days after transient focal ischemia. Moreover, in their study, highly relevant information about the functional activity of NF-κB after transient ischemia was obtained in κB5 transgenic mice, which have chimeric human/mice β-

globin reporter gene under exclusive control of three NF-κB binding sites (Schneider et al., 1999). Authors of the study found that in κB5 mice brain ischemia followed by 20 hours of reperfusion increased the expression of NF-κB driven β-globin reporter gene by over 300 %, confirming that NF-κB is also functionally activated.

NF-κB activity after permanent focal brain ischemia (without reperfusion) has not been previously established. Especially, no studies about functional and transcriptional activity of NF-κB in permanent focal ischemia have been described. Seegers and coworkers (2000) found increased p65 immunoreactivity in neurons located in the penumbra region 7 hours after permanent ischemia, but did not provide any information about the functional activity of NF-κB. Also, Irving and coworkers (2000) found increased NF-κB binding with EMSA 3 hours after the onset of permanent focal ischemia, but decreased NF-κB binding from 6 to 48 hours after the onset of ischemia. We, however, found that focal ischemia without reperfusion (PMAO model) is capable of activating NF-κB and inducing its transcriptional activity by 260% in the κB5 mice brain 24 hours after the insult. This is a novel finding since NF-κB has been proposed to get activated by reactive oxygen species (ROS) (Bowie and O'Neill, 2000), which are highly elevated in brain tissue in focal ischemia with reperfusion (Chan, 1994). Therefore, our results in focal ischemia model without reperfusion suggest that NF-κB activation and transcriptional activity does not require reperfusion or excessive ROS production. Also, we found that glutamate excitotoxicity induced a strong increase in p50 immunoreactivity and disappearance of cytosolic p50 protein in cortical neurons without any significant ROS production *in vitro*, further supporting the idea that NF-κB activation in neurons does not necessarily require ROS. It is, however, also possible that NF-κB activation

and its contribution to brain damage in permanent focal ischemia model may occur via another pathway. For example, NF-κB activation can be mediated via cytokine binding to their receptors, such as described for cytokines TNF-α and IL-1β (Grilli et al., 1996a; Hu, 2003; Nadjar et al., 2003). Both TNF-α and IL-1β are over-expressed in ischemic brain and have been shown to enhance ischemic cell death (Buttini et al., 1994; Gong et al., 1998; Hill et al., 1999; Liu et al., 1994). The enhanced expression of these cytokines may promote their own expression (positive feedback) by enhancing transcriptional activity of NF-κB, thereby promoting further damage in a self-inflicted manner. Even though our results about NF-κB activity in permanent focal ischemia are not in line with the study by Irving and coworkers (2000), our model does not only provide information about NF-κB binding to its consensus sequence, but also more relevant and direct information about the transcriptional activity of NF-κB in the ischemic brain.

We found induction and nuclear localization of p65 immunoreactivity in human post-mortem samples obtained from 3 patients who had died at stroke. Immunohistochemical staining showed that during the first 28 hours after the onset of stroke, NF-κB (p65) immunoreactivity was localized to both cytoplasm and nuclei of neurons located at the penumbra. Nuclear localization of NF-κB (p65) dominated immunoreactivity at 38 hours after the onset of stroke. These data puts NF-κB into a clinical context and show that NF-κB is activated also in human stroke.

6.2 PDTC inhibits NF-κB

We found that in the TMCAO model, intraperitoneally administered PDTC 2 hours after the insult and again 12 hours later was capable of inhibiting NF-κB p65 subunit immunoreactivity and nuclear localization in neurons located to penumbra region in the ipsilateral hemisphere 24 hours after the insult. Moreover, increased binding of NF-κB at 6 and 24 hours after TMCAO to its radiolabeled consensus sequences in EMSA experiments was inhibited in PDTC treated rats after ischemia. In permanent focal ischemia model, PDTC inhibited effectively NF-κB driven transcriptional activity in kB5 –transgenic mice. In neonatal brain PDTC was able to reduce NF-κB p50 immunoreactivity and nuclear localization in neurons after hypoxic-ishemic insult. Finally, PDTC was also able to inhibit NF-κB (p50) immunoreactivity and nuclear translocation in NMDA excitotoxicity model and hypoxia-reoxygenation models *in vitro*.

Pyrrolidine dithiocarbamate is an established inhibitor of NF-κB and antioxidant (Bowie and O'Neill, 2000). The ability of dithiocarbamates to inhibit NF-κB may be related to the fact that these compounds are also known to be powerful redox-active agents which are able to alter the intracellular redox potential and scavenge reactive oxygen and nitrogen species through their thiol groups (Bessho et al., 1994; Orrenius et al., 1996). In line with the hypothesis that PDTC acts as an antioxidant, our *in vitro* experiments show that hemin-induced production of ROS in neuronal culture was prevented by PDTC treatment. However, in our NMDA excitotoxicity model in which ROS production is not elevated, PDTC prevented nuclear translocation of p50 immunoreactivity and prevented the disappearance of p50 protein from the cytosolic fraction. These observations favor the idea that PDTC inhibits NF-κB independent of

its antioxidative functions. Recently, PDTC was found to inhibit NF-κB activity by inhibiting IκB -ubiquitine ligase activity (Hayakawa et al., 2003). In NF-κB activation, phosphorylated inhibitory IκBs are polyubiquitylated by a specific ubiquitin ligase (Karin and Ben-Neriah, 2000). Upon ubiquitylation, the IκB proteins are rapidly degraded by the proteasome, thereby freeing NF-κB, which translocates to the nucleus where it activates transcription. We cannot exclude the possibility that PDTC inhibits NF-κB through its antioxidative properties, since in our experiments, as well as in other studies, PDTC has been shown to be a potent antioxidant (Zhu et al., 2002). Moreover, PDTC has been shown to be a potent chelator of metals such as copper and zinc (Chen et al., 2001a; Erl et al., 2000; Furuta et al., 2002; Kim et al., 1999b). Indeed, it has recently been suggested that PDTC inhibits NF-κB activation by increasing intracellular Zn^{2+} (Kim et al., 1999a). However, increased levels of metal ions such as Zn^{2+}, Cu^{2+} and Fe^{2+} inside the cell would be expected to increase rather than decrease the formation of hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) through Fenton reaction (Jimenez Del Rio and Velez-Pardo, 2004), which are postulated to be important pathway for NF-κB activation (Bowie and O'Neill, 2000).

At present we have no direct evidence about the exact mechanism how PDTC inhibits NF-κB in neurons after ischemic, hypoxic, or excitotoxic insults. Altogether, evidence presented here suggests, however, that NF-κB inhibition with PDTC is highly effective. This inhibition may be mediated by direct inhibition of ubiquitin ligase activity (Hayakawa et al., 2003), by antioxidant effect (Han et al., 2001), or involvement of a yet unknown mechanism (Brennan and O'Neill, 1995). Further experiments are warranted to address the exact mechanism of how PDTC inhibits NF-κB in the brain.

6.3 PDTC inhibits ischemia-induced proinflammatory gene expression and apoptotic cleaved caspase-3 level

In our experiments, expression of several inflammatory mediators was increased in the brain in PMCAO and TMCAO models of adults. Moreover, we found that apoptotic cleaved caspase-3 levels were induced in a neonatal model of hypoxia/ischemia. PDTC treatment inhibited the expression of proinflammatory TNF−α and IL-1β protein in brain at 24, but not 12 hours after the transient focal ischemia. Also, PDTC inhibited the COX-2 protein expression at 24 hours as determined by immunoblotting and immunohistochemistry in PMCAO and TMCAO models. Finally, we found that PDTC inhibited the early expression of apoptotic cleaved caspase-3 protein level in the neonatal brain after hypoxic/ischemic insult.

The enhanced expression of inflammatory mediators TNF-α, IL-1β and COX-2 has been previously established in several ischemia models (Buttini et al., 1996; Buttini et al., 1994; Nogawa et al., 1997; Sairanen et al., 1997; Wang et al., 2004; Wang et al., 1994). These inflammatory molecules have been shown to be transcriptionally regulated by transcription factor NF-κB (Hiscott et al., 1993; Kaltschmidt et al., 2002; Shakhov et al., 1990). However, so far the role of NF-κB in mediating the expression of these mediators in brain ischemia has not been established, although proposed (Barone and Feuerstein, 1999). In our experiments, special care was taken to prepare relevant surviving tissue (penumbra) from brain samples. We were not able to determine the cellular source of TNF-α and IL-1β because the expression was measured from tissue homogenates. We, however, found that COX-2 immunoreactivity was localized to neurons in the penumbra areas after permanent and transient focal ischemia. Increased expression of TNF-α (Liu et al., 1994) and COX-2

(Nogawa et al., 1997) has been previously shown to also occur in neurons, whereas IL-1β has been shown to be expressed by endothelial cells, microglia, and macrophages after brain ischemia (Bhat et al., 1996; Davies et al., 1999; Zhang et al., 1998). Since our immunohistochemical evidence points out that NF-κB was activated in neurons and was co-localized with COX-2 after transient ischemia, it is possible that increased neuronal expression of inflammatory mediators (including TNF-α and IL-1β) and their reduction with PDTC is mediated via inhibition of NF-κB in our studies. We cannot, however, rule out alternative explanations, such as the inhibitory effect of PDTC on the expression of these inflammatory molecules occurs in brain cells other than neurons. Indeed, we have also noticed that glial IL-1β is upregulated in the penumbra tissue after permanent focal ischemia and this glial IL-1β immunoreactivity is reduced in PDTC treated animals (Nurmi 2004, unpublished observations). We did not, however, see any clear effect with PDTC on microglial activation in the transient focal ischemia model, suggesting that microglial cells may not be affected by PDTC in our model.

In a neonatal model of hypoxia-ischemia, PDTC treatment transiently reduced the cleaved caspase-3 levels in the brain 24 hours after hypoxic-ischemic insult. We also found that NF-κB was activated and effectively inhibited by PDTC in neonatal brain six hours after the insult. Dithiocarbamates have been shown to modulate caspases through thiol modification and have been shown to be potent inhibitors of caspase-1 and caspase-3 (Nobel et al., 1997a; Nobel et al., 1997b). Apoptosis plays an important role in neonatal HI (Edwards et al., 1997; Mehmet et al., 1998; Yue et al., 1997). Especially caspase-3 has been found to be activated in neonatal animal models of HI and suggested to contribute to neuronal cell death. (Cheng et al., 1998; Hu et al., 2000; Nakajima et al., 2000) The importance of caspases in neonatal hypoxia-

ischemia model has been highlighted in studies applying caspase inhibitors which may be able to provide neuroprotection (Cheng et al., 1998). Our result that PDTC reduces, at least transiently, cleaved caspase-3 level is difficult to interpret. Changes in cleaved caspase-3 levels in HI may occur independently of NF-κB favoring the idea that PDTC modulates mechanisms regulating caspase-3 cleavage to its active form. For example, this could occur through thiol modulation of caspases as shown by Nobel and coworkers (1997a; 1997b). It is also possible that NF-κB mediated apoptotic signaling is suppressed by PDTC, inhibiting pathways upstream caspase-3 activation. For example, binding of tumor necrosis factor (TNF-α) to its death receptor on the plasma membrane induces the receptor trimerization and recruits a number of death domain-containing molecules to form the receptor complex. Ultimately, this complex promotes activation of downstream caspase cascade and induces degradation of inhibitory IκBα and subsequent NF-κB activation (Hu, 2003). Future studies are needed to clarify the mechanism behind the reduction of cleaved caspase-3 level by PDTC in neonatal hypoxia-ischemia model.

6.4 Genetic deletion of p50 and PDTC protects against cerebral ischemia models in adult and neonatal rodents

Previously, p50 knock out mice (p50-/-) subjected to transient focal ischemia have been reported to develop smaller brain infarcts (Schneider et al., 1999). Importantly, we found that the same p50-/- mouse strain was also protected against permanent focal ischemia without reperfusion. Moreover, by treating wild type mice with pyrrolidine dithiocarbamate (PDTC), a significant protection against permanent focal ischemia was seen. The protective effect of PDTC in permanent focal ishemia model

without reperfusion may be of clinical interest, since in many cases, spontaneous reperfusion does not occur until 24 hours after the onset of human stroke (Lindsberg and Kaste, 2003). Therefore, our results suggest that NF-κB contributes to development of brain damage and inhibition of NF-κB with PDTC or its derivatives may have clinical relevance. Indeed, dithiocarbamates have been demonstrated to have beneficial effects in other clinical settings (Reisinger et al., 1990).

We found that PDTC treatment was highly protective in transient focal and global ischemia model in adult animals as well as in hypoxia-ischemia model in neonates with a wide therapeutic time window. Even when PDTC was administered 6 hours after the onset of ischemia, significant protection was obtained. From the clinical point of view, a wide therapeutic time window is essential in stroke treatment, since the majority of stroke patients arrive in the emergency unit hours after the onset of ischemic attack, after which several potential neuroprotective compounds are no longer effective. As discussed in previous chapters, NF-κB was effectively inhibited by PDTC treatment. This inhibition was associated with reduced expression of NF-κB regulated inflammatory molecules in the ischemic brain, all of which have been shown to contribute to the development of brain damage in experimental settings (Buttini et al., 1996; Buttini et al., 1994; Nogawa et al., 1997; Wang et al., 2004; Wang et al., 1994). Induction of these NF-κB regulated molecules has been established also in clinical cases of stroke (Mazzotta et al., 2004; Sairanen et al., 1998; Tomimoto et al., 2000). In addition, previous studies have proposed that inhibition of NF-κB may have beneficial effects in focal and global cerebral ischemia (Shen et al., 2003; Williams et al., 2003).

Results in our studies suggest that neuroprotection provided by PDTC treatment may be mediated by downregulation of expression of NF-κB regulated

proinflammatory molecules. This is supported by our experimental evidence where NF-κB activation was found to precede or coincide with the production of inflammatory mediators and that inhibition of NF-κB with PDTC was seen effectively both *in vivo* and *in vitro*. This is in line with experimental studies where pharmacological interventions against inflammatory molecules such as COX-2 and iNOS, which have been found protective, even when administered as late as 24 hours after the insult (Sugimoto and Iadecola, 2003). Moreover, PDTC has been shown to effectively inhibit NF-κB, peripheral inflammatory response (neutrophil infiltration) and cytokine production in other disease models (Boyle et al., 1998; Cuzzocrea et al., 2002; Rangan et al., 1999; Yoshida et al., 1999). It should be noted, however, that complete inhibition of NF-κB may interfere with the expression of potential beneficial genes after stroke. These genes may promote survival and have antiapoptotic effects after the acute phase of NF-κB mediated transcription (Mattson and Camandola, 2001). Indeed, this NF-κB mediated survival gene induction after stroke may be of significance, but not necessarily if excessive amounts of apoptotic and inflammatory mediators are produced.

PDTC has antioxidant properties and several antioxidants have been found to be protective in brain ischemia models (Fujimoto et al., 1984; Lin et al., 2004; Suzuki et al., 1984). Recently, dithiocarbamate derivative diethyl dithiocarbamate (DDTC), was found to inhibit NF-κB, but increased the brain damage in transient focal ischemia model (Hill et al., 2001). In addition, we found that proline dithiocarbamate (Pro-DTC), another dithiocarbmate derivative was as protective as PDTC *in vitro*, but did not provide protection against permanent focal ischemia *in vivo*. It has been previously shown that DDTC and its metabolite disulfiram may exert neurotoxic properties (Ningaraj and Rao, 1998; Vaccari et al., 1998; Vaccari et al., 1996),

whereas Pro-DTC does not even cross the blood brain barrier (Lille et al., 2001). This highlights how different derivatives of the same compounds may differentially modulate NF-κB activity or provide neuroprotection.

We used relatively high concentrations (from 50-200 mg/kg) of PDTC in our *in vivo* experiments. However, our treatment protocol was composed of a single intraperitoneal (i.p.) injection (50 mg/kg of PDTC) in neonates to two i.p. injections (100-200mg/kg of PDTC) in adults. We did not notice any consistent or permanent changes in blood pH, partial oxygen pressure (pO_2), partial carbon dioxide pressure (pCO_2), cerebral blood flow, or body temperature at the time points studied. We, however, noticed a transient decrease in blood pH at 6 hours and an increase in blood pCO_2 at 24 hours in PDTC treated animals in transient focal ischemia model. These changes are likely not relevant from the neuroprotection point of view, since decreased blood pH and increased blood pCO_2 would propably have no beneficial effect. Therefore, potential side effects of PDTC or other dithiocarbamates may be acceptable and tolerable in acute treatment protocols. Also, the route of administration of PDTC or other dithiocarbamate derivatives could be more effective (intravenous vs. intraperitoneal) and lower doses of compound could be as effective as we observed after i.p. injections in our study.

Taken together, our results highly suggest that PDTC has neuroprotective effects in a permanent and transient focal ischemia model, global ischemia model, and in a neonatal model of hypoxic-ischemic brain injury. The protective effect of PDTC may be explained by NF-κB inhibition and the resulting reduction in inflammatory gene expression. Moreover, PDTC may exert antiapoptotic effects distinct from NF-κB modulation.

7 SUMMARY AND CONCLUSIONS

This was designed to study the role of NF-κB in brain ischemia models of adult and neonatal brain. The effect of NF-κB inhibitor and antioxidant, pyrrolidine dithiocarbamate (PDTC), was studied using different animal and *in vitro* models of ischemic brain injury. The following results were obtained:

1) Focal cerebral ischemia in adults and hypoxic-ischemic insult in the neonates induces NF-κB activation and transcriptional activity in the brain, which can be effectively inhibited by PDTC.
2) PDTC reduces the protein expression of several brain ischemia-induced inflammatory molecules as well as apoptotic cleaved caspase-3 levels possibly by inhibiting NF-κB transcriptional activity.
3) Genetic deletion of the p50 subunit of NF-κB or treatment with PDTC provides neuroprotection in various animal models of brain injury.

In summary, PDTC provides strain and model independent protection in adult and immature brain, which is associated with inhibition of NF-κB, and anti-inflammatory and antiapoptotic properties. Inhibition of NF-κB may be an important approach in future stroke treatment strategies.

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