Oxidative stress and antioxidant strategies in experimental cerebral ischemia

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List of abbreviations

2VO: 2 vessel occlusion

aCD8: anti-CD8 depleting antibody

AD: Alzheimer's disease

CBF: cerebral blood flow

CCAs: common carotid arteries

COX: cyclooxygenase

CNS: central nervous system

DMSO: dimethyl sulphoxide

eNOS: endothelial nitric oxide synthase

H_2O_2: hydrogen peroxide

H_2-RA: 2.1% hydrogen gas containing room air

IgG2: isotype control antibody

iNOS: inducible nitric oxide synthase

LSD: least significant difference

MABP: mean arterial blood pressure

MCA: middle cerebral artery

MCAO: middle cerebral artery occlusion

MnSOD: manganase superoxide dysmutase

MPO: myeloperoxidase

NF-κB: nuclear factor-κB

NMDA: N-methyl-D-aspartate

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NOS: nitric oxide synthase

NPS: normal porcine serum

$O_2^{\bullet-}$: superoxide anion

$OH^{\bullet}$: hydroxyl radical

$ONOO^-$: peroxynitrite

PBS: phosphate-buffered saline

PBS-T: phosphate-buffered saline containing 0.2% Tween-20

PPAR: peroxisome proliferator-activated receptor

RNS: reactive nitrogen species

ROS: reactive oxygen species

RSG: rosiglitazone

RT: room temperature

RT-PCR: real-time polymerase chain reaction

SDS: sodiumdodecylsulfate

SHAM: sham-operated

SOD: superoxide dysmutase

TBS: tris buffered saline

TGI: transient global ischemia

TNFα: tumor necrosis factor α

α-TTP: α-tocopherol-transfer-protein

Összefoglalás

Az oxidatív stressz bizonyítottan szerepet játszik az iszkémiás neuronkárosodás patomechanizmusában. Kísérleteink során különböző agyi iszkémia modellekben vizsgáltuk egyes pro- és antioxidáns enzimek szintjét, illetve jellemeztük bizonyos antioxidáns terápiák hatékonyságát.

A két arteria carotis communis permanens lekötésével (2VO=2 vessel occlusion) enyhe krónikus előagyi iszkémiát hoztunk létre patkányban, melynek különböző fázisaiban (1 naptól 1 évig) vizsgáltuk a ciklooxigenáz-2 (COX-2), a 3 nitrogén-monoxid-szintáz (NOS) izoforma és a mangán szuperoxid-dizmutáz enzimszintek változásait. Ugyanezen enzimeket vizsgáltuk 3 nappal 10 perces súlyos előagyi iszkémiát követően (TGI = tranziens globális iszkémia), melyet 2VO-val kombinált hipovolémiás hipotenzióval indukáltunk. A 2VO modellben antioxidáns terápiaként az α-tokoferol, míg TGI-ben a hidrogén protektív hatásait jellemeztük. A leukocita infiltráció oxidatív stresszben betöltött szerepének vizsgálatához az arteria cerebri media okklúziójával fokális iszkémiát hoztunk létre, melyben a CD8+ T sejtek depléciójának neuronkárosodásra és indukálható NOS (iNOS) expresszióra gyakorolt hatását jellemeztük.

Mindkét előagyi iszkémia modell akut fázisában COX-2 upregulációt és csökkent neuronális NOS szintet detektáltunk a hippokampuszban, melyek rendre kifejezettebb excitotoxicitásra és neuronpusztulásra utalnak. A 2VO modellben ezen válaszok enyhébben és nagyobb késéssel jelentkeztek, mely utalhat a TGI-ben bekövetkező súlyosabb neuronpusztulásra. A 2VO modell késői fázisában (12 hónap) az endoteliális NOS upregulációja hozzájárulhat az agyi perfúzió helyreállításához. Csak fokális iszkémiában detektáltunk iNOS upregulációt, melyet a CD8+ T sejtek depléciója csökkentett, ezzel redukálva az infarktus méretét és javítva a szenzomotoros funkciókat. Ezek alapján az iNOS káros hatású fokális agyi iszkémiában, overexpressziójáért főként az infiltráló fehérvérsejtek – mint a CD8+ T sejtek – felelősek.

Az α-tokoferol kezelés csökkentette a neuronkárosodást és javította a térbeli tanulási képességet 2VO-val modellezett krónikus agyi hipoperfúzióban, míg a hidrogén kivédte a TGI vizsgált enzimszintekre gyakorolt hatását. Ezek alapján az α-tocoferol hatékony lehet krónikus agyi hipoperfúziós kórképekben a kognitív funkciók romlásának megelőzésében. A hidrogénnel dúsított levegő inhalációja pedig a gyors diffúziónak és kiürülésnek

köszönhetően akut cerebrovaszkuláris események reperfúziós szakaszában bírhat terápiás jelentőséggel.

Summary

Oxidative stress plays an important role in the pathomechanism of ischemic neuronal death. In our experiments we investigated the changes of pro- and antioxidant enzyme levels in different models of cerebral ischemia, and examined the potential protective effects of certain antioxidant treatment strategies in these models.

We characterized the changes of cyclooxygenase-2 (COX-2), the 3 nitric oxide synthase (NOS) isoforms and manganase superoxide dysmutase levels in the hippocampus and in the cortex in different phases (between 1 day and 12 months) of mild forebrain ischemia induced by the permanent occlusion of the common carotid arteries in rats (2VO=2 vessel occlusion) and 3 days following 10 min severe forebrain ischemia (TGI = transient global ischemia) induced by 2VO combined with hypovolemic hypotension in rats. We investigated the effects of the vitamin E component α-tocopherol in the 2VO model, and hydrogen therapy in TGI. To examine the participation of leukocyte infiltration in oxidative stress, we induced focal ischemia by medial cerebral artery occlusion in mice and investigated the effect of CD8+ T cell depletion on neuronal damage and the expression of inducible NOS (iNOS).

In the acute phase of both forebrain ischemia models, we described COX-2 upregulation indicating the presence of excitotoxicity and decreased neuronal NOS expression referring to neuronal loss in the hippocampus, which is the most vulnerable part of the brain to oxidative damage. These responses were more moderate and developed with a delayed dynamics following 2VO suggesting a more severe neuronal damage in TGI. In the late phase (12 months) of the 2VO model, endothelial NOS is upregulated and can contribute to the compensation of cerebral perfusion. iNOS was induced only in focal ischemia; CD8+ T cell depletion attenuated its upregulation resulting in smaller infarct size and improved sensorimotor functions. These results suggest a deleterious effect of iNOS in focal ischemia, where its overexpression predominates in infiltrating leukocytes, such as CD8+ T cells.

α-Tocopherol treatment prevented neuronal loss and attenuated learning deficit in the 2VO model of chronic hypoperfusion seen is Alzheimer disease (AD) and normal aging. Hydrogen containing room air inhalation prevented the changes of the investigated enzyme levels induced by TGI that occurs in patients during acute severe hypotension of cardiac origin. Therefore, α-tocopherol may prevent the development of AD and normal aging-coupled memory dysfunctions, while hydrogen therapy should be considered in the acute management of cardiovascular emergencies and / or cardiac surgery.

Introduction

1. Cerebrovascular disorders and their animal models

According to WHO data, stroke is the 3rd leading cause of death worldwide and the major cause of long-term disability in industrialized countries. Besides stroke syndrome, chronic cerebral hypoperfusion leads to the development of cognitive disorders, such as dementia that has a prevalence of 6.4% in Europe which is increasing continously[46]. Therefore the acute management and the long term medical attendance of cerebrovascular patients is a growing challenge.

Cerebral hypoperfusion leads to the disruption of oxygen and glucose supply of the brain termed as cerebral ischemia. Due to its high metabolic rate and limited capacity to regenerate, the brain exhibits a higher sensitivity to ischemia than other organs. The localization, the degree, and the duration of the perfusion disturbance determine the phenotype of the consequent neurological disorder. Accordingly, the disruption of cerebral blood flow (CBF) can be permanent or transient, it can affect a distinct brain region (focal ischemia) leading to symptoms related to the ischemic area, or the whole brain (global ischemia), where the damage is seen in the most vulnerable brain regions. Several animal models have been developed to simulate the different human cerebrovascular disorders and have provided insight into the morphology and the pathomechanism of brain injury.

Chronic cerebral hypoperfusion is defined as a moderate but persistent reduction of CBF and has been associated with a cognitive decline during aging, senescence or the memory dysfunction in Alzheimer's disease (AD) and vascular dementia[32,43,104]. Its well-characterized model is the permanent bilateral occlusion of the common carotid arteries (CCAs) in rats that induces mild forebrain ischemia and is called the 2VO (= 2 vessel occlusion) model[44].

Transient global ischemia (TGI) occurs commonly in patients during acute severe hypotension caused by cardiac arrest, arrhythmia, or complex cardiac surgery[123,144,150]. The spectrum of neurological symptoms includes cognitive impairment, seizures, ischemic stroke, and coma[100,114], making TGI a major complication following disorders mentioned above. TGI can be experimentally induced by (1) 4VO in rats involving permanent coagulation of the vertebral arteries and temporary ligation of the CCAs, (2) transitional 2VO combined with hypotension in rats, or (3) 2VO without hypotension – due to the incomplete Willis circle – in

gerbils. The latter two models result in more profound forebrain ischemia[83]. The applied insult is generally short, between 3 and 30 min, 10 min being the most often used time period.

The stroke syndrome can be caused either by focal cerebral ischemia or by intracerebral hemorrhage. Ischemic stroke yields more than 80% of all strokes; it is caused by thrombotic or embolic occlusion of one of the main or secondary cerebral arteries. In rodents, experimental ischemic stroke can be induced by transient (allowing reperfusion thus modeling thrombolysis) or permanent occlusion of the middle cerebral artery (MCA) hence called the MCAO (MCA occlusion) model.

2. Ischemia induced neuronal damage

Ischemic cell death is initiated by changes that result directly from inhibition of oxidative phosphorylation. These changes include decreased pH, decreased amount of ATP, membrane depolarization, increased cell Na^+ and Ca^{2+}. These lead to activation of damaging processes including proteases, phospholipases, and free radical actions that produce long-term changes in macromolecules. The consequential ischemic neuronal death can be necrosis and apoptosis. Necrosis is typically characterized by lack of the energy substrate[155], where the lysis of the plasma membrane and cell organelles occur provoking inflammation in the surrounding tissue[163]. In contrast, apoptosis takes place in the presence of ATP, it involves ordered physiological processes in that new structures are formed that allow the cells to die with minimal release of intracellular content[61,77]. Necrotic cell death most probably predominates in the acute phase of cerebral ischemia when ATP is rapidly depleted, while the delayed neuronal death could be apoptotic[7,19]. The delay of ischemic cell death depends on the severity of the insult: the greater the CBF decrease and energy deprivation the shorter the delay[83].

In the acute phase of 2VO model, CBF drops suddenly to 35-60% of control level, affecting mainly the hippocampus, the parietal and the frontal cortex. The CBF values already starts to gradually recover at 1 week, only a slight reduction has been reported between 8 weeks and 3 months, finally CBF returns to the control level at 6 months[26,125,128,138,152,153,156]. Although, this flow pattern does not match with human disorders, the period between 1 and 8 weeks can be used to simulate the chronic hypoperfusion induced brain damage observed in human dementia and aging. Compared to the 2VO model, a more severe disruption of blood supply can be seen in the TGI model, where CBF falls to ~15% of control levels in the hippocampus, neocortex and striatum[149]. Although CBF and ATP levels recover quite well

during reperfusion in this model[132], tissue metabolic rate is depressed for at least 6 h after global ischemia[107].

In both the 2VO and TGI model, hypoperfusion is global affecting the whole forebrain, while neuronal cell death develops only in the most vulnerable brain regions, such as the hippocampus[137]. Delayed pyramidal cell loss in the hippocampal CA1 region is a cardinal and well-studied feature of global ischemia, however – in accordance with the different degree of CBF decrease – it develops with different delay in these 2 models. In 2VO, there was no loss of neurons during the first week of 2VO[126], but at 2 weeks following ischemia induction 6-29% of the animals exhibited hippocampal injury in the CA1 subfield[41,126,138]. In contrast, hippocampal neuronal damage can be detected already from 24 h after 10 min TGI[173], and most of the pyramidal cells in the CA1 region undergo delayed cell death by 3 days[147]. Hippocampal injury is accompanied by hippocampus-related learning and memory deficit in both models[43,99,127].

In the MCAO model, there is a core ischemic region where blood flow is reduced to <15% and a penumbral region where CBF is <40% of control level[52,119]. Unlike global ischemia, which leads to neuronal cell death in distinct cell populations, focal ischemia produces a contiguous mass of necrotic brain tissue termed the infarct, where rather than measuring damage by counting dead cells, damage is generally expressed as the volume of the infarct. The infarct begins in the core and reaches the penumbra already by 24 h after ischemia onset. Between 24 and 72 h following MCAO, the infarct continues to grow in the penumbral region at a much slower rate[50]. The infarction is located in the cortical area of the MCA territory and thus it is associated with sensorimotor deficits[96,174].

3. Role of neuroinflammation in neuronal damage

Neuroinflammation induced by cerebral ischemia is involved in secondary brain damage, however by removing cell debris it is also required for the regenerative process of surviving neurons. The inflammatory process involves the rapid activation of resident cells (mainly microglia) that can induce the infiltration of circulating leukocytes, such as neutrophils, T cells and monocytes/macrophages. Leukocyte invasion into the brain occurs primarily in brain injuries that are also accompanied by the breakdown of the blood-brain-barrier, otherwise microglia are the prominent responding cellular elements[56,146].

In both the 2VO and the TGI models, the microglial activation in the hippocampus occurs within minutes and still observed several weeks following ischemia induction[1,42,110]. Inhibitors of microglial activation prevent hippocampal damage and attenuate cognitive

impairment[14,170]. Microglia react rapidly to cerebral ischemia by synthesizing and releasing proinflammatory cytokines, such as interleukines and tumor necrosis factor α (TNFα), and reactive oxygen species (ROS) which are considered harmful for neurons at high concentrations[88,151]. By producing ROS, microglia contributes to oxidative damage as the neuroprotective effect of microglia inhibition is accompanied by the reduction of oxidative stress[14]. Although both 2VO and TGI evoke a systemic inflammatory response identified by the formation of leukocyte-platelet aggregates and by the activation of neutrophil granulocytes[89,134], most likely invading leukocytes do not have pronounced role in the mechanism of brain injury in global ischemia. The latter is supported by observations, where accumulation of leukocytes was observed only if TGI was extended to 40 min or longer[3] and preischemic induction of neutropenia did not attenuate the damage to CA1 neurons[139].

In contrast to the relatively minor role of peripheral immune cells in the mechanism of neuroinflammation in global ischemia, the onset of ischemia is followed by significant influx of these cells after MCAO. The rapid and excessive activation of resident microglia leads to the consequential release of proinflammatory mediators[2,86] inducing the expression of adhesion molecules on both cerebral endothelial cells and on leukocytes, thus promoting the adhesion and transendothelial migration of circulating leukocytes[168]. The infiltrating leukocytes release cytokines, chemokines and excessive amount of ROS that amplify the brain inflammatory process and the neuronal damage. In the course of brain invasion, the initial rapid recruitment of neutrophils is followed by the accumulation of lymphocytes and monocytes[145]. The deleterious nature of this inflammatory process was proven by observations demonstrating that ischemic injury can be attenuated by the preischemic induction of neutropenia[74] and/or the pharmacological blockage of adhesion molecules or their receptors[35,48,96]. However, less is known about the contribution of specific leukocyte subpopulations to post-ischemic neuroinflammation and neuronal damage.

4. Oxidative stress in cerebral ischemia

Oxidative stress plays an important role in the pathomechanism of ischemic neuronal death. Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons that give considerable reactivity to the compound. However, not all ROS and reactive nitrogen species (RNS) are free radicals. ROS and RNS are also produced during the normal cellular metabolism by prooxidant enzymes (NADPH oxidase, xanthine oxidase, cyclooxygenase: COX), or nonenzymatically by mitochondria[10]. The production of ROS/RNS is balanced by the the scavenging antioxidant system. This system consists of

antioxidant enzymes (superoxide dysmutase: SOD, glutathion-peroxidase, catalase), enzyme-dependent antioxidant molecules (glutathion, NADPH) and enzyme-independent free radical scavengers (vitamins). The delicate balance between pro- and antioxidant systems assures the low concentrations of ROS/RNS required for their physiological functions. For instance, superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) function as regulatory signaling molecules and regulate apoptosis, cell proliferation and differentiation[98,135]; at higher concentrations, H_2O_2 is converted into hypochlorous acid that defends against bacterial invasion[162]; nitric oxide (NO) functions as a neurotransmitter and is essential for the dilation of blood vessels[113].

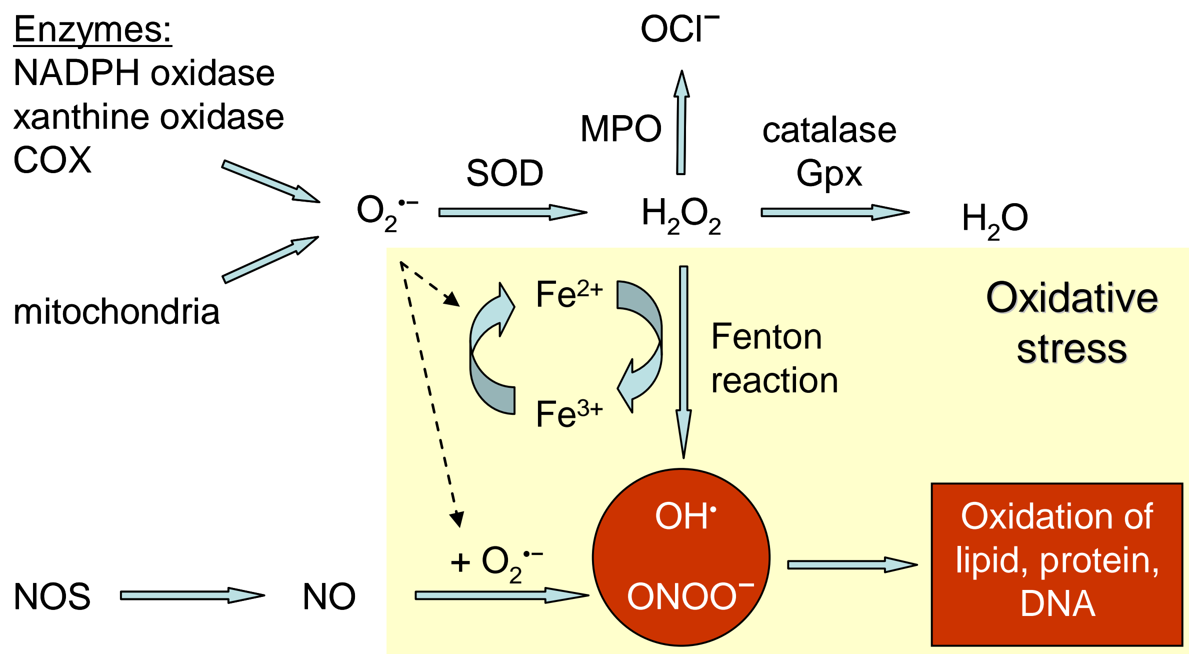


Fig. 1. Schematic representation of ROS/RNS production. Superoxide anion radical ($O_2^{\bullet -}$) can be produced by enzymes (such as NADPH oxidase, xanthine oxidase or cyclooxygenase: COX), or nonenzymatically by the mitochondrial electron transport chain. $O_2^{\bullet -}$ is converted enzymatically by the superoxid dismutase (SOD) into hydrogen peroxide (H_2O_2), which is detoxified into water (H_2O) by either glutathione peroxidase (Gpx) or catalase, or converted into hypochlorous acid (OCl^-) by myeloperoxidase (MPO) in phagocytes. During oxidative stress, excessive generation of $O_2^{\bullet -}$ leads to the increased production of H_2O_2 that may react with cellular metals, such as Fe^{2+} or Cu^+ ions producing the cytotoxic hydroxyl radical ($OH^\bullet$) via the Fenton reaction[59]. By releasing Fe^{2+} from Fe^{3+} containing enzymes, $O_2^{\bullet -}$ can further facilitate the production of $OH^\bullet$ [97]. Nitric oxide (NO) is produced by different types of nitric oxide synthase (NOS). Although NO is a free radical, cytotoxicity attributed to NO is rather due to peroxynitrite ($ONOO^-$) produced by the interaction between NO and $O_2^{\bullet -}$ anion[18]. Peroxynitrite is cytotoxic due to its direct oxidative reactions with macromolecules, and because its cleavage is an additional source of $OH^\bullet$ [85].

During cerebral ischemia, the activation of prooxidants and/or the reduction of antioxidant defense can both contribute to the elevation of ROS levels causing oxidative stress. On one hand, ischemia-induced elevated intracellular Ca^{2+} concentrations increase the production of free radicals due to the activation of prooxidant enzymes and the disruption of the mitochondrial respiratory chain[105]. In addition, the ischemia-induced inflammatory

processes also contribute to the release of ROS/RNS. On the other hand, the antioxidant defense is perturbed because of the increased consumption and the inadequate replenishment of antioxidant molecules in ischemic brain tissue. The imbalance of pro- and antioxidant system leads to increased production of highly reactive ROS/RNS such as hydroxyl radical ($OH^{\bullet}$) and peroxynitrite ($ONOO^{-}$) leading to the oxidative damage of macromolecules such as lipids, proteins and nucleic acids, impairing the functional integrity of the cell. The brain is particularly vulnerable to oxidative stress for several reasons. 1) high oxygen consumption; 2) enzymes of neurotransmitter metabolism generating $OH^{\bullet}$ as a by-product, such as monoamine oxidase and tyrosine hydroxylase[131]; 3) in the cell membrane higher ratio of peroxidisable polyunsaturated fatty acids side chains, which are especially sensitive to free radical attack[59]; 4) decreased antioxidant capacity[64] 5) higher amount of iron that can lead to $OH^{\bullet}$ production via the Fenton reaction[59]. Therefore antioxidant therapies could be efficiently involved in the treatment of cerebral ischemia. However, further information are needed about the contribution of the distinct pro- and antioxidant enzymes to oxidative damage and the temporal pattern of the development of oxidative stress in cerebral ischemia.

4.1. Role of the COX system in cerebral ischemia

COX enzymes (COX-1 and COX-2) convert arachidonic acid to prostaglandinH_2 producing $O_2^{\bullet-}$ during this process. By virtue of their vasodilatory effects, prostaglandins can help maintain the cerebral perfusion[54], while $O_2^{\bullet-}$ gives rise to oxidative damage. Both COX isoforms are constitutively expressed in the central nervous system (CNS), and participate in homeostatic processes including regulation of tissue perfusion[67], synaptic plasticity and memory functions. Accordingly, selective COX-2 inhibitors reduce high-frequency stimulation induced long-term potentiation at hippocampal perforant path-dentate granule cell synapses[21]. Besides the constitutive expression, COX-2 is also inducible by a variety of stimuli ranging from pro-inflammatory factors (e.g. cytokines, endotoxin), seizure activity, cerebral ischemia to brain injury[157]. COX-2 is upregulated in the brain of AD and stroke patients and various animal models of acute brain ischemia[17,29,34,122]. COX-2 overexpression is located in neurons after ischemic injury, and has been associated with excitotoxicity mediated by N-methyl-D-aspartate (NMDA) receptors[108]. COX-2-selective inhibitors prevent cerebral cortical neurons from undergoing Aβ-induced apoptosis[166], reduce the infarct size in the MCAO model in rats[16], and attenuate white matter damage in chronic cerebral ischemia[159]. Taken together these observations, COX-2 has physiological roles in the CNS; however, its overexpression might be harmful for the ischemic brain.

4.2. Role of the nitric oxide synthase (NOS) system in cerebral ischemia

NOS enzymes catalyze the production of NO from L-arginine. There are three isoforms of NOS as the constitutive isoforms presented in vascular endothelial cells (eNOS) and in the nervous system (nNOS), and the inducible isoform of NOS (iNOS). The NO released from endothelial cells regulates local blood flow, platelet aggregation and neutrophil adherence[71,136]. The nNOS isoform occurs in brain neurons and generates NO acting as a neurotransmitter[101]. The iNOS isoform is induced by exposure to cytokines and lipopolysacharid, and expressed by numerous cell types such as neutrophils, microglial cells, astrocytes and neurons, as a consequence of the inflammatory processes[84]. The overproduction of NO by NOS occurs in a number of clinical disorders including acute cerebral ischemia and chronic neurodegenerative diseases such as AD and Parkinson's disease, and aging coupled dementia[30,65]. On one hand, NO is a potent vasodilatator and an inhibitor of platelet aggregation and leukocyte adhesion, as a result it might improve post-ischemic blood flow by enhancing collateral circulation and preventing microvascular plugging[71,136]. On the other hand, NO promotes oxidative damage by reacting with $O_2^{\bullet-}$ to form $ONOO^-$ [18]. Therefore, NO can be protective or destructive to the ischemic brain depending on the amount, the temporal pattern and the site of NO production.

At the onset of ischemia, NO overproduction is driven by the upregulation of nNOS and eNOS[6,172,175], at later times iNOS is responsible for the synthesis of NO[69]. Post-ischemic iNOS induction is likely to be initiated by cytokines that accumulate in the ischemic brain and the consequential inflammatory process; therefore it produces large amounts of NO for a long period of time, leading to cytotoxicity[65]. Under ischemic conditions substrate supply is limited, and NOS produces a mixture of $O_2^{\bullet-}$ and NO that react to form $ONOO^-$ and result in cytotoxicity[164]. As eNOS is in the vascular endothelium, mostly nNOS and iNOS are subject to substrate or cofactor limitation described above. In line with these different features of the 3 NOS isotype, MCA occlusion in eNOS knockout mice leads to larger infarcts[63], while nNOS and iNOS inhibitors reduces cerebral ischemic damage[68,169].

4.3. Role of manganese SOD (MnSOD) in cerebral ischemia

SOD is involved in the enzymatic antioxidant defense system of the cells by catalyzing the dismutation of the $O_2^{\bullet-}$constantly produced by the mitochondria and enzymes like COX. This action is biologically necessary for the survival of the organism because although the $O_2^{\bullet-}$ spontaneously dismutes to oxygen and hydrogen peroxide quite rapidly, it reacts even faster with certain targets such as the $NO^{\bullet}$ radical forming $ONOO^-$ [18]. SOD has three

isoforms: SOD1 is located in the cytoplasm, SOD2 is in the mitochondria, and SOD3 is extracellular. SOD1 and SOD3 contain copper and zinc (Cu/ZnSOD), while SOD2 has manganese (MnSOD) in its active site[171]. As mitochondria play a pivotal role in the regulation of apoptosis[87], and are the major subcellular source of the $O_2^{\bullet-}$ [130], MnSOD levels can influence the mitochondrial control of apoptosis under ischemic conditions.

5. Antioxidant strategies

In order to determine whether free radicals may constitute a valuable therapeutic target, several antioxidant strategies have been tested in different models of cerebral ischemia. The antioxidants used in our studies are presented in the following.

5.1. α-Tocopherol

α-Tocopherol is a member of the lipid-soluble vitamin E complex and is best known as an antioxidant. Vitamin E is essential for the physiological function of the CNS, vitamin E deficiency results in primarily neurologic symptoms (loss of deep tendon reflexes, cerebellar ataxia, dysarthria, retinitis pigmentosa) and observed in genetic disorders affecting proteins that have important roles in the metabolism of vitamin E[55,62,129]. Among the components of Vitamin E (α-, β-, γ-, δ-tocopherols and tocotrienols), α-tocopherol is selectively sorted out by the α-tocopherol-transfer-protein (α-TTP) for incorporation into very low density lipoprotein and reaches the highest plasma concentration, while most of the other vitamin E components are excreted via the bile[11]. In the same time, in case of vitamin supplementation α-TTP and other transfer and binding proteins may also limit the increase of plasma α-tocopherol concentration. In line, the normal α-tocopherol concentration (~25 µmol/L) of healthy subjects did not increase > 2–3 fold, irrespective of the amount or duration of supplementation[37], decreasing the danger of intoxication. The recommended α-tocopherol intake is increasing by years and has been lately established at 15 mg/day.

Due to its lipid solubility, α-tocopherol is located in the lipoprotein membranes of the cell; therefore it can prevent oxidation of membrane lipid molecules. The peroxidation of lipids (LH) is a free radical chain reaction, where lipid radical ($L^\bullet$) and lipid peroxyl radical ($LOO^\bullet$) are formed, the chemical reaction can be summarized by the following scheme[72]:

Initiation: $OH^\bullet + LH \rightarrow L^\bullet + H_2O$

Propagation: $L^\bullet + O_2 \rightarrow LOO^\bullet$ and $LOO^\bullet + LH \rightarrow LOOH + L^\bullet$.

α-tocopherol (α-TOH) serves as a H donor for the lipid and lipid peroxyl radicals, the developing tocopheroxyl radical (α-TO$^\bullet$) is relatively stable and unreactive due to the delocalization of the unpaired electron within the molecule, thus the chain reaction is disrupted[72]:

$$L^\bullet + \alpha\text{-TOH} \rightarrow \alpha\text{-TO}^\bullet + LH \quad \text{and} \quad LOO^\bullet + \alpha\text{-TOH} \rightarrow LOOH + \alpha\text{-TO}^\bullet.$$

The regeneration of α-tocopherol from tocopheroxyl radical is performed by other antioxidant vitamins (such as vitamin A, vitamin C) and ubiquinol[9,72]. Therefore, α-tocopherol is the first line of the enzyme-independent antioxidant defense; furthermore, besides lipid peroxidation it also prevents protein oxidation[20].

α-Tocopherol has been widely investigated as dietary supplement for the prevention of cardio- and cerebrovascular diseases. Besides its free radical scavenging properties, it exerts cardiovascular protective action also through the attenuation of inflammatory reactions such as the release of proinflammatory cytokines. As a result, it hampers the adhesion of leukocytes to the endothelial cells, the aggregation of platelets and the consequent formation of atherosclerotic plaques[143]. Additionally, α-tocopherol induces vasodilation through modulation of the eicosanoid metabolism and the release of prostacyclin[160]. In the CNS, α-tocopherol reduces lipid peroxidation and protein oxidation in ischemia /reperfusion or beta-amyloid-induced oxidative brain damage, as proved by the decreased levels of malondialdehyde and protein carbonyl following α-tocopherol treatment[20,76]. Conceivably, these mechanisms are responsible for the observed reduction of infarct volume and improved neurological performance scores after MCAO in mice[20,109].

5.2. Hydrogen

In the field of biological medicine, hydrogen has been considered a physiologically inert gas and it is often used in diving medicine. Recent studies revealed its antioxidant and anti-inflammatory properties giving potential to medical application. Molecular hydrogen is produced constantly under physiological conditions in the human body by intestinal bacteria[60] that can be responsible for the basal level of circulating hydrogen detected in mammals[27]. Exogenous hydrogen can be administered via inhalation of gas[124], by hydrogen-rich water per os[116], hydrogen-saturated saline intravenously[177] or intraperitoneally[12]. Hydrogen gas is highly flammable and burns in the air above the concentration of 4.6%. The hydrogen concentration of the inhalated gas mixture used in studies is 1-4%, and 2% already exerts the maximal protective effect[124], making its medical application safe. The hydrogen content of arterial blood increases in proportion to hydrogen concentration of the inhaled gas mixture,

the amount of hydrogen dissolved in the venous blood is less than that in the arterial blood, suggesting that hydrogen is incorporated into tissues[124]. As the hydrogen molecule is small and neutral, it should easily penetrate the cellular and intracellular membranes that are normally preventing water-soluble anti-oxidants from entering cells and organelles such as the mitochondria, the major source of ROS production.

Oshawa et al showed that in cultured cells hydrogen selectively reduced $OH^{\bullet}$ by forming water, but it did not influence the level of $O_2^{\bullet-}$ and H_2O_2 [124]. Due to $OH^{\bullet}$-scavenging, hydrogen reduces oxidative damage to DNA, lipids and proteins as evidenced by decreased levels of 8-hydroxydeoxyguanosine, malondialdehyde and protein carbonyl in studies described below. Additional cytoprotective effects can be due to its anti-apoptotic effect[13], inhibition of inflammatory mediators[79,165] or downregulation of the nuclear factor-κB (NF-κB) signaling pathway[23]. Owing to these antioxidant and other cytoprotective effects, hydrogen therapy is beneficial in different disease models, including ischemia-reperfusion injury, inflammation, toxicity or trauma. In the CNS, hydrogen reduces apoptosis in neonatal hypoxia-ischemia in rats[12], preserved cerebrovascular reactivity to hypercapnia and reduced neuronal injury induced by asphyxia–reventilation[38], in a rat model of MCAO, reduced the infarct size[22,124] and improved neurological function[22]. Hydrogen also protected against nigrostriatal degeneration in a rat model of Parkinson's disease[49], and prevented β-amyloid-induced neuroinflammation and oxidative stress in line with improving memory dysfunction[93].

5.3. Rosiglitazone (RSG)

RSG, a synthetic agonist of peroxisome proliferator-activated receptor-γ (PPARγ), is widely used as an insulin sensitizer for the treatment of type 2 diabetes mellitus. PPARs are members of nuclear hormone receptor superfamily that function as ligand-activated transcriptional factors controlling lipid and lipoprotein metabolism, glucose homeostasis, cell proliferation and apoptosis[4,40]. 15-d-Prostaglandin J2 is the endogenous agonist ligand of PPARγ, while several thiazolidinediones including RSG are potent synthetic agonists[80]. Three PPAR subtypes: PPARα, PPARβ/δ and PPARγ, have been isolated. PPARγ is widely expressed in neurons and glia of the CNS. PPARγ regulates cytokine production and adhesion molecule expression, by interfering with the NF-κB and activator protein-1 signaling pathways, resulting in down-regulation of inflammatory processes[36]. RSG was also shown to have antioxidant properties; it reduced cyclooxygenase-2 (COX-2) expression and the production of ROS and cytokines, leading to attenuated neuronal damage in the hippocampus

in TGI[90]. Indeed, increased PPARγ activity reduced infarct volume and improves neurologic function following MCAO in rats[148] indicating its neuroprotective potential.

6. Aim of the study

The major aim of our studies was to provide novel experimental data on the changes of the pro- and antioxidant enzyme levels in cerebral ischemia and also to test the efficacy of various treatment options all targeting oxidative stress. In the course of our experiments various ischemia models were used, and the study outcome measures also differed in part due to the availability of methods.

1. We characterized the changes of COX-2, the 3 NOS isoform and MnSOD levels in the hippocampus and in the cortex in chronic mild forebrain ischemia induced by 2VO and in transient severe forebrain ischemia using the TGI model.

2. We evaluated the effects of α-tocopherol on neurological function, neuronal damage and microglial activation in the 2VO model, and tested the potential protective effects of hydrogen in comparison with RSG in the TGI model.

3. To examine the participation of leukocyte infiltration in ischemic brain injury and oxidative stress, we investigated the effect of CD8+ T cell depletion on neuronal damage and the expression of iNOS in a mouse MCAO model of focal cerebral ischemia.

Materials and methods

1. Animal models of cerebral ischemia

1.1. Mild forebrain ischemia: the 2VO model

Ischemia was induced in rats by permanent occlusion of the CCAs (2VO). Briefly, the animals were anesthetized with 400 mg/kg chloral hydrate i.p., followed by 0.05 ml of 0.1% atropine i.m. (0.14–0.18 mg/kg) to avoid an increase in vagal parasympathetic tone. A ventral cervical incision was made in the midline to expose the CCAs, which were gently separated from their sheaths and vagal nerves, and permanently ligated with surgical sutures. In the sham-operated (SHAM) rats, the arteries were similarly exposed, but not ligated. Lidocaine (1%) was applied as local anesthetic.

1.2. Severe forebrain ischemia: the TGI model

TGI was induced in rats by transient occlusion of the CCAs combined with simultaneous hypovolemic hypotension. The animals were anesthetized with 1.5-2% halothane in $N_2O:O_2$ (2:1) and were breathing spontaneously during the surgery, their body temperature was maintained with a heating pad. CCAs were exposed through a ventral cervical incision in the midline, gently separated from their sheaths and vagal nerves. Mean arterial blood pressure (MABP), pH and the partial pressure of O_2 and CO_2 were monitored via an arterial cannula inserted into the left femoral artery. TGI was induced by clamping both CCAs in combination with lowering the MABP to 40 mmHg by withdrawal of blood (6.3±1.4 ml, 1.5ml/min) with heparanized syringes through a venous cannula inserted into the left femoral vein. Ten minutes after MABP had reached 40 mmHg, blood was reinfused, and the CCAs clamps were removed to allow reperfusion of the brain. In the SHAM group, preparation of the CCAs and catheterization of the femoral vessels was performed, without the ligation of CCAs or the induction of transient hypotension.

1.3. Focal ischemia: the permanent MCAO model

Focal ischemia was induced in mice by transtemporal electrocoagulation of the left MCA distal to the lenticulostriate arteries. Mice were anesthetized with 1.5-2% halothane in $N_2O:O_2$ (2:1). After a 1 cm skin incision between left eye and ear, temporal muscle was removed and a burr hole was drilled through the temporal skull. The dura mater was removed and the MCA permanently occluded using bipolar electrocoagulation forceps (ERBOTOM, Erbe, Germany).

2. Brain samples and Western blot

Western blot was used to determine the ischemia-induced changes of COX-2, the three NOS isoform and MnSOD enzyme levels. Rats were deeply anesthetized with an overdose of chloral hydrate i.p. and were perfused transcardially with 100 ml cold (4 ^{o}C) saline. The brains were removed and samples were quickly taken from the hippocampus and the frontal cortex. The samples were immediately frozen in liquid nitrogene and stored at -80 ^{o}C until further processing. The frozen samples were homogenized in a 10-fold amount (1:10 mg/µl) of 50 mM Tris buffered saline (TBS) containing 0.15 M NaCl, 2 mM phenylmethylsulfonylfluoride, 2 mM activated Na_3VO_4, 2 mM EDTA, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 1% Nonidet–P-40 and 0.1% sodium deoxycholate by using a glass–teflon potter, and were sonicated at 40 kHz (10 s) with the same amount of 0.1% sodiumdodecylsulfate (SDS) (1:1 sample / 0.1% SDS). The hippocampus and cortex homogenates were centrifuged at 10,000 x *g* for 5 min at 4 °C and the supernatants were used for further analysis. Protein concentrations of supernatants were determined by a microplate reader, using the method of Lowry et al.[102].

Each supernatant fraction was mixed in a ratio of 1:1 with Laemmli sample buffer (Bio-Rad, USA), containing 62.5 mM Tris-HCl, 2% SDS, 25% glycerol and 0.01% bromophenol blue (pH 6.8), and heated to 100 °C for 3 min. Proteins (20 µg per lane) were separated with 10% SDS-polyacrylamide gel and then electrotransferred to nitrocellulose membrane (Amersham, USA) by using the Bio-Rad Mini-Protean Basic system. In all instances, the membranes were stained with Ponceau stain (Santa Cruz Biotechnology Inc., USA), to verify the uniformity of protein loading and the transfer efficiency across the samples. The membranes were next quenched with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.2% Tween-20 (PBS-T) for 1 h at room temperature (RT).

The blotted membranes were incubated with COX-2 (polyclonal, 1:250, Cayman Chemicals, USA), eNOS (polyclonal, 1:250, Santa Cruz Biotechnology Inc., USA), iNOS (polyclonal, 1:250, Santa Cruz Biotechnology Inc., USA) and nNOS (polyclonal, 1:250, Cayman Chemicals, USA) antibodies overnight at 4 °C or with MnSOD antibody (polyclonal, 1:500, Stressgen Bioreagents Corp., USA) for 2 h at RT. After rinsing in PBS-T (3×10 min), the membranes were incubated for 1 h with horseradish peroxidase conjugated goat anti-rabbit IgG (1:10,000 Sigma-Aldrich, USA). The immunoreactive bands were visualized by using the Supersignal West Pico Chemiluminescent Reagent (Pierce, USA). Finally, the membranes were exposed to an autoradiographic film (Amersham, USA). The membranes

were stripped (Western Blot Stripping Buffer, Pierce, USA) and incubated with anti-β-actin monoclonal antibody (1:5000; Sigma-Aldrich, USA) for 90 min at RT, followed by goat anti-mouse (1:10,000 Sigma-Aldrich, USA) secondary antibody coupled to horseradish peroxidase for 1 h at RT in order to assess the gel-loading homogeneity. The optical densities of the bands were quantified by using a computerized image analysis system (ImageJ Program, USA). Data were normalized for β-actin and are expressed as % changes from control protein levels.

3. Composition of experiments

Table 1a. Study 1.

Groups (n)	Elapsed time following operation						
Surgery	1	3	1	2	3	6	12
	day(s)		week(s)		month(s)		
naive	4	4	4	4	4	-	-
SHAM	3	5	4	4	6	5	6
2VO	5	5	4	4	4	4	5

Table 1b. Study 2.

Groups (n)	Treatment			
	Pre		Post	
Surgery	oil	toc	oil	toc
SHAM	7	9	10	8
2VO	9	7	9	6

Table 1c. Study 3.

Groups (n)	Treatment			
Surgery	NT	H_2-RA	vehicle	RSG
Naive	5	-	-	-
SHAM	6	8	6	6
TGI	6	8	5	7

Table 1d. Study 4.

Groups (n)	Duration of PFI	Treatment	
		IgG2	aCD8
Corner test, Histology	1 day	10	10
	3 days	10	10
	7 days	9	10
RT-PCR	1 day	5	5
	5 days	5	5

Abbreviations: 2VO: 2 vessel occlusion, aCD8: anti-CD8 depleting antibody, H_2-RA: 2.1% hydrogen gas containing room air, IgG2: antibody isotype control, NT: non-treated, oil: soybean oil, PFI: permanent focal ischemia, RSG: rosiglitazone, TGI: transient global ischemia, toc: α-tocopherol, SHAM: respective sham-operation

3.1. Study 1. Changes of pro- and antioxidant enzyme levels in the 2VO model

To characterize the temporal pattern of pro- and antioxidant enzyme level changes, rats were perfused after different survival periods (1 day, 3 days, 1 week, 2 weeks, 3 months, 6 months, and 12 months) following 2VO/sham-operation, and Western blot was performed on the hippocampus and cortex samples. Non-operated rats were involved as naive controls. The number of animals in the different experimental subgroups at different survival periods is shown on Table 1a. COX-2, eNOS, nNOS, iNOS and MnSOD levels were determined with Western blotting. The data were expressed as percentages of the naive control levels (1 day – 3 month groups), or (when naive animals were not included) as percentages of the data for the SHAM animals (6 and 12 month groups). One-way ANOVA was performed for surgery

(SHAM vs. 2VO) followed by a least significant difference (LSD) post-hoc test. The accepted significance level was $p<0.05$ in all kinds of statistical comparisons used in our study.

3.2. Study 2. Effects of α-tocopherol treatment in the 2VO model

We evaluated the effects of α-tocopherol treatment on neurological function, neuronal damage and microglial activation 2 weeks following 2VO.

3.2.1. α-Tocopherol treatment

The animals received i.p. injections of 100 mg/kg α-tocopherol (Sigma, St. Louis, MO, U.S.A.), while the control rats received its solvent soybean oil in equal volume (~0.4 ml, Sigma, St. Louis, MO, U.S.A). The treatments were performed on 5 consecutive days before or after 2VO induction (i.e. pre-treatment or post-treatment). In the pre-treatment, the final injection was administered 1 day before vessel occlusion; in the post-treatment, the first injection was given directly after surgery. Table 1b. demonstrates the number of rats in the 8 experimental groups based on the various surgery and treatment strategies.

3.2.2. Spatial learning: Morris water maze

One week following the 2VO/sham-operation, the spatial learning capacity of the animals was assessed in the Morris water maze. Briefly, in this learning paradigm the rats have to find a hidden platform within 2 min after they had been placed in a circular pool 160 cm in diameter. The animals enter the pool from one of the 4 standard entry points, randomly selected over the trials. Rats that fail to find the platform within 2 min are guided to it. Visual cues and an auditory source guide the animals in their learning. All animals performed two trials per day (4 h apart) for 5 consecutive days. The swimming paths were recorded by a computerized video imaging analysis system (Etho Vision, Noldus Information Technology BV,Wageningen, The Netherlands). In each trial, the distance travelled until reaching the platform was analyzed. The daily performance was expressed as the average swimming distance of the 2 trials per day. Repeated two-way ANOVA was performed for surgery (SHAM vs. 2VO) and for treatment (soybean oil vs. α-tocopherol). One-way ANOVA was performed for individual daily comparisons followed by the LSD post-hoc test. Additionally, in trials 2–10, the numbers of animals that could not find the platform in 2 min (severely impaired rats) were counted and summated for the 9 trials. The number of these observations was expressed as percentage of the total observations. Statistical analysis was performed with a non-parametric chi-square test.

3.2.3. Histological analysis

Two weeks after 2VO induction (shortly after the completion of the Morris water maze tests), the animals were anesthetized with an overdose of chloral hydrate (i.p.). The rats were perfused transcardially with 100 ml of saline followed by 400 ml 4% of paraformaldehyde. The brains were removed, postfixed in 4% paraformaldehyde solution for 1 h, and then stored in 0.1 M PBS containing 0.1% sodium azide. Free-floating coronal sections at the level of the dorsal hippocampus were cut at 20-μm thickness on a cryostat microtome. Two sets of sections per animal containing the dorsal hippocampus were selected for staining.

The first set of sections were mounted and air-dried on gelatin-coated microscopic slides and stained with cresyl violet. Photomicrographs of the hippocampus CA1 stratum pyramidale were taken with a computerized image analysis system to identify neuronal damage (Olympus BX50 microscope, DP50 digital camera, ImagePro Plus software, Media Cybernetics, U.S.A.).

The second set of sections was immunocytochemically stained for OX-42 labeling microglia activation. After pre-treatment with 0.5% Triton X-100 and 3% H_2O_2 in 0.01 M PBS, and pre-incubation in 20% normal porcine serum (NPS), the sections were treated with Avidin (Vector), followed by Biotin (Vector). The sections were incubated overnight in biotinylated mouse anti-OX-42 antibody (CD11b, Serotec), 1:500, 20% NPS, and 0.03% merthiolate in 0.01 M PBS. Then the sections were incubated in a solution of STA-PER (Jackson), 1% NPS, and 0.03% merthiolate in TBS. The color reaction was developed with nickel-diaminobenzidine and H_2O_2. Finally, the sections were mounted on gelatin-coated microscopic slides, air-dried, and coverslipped with dibutyl phthalate in xylene. In the dorsal hippocampus, the surface areas of OX-42-immunoreactive microglia were quantified by using a computerized image analysis system (Olympus BX50 microscope, DP50 digital camera, ImagePro Plus software, Media Cybernetics, U.S.A. The analysis was performed on three consecutive coronal sections at bregma-3.60 mm (Paxinos and Watson, 1986). The stratum radiatum and stratum oriens of the hippocampal CA1 region were delineated manually at 10x magnification, after background subtraction and gray scale threshold determination. The area covered by immunoreactive microglia was computed as a percentage of the total area delineated. The measurements were performed bilaterally on the 3 brain sections, the 6 values per animal per area were averaged, and this average was used for the further statistical analysis. Multivariate ANOVA was performed for surgery (SHAM vs. 2VO), the time of treatment (pre-treatment vs. post-treatment) and the type of treatment (vehicle vs. α-tocopherol).

3.3. Study 3. Effect of hydrogen and RSG treatment in TGI

In the TGI model, we investigated the changes of pro- and antioxidant enzyme levels in the hippocampus and the cortex 3 days after surgery, and tested the potential protective effects of hydrogen in comparison with RSG.

3.3.1. Hydrogen inhalation and RSG treatment

The TGI and the SHAM groups were further divided into four subgroups based on the various pharmacological treatments, and a non-operated (naive) group was added. Table 1c. demonstrates the number of animals in the 9 experimental groups. The first set of animals received no treatment. In the second group of rats, the gas mixture used for anesthesia was changed at the beginning of reperfusion and used for 30 minutes: 1.5-2% halothane was evaporated in 2.1% hydrogen gas containing room air (H_2-RA). The third set of animals received the vehicle: dimethyl sulphoxide (DMSO) in equal volume (~60 μl) to the drug solution applied to the last group, which was treated with 6 mg/kg RSG dissolved in DMSO (30 mg/ml). The RSG/DMSO solutions were diluted in 0.5 ml venous blood and administered i.v. (v = 6 ml/min) 15 minutes before TGI. COX-2, eNOS, nNOS, and MnSOD levels were determined using Western blotting. Two-way ANOVA was performed for surgery (naive vs. SHAM vs. TGI) and for treatment (non-treated vs. H_2-RA vs. vehicle vs. RSG), followed by the LSD post-hoc test.

3.4. Study 4. The role of CD8+ T lymphocytes in focal ischemia

We investigated how the depletion of the CD8+ T lymphocytes - one type of the infiltrating leukocytes - affected neurological functions, brain infarct size and iNOS expression indicating oxidative stress after MCAO-induced focal ischemia in mice.

3.4.1. Depletion of CD8+ T lymphocytes

CD8+ T lymphocytes were depleted 24 h prior MCAO by i.p. injection of rat anti-mouse CD8 (300 μg, Ly-2, clone 53-6.7, BioXCell) antibody (aCD8) diluted in sterile PBS. Control animals received isotype contorol antibody (IgG2). Experimental groups are shown in Table 1d.

3.4.2. Sensorimotor dysfunction: Corner-test

Corner test was performed 1 day before, 1, 3 and 7 days after ischemia induction to describe the sensorimotor dysfunctions. The corner is formed by two boards each with a dimension of 30 x 20 x 1 cm which are attached at a 30° angle with a small opening along the

joint to encourage the mouse entering the corner. The mouse is placed between the two boards facing the corner. The mouse enters deep into the corner then rears forward and upward due to the vibrissal stimulation, then turns back to face the open end. Twelve turns were scored for each test, the percentage ratio of right turns of all turns was calculated and normalized on the performance prior surgery of each individual mice. Turning movements that were not part of a rearing movement were not scored. Non-parametric T test was performed for treatment (IgG2 vs. aCD8) at each timepoint.

3.4.3. Infarct volume assessment

The infarct volume was determined on cryosections stained with the high-contrast silver staining technique. In brief, mice were deeply anesthetized with 250 mg/kg tribromoethanol i.p. and perfused transcardially with cold saline 1, 3 and 7 days following MCAO. Brains were removed, immediately frozen and 20 µm thick coronal cryosections were cut every 400 µm. The slides were submerged for 2 minutes into a silver impregnation solution (see below), which was shaken vigorously. Then the slides were washed in distilled water 6 times for 1 minute before they were transferred to a developer solution for 3 minutes (see below). After the slides had been washed in distilled water 3 times for 1 minute, they were air dried, scanned at 600 dpi, and the infarct areas measured using a public domain image analysis program (Scion Image). The total infarct volume was obtained by integrating measured areas and distance between sections. Correction for brain edema was applied by subtraction of the ipsilateral minus contralateral hemisphere volume from the directly measured infarct volume. One-way ANOVA was performed for treatment (IgG2 vs. aCD8), followed by the LSD post hoc test.

Impregnation solution: 45 ml saturated lithium carbonate solution was added to 23 ml 10% silver nitrate solution. The formed precipitate was dissolved during continuous stirring by addition of a ~3 ml 25% ammonium hydroxid, finally the solution was diluted 6x with distilled water. Developer solution: For 420 ml solution, 280 ml distilled water, 80 ml 37% formaldehyde solution and 60 ml acetone was mixed, then 1.2 g hydroquinone and 4.4 g trisodium citrate dihydrate was dissolved. Thereafter, this solution was exposed to room air until it became copper colored (30 to 60 minutes). All solutions were prepared daily.

3.4.4. Leukocyte infiltration

Immunohistochemistry was performed 1, 3 and 7 days following MCAO on coronal cryostat sections (12 µm) to determine the invasion of T lymphocytes and granulocytes into the infarcted hemisphere.

After pre-treatment with 4% paraformaldehyde for 60 min, endogenous peroxidase was blocked by Peroxidase Blocking solution (DAKO, Glostrup, Denmark). Sections were then incubated with primary antibodies against CD3 (Clone 3H698, Zytomed, Berlin, Germany) or myeloperoxidase (MPO, Clone RP-053; Zytomed, Berlin, Germany) for 60 min at 21 °C. Immunoreactivity was visualized by a universal immunoenzyme polymer method (Nichirei Biosciences, Tokyo, Japan) and sections were developed in diaminobenzidine (Lab Vision Cooperation, Fermont, CA, USA). Positive cells were counted in the ischemic hemisphere of the two treatment groups. One way ANOVA was performed for treatment (IgG2 vs. aCD8) followed by the LSD post hoc test.

3.4.5. Real-time polymerase chain reaction (RT-PCR)

We isolated RNA from separated cerebral hemispheres with RNApure (Peqlab) 1 and 5 days following MCAO. We performed reverse transcription with the High Capacity cDNA Archive Kit (Applied Biosystems) and RT-PCR with SYBR-Green assays (Applied Biosystems) on an ABI7500 RT-PCR System (Applied Biosystems). The primers were purchased as ready-to-use primer sets (Super Array). All assays were performed in duplicates. The results were normalized to the level of the housekeeping gene encoding peptidylprolyl isomerase A (cyclophilin). The expression of cyclophilin was not influenced either by MCAO or the anti-CD8 treatment. Two-way ANOVA was performed for hemispheres (ischemic vs. non-ischemic) and treatment (IgG2 vs. aCD8) at each timepoint, followed by LSD post hoc test.

Results

1. Study 1: Changes of pro- and antioxidant enzyme levels in the 2VO model

The cerebral COX-2 expression underwent dynamic changes in the 1-year period after 2VO. In the hippocampus, the COX-2 levels progressively increased from 3 days to 1 week after ischemia onset, reaching significant elevation (150±17% of the naive control level) by 1 week after 2VO (Fig. 2A, 2B). In the cortex, similar changes were not observed (data not shown). Two weeks and 3 months after surgery, 2VO animals exhibited a lower amount of COX-2 than did the SHAM rats in both cerebral regions, with a significant 43±5% reduction in the cortex at 3 months. Six and 12 months following the operation, we did not observe any difference between the groups in either brain regions.

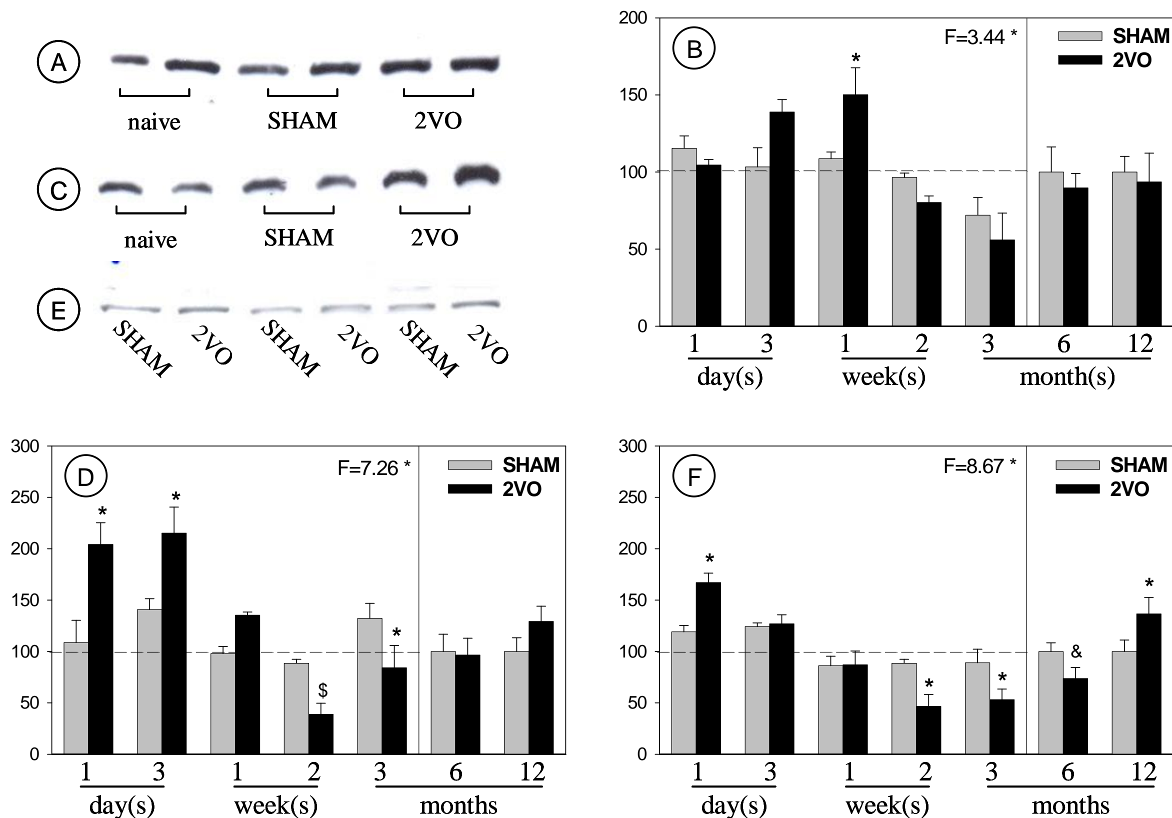


Fig. 2. Panel A: Representative blot of COX-2 from the hippocampus 3 days after the operation. Panel B: Protein levels of COX-2 in the hippocampus. Panel C: Representative blot of eNOS from the hippocampus 3 days after the operation. Panel D: Protein levels of eNOS in the hippocampus. Panel E: Representative blot of eNOS from the cortex 12 months after the operation. Panel E: Protein levels of eNOS in the cortex.
Optical densities of bands are expressed as percentages of the naive control levels (1 day – 3 months groups), or as percentages of the data for the SHAM animals (6 and 12 months groups). Data are expressed as means ± S.E.M. One-way ANOVA was performed for surgery (SHAM vs. 2VO), followed by LSD post-hoc test. *: $p<0.05$, $: $p=0.052$, &: $p=0.058$. Abbreviations: 2VO: 2 vessel occlusion, COX-2: cyclooxygenase-2, eNOS: endothelial nitric oxide synthase, SHAM: sham-operation.

The eNOS levels increased in both the hippocampus and the cortex in the very early stage of hypoperfusion (Fig. 1C-F). In the hippocampus, the eNOS expression was significantly higher in the 2VO group as compared with the SHAM animals 1 day (204±21% vs. 108±22%, 2VO vs. SHAM, respectively) and 3 days (215±25% vs. 141±11%, 2VO vs. SHAM, respectively) after the surgery, but the response mostly receded by the end of the first week of survival. In the cortex, the eNOS expression increased significantly to 167±9% 1 day following surgery in the 2VO group, whereas 3 days and 1 week after the operation there was no difference between the 2VO and SHAM animals. Hypoperfusion after 2 weeks or 3 months caused significant reductions in the eNOS levels in both the hippocampus and the cortex, and in both areas they returned to baseline levels by 6 months after 2VO. Interestingly, in the 2VO animals eNOS levels tended to increase at 12 months, the 36±16% increase in the cortex was even statistically significant.

In the hippocampus, 3-day and 1-week hypoperfusion caused moderate reductions in the nNOS enzyme levels (Table 2). In the cortex, the 3-day hypoperfusion significantly increased the amount of nNOS compared to the SHAM animals (168±13% vs. 121±8%, 2VO vs. SHAM, respectively). In the later phase (2 weeks – 12 months), there was no difference between the 2VO and control groups either in the hippocampus or in the cortex.

Table 2. Quantitative analysis of nNOS enzyme level

Duration of ischemia	Hippocampus		Cortex	
	SHAM	2VO	SHAM	2VO
1 day	106 ± 2	100 ± 6	140 ± 17	150 ± 17
3 days	114 ± 13	76 ± 2	121 ± 8	168 ± 13 *
1 week	75 ± 8	68 ± 22	86 ± 5	78 ± 13
2 weeks	92 ± 8	95 ± 7	86 ± 5	78 ± 7
3 months	120 ± 22	112 ± 28	97 ± 7	97 ± 4
6 months	100 ± 13	85 ± 11	100 ± 7	100 ± 9
12 months	100 ± 7	139 ± 15	100 ± 5	103 ± 6

Optical densities of bands are expressed as percentages of naive control levels (1 day – 3 months groups), or as the percentages of the data for the SHAM animals (6 and 12 months groups). Data are expressed as mean ± S.E.M. One-way ANOVA was performed for surgery (SHAM vs. 2VO) followed by the LSD post-hoc test. * $p < 0.05$. Abbreviations: 2VO: 2 vessel occlusion, nNOS: neuronal nitric oxide synthase, SHAM: sham-operation.

In both the hippocampus (Fig. 3) and the cortex (data not shown), the expression of iNOS was reduced in the 2VO animals 2 weeks after the operation.

Chronic hypoperfusion did not significantly affect MnSOD expression levels in either region investigated (data not shown).

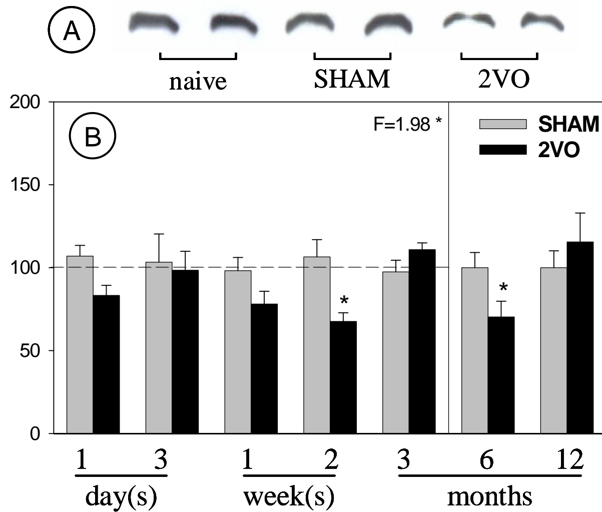


Fig. 3. Panel A: Representative blot of iNOS from the hippocampus 2 weeks after the operation. Panel B: Protein levels of iNOS in the hippocampus. Optical densities of bands are expressed as percentages of the naive control levels (1 day – 3 months groups), or as percentages of the data for the SHAM animals (6 and 12 months groups). Data are expressed as means ± S.E.M. One-way ANOVA was performed for surgery (SHAM vs. 2VO), followed by LSD post-hoc test. * p<0.05. Abbreviations: 2VO: 2 vessel occlusion, iNOS: inducible nitric oxide synthase, SHAM: sham-operation.

2. Study 2: Effects of α-tocopherol treatment in the 2VO model

2.1. Spatial learning

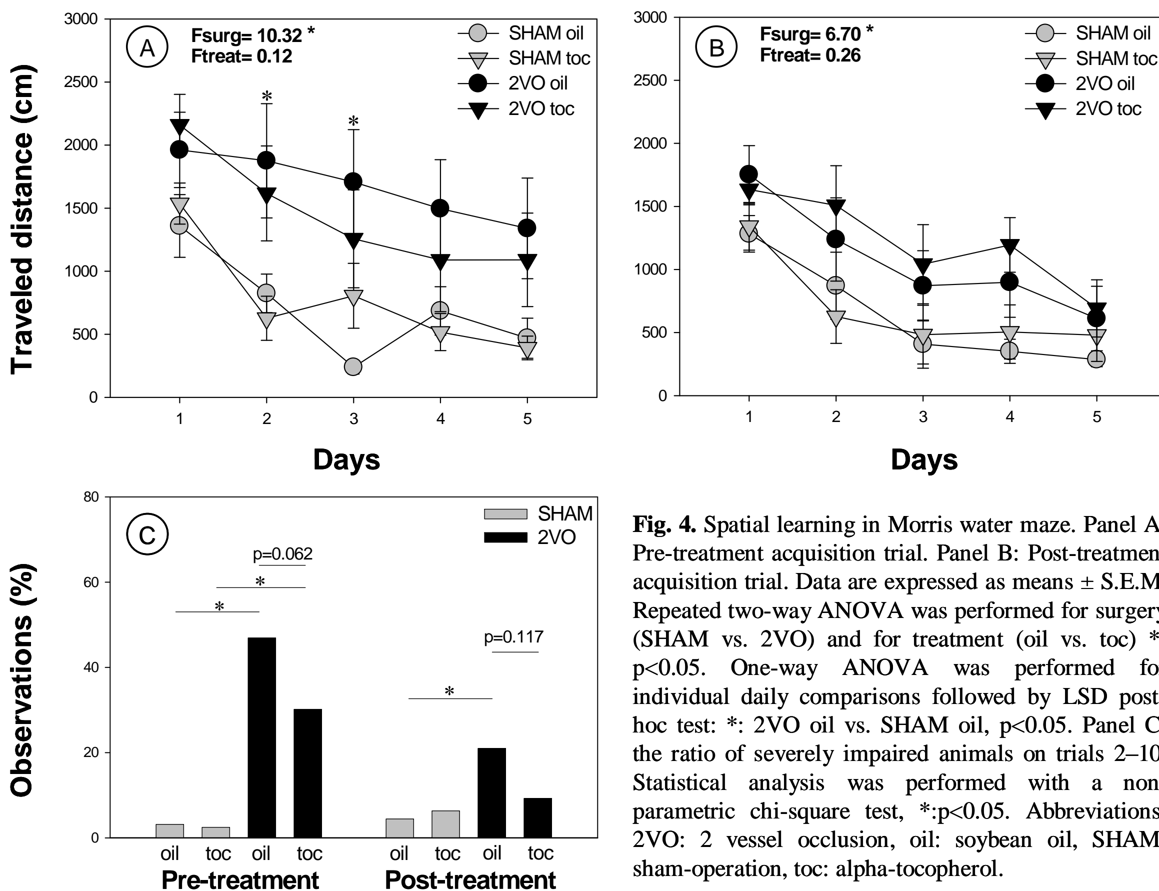


Fig. 4. Spatial learning in Morris water maze. Panel A: Pre-treatment acquisition trial. Panel B: Post-treatment acquisition trial. Data are expressed as means ± S.E.M. Repeated two-way ANOVA was performed for surgery (SHAM vs. 2VO) and for treatment (oil vs. toc) *: p<0.05. One-way ANOVA was performed for individual daily comparisons followed by LSD post-hoc test: *: 2VO oil vs. SHAM oil, p<0.05. Panel C: the ratio of severely impaired animals on trials 2–10. Statistical analysis was performed with a non-parametric chi-square test, *:p<0.05. Abbreviations: 2VO: 2 vessel occlusion, oil: soybean oil, SHAM: sham-operation, toc: alpha-tocopherol.

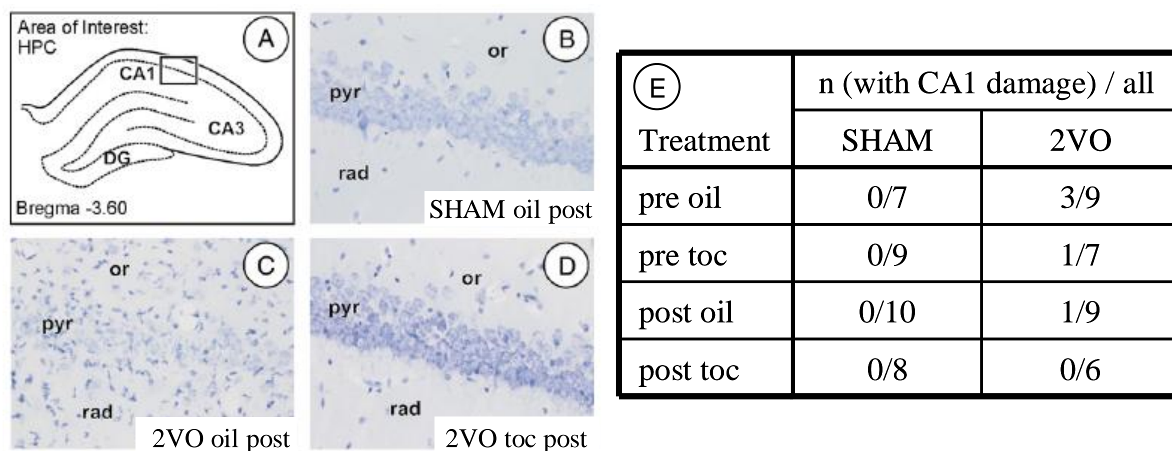
SHAM groups performed equally in the Morris maze spatial orientation test (Fig. 4A, 4B). The distance traveled by 2VO groups was significantly longer than that for the SHAM groups, particularly in the pretreated groups, where soybean oil treated 2VO animals displayed significant learning impairment on days 2 and 3 compared to the SHAM groups. At

the same time, performance of the α-tocopherol treated 2VO animals did not differ significantly from the SHAM groups. Among the post-treated groups, there were smaller differences found.

Analysis of the percentage of animals that failed to find the hidden platform provided bigger differences between the groups. Both after pre- and post-treatment, significantly higher proportion of 2VO oil rats showed severe impairment (47–21%) in comparison with SHAM groups (3–6%). The α-tocopherol treatment decreased the ratio of 2VO rats that failed to find the platform from 47% to 30% after pre-treatment, and from 21% to 9% after post-treatment (Fig. 4C).

2.2. Neuronal loss

In some 2VO rats, unilateral damage of the CA1 pyramidal cell layer was seen on cresyl violet stained hippocampal sections (Fig. 5C), whereas this area was intact in all SHAM animals (Fig. 5B). In the oil-treated 2VO groups, 4 of 18 animals showed unilateral CA1 lesions, while only 1 of the 13 α-tocopherol-treated 2VO rats revealed similar pathology. CA1 lesions were not detected in any of the α-tocopherol post-treated animals (Fig. 5E).



Treatment	n (with CA1 damage) / all	
	SHAM	2VO
pre oil	0/7	3/9
pre toc	0/9	1/7
post oil	0/10	1/9
post toc	0/8	0/6

Fig. 5. Photographs demonstrating damage to the hippocampus CA1 pyramidal cell layer stained with cresyl violet. Panel E presents the number of animals that displayed CA1 damage in the various experimental groups. Abbreviations: 2VO: 2 vessel occlusion, DG: dentate gyrus, HPC: hippocampus, oil: soybean oil, or: stratum oriens, post: post-treatment, pre: pre-treatment, pyr: stratum pyramidale, rad: stratum radiatum, SHAM: sham-operation, toc: α-tocopherol.

2.3. Microglial activation

OX-42 immunocytochemistry and morphometry were performed to label the activation of microglial cells. The OX-42 signal was low in the SHAM groups (Fig. 6B). 2VO increased microglial activation in the soybean oil treated animals, both in pre-and post-treatment groups

(Fig. 6C, 6E). In contrast, both α-tocopherol pre- and post-treatment weakened the OX-42 signal in the 2VO rats showing reduced microglial activation (Fig. 6D, 6E).

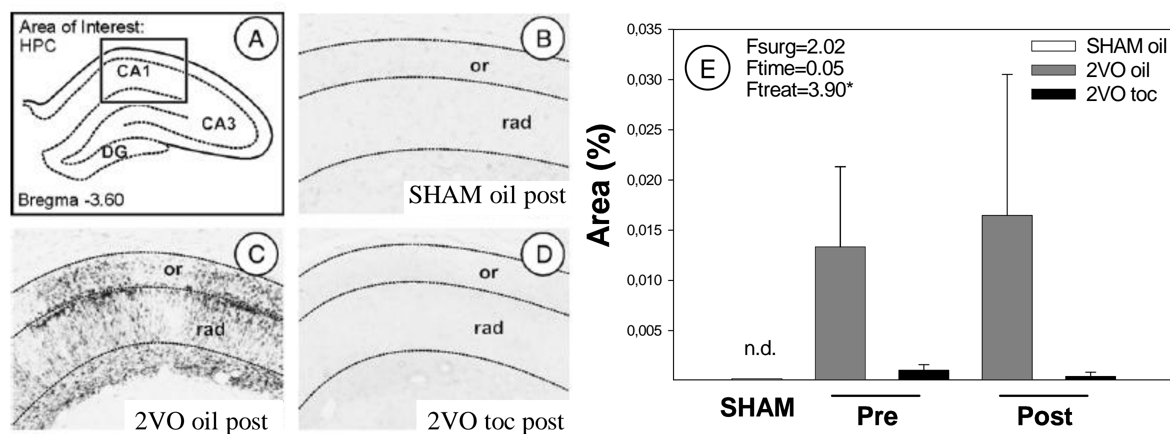


Fig. 6. Microglial activation in the hippocampus CA1 region labeled immunocytochemistry for OX-42. Data are expressed as means ± S.E.M. Multivariate ANOVA was performed for surgery (SHAM vs. 2VO), the time of treatment (pre vs. post) and the type of treatment (oil vs. toc), *: p<0.05. Abbreviations: 2VO: 2 vessel occlusion, DG: dentate gyrus, HPC: hippocampus, n.d.: not detectable, oil: soybean oil, or: stratum oriens, post: post-treatment, pre: pre-treatment, rad: stratum radiatum, SHAM: sham-operation, toc: α-tocopherol.

3. Study 3: Effect of hydrogen and RSG treatment in TGI

The physiological parameters (MABP, pH, partial pressure of CO_2 and O_2) were within the normal range and were not statistically different among the experimental groups (data not shown).

In the hippocampus, TGI caused a significant increase of COX-2 levels in the non-treated and the vehicle-treated groups as compared with their respective SHAM groups. Both H_2-RA inhalation and RSG treatment prevented the TGI-induced upregulation of COX-2. Moreover, RSG also decreased the basal expression of this enzyme (Fig. 7A, 7B). In the cortex, TGI did not influence COX-2 expression, while RSG applied in combination with TGI induced COX-2 downregulation as compared with the non-treated TGI group (Table 3).

In the hippocampus, TGI caused significant reduction in nNOS levels in the non-treated group compared to SHAM animals (56.0 ± 17.5% vs. 91.5 ± 12.5%, TGI vs. SHAM). Both H_2-RA inhalation and RSG treatment prevented the downregulation of nNOS, while there was no significant difference between the non-treated and vehicle-treated TGI groups (Fig. 7C, 7D). TGI had no effect on nNOS expression in the cortex (Table 3).

The expression of eNOS and MnSOD were not affected by TGI either in the hippocampus (data not shown) or the cortex. (Table 3). All applied treatments including the vehicle decreased MnSOD expression in the hippocampus, while in the cortex only RSG influenced its level (Fig. 7E and Table 3).

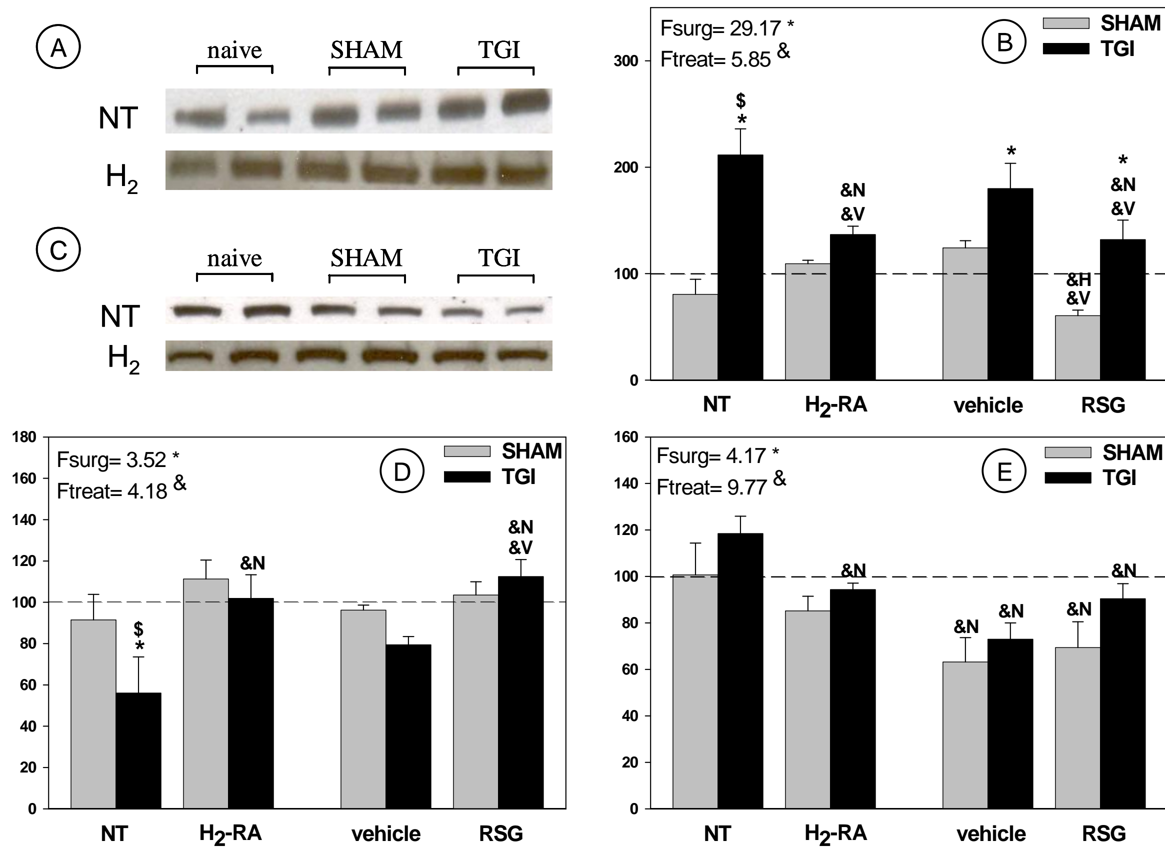


Fig. 7. Panel A, C: Representative blot of COX-2 (A) and nNOS (C) from the hippocampus of non-treated and H_2-RA rats. Panel B, D, E: Protein levels of COX-2 (B), nNOS (D) and MnSOD (E) in the hippocampus. Data are expressed as mean ± S.E.M. Two-way ANOVA was performed for surgery (naive vs. SHAM vs. TGI) and for treatment (NT vs. H_2-RA vs. vehicle vs. RSG), followed by the LSD post-hoc test. $, *, &: $p < 0.05$; $: vs. naive; *: vs. SHAM; &N: vs. corresponding NT group (SHAM vs. SHAM or TGI vs. TGI); &H: vs. corresponding H_2-RA group; &V: vs. corresponding vehicle group. Abbreviations: COX-2: cyclooxygenase-2, H_2-RA: 2.1% hydrogen gas containing room air, MnSOD: manganese superoxide dismutase, nNOS: neuronal nitric oxide synthase, NT: non-treated, RSG: rosiglitazone, SHAM: sham-operated, TGI: transient global ischemia.

Table 3. Quantitative analysis of enzyme expressions in the frontal cortex

Treatm.	Surgery	COX-2	nNOS	eNOS	MnSOD
NT	SHAM	113 ± 7	91 ± 7	87 ± 13	106 ± 11
	TGI	105 ± 13	84 ± 11	104 ± 9	100 ± 6
H_2-RA	SHAM	104 ± 13	110 ± 6	102 ± 6	97 ± 7
	TGI	117 ± 19	104 ± 18	99 ± 4	97 ± 6
vehicle	SHAM	195 ± 11	109 ± 9	114 ± 11	90 ± 3
	TGI	197 ± 22 *N,H,R	127 ± 6 *C	144 ± 5 *S,N,H	107 ± 6
RSG	SHAM	85 ± 14	95 ± 13	87 ± 14	78 ± 7 *N
	TGI	67 ± 9 *N,H	102 ± 8	117 ± 20	87 ± 5 *V

Optical densities of bands are expressed as percentages of the naive control levels. Values are expressed as means ± S.E.M. Two-way ANOVA was performed for surgery (naive vs. SHAM vs. TGI) and for treatment (NT vs. H_2-RA vs. vehicle vs. RSG) followed by LSD post-hoc test. S, N, H, V, R: $p < 0.05$; S: vs. corresponding SHAM; N: vs. corresponding NT group (SHAM vs. SHAM or TGI vs. TGI); H: vs. corresponding H_2-RA group; V: vs. corresponding vehicle group, R: vs. corresponding RSG group.

Abbreviations: COX-2: cyclooxygenase-2, eNOS: endothelial nitric oxide synthase, H_2-AR: 2.1% hydrogen supplemented room air, MnSOD: manganese superoxide dismutase, nNOS: neuronal nitric oxide synthase, NT: non-treated, RSG: rosiglitazone, SHAM: sham-operated, TGI: transient global ischemia.

4. Study 4: The role of CD8+ T lymphocytes in focal ischemia

4.1. Sensorimotor dysfunction

In both the control and CD8+ lymphocyte depleted groups, the ratio of right turns elevated compared to the pre-operative state until 3 days following MCAO (Fig. 8). Seven days after surgery, this deviation was still observed in the control group, while the sensorimotor function of the CD8+ lymphocyte depleted mice improved significantly compared to the IgG2 treated control animals.

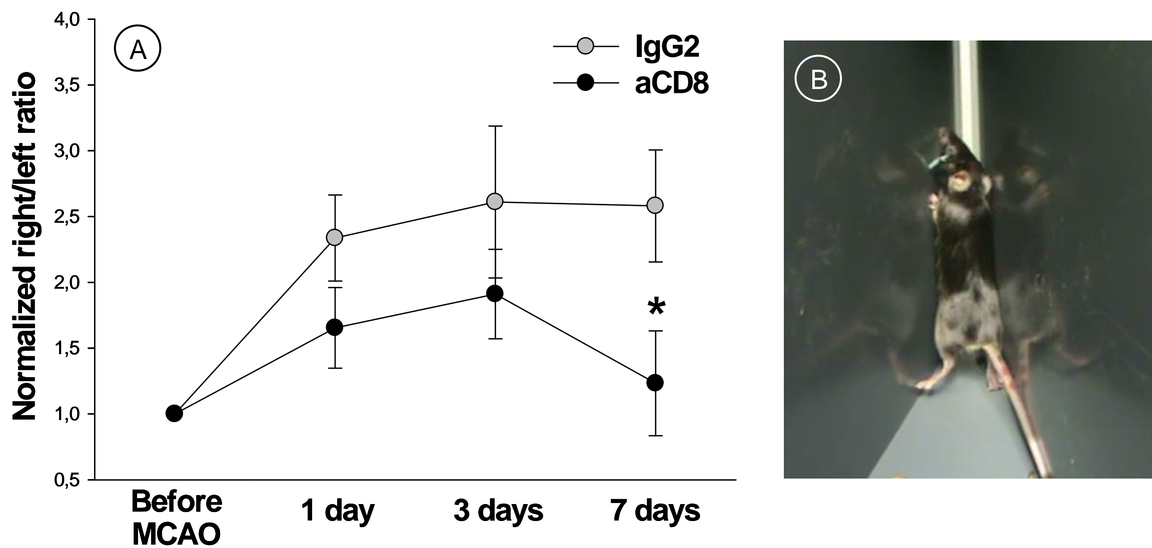


Fig. 8. Sensorimotor dysfunction in the corner test. Panel A: Ration of right/left turns normalized on the performance prior surgery of each individual mice. Values are expressed as means ± S.E.M. Non-parametric T test was performed for treatment (IgG2 vs aCD8) at each timepoint, * $p<0.05$. Panel B: Photograph of the corner that was used for the measurements. Abbreviations: aCD8: anti-CD8 depleting antibody, IgG2: isotype control antibody.

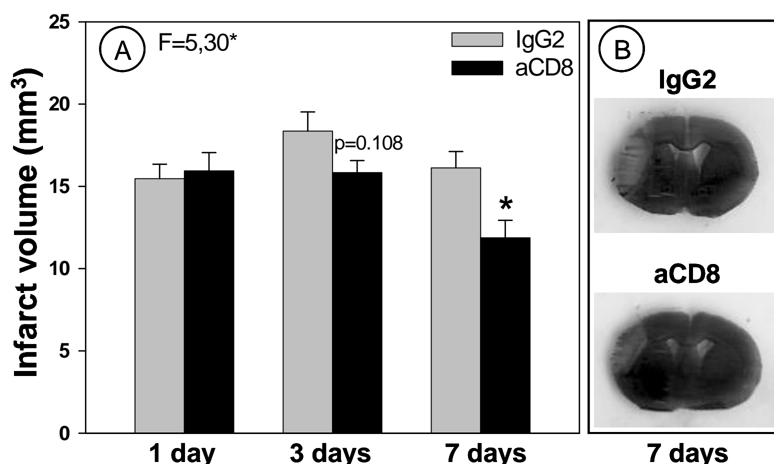


Fig. 9. Panel A: Infarct volume (mm3). Data are expressed as means ± S.E.M. One-way ANOVA was performed for treatment (IgG2 vs. aCD8), followed by LSD post hoc test. * $p<0.05$. Panel B: Representative silver stained cryosections from IgG2 and aCD8 groups 7 days following MCAO. Abbreviations: aCD8: anti-CD8 depleting antibody, IgG2: isotype control antibody.

4.2. Infarct volume

Infarct volumes did not differ between control and depleted groups 24 h after MCAO (Fig. 9). Three days following surgery, sections from aCD8 treated mice revealed slightly smaller infarcted volume compared to the IgG2 group, this difference became significant 7 days following surgery.

4.3. Leukocyte infiltration

Immunohistochemistry was performed to determine the invasion of T lymphocytes and granulocytes into the infarcted hemisphere. In both groups, only 2-3 CD3+ T cells were counted on the infarcted side per section 1 day after MCAO. The number of invading T lymphocytes in the infarcted hemisphere of the brain was more rapidly increasing in the IgG2 group resulting in significant difference between the control and CD8 depleted groups 3 and 7 days following MCAO. Invasion of MPO+ granulocytes appeared already on the first day of ischemia, and was maintained till the end of the first week. Depletion of CD8+ T cells did not influence the infiltration rate of granulocytes at any timepoint investigated.

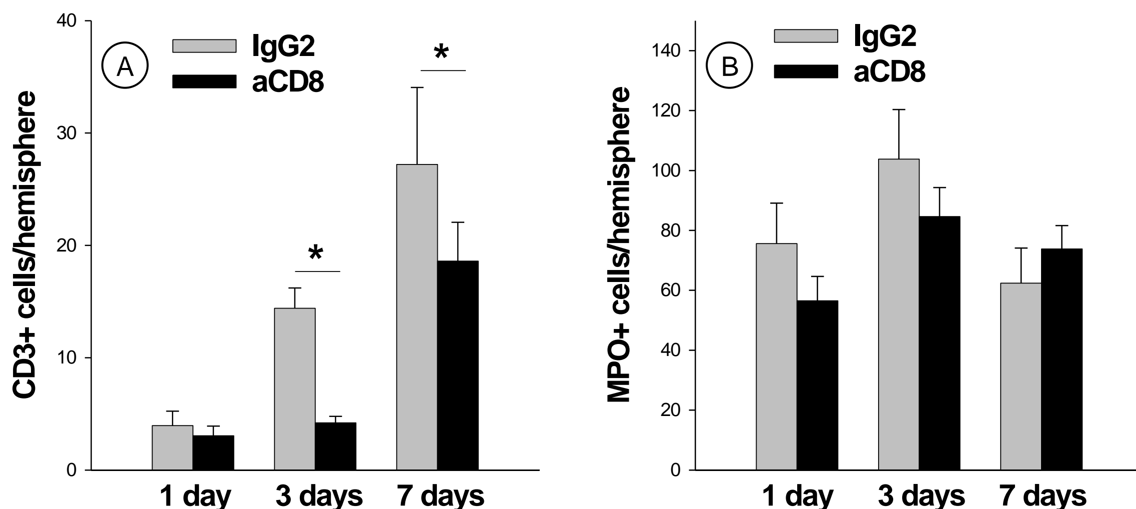


Fig. 10. Leukocyte inflitration of the ischemic brain. Panel A: Invading CD3+ T cell number per ischemic hemisphere of a 12μm section. Panel B: Invading MPO+ cell (granulocyte) number per ischemic hemisphere of a 12μm section. Data are expressed as means ± S.E.M. One way ANOVA was performed for treatment (IgG2 vs aCD8) followed by LSD posthoc test. * $p<0.05$. Abbreviations: aCD8: anti-CD8 depleting antibody, IgG2: isotype control antibody, MPO: myeloperoxidase.

4.4. Gene expression

RT-PCR was used to determine the focal ischemia-induced changes of mRNA expression of TNFα and iNOS in the ischemic and non-ischemic hemisphere. MCAO substantially upregulated TNFα and iNOS in the ischemic hemisphere of control mice. One

day after surgery, only TNFα showed ischemia-induced upregulation which was not influenced by CD8+ T cell depletion. Five days following MCAO, the expression of TNFα mRNA was further increased in control mice, while depletion of CD8+ T cells prevented the further increase of the mRNA of this cytokine. Similarly, iNOS expression was also induced in the control group 5 days following MCAO, while in the CD8 depleted group, iNOS mRNA level did not differ significantly between the ischemic and non-ischemic hemisphere.

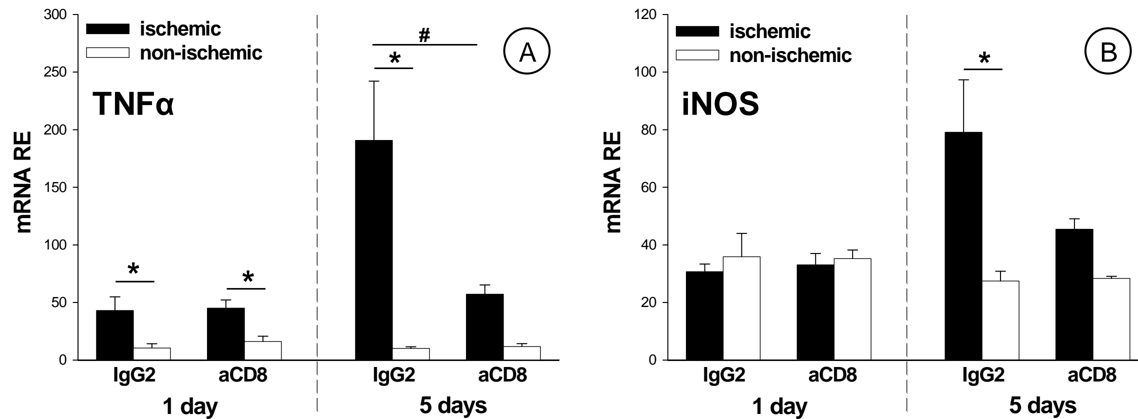


Fig. 8. Cerebral expression of TNFα (Panel A) and iNOS (Panel B) mRNA. Data are expressed as means ± S.E.M. Two-way ANOVA was performed for hemispheres (ischemic vs. non-ischemic) and treatment (IgG2 vs. aCD8) at each timepoint, followed by LSD post hoc test. *, #: $p<0.05$. Abbreviations: aCD8: anti-CD8 depleting antibody, IgG2: isotype antibody control, iNOS: inducible nitric oxide synthase, mRNA RE: messenger RNA relative expression (normalized on cyclophilin), TNFα: tumor necrosis factor α.

Discussion

1. Changes of pro- and antioxidant enzyme levels in global ischemia

We described the changes of some of the pro- and antioxidant enzyme levels in the hippocampus and in the cortex in 2 different ischemia models: in chronic mild forebrain ischemia induced by 2VO and in transient severe forebrain ischemia using the TGI model. Based on the prolonged changes of CBF seen in chronic ischemia, we characterized these enzyme level changes at different time points (between 1 day and 12 months) following 2VO. In the TGI model, we investigated the pro- and antioxidant enzyme levels 3 days after surgery, as most of the pyramidal neurons in the hippocampal CA1 region were shown to undergo delayed cell death by this timepoint[147].

This study revealed the following findings: in chronic mild ischemia induced by 2VO, enzyme level changes occurred mainly in the early phase of hypoperfusion, while eNOS upregulation seen in the later phase can contribute to restore local CBF. In the acute phase of both investigated ischemia models, we demonstrated similar enzyme level changes affecting mainly the hippocampus, however, these alterations were more moderate and developed with a delayed dynamics following 2VO compared to TGI.

1.1. COX-2 enzyme levels in global ischemia

COX-2 levels changed similarly in the acute phase following 2VO and TGI: in both models they increased in the hippocampus, while they were unaffected in the cortex. However, the temporal pattern of COX-2 induction differed in these models. COX-2 increase started at 3 days following 2VO and reached a significant elevation only at 1 week. In TGI, however, COX-2 increase was already significant at 3 days survival. Furthermore, this elevation was higher than the maximal upregulation seen in 2VO animals (211.5±24.5% vs. 150±17%). Taken together, COX-2 upregulation is more moderate and develops with a delayed dynamics following 2VO compared to the TGI model. Our findings are in accordance with previous studies also showing COX-2 induction in different models of cerebral ischemia[8,25,117,122]. In TGI models, COX-2 levels in the CA1 hippocampal layer were found to peak at 1 day following ischemia, and – in line with our findings – were still observed at 3 days[25,117]. Glutamate excitotoxicity induced by ischemia can explain COX-2 upregulation, since the acute induction of COX-2 mRNA and protein levels was reduced by an NMDA receptor antagonist[108]. Conversely, ischemia-induced COX-2 production was further triggered by glutamate-induced activation of NMDA-receptors[66].

Both in focal ischemia models and in TGI, specific COX-2 inhibitors reduced the degree of ischemic brain damage[28,34,115,117,122]. Whether COX-2 inhibition is also beneficial in 2VO appears to be more contradictory, as NS-398 (a selective COX-2 inhibitor) although improved the spatial learning ability, but also simultaneously increased the extent of neuronal damage and the proliferation of astrocytes in the hippocampus 2 weeks after 2VO[73]. Additionally, cilostazol exerted a brain-protective effect through COX-2 upregulation in the 2VO rat model[161]. Our findings regarding the different temporal pattern of COX-2 induction following 2VO and TGI may explain why COX-2 inhibitors achieve different effects in these ischemia models.

In our study, two weeks and 3 months after 2VO, COX-2 expression was decreased in the hippocampus and in the cortex. We suggest that the decreased COX-2 expression is an aspecific response to the hypoperfusion because similar decreases were observed in the expressions of other enzymes investigated in our model. The reductions in the enzyme levels can be ascribed to failure of the substrate and energy supply required for enzyme synthesis, or the neuronal loss caused by ischemia. The latter assumption is supported by previous findings that 2 weeks after 2VO, neuronal loss was observed in the hippocampal CA1 region[41], and 3-month hypoperfusion resulted in a loss of about 50% of COX-2-positive neurons without any alteration in the COX-2 expression of the surviving cells[42]. The restoration of COX-2 expression 6 and 12 months after 2VO onset points to compensatory mechanisms of the brain.

1.2. eNOS enzyme levels in global ischemia

At 1 and 3 days after 2VO, eNOS levels were increased in both the hippocampus and the cortex. In contrast, there was no change in eNOS level 3 days following TGI, however our findings cannot exclude the presence of eNOS upregulation at an earlier timepoint. Several studies reported the upregulation of eNOS in different models of cerebral ischemia[92,112,158], and variations in the temporal expression between different ischemia models were also described[92,112]. Elevated eNOS levels could be due to several factors, including changing shear stress in the microvasculature[51]. NO generated by eNOS may counteract the diminished blood supply by inducing vasodilation[71], and inhibiting platelet and neutrophil adhesion to the endothelium[136]. Accordingly, MCAO elicited larger infarcts in eNOS knockout mice as compared with wild-type animals[63].

In the present study, 2 weeks and 3 months after 2VO eNOS enzyme levels were decreased, but were restored at 6 months. De la Torre et al.[33] likewise observed a marked depletion of eNOS in the hippocampal endothelial cells 6 months after 2VO induction. They

attributed the reduction of eNOS levels to the disturbed CBF. At the same time, perivascular cells such as pericytes, glial cells and adjacent neurons displayed an eNOS overexpression, which may serve as a compensatory reaction to the eNOS depletion within the endothelial cells[33]. This latter finding can explain our observation that overall hippocampal eNOS levels returned to the baseline at 6 months after 2VO induction. In fact, eNOS was upregulated 12 months after 2VO in both the hippocampus and the cortex, perhaps reflecting an increased capillary density shown by Hai et al.[58] and/or the upregulation of the enzyme in the perivascular cells, so as to compensate cerebral hypoperfusion.

1.3. nNOS enzyme levels in global ischemia

Three days after 2VO, we found an elevated nNOS expression in the cortex, in contrast to the hippocampus, where nNOS levels were slightly decreased. In the TGI model, nNOS expression was significantly reduced in the hippocampus 3 days following surgery, but in this model no change was detected in the cortex. Zhang et al.[175] showed that the number of nNOS containing neurons was significantly increased in the ipsilateral cortex up to 48 h following MCAO. At the same time, nNOS level in the ipsilateral hippocampus began to decrease already at 6 h, and remained depressed for at least 72 h after the insult. nNOS levels are affected by opposing mechanisms following ischemic stress: 1) nNOS appears to be upregulated in ischemic zones shortly after the insult; 2) but >24 h after ischemia, nNOS-containing neurons are being lost decreasing the tissue nNOS levels[68]. Therefore, reduced hippocampal nNOS protein levels detected in our experiments likely indicate the loss of nNOS immunopositive neurons. The more pronounced decrease of nNOS level in TGI may reflect the more severe neuronal loss compared to the 2VO model. This suggestion is supported by previous studies where no conspicuous loss of neurons was found during the first week after 2VO induction[126] whereas 10 min TGI caused severe pyramidal cell death in the CA1 region at 3 days following ischemia[147].

1.4. MnSOD enzyme levels in global ischemia

MnSOD levels were affected by neither 2VO nor TGI. The effect of cerebral ischemia on MnSOD levels is controversial. On one hand, several studies described the upregulation of MnSOD in transient ischemia models[120], while others reported reduced MnSOD level caused by reperfusion[78]. Similar to the equivocal findings on MnSOD protein levels, the importance of MnSOD activity in cerebral ischemia is also debated. One group of investigators reported that the overexpression of MnSOD or administered SOD mimetics reduced ischemic brain injury[81,141], whereas others found that MnSOD overexpression failed to reduce the level of

ischemic brain damage and to improve the neurological outcome[47]. Taken together these findings and our results, most likely MnSOD does not play a major role in the regulation of oxidative processes during global ischemia.

1.5. Conclusion of pro- and antioxidant enzyme level changes

The expression of enzymes involved in oxidative processes is dynamically altered following 2VO. The changes within the first week of hypoperfusion coincide with the acute ischemic phase of the 2VO model, during which the CBF drops suddenly and remains low. In the oligemic phase of hypoperfusion between 2 weeks and 3 months after 2VO onset, enzyme levels are reduced, which is probably caused by the ongoing cellular loss and the diminished nutrition of the surviving cells leading to decreased protein synthesis Later, as CBF is being gradually restored, enzyme expression returns to the baseline ~6 months after 2VO induction. The upregulation of eNOS 12 months following 2VO onset can be associated with the microvascular compensation to restore local CBF.

In the acute phase of both forebrain ischemia models, changes in the investigated enzyme levels are more profound in the hippocampus, which is the most vulnerable region of the brain to oxidative damage and glutamate excitotoxicity[137]. Specifically, we demonstrated COX-2 upregulation and decreased nNOS expression indicating excitotoxicity and neuronal loss in the hippocampus, respectively. However, these alterations are more moderate and develop with a delayed dynamics following 2VO compared to TGI. These findings are in accordance with the larger decrease in CBF during the ischemic phase and the consequent more severe neuronal damage in the latter experimental model.

2. Effect of antioxidant treatments in global ischemia

We examined the potential protective effects of the antioxidant α-tocopherol 2 weeks following 2VO, as neuronal damage develops by 2 weeks after 2VO[126], and this timepoint simulates well the neuronal damage seen in human chronic cerebral hypoperfusion[44]. We also described the effect of H_2-RA inhalation 3 days following TGI in comparison with the RSG treatment that has well characterized neuroprotection in this model and was used as a reference drug. The 3-day survival period was chosen according to the findings of Sugawara et al.[147], who found severe hippocampal pyramidal cell loss 3 days after TGI.

2.1. α-Tocopherol

The most important novel finding of our study is that α-tocopherol is neuroprotective against 2VO-induced neuronal injury even when administered as a post-treatment.

The present study confirms the previously described, 2VO-induced spatial learning impairment assessed in the Morris water maze 2 weeks after the onset of 2VO in rats[41,44]. As a novel finding, α-tocopherol treatment attenuated the learning impairment. Indeed, α-tocopherol similarly ameliorated beta-amyloid-induced learning dysfunction of rats and mice in the water maze paradigm[76,167]. This beneficial effect was probably due to the antioxidant capacity of α-tocopherol as evidenced by reduced lipid peroxidation and protein oxidation in the beta-amyloid study[76]. Since oxidative stress probably plays a role in 2VO-induced neurodegenerative processes[44], antioxidant actions of α-tocopherol are likely responsible for the attenuated learning dysfunction in this model as well.

In addition to attenuating spatial learning deficit, α-tocopherol preserves also neuronal integrity, as we showed that α-tocopherol prevented the loss of cresyl violet-stained pyramidal cells in the hippocampal CA1 region. Our results correspond well with the previously reported finding, in which α-tocopherol decreased infarct size in a mouse MCAO model[109].

α-tocopherol also prevents 2VO-induced marked microglial activation. The action of α-tocopherol on ischemia-induced microglial activation has so far not been clarified, but α-tocopherol attenuated the production of cytokines such as TNFα, interleukin-1beta and interleukin-6 in cultured microglia[39,53,94]. Furthermore, when microglia stimulated with lipopolysaccharide and treated with α-tocopherol were co-cultured with neurons, α-tocopherol prevented the neuronal damage caused by the activated microglia[94]. These findings indicate that the suppression of microglial reactions contributes to the neuroprotective effects of α-tocopherol.

In summary, α-tocopherol improves spatial learning, prevents neuronal cell loss and attenuates microglial activation in a rat model of chronic cerebral hypoperfusion. Considering the well-known antioxidant capacity of α-tocopherol, we suggest that α-tocopherol achieves its neuroprotective effects by combatting cerebral hypoperfusion-induced oxidative stress and microglial activation.

2.2. Inhalation of H_2-RA and RSG treatment in TGI

The major finding of the present study is that both RSG treatment and H_2-RA inhalation prevent the TGI-induced enzyme level changes in the rat hippocampus, additionally, RSG decreases the basal COX-2 levels.

In our study, TGI increased COX-2 levels in the hippocampus indicating the presence of excitotoxicity. This COX-2 upregulation was prevented by both H_2-RA inhalation and RSG treatment. Moreover, RSG decreased the basal COX-2 production both in the hippocampus and the cortex. Previous studies showed that RSG treatment decreased COX-2 expression in neurons within the peri-infarct tissue, and this was associated with increased neuronal survival and smaller infarct areas[154]. However, long lasting downregulation of the basal COX-2 level may decrease the beneficial effects of prostanoids[106]. Accordingly, chronic treatment with COX-2 inhibitors is accompanied by higher risk of cardiovascular events[111], and type II diabetes studies revealed higher incidence of cardio- and cerebrovascular disorders in patients treated with RSG[121]. The action of hydrogen on COX-2 expression has so far not been investigated. In neuronal cell culture, hydrogen selectively reduced hydroxyl radical level[124] that was reported to induce COX-2 upregulation[45]. Scavenging hydroxyl radicals may provide a mechanism how hydrogen can prevent COX-2 induction in our study, as the well known hydroxyl radical scavenger mannitol also reduced COX-2 expression[82].

In our experiments, TGI induced reduction of nNOS level in the hippocampus, referring to neuronal loss. Both hydrogen and RSG prevent the TGI-induced nNOS downregulation indirectly indicating the neuroprotective effect of these compounds. The latter suggestion corresponds with the findings, where hydrogen ameliorated cellular injury caused by I/R both in vitro and in vivo[124], and RSG attenuated TGI-induced neuronal damage in the hippocampal CA1 region[24].

In our study, MnSOD was not affected by TGI, while all the applied treatments including the vehicle DMSO alone decreased the basal expression of MnSOD. MnSOD is an inducible antioxidant enzyme, the synthesis of which is upregulated by ROS[120]. Similarly to hydrogen and RSG, DMSO was also proven to have antioxidant properties[142]. Therefore, decreasing ROS offers a possible mechanism for these compounds to downregulate the intrinsic antioxidant MnSOD. Similar finding on influencing the endogen antioxidant system was reported, where the antioxidant α-tocopherol reduced MnSOD activity in beta-amyloid induced neuronal damage[76].

In the present study, we first described the effect of hydrogen therapy in a rat model of TGI. In conclusion, TGI-induced neuronal damage is characterized by COX-2 upregulation

indicating the presence of excitotoxicity and decreased nNOS expression referring to neuronal loss. Both H_2-RA inhalation and RSG treatment prevented the TGI-induced changes of the investigated enzyme levels. RSG has been previously proven to be neuroprotective in TGI[24,90]. Similar to RSG, hydrogen also decreases TGI-induced neuronal damage but without the downregulation of the basal COX-2 expression.

2.3. Conclusion of the applied antioxidant treatments

α-Tocopherol treatment prevents neuronal loss and attenuates learning deficit in the 2VO rat model of chronic cerebral hypoperfusion. Therefore, α-tocopherol may prevent the development of AD and normal aging-coupled memory dysfunctions, or post-stroke dementia.

H_2-RA inhalation and RSG treatment prevents the TGI-induced changes of the investigated enzyme levels. Although, RSG was proven to be neuroprotective in cerebral ischemia, its chronic application may be harmful due to the downregulation of the basal COX-2 expression. In contrast, the inhalation of H_2-RA does not influence the basal COX-2 levels and has no described harmful side effects. Therefore, after further investigations, hydrogen therapy should be considered in the acute management of cardiovascular emergencies and / or cardiac surgery.

3. The role of inflammation in neuronal damage and oxidative changes in focal ischemia

To examine the participation of leukocyte infiltration in ischemic brain injury and oxidative stress, we investigated the effect of CD8+ T cell depletion in a mouse MCAO model of focal cerebral ischemia. The main outcome measures were sensorimotor dysfunction, infarct size, and iNOS expression indicating oxidative damage.

The most important finding of our study is that depletion of CD8+ T lymphocytes reduces infarct size and improves the neurological function following MCAO, this effect is accompanied by decreased iNOS expression.

3.1. The role of CD8+ T cells in focal ischemia

Similarly to previously described findings[95,174], MCAO resulted in sensorimotor dysfunction assessed in the corner test that measures sensory and motor asymmetries associated with cortical or striatal dysfunction. Depletion of CD8+ T cells attenuated the ischemia induced sensorimotor dysfunction resulting in significant difference between control and depleted groups 7 days following MCAO. The results of corner test were coherent with

the infarct volume alterations. Three days following ischemia induction, infarct volume of CD8 depleted mice was slightly smaller compared to the control group; this difference became significant 7 days after MCAO. Similar effect of CD8 depleteion on the infarct size was shown by Liesz et al.[96] without the assessment of neurological performance or the mechanism of action.

In accordance with previous findings[145], in our study granulocyte recruitment was already observed 1 day following MCAO and persisted till the end of the first week of ischemia, while CD3+ T cell invasion was first increased 3 days following MCAO and it further elevated by day 7. The depletion of CD8+ T lymphocytes did not influence the invasion of granulocytes, while T cell recruitment was significantly attenuated in the CD8 depleted group. Most likely, the remaining T cell infiltration in the CD8 depleted group is due to the CD4+ T cells, that give ~70% of circulating T lymphocytes[91].

In the present study, focal ischemia increased the expression of TNFα mRNA 1 day, and more profoundly 5 days following MCAO. Previous studies also showed the upregulation of TNFα early after ischemia induction[5,31,95,96,118]. The main sources of TNFα in the brain are microglia[57] that get activated within minutes following MCAO[75]. However, CD8+ T lymphocytes were also proven to produce TNFα[133]. Therefore, the elevation of TNFα expression seen in our experiment can signal microglial activation 1 day following ischemia induction, while CD8+ T cells can also participate in the later increase of cytokine expression, as depletion of CD8+ cells prevented TNFα upregulation 5 days after MCAO.

In contrast to TNFα, the expression of iNOS mRNA was not affected by MCAO 1 day following surgery. Later on, it was significantly elevated on day 5. In accordance with our results, iNOS expression was elevated 2-6 days in the postischemic period, and was considered to participate in the late phase of tissue damage[70]. Concerning the cellular source of iNOS, it was shown to be expressed by microglia/monocytes, astrocytes and invading neutrophils in the brain[15], and by infiltrating CD8+ T cells in other tissues such as the liver[140]. In our experiment, CD8+ T cells can contribute to the elevated iNOS mRNA level 5 days after surgery as CD8+ T cell depletion attenuated iNOS upregulation at this timepoint.

Both TNFα and iNOS are considered as mediators of focal ischemic brain injury. Intracerebral injection of TNFα exacerbated ischemic injury[5], conversely, anti-TNFα monoclonal antibody or soluble TNF-receptor treatment reduced brain damage[5,31,95,118]. The iNOS inhibitor aminoguanidine attenuated post-ischemic iNOS activity and reduced infarct size after MCAO[70,172]. In line with these findings, the attenuation of TNFα and iNOS

upregulation was accompanied by reduced infarct size and improved sensorimotor functions in the present study.

In conclusion, depletion of CD8+ T lymphocytes reduced infarct size and improved the neurological function 7 days following permanent MCAO, suggesting a delayed deleterious effect of CD8+ T cells in focal ischemia. Most likely, production of cytotoxic cytokines such as TNFα and induction of oxidative damage caused by iNOS upregulation play an important role in the molecular mechanisms of brain damage caused by CD8+ T lymphocytes.

3.2. iNOS expression in cerebral ischemia

We investigated iNOS expression in permanent global ischemia induced by 2VO in rats and in permanent focal ischemia induced by MCAO in mice. In global ischemia, only a slight reduction was observed both in the hippocampus and the cortex 2 weeks after 2VO onset, while in focal ischemia, marked iNOS induction was seen 5 days following MCAO. These results suggest the role of iNOS in focal ischemia, where besides microglia activation infiltrating leukocytes contribute to post-ischemic brain damage. Although microglia were proven to express iNOS during ischemic conditions[103], most likely microglia do not play important role in iNOS expression in the investigated ischemia models, as i) in the 2VO model, iNOS protein level was even decreased in the hippocampus 2 weeks following ischemia induction despite the hippocampal microglial activation proven by OX-42 labeling at the same timepoint; ii) in the MCAO model, the microglial activation suggested by significant TNFα upregulation at 1 day did not induce iNOS expression. In all probability, iNOS overexpression predominates in infiltrating leukocytes, as the depletion of CD8+ T cells attenuated iNOS upregulation.

4. Final conclusion

4.1. Oxidative changes in cerebral ischemia

According to our results, in chronic mild ischemia induced by 2VO, enzyme level changes occur mainly in the early phase of hypoperfusion, while in the later phase, eNOS upregulation can contribute to restore local CBF. In the acute phase of both chronic mild and transient severe global ischemia, changes of the investigated enzyme levels are similar and affect mainly the hippocampus. Specifically, we demonstrated COX-2 upregulation and decreased nNOS expression indicating excitotoxicity and neuronal loss, respectively. These alterations are more moderate and develop with a delayed dynamics following 2VO

compared to TGI, that is in accordance with the severity of CBF disturbance and the consequential neuronal damage described in these models.

In focal ischemia, brain invading leukocytes also contribute to the development of oxidative stress. iNOS is induced only in focal ischemia, where CD8+ T cell depletion attenuated its upregulation resulting in smaller infarct size and improved sensorimotor functions. These results suggest a deleterious effect of iNOS in focal ischemia, where its overexpression predominates in infiltrating leukocytes, such as CD8+ T cells.

4.2. Antioxidant strategies

Several antioxidant strategies have been investigated in cerebral ischemia, including the upregulation of intrinsic antioxidant enzymes or administration of their agonists, inhibition of prooxidant enzymes and application of free radical scavengers. As several ROS/RNS are needed for physiological functions, therapies targeting a defined pro- or antioxidant enzyme can result in imbalance between different reactive agents leading to controversial results regarding neuroprotection. Therefore, strong cytotoxic radicals such as $OH^{\bullet}$ and $ONOO^{-}$ must be specifically neutralized without compromising the essential biological activities of other ROS/RNS. Alternatively, the deleterious processes induced by these reactive molecules such as the oxidation of macromolecules should be prevented. Besides its specificity, the antioxidant therapy should have no toxic side effects.

Here we demonstrate the antioxidant effects of 2 compounds that fulfill the requirements mentioned above: α-tocopherol and hydrogen. α-Tocopherol is located in the membranes of cells and cell organelles and it terminates oxidative chain reactions[72], while hydrogen selectively scavenges the hydroxyl radical[124]. In case of α-tocopherol, transfer and binding proteins limit the increase of its plasma concentration[37]. Hydrogen has been considered physiologically inert, it is produced by the gut flora then exhaled, or further metabolized[60]. Therefore, the application of these compounds is not accompanied by the risk of intoxication.

We first described the protective effect of α-tocopherol in a 2VO rat model of chronic cerebral hypoperfusion. Due to its simple per os usage, α-tocopherol can have relevance as a preventive dietary supplement in cerebral hypoperfusion related memory dysfunctions. We first investigated the effect of hydrogen supplemented air inhalation in TGI. Although inhaled molecular hydrogen is not practical for daily use or continous administration, due to its rapid gaseous diffusion, hydrogen therapy should be considered in the acute management of cardiovascular emergencies and / or cardiac surgery.

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