Aus der Klinik für Neurologie
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DISSERTATION

Effects of peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists and physical activity on long-term stroke outcome in a mouse model

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List of Abbreviations

ACE .....Angiotensin-converting enzyme
AHA .....American Heart Association
AP-1.....Activator protein 1
BrdU 5-bromo-2-deoxyuridine
CNS .....Central nervous system
CoA.....Coenzyme A
COX-2.....Cyclooxygenase-2
CRP .....C-reactive protein
DG .....Dentate gyrus
DMEM.....Dulbecco´s modified Eagle´s medium
eNOS.....Endothelial nitric oxide synthase
EPCs .....Endothelial progenitor cells
FCS .....Fetal calf serum
H&E .....Hematoxylin and eosin
HMG-CoA....Hydroxymethylglutaryl-coenzyme A
hs-CRP.....High-sensitivity C-reactive protein
i.p.....Intraperitoneal
IL-1 .....Interleukin-1
IL-1β Interleukin-1β
IL-6 .....Interleukin-6
iNOS.....Inducible nitric oxide synthase
LFA-1 .....Late functional antigen 1
L-NAME.....N-nitro-L-arginine methyl ester
LV .....Lateral ventricles
MABP .....Mean arterial blood pressure
MCA.....Middle cerebral artery
MCAo.....Middle cerebral artery occlusion
MCP-1 .....Monocyte chemoattractant protein-1
MHC .....Major histocompatibility complex
MMP .....Matrix metalloproteinase
NC .....Normal chow
NF-kB .....Nuclear factor- kappa B
NO .....Nitric Oxide

NOS.....NO synthase

PP.....Pyrophosphate

PPAR-γ .....Peroxisome proliferator-activated receptor-gamma

PPARs .....Peroxisome proliferator-activated receptors

PPREs.....PPAR response elements

PSE .....Plant sterol esters

rCBF .....Regional absolute cerebral blood flow

RMS.....Rostral migratory stream

RXR.....Retinoid X receptor

SGZ .....Subgranular zone

SVZ.....Subventricular zone

TIA.....Transient ischemic attack

TNF.....Tumor necrosis factor

1. Summary

Inflammation, as one of the major risk factors for stroke, unifies mechanisms in ischemic stroke pathogenesis, and provides new avenues for stroke prevention— physical exercise, peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists, statins, and angiotensin-converting enzyme (ACE) inhibitors. These new stroke prevention therapies may contribute to reduced inflammation, and stabilize the atherosclerotic plaque, or act via other protective mechanisms. Stroke outcome is modulated by the interaction of the injured brain with the immune system.

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists (thiazolidinediones) have anti-inflammatory effects and improve endothelium function. Here, we analyzed the effects of pioglitazone on short- and longer-term outcome after mild transient brain ischemia. 129/SV mice were subjected to 30 min filamentous middle cerebral artery occlusion (MCAo), followed by reperfusion. Post event, animals were treated with daily intraperitoneal (i.p.) pioglitazone (20 mg/kg body weight) or vehicle. Pioglitazone given acutely after transient brain ischemia / reperfusion reduced lesion size and the number of Iba1-expressing microglia in the ischemic striatum at three days. *In vitro*, pioglitazone attenuated migration and proliferation of primary mouse microglia. However, analysis at 6 weeks after MCAo/reperfusion no longer yielded an effect of pioglitazone on either lesion size or Iba1+ cell counts. Regarding functional longer-term outcome, we also did not detect a beneficial effect of pioglitazone on motor function measured either on the pole test or the wire hanging test or on learning and memory in the Morris water maze. Our study thus underscores the importance of extending experimental stroke studies to an analysis of longer-term outcome.

Clinical and experimental evidence indicates that regular physical activity (1) upregulates endothelial nitric oxide synthase (eNOS); (2) improves endothelium-dependent vasodilation; (3) protects from vascular disease in an acute model of ischemic stroke; (4) furthermore, improved neo-vascularization and long-term functional and histological protection through regular physical activity could be shown.

Here, we tested the hypothesis that the long-term stroke-protective effects of regular physical activity are mediated via up regulation of eNOS and enhanced neovascularization in a chronic stroke model. To do so, we used N-nitro-L-arginine methyl ester (L-NAME), a pharmacologic inhibitor of NOS and the antiangiogenic agent endostatin. Here, we compared groups of animals subjected to voluntary exercise vs a sedentary lifestyle. After 3 weeks of physical training animals were exposed to mild cerebral ischemia induced by 30 min occlusion of the left middle cerebral artery (MCAo) followed by reperfusion. Then animals were put back to their home cages and treatment was continued as before. A subset of animals from each group was treated either with endostatin or L-NAME respectively. Four weeks after MCAo brain damage in ischemic mice was evaluated by computer-assisted infarct volumetry. We showed abolished neuroprotection after exercise either with co-treatment of L-NAME or endostatin. Our results demonstrate that eNOS upregulation and angiogenesis are implicated in the long-term neuroprotective effects of physical activity.

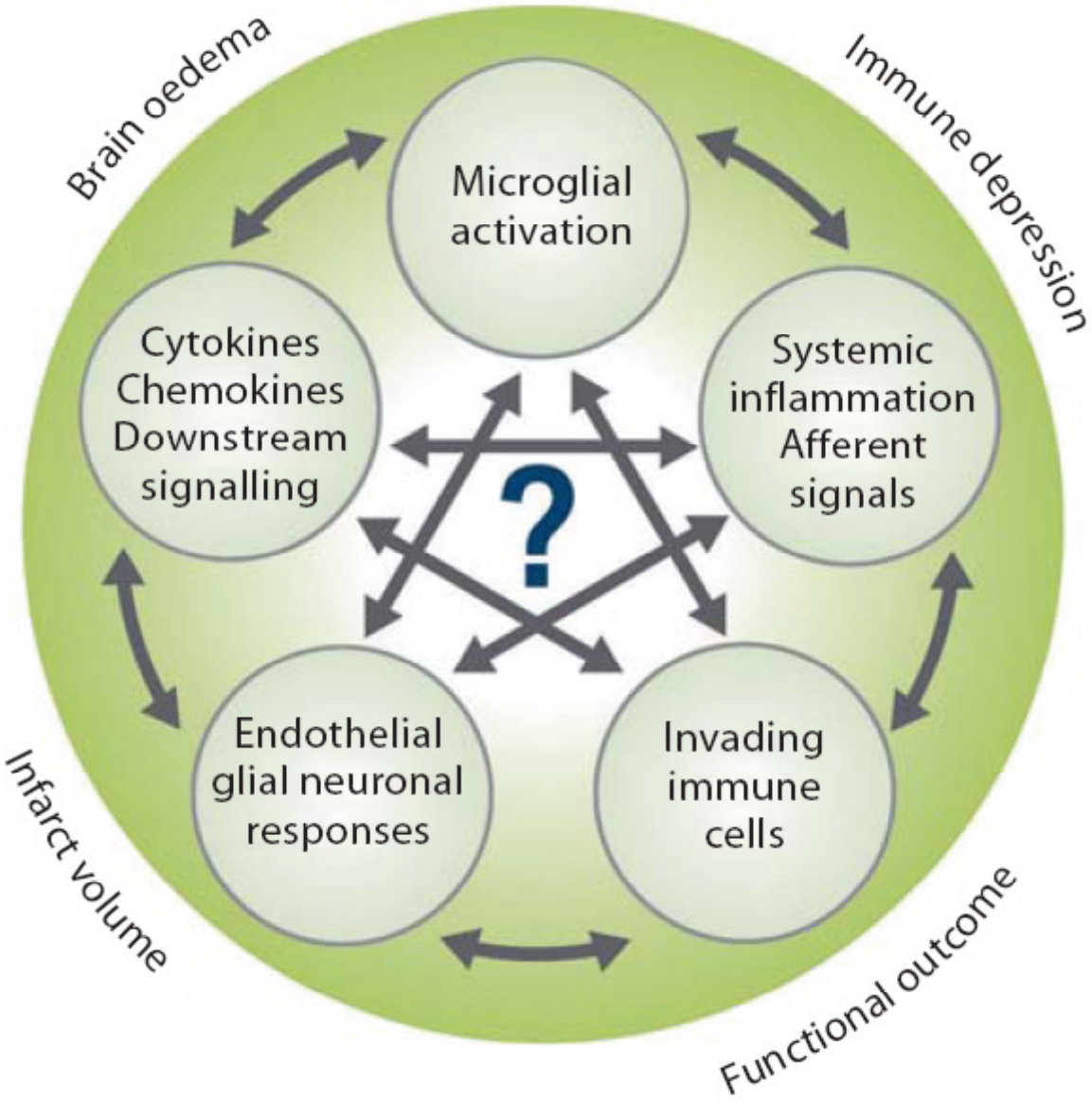
2. Introduction

Stroke, as one of the major causes of mortality, is responsible for 9.5% of all deaths and 5.1 million of the 16.7 million cardiovascular disease deaths worldwide (Bonita, et al., 2001). In China and Japan, stroke is the leading cause of death. The absolute numbers of strokes in China equal those of the entire developed world. Indeed, globally, about two thirds or more of stroke deaths occur in the developing world (Sacco, 1999). Throughout the world, the stroke prevention gap widens: lack of stroke risk factor profiling; unfavorable prevention programs; lack of awareness of stroke risk factors and warning signs in the public wrong application (Ferguson, et al., 1999, Goldstein, 1999). Indeed, stroke can be prevented because it has a high prevalence, well-defined modifiable risk factors, and effective preventive treatments exist (Goldstein, et al., 2001, Gorelick, 2002, Gorelick, et al., 1999, Hankey and Warlow, 1999).

Tissues of multicellular organisms respond to injury via the basic mechanism of inflammation. The neurovascular unit is an important site of inflammation after stroke. It consists of a complex cellular system, such as circulating blood elements, endothelial cells, pericytes, perivascular antigen-presenting cells, astrocytic end-feet and neurones (Lo, et al., 2003). At the molecular level, a complex cascade of mediators is involved in inflammation. Current studies show that there are five key mediators which act in inflammation (Figure 1).

1) Activated microglia cells and macrophages possess the ability to migrate, differentiate, phagocytose and to release a wide variety of molecules involved in inflammation (Eglitis and Mezey, 1997, Priller, et al., 2001). Indeed, microglia cells play an important role in the development of ischemic infarct (Yrjanheikki, et al., 1999).
2) Inflammation in cerebral ischemic stroke depends on the regulation of gene expression. Signal transduction pathways include oestrogen receptor, activator protein 1 (AP-1) and Nuclear factor- kappa B (NF-kB) (Kaczmarek, et al., 2002). They act in the inflammatory response by binding to response elements in the promoter region of multiple genes. Therefore, transcription factor signalling seems to provide defined molecular targets in ischemic brain injury (Herrmann, et al., 2005).
3) Nitric Oxide (NO) generated by endothelial NO synthase (eNOS) protects from stroke by maintaining cerebral blood flow and lowering infarct volume (Endres, et al., 2004).
4) Poststroke inflammation exerts its effects via a complex network of cytokines including Interleukin-1 (IL-1), Interleukin-6 (IL-6) and tumor necrosis factor (TNF) (Allan, et al., 2005, Emsley, et al., 2005).

5) Neuroinflammation in stroke induces BBB breakdown and neuronal damage. However, it protects from stroke injury.



Endres et al., 2008

Figure 1. Five key players of the inflammatory response in stroke.

These players' interrelation is unclear. Which ones contribute to better stroke outcome?

2.1. Ischemic stroke and middle cerebral artery occlusion (MCAo)

Via a complex network of arteries and vessels, blood is carried to the brain (Figure 2). A stroke happens when the blood supply to a part of the brain is interrupted suddenly or when a blood vessel bursts in the brain, spilling blood into the space surrounding brain cells. Brain cells are damaged when they do not receive oxygen and nutrients from the blood any more. Most strokes occur when cerebral arteries are abruptly blocked, which results in 'ischemic' stroke. Ischemic stroke is one of the leading causes of death and adult disability world wide. With an incidence of approximately 250-400 in 100 000 and a mortality rate of around 30%, stroke remains the third leading cause of death in industrialized countries. In the United States alone, four million people suffer from stroke and face its debilitating consequences (Dirnagl, et al., 1999). The poor prognosis of stroke patients is largely due to a lack of effective therapies. Several lines of therapeutic interventions for the treatment of stroke were investigated. Many neuroprotective drugs that have shown beneficial effects in experimental studies failed in clinical trials, some even worsened stroke outcome. However, more than 180 neuroprotectants have failed to show significant benefits on ischemic stroke in clinical trials (Endres, et al., 2004). The reasons for this apparent discrepancy between successful use in preclinical experimental research and subsequent clinical failure are multifactorial (Culman, et al., 2007, Dirnagl, 2006, Green and Shuaib, 2006).

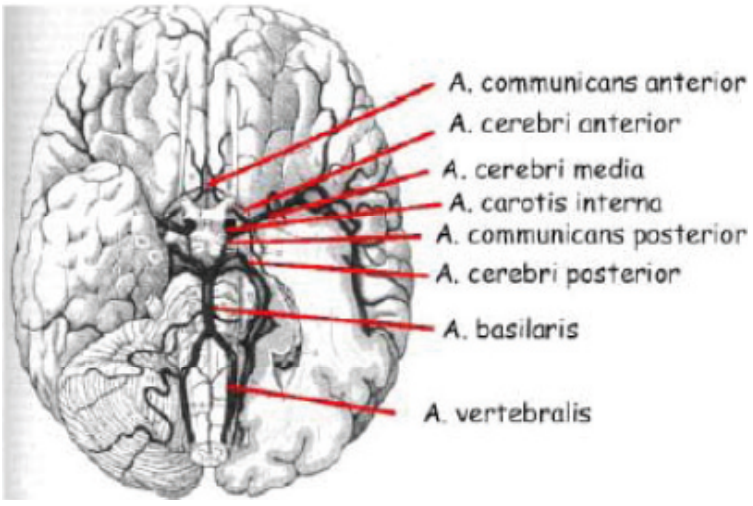
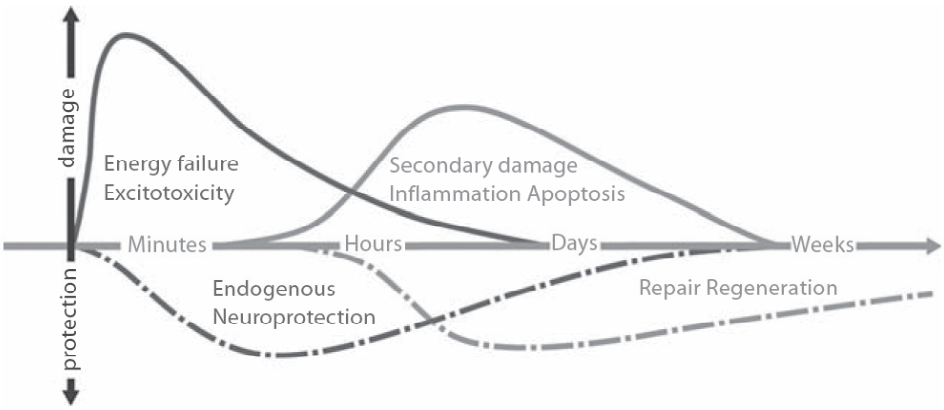


Figure 2. A basal view of brain arteries

Once a brain vessel is occluded, a complex series of cellular and molecular events rapidly act. Cells swell and depolarize, excitatory amino acids and K^+ ions are released when intracellular Ca^{2+} levels soar ('excitotoxicity'). It is this dramatic acute phase of focal cerebral ischaemia which was the focus of a large number of clinical trials of neuroprotective agents that ultimately failed (Grotta, 2002, O'Collins, et al., 2006, Richard Green, et al., 2003). However, after the early damage, the lesion may indeed continue to grow several hours and even days after the injury of ischaemia. To widen the time window for stroke treatment, we need to target the underlying mechanisms. We know that the brain mounts a potent action against many of the deleterious secondary mechanisms, but this reaction of the brain to the ischemic insult is only partially successful (Figure 3).



Endres et al., 2008

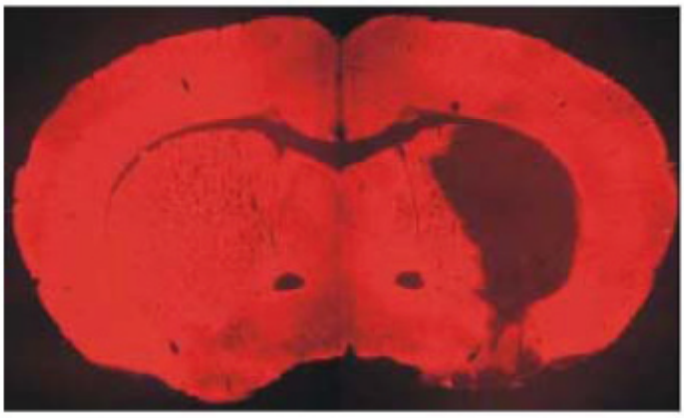
Figure 3. Simplified pathobiology of stroke.

Focal cerebral ischemia activates a complex series of mechanisms that damage brain cells. In infarcted tissue, destruction has overwhelmed protection, although tissue around the ischemic core might have been spared by protection of restored substrate delivery and cellular mechanisms. The x-axis illustrates the evolution of the cascades over time and the y-axis reflects the impact of each element of the destructive (top) and protective (bottom) cascades on final outcome. The upper lines envelop main pathophysiological properties of tissue destruction in stroke (grouped for the acute mechanisms of excitotoxicity and the delayed mechanisms of inflammation and apoptosis) and the lower broken lines envelop corresponding protective tissue responses (grouped for the acute mechanisms of endogenous neuroprotection and the delayed mechanisms of repair / regeneration).

The pathophysiological mechanisms of cerebral ischaemia and their interactions are exceedingly complex, and many act as a 'double-edged sword', with both beneficial and negative effects (Figure 3). Glutamate is a main player in excitotoxicity (Martin, et al., 1994). However, it is essential for normal brain function and a major driving force of reorganization and synaptogenesis after brain injury (Bernabeu and Sharp,

2000). Nitric oxide (NO) derived from endothelia seems to increase blood flow while neuronal and inducible NO synthase (NOS) may benefit formation of peroxynitrite and hydroxyl anions (Endres, et al., 2004, Iadecola, 1997). Inflammation aggravates ischemic injury, but also offers the necessary environment for regeneration and repair (Kerschensteiner, et al., 1999). Formation of a glial scar may exacerbate the lesion and impede its progression, while it has also protective effect as a barrier for axonal sprouting (Nedergaard and Dirnagl, 2005). Apoptosis leads to lesion growth, but also reduces inflammation (Zipfel, et al., 2000). Stroke causes immunodepression but at the same time increases susceptibility to infection (Meisel, et al., 2005).

The middle cerebral artery is the largest branch of the internal carotid. The artery supplies a portion of the frontal lobe and the lateral surface of the temporal and parietal lobes, such as the primary motor and sensory areas of the hand, arm, face and throat and in the dominant hemisphere, the areas for speech. The middle cerebral artery is the artery most often occluded in stroke. Focal brain ischemia induced in mice by occlusion of the middle cerebral artery (MCA) is a widely used experimental paradigm of human brain infarct (Ardehali and Rondouin, 2003). Here, a model of mild focal brain ischemia by 30 min filamentous occlusion of the MCA in the mouse was developed. In this model, neuronal cell death evolves over days within the caudato-putamen while glial cells in the insult survive (Figure 4) (Endres, et al., 1998, Katchanov, et al., 2001). Medium spiny projection neurons are affected exclusively by neuronal death, whereas all types of interneurons stay intact (Katchanov, et al., 2003). Moreover, mortality is much higher in mouse models of severe ischemia, amounting to as much as 50% to 70% at 7 days survival paradigms (Huang, et al., 2001, Kondo, et al., 1997, Prass, et al., 2003), whereas survival rates in our mild model are almost 90% at 6 weeks. This model is ideally suited to study long-term sequelae after stroke.



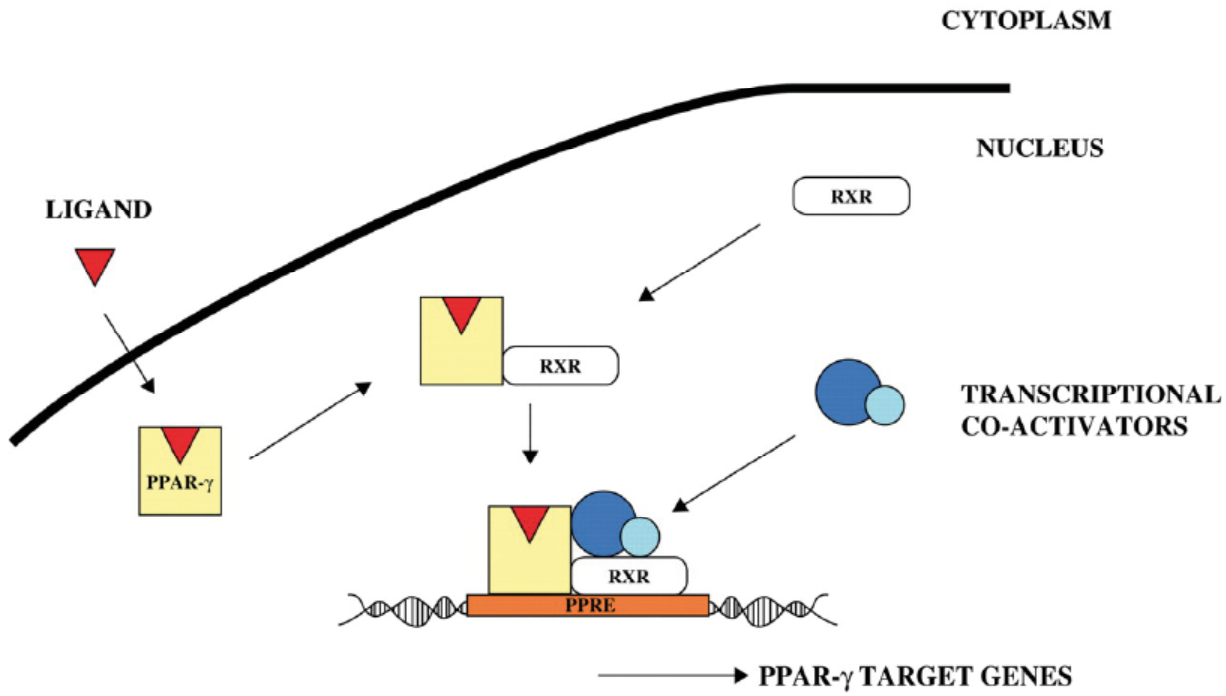
Katchanov et al., 2001

Figure 4. Representative examples of MAP2 immunohistochemistry 40-µm coronal brain section 4 weeks after 30 min MCAo

129/SV mice were exposed to 30 minutes of MCAo/reperfusion and sacrificed 4 weeks later. The section shows neuronal loss in the ischemic infarct which is confined to the caudate putamen.

2.2. Peroxisome proliferator-activated receptors (PPARs) and pioglitazone

PPARs are ligand-activated transcription factors and belong to the nuclear hormone receptor superfamily (Evans, 1988). Three PPAR isoforms have been identified (alpha, delta/beta and gamma). PPAR-γ regulates gene expression by forming heterodimers with the retinoid X receptor before binding to PPAR response elements in the promoter region of their target genes (Figure 5). The gene encoding PPAR-γ is located on chromosome 3 at position 3p25. Stroke enhances the expression of PPAR-γ mRNA and protein in neurons and microglia (Lin, et al., 2006, Ou, et al., 2006, Victor, et al., 2006, Zhao, et al., 2006). However, this increased PPAR-γ expression may not be functionally relevant because cerebral ischemia reduces DNA binding of PPAR-γ. PPAR-γ agonists increase PPAR-γ DNA binding, which can be fully reversed by selective PPAR-γ antagonists (Culman, et al., 2007, Zhao, et al., 2006).



Abdelrahman et al., 2005

Figure 5. Activation of PPAR-γ.

Heterodimerisation of PPAR- γ and the retinoid X receptor (RXR) produce an active transcription complex after ligand binding. This complex associates with transcriptional co-activators while binding to sequence specific PPAR response elements (PPREs) located in target genes.

Pioglitazone is a synthetic PPAR-γ agonist, acts as an insulin sensitizer and is used in the treatment of type 2 diabetes. In the last few years, it has, however, become evident that the therapeutic effects of PPAR-γ ligands may reach far beyond their use as insulin sensitizers. For instance, neuron sparing within the substantia nigra was promoted by pioglitazone in models of Parkinson's disease (Breidert, et al., 2002, Dehmer, et al., 2004). Pioglitazone has also been shown to confer neuroprotection in transgenic mouse models of amyotrophic lateral sclerosis (Kiaei, et al., 2005, Schutz, et al., 2005) and has been used as a modulator of CNS inflammation to counteract neurodegeneration, as demonstrated in an animal model of Alzheimer's disease (Roses, et al., 2007). Several studies report enhanced neuroprotection by pioglitazone in animal models of intracerebral hemorrhage (Zhao, et al., 2006). After traumatic spinal cord injury, pioglitazone treatment also improves anatomical and locomotor recovery (McTigue, et al., 2007).

Recently, PPAR-γ has been implicated as a regulator of cellular inflammatory responses. The inflammatory response in stroke involves leucocytes, endothelial and glial cells as well

as neurons. At the molecular level, inflammation involves a complex cascade of mediators (Endres, et al., 2008). So far, a detailed characterization of how neuroplastic changes following brain ischemia are influenced by treatment with PPAR-γ agonists is largely lacking.

Preclinical studies have demonstrated a neuroprotective effect of PPAR-γ agonists in models of acute cerebral ischemia (Collino, et al., 2006, Luo, et al., 2006, Shimazu, et al., 2005, Zhao, et al., 2005). The beneficial effects of pioglitazone are mediated through intracerebral activation of PPAR-γ and inhibition of inflammatory responses at early time points after stroke (Zhao, et al., 2005). However, so far, all experimental studies have focussed on short-term survival whereas long-term effects of pioglitazone treatment after brain ischemia have not been assessed.

Here, we confirm the neuroprotective effects of acute pioglitazone treatment on animals in a short-term survival paradigm. However, analysis of longer-term stroke outcome is this study's main focus. We report that pioglitazone treatment did not yield a beneficial effect on lesion size or functional outcome at later time points after MCAo/reperfusion. Our study thus underscores the importance of studying longer-term outcome in stroke experiments.

2.3. Physical exercise and eNOS

Physical exercise is associated with improving collateralization in patients with cerebrovascular and cardiovascular events, exercise capacity and endothelial function. Physical activity also improves mood, body weight, blood pressure, insulin sensitivity, and hemostatic and inflammatory diseases (Laufs, et al., 2004). In an acute mouse model of ischemic stroke (Figure 6A) voluntary training on running wheels decreases cerebral infarct size and functional deficits, contributes to better endothelium-dependent vasorelaxation, and augments cerebral blood flow in wild-type mice. The main mechanism of physical activity protecting against cerebral ischemic stroke seems to relate to the upregulation of endothelial nitric oxide synthase (eNOS) in the vasculature. Endothelium-derived NO has the following properties: vasodilatory, anti-inflammatory, antithrombotic and antiproliferative. The neuroprotective effects of physical training are not observed in eNOS-knockout mice (Endres, et al., 2004).

Recent research has demonstrated that vascular function seems to be significantly modulated by circulating cells derived from the bone marrow while it also depends on cells that reside within the vessel wall. This circulating bone marrow-derived cell population has been named endothelial progenitor cells (EPCs). In addition to regulating endothelium function eNOS plays an essential role for neo-angiogenesis and regulation of stem and progenitor cells which may be important mechanisms for long-term tissue recovery in the ischemic brain (Aicher, et al., 2003, Endres, et al., 2004, Matsunaga, et al., 2000, Murohara, et al., 1998). Cerebral ischemia promotes new blood vessel formation. Bone marrow-derived EPCs participate in this process, which may be relevant for functional recovery and specific therapeutic approaches (Beck, et al., 2003, Krupinski, et al., 1994, Wei, et al., 2001, Zhang, et al., 2002). Physical exercise contributes to increased production and increased numbers of circulating EPCs (Endres, et al., 2004).

The purpose of this study was to determine whether physical activity improves long-term stroke outcome and whether the protective and beneficial effects of physical activity are mediated via upregulation of eNOS and angiogenesis (Figure 6B). Therefore, the following groups of animals were compared exercise vs sedentary with and without L-NAME and exercise vs sedentary with and without endostatin. All animals were subjected to a well established so called chronic model of cerebral ischemia induced by a 30 min occlusion of the MCA followed by reperfusion. Here, we tested the hypothesis whether the protective effects of physical exercise on chronic stroke outcome are mediated by endothelial NO synthase upregulation. NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) was dissolved in the drinking water. In order to test the hypothesis that neovascularization is essential to neuroprotection, a natural antiangiogenic agent, endostatin, was used. Endostatin is the cleaved carboxy-terminal globular domain of Type XVIII collagen and is comprised of 184 amino acids. Endostatin is known to inhibit endothelial cell migration *in vivo* and *in vitro*, to induce endothelial cell apoptosis and to have a direct inhibitory effect on EPCs (Capillo, et al., 2003, O'Reilly, et al., 1997).

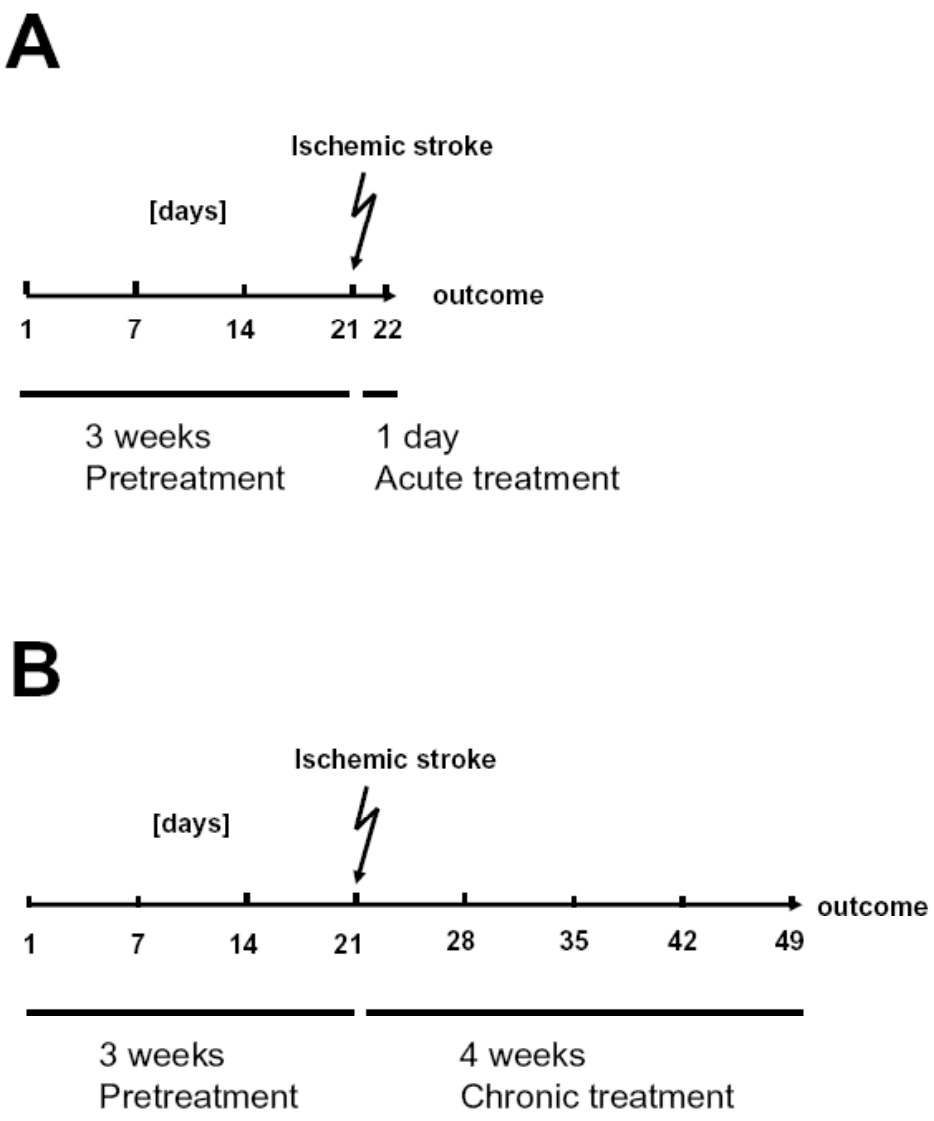


Figure 6. Physical exercise and stroke outcome.
After 3 weeks of running, animals were subjected to 30min MCAo/reperfusion. Post-treatment exercise continues after the operation. A. Mice were killed at 1 day after MCAo. B. Animals were sacrificed at 4 weeks after MCAo.

2.4. Plant sterol esters (PSE) supplementation

Margarines, as “Functional foods” containing plant sterol esters (PSE), have the reputation to prevent cardiovascular diseases and are widely used as a nonprescription approach to decrease plasma cholesterol levels. The chemical structure of phytosterols is different from that of cholesterol by the presence of modified side chains at carbon C-24 (John, et al., 2007). Phytosterols are not synthesized by mammals. Sitosterol and campesterol

make up the bulk of plant sterols. Their main dietary sources are fat-rich vegetables, such as vegetable oils, fruits, and nuts. Their primary mechanism of reduceing blood cholesterol levels is to replace cholesterol competitively in bile salt micelles, which reduces absorption of unesterified cholesterol from the small intestine (John, et al., 2007, von Bergmann, et al., 2005). In humans, 0.8 to 4.0 g of PSE daily has been shown to decrease by 10% to 15% low-density lipoprotein cholesterol concentrations (von Bergmann, et al., 2005). The precise molecular mechanisms for sterol absorption remain to be elucidated. However, cholesterol and plant sterol absorption both need NPC1L1 protein (Altmann, et al., 2004).

Plant sterol–enriched chow lowers cholesterol concentrations with reduced plaque volume in animal models of atherosclerosis (Moghadasian, et al., 1997, Ntanios, et al., 2003). However, the net effect of plant sterols on vascular disease seems to be related to the following 3 lines:

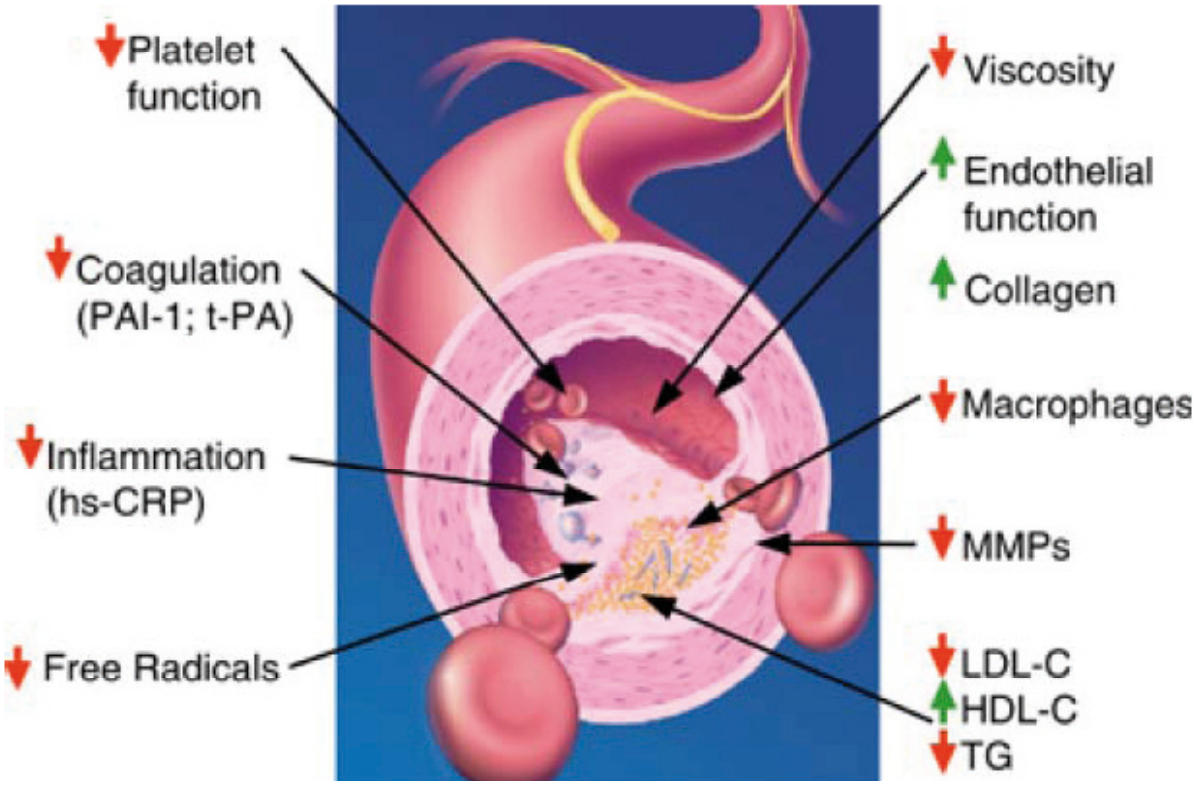
1. People with the autosomal-recessive disease phytosterolemia have increased plant sterols plasma concentrations, which results in hyperabsorption and reduced biliary elimination. These patients have a defect in either the ABCG5 or ABCG8 transporter genes, suffer from premature atherosclerosis and higher risk of coronary heart and aortic valve disease (Berge, et al., 2000, Glueck, et al., 1991).
2. Plant sterols have been found in atherosclerotic lesions from normal cholesterol absorption individuals (Miettinen, et al., 2005).
3. Epidemiological studies have shown that increased plant sterol concentrations are associated with higher risk of vascular disease (Assmann, et al., 2006, Rajaratnam, et al., 2000, Sudhop, et al., 2002).

Except the widespread use of sterol-enriched margarine, the effects of plant sterols on vascular disease remain unclear. Therefore our study focussed on effects of PSE on cerebral ischemia in a well established mild focal ischemia animal model.

2.5. Statin and stroke outcome

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) decrease cholesterol levels. However statins also possess a number of cholesterol-independent, pleiotropic effects such as anti-inflammatory effects (Davignon, 2004, Laufs, 2003).

Pleiotropic effects are not what statins were originally developed for. Whether pleiotropic effects are of clinical benefit may largely depend on the pathophysiology of a specific disease in a particular individual. The effects of statins on the vasculature involve lipoprotein metabolism, anti-inflammatory and anti-thrombotic effects. In addition to decreasing high-sensitivity C-reactive protein (hs-CRP) levels and other markers of inflammation including CD40, statins not only improve endothelium-dependent vasorelaxation but also increase the number of circulating endothelial progenitor cells. These effects act together (Endres, 2005) (Figure 7).

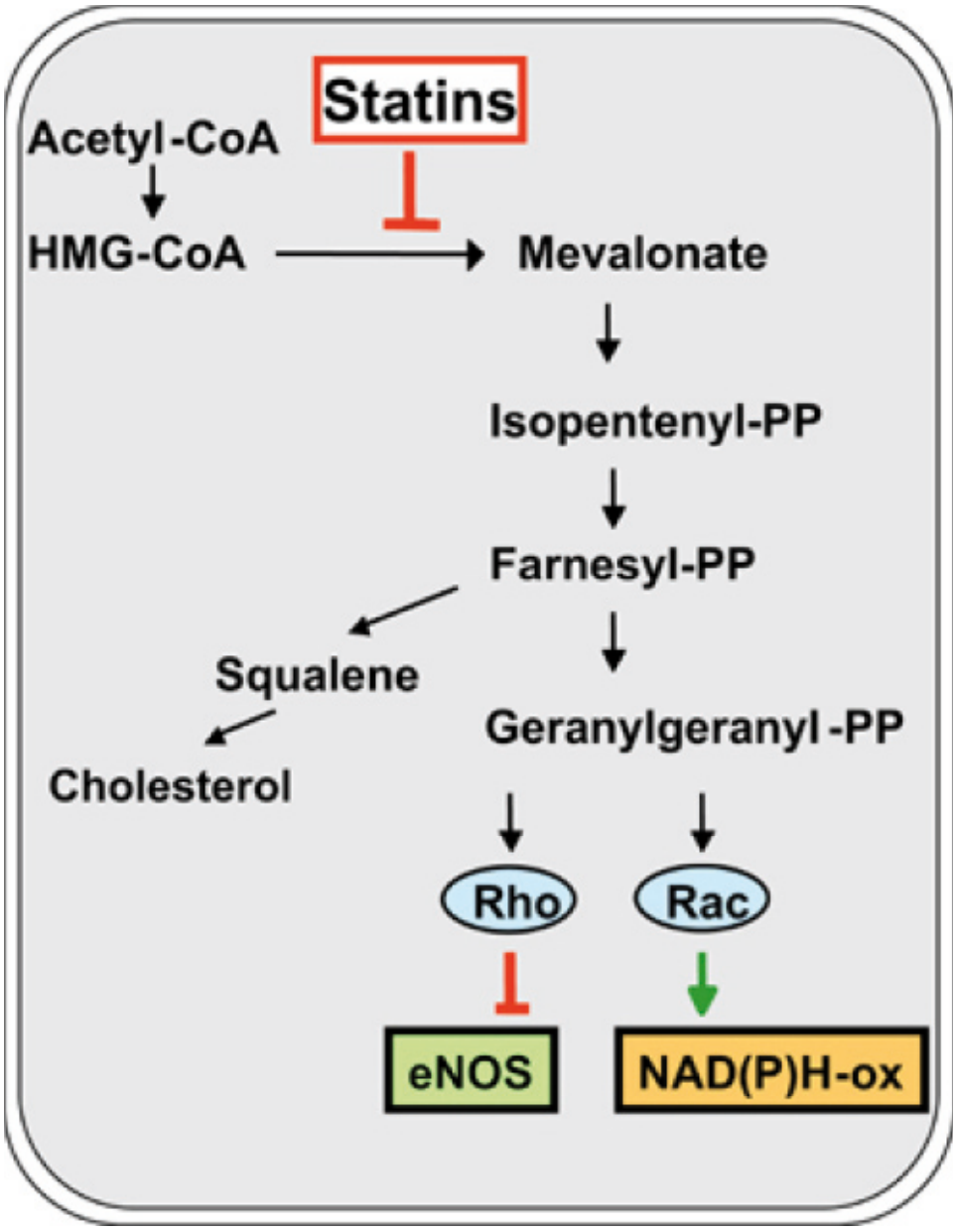


Endres 2005

Figure 7. Pleiotropic effects of statins.

Cholesterol-independent vasoprotective effects of statins. PAI-1: Plasminogen activator inhibitor-1; t-PA : tissue-plasminogen activator; hs-CRP: high-sensitivity C-reactive protein; MMPs: matrix metalloproteinases; LDL-C low-density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TG: triglycerides.

Inhibition of the mevalonate pathway mediates pleiotropic mechanisms. Upregulation of endothelial NO synthase (via rho GTPase inhibition) and downregulation of NADPH oxidase (via rac GTPase inhibition) are shown in Figure 8 (Endres and Laufs, 2004).



Endres 2004

Figure 8. Schematic diagram of the mevalonate pathway for cholesterol synthesis. Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) block the conversion of HMG-CoA to mevalonate. Therefore, the synthesis of both cholesterol and isoprenoid intermediates is inhibited. Statins inactivate small G-proteins Rho and Rac via inhibition of geranylgeranylation. Inhibition of Rho signalling leads to upregulation of endothelial nitric oxide (NO) synthase (eNOS) resulting in increased NO bioavailability. Inhibition of Rac GTPase inactivates NAD(P)H-oxidase activity and superoxide production. Coenzyme A (CoA) and pyrophosphate (PP).

Cholesterol-independent pleiotropic actions of statins may play a beneficial role in ischemic stroke. While increased cholesterol is a main risk factor for coronary heart disease, the link between cholesterol and stroke is unknown (Taylor and Landau, 1990). Large meta-

analyses have shown no correlation between high cholesterol levels and stroke incidence (Endres, 2006). Smaller studies have demonstrated that elevated cholesterol levels may increase the risk for atherothrombotic stroke. However, decreased cholesterol levels are associated with hemorrhagic stroke. Based on previous studies, cholesterol is not included in the risk factors for ischemic stroke according to the American Heart Association (AHA). Therefore, the result that statins reduce the incidence of myocardial infarction and stroke (Endres, 2005) is somewhat surprising. Indeed, available data suggest that protective effects of statins are via direct actions on endothelial function, anti-thrombotic and anti-inflammatory mechanisms, as well as via lowering cholesterol.

Increasing evidence suggests that statins decrease a number of markers of inflammation, such as C-reactive protein (CRP), soluble intercellular adhesion protein or interleukin-6 (Albert, et al., 2001, Ridker, et al., 2001). Independent of HMG-CoA reductase inhibition, statins interfere with the interaction of Late functional antigen 1 (LFA-1) and intercellular adhesion molecule 1 (Weitz-Schmidt, et al., 2001). In the central nervous system (CNS), statins may downregulate inducible NOS and cytokines (Pahan, et al., 1997). Additionly, statins directly inhibit the interferon-γ-induced expression of major histocompatibility complex (MHC) class II on B-cells and macrophages (Kwak, et al., 2000), which may suggest that statins play a role in immunomodulation (Palinski, 2000). These immunomodulatory effects may contribute to reduced organ rejection after transplantation (Kobashigawa, et al., 1995) or may lead to better outcome from immune-mediated diseases including rheumatoid arthritis or multiple sclerosis (Aktas, et al., 2003, Youssef, et al., 2002).

Experimental studies have demonstrated that statins not only reduce lesion volume but also improve outcome from cerebral ischemia/reperfusion (Endres, et al., 1998). The protective mechanism relates to increased endothelial NO synthase and cerebral blood flow augmentation (Endres, 2005, Endres, et al., 1998).

2.6. Angiotensin-converting enzyme (ACE) inhibitors

Hypertension is one of the most important modifiable risk factors for stroke (Goldstein, et al., 2001, Gorelick, et al., 1999). Up to 50% of strokes occur because of hypertension. Hypertensives are at about 3 to 4 times higher risk of stroke than nonhypertensives, and

borderline hypertensives are at around 1.5 times the risk of nonhypertensives (Gorelick, 1995). Both systolic and diastolic hypertension increases stroke risk. It has been demonstrated that decreasing diastolic blood pressure for a long period of time by as little as 5 to 6 mm Hg reduces the risk of a first stroke by 35% to 40% (Collins, et al., 1990, MacMahon, et al., 1990). Furthermore, decreasing diastolic blood pressure by 5 to 6 mm Hg and systolic blood pressure by 10 to 12 mm Hg for a period of 2 to 3 years in stroke and transient ischemic attack (TIA) patients could lower the annual risk of a recurrent stroke from 7.0% to 4.8% (Gueyffier, et al., 1997, Hankey and Warlow, 1999). Clearly, the risk of stroke is associated with increased blood pressure. Importantly, individuals with high-normal blood pressure or mild hypertension are also at increased risk of stroke.

Hypertension may predispose to stroke by inducing atherosclerosis of the aorta and of large cerebral arteries, resulting in arteriosclerosis and lipohyalinosis of small-diameter penetrating arteries, and causing heart diseases (Rossi, et al., 1995). Hemorrhagic stroke also has a direct relationship with blood pressure.

Inflammation is one of the important novel risk factor candidates for atherosclerosis (Kullo, et al., 2000, Ridker, et al., 2001). Many of these key novel candidate risk factors have overlapping mechanisms, important synergistic effects with the atherosclerotic process, or complementary actions in the arterial thrombosis development. Inflammatory markers, including C-reactive protein (CRP) and fibrinogen, originate in the liver (Rader, 2000). They are activated by systemic cytokines such as interleukin 1ß, interleukin 6, and tumor necrosis factor-α. Cytokines are intercellular signaling polypeptides, which are produced at extrahepatic sites (e.g. the heart, vessel walls, macrophages, and adipose tissue). During the inflammatory process, cytokines are produced as stimulators of acute-phase proteins in both acute and chronic inflammatory disorders (Gabay and Kushner, 1999). Acute-phase proteins seem to play a role both in the initiation and in the termination of the inflammatory response.

In animal models of atherosclerosis, ACE inhibitors exerted consistent protective effects on plaque progression (Halkin and Keren, 2002). One major mechanism was via reducted monocyte chemoattractant protein-1 (MCP-1) expression and concomitant macrophage plaque infiltration. Decreased MCP-1 levels have also been measured in individuals with myocardial infarction treated by ACE inhibitors (MacMahon, et al., 2000).

ACE inhibitors have been used for the treatment of high blood pressure in the United States since the1970s (Black, 1996). They act on the renin-angiotensin-aldosterone system by

inhibiting the conversion of angiotensin I to angiotensin II by inhibiting ACE. Angiotensin II increases blood pressure because it is a potent peripheral vasoconstrictor, a stimulator of aldosterone from the adrenal cortex, which also has a negative influence on renin secretion. ACE is identical to Kinase II, which catalyzes the breakdown of bradykinin. Bradykinin is a one of the most potent vasodepressors. Blocking ACE with ACE inhibitors increases bradykinin concentrations (Stoll and Bendszus, 2006).

ACE inhibitors have been demonstrated to lower stroke risk in high-risk individuals with vascular disease or diabetes mellitus plus other risk factors and in those with ischemic or hemorrhagic stroke and higher or normal blood pressure (Stoll and Bendszus, 2006).

2.7. Functional approach: behavior tests

Stroke patients often display cognitive deficits and behavioral abnormalities in addition to a wide range of motor and sensory deficits (Caplan, et al., 1990). However, most preclinical experiments focused on morphometric assessment of infarct volume as the only stroke outcome. Although it is logical to assume that the infarct volume measurement is closely correlated with functional outcome, impairment or preservation of complex behavior may reflect other changes at a subcellular level and in synaptic or electrophysiologic function, which may reflect diffuse morphological changes that can not be quantified by counting dead neurons or tracing a cursor around an infarcted region (Aronowski, et al., 1996). Previous studies have demonstrated the occurrence of cognitive deficits in animal models of cerebral ischemia (Gupta, et al., 2002, Hirakawa, et al., 1994, Yonemori, et al., 1996). However, data regarding transient ischemia in the mouse are limited (Winter, et al., 2004).

In the long-term survival groups we assessed a group of complex behavioral outcomes with tests for overall motor function, spatial learning and memory. We used our established animal model, which is well suited for behavioral analyses, because mortality is very low in this model (compared with > 50% with longer MCA occlusion) and animals with 30 min occlusion have only mild sensorimotor neurologic deficits (usually mild forepaw flexion) and no locomotor disabilities at 72 hours until several weeks after the injury (Winter, et al., 2004).

Balance and grip strength were assessed on the Wire Hanging test. Motor deficits were measured on the pole test. In the Morris water maze task, spatial learning was assessed. Following a standard place task over 7 days, we additionally performed a probe trial and platform task experiment to assess strategy switching and relearning (Bert, et al., 2002, Harker and Whishaw, 2002).

3. Materials and methods

3.1. Animals

All experimental procedures were performed according to national and institutional guidelines for the care and use of laboratory animals and were approved by an official committee. 129/SV wild-type mice (18-22 g) (BfR, Berlin, Germany) were housed with ad libitum access to food and water.

3.2. Drug administration

Once daily treatment with intraperitoneal pioglitazone (20 mg/kg body weight; Takeda Pharmaceuticals) or vehicle was initiated either acutely after occlusion of the middle cerebral artery (MCA) and reperfusion (or after sham operation) or one day after MCA occlusion and reperfusion (Figure 9). Treatment was continued at this dosage (once daily) until sacrifice either on day 3 or day 42 after MCAo. 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, Deisenhofen, Germany) was administered at a dose of 50 mg/kg body weight at a concentration of 10 mg/ml in sterile 0.9% NaCl solution via intraperitoneal injections twice daily after cerebral ischemia for 3 days or 7 days (Figure 9). For the indicated mice, N-nitro-L-arginine methyl ester (L-NAME; Sigma) was added to the drinking water (daily dose, 50mg/kg; concentration in drinking water, 1.5 mg/mL) to inhibit NOS starting from the beginning of the experiments. Inhibition of neovascularization was achieved by administering endostatin (10 µg; subcutaneously; Calbiochem-Novabiochem, Darmstadt, Germany) once daily for 14 days starting from MCAo.

3.3. Physical Exercise

Some animals were housed in polyethylene cages that were equipped with stainless steel running wheels (24 cm diameter, Bewegungskäfig, Tecniplast, Hohenpeissenberg, Germany) equipped with a counter. Mice ran voluntarily and predominantly at night. The mean running distance was 4,300 ± 200m/24 hours.

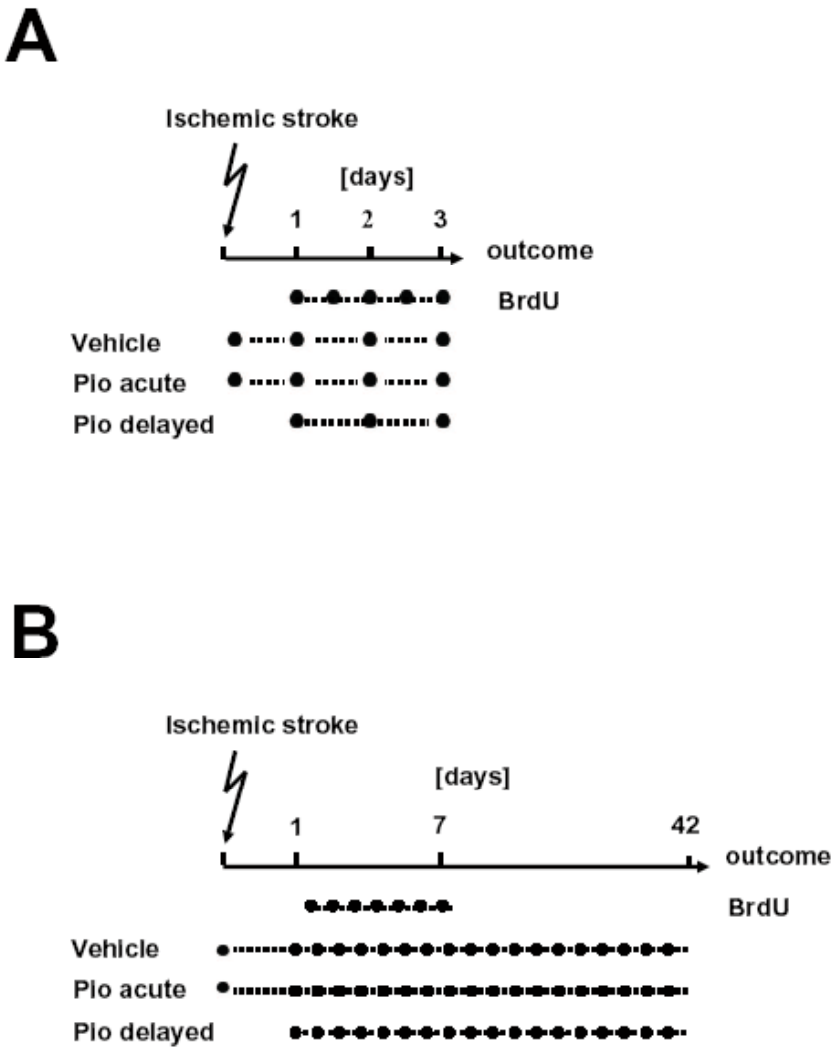


Figure 9. Animals and drug administration
A. 3 days short-term survival paradigm. Here, BrdU was injected twice daily from day 1 to day 3. B. 6 weeks survival paradigm. BrdU was injected twice daily from day 1 to day 7. Experimental groups were: (i) sham + vehicle; (ii) sham + 'pioglitazone acute' (first injection of pioglitazone on the same day as sham operation); (iii) MCAo + vehicle; (iv) MCAo + 'pioglitazone acute' (first injection of pioglitazone on the same day as MCAo); (v) MCAo + 'pioglitazone delayed' (first injection of pioglitazone 24 hours after MCAo).

3.4. Microglial cell culture

Microglial cultures were prepared from brains of newborn NMRI mice, using techniques as described previously (Prinz, et al., 1999). Cultures typically contain > 95 % of microglial cells, as validated with Griffonia simplicifolia isolectin B4 (Sigma, Deisenhofen, Germany),

a specific marker for microglia. Cultures were used for experiments 1 to 5 d after plating. In brief, the forebrain was carefully freed of blood vessels and meninges. Cortical tissue was trypsinized for 2 min, dissociated with a fire-polished pipette and washed twice. Mixed glial cells were cultured for 9 to 12 days in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin, with medium changes every third day. Microglial cells were then separated from the underlying astrocytic layer by gentle shaking of the flask for one hour at 37 °C in a shaker-incubator (100 rpm). The cells were used in microchemotaxis assay.

3.5. Microchemotaxis assay

Cell migration assays in response to ATP (300 µM) and pioglitazone were performed in 48-well microchemotaxis chambers (Neutroprobe, Bethesda, MD) (Nolte, et al., 1996). For co-stimulation experiments, we stimulated microglia with ATP (300 µM) in combination with different concentrations of pioglitazone. DMEM alone was used as a negative control, and 300 µM ATP (Sigma, Deisenhofen, Germany) as positive control. Upper and lower wells were separated by polycarbonate filter (5 µM pore size; Poretics, Livermore, CA). Microglial cells (4×10^4) in 50 µl serum-free DMEM medium were added to the upper wells, and the chamber was incubated at 37 °C and 5 % CO_2 for 180 min. Rate of microglial migration was calculated by counting cells in four random fields of each well using a 40 x bright field objective. The induced migration was normalized to the migration rate under control or basal conditions.

3.6. Cresyl violet assay

Microglial cell proliferation was determined by using Cresyl Violet assay. Microglia cells (plated in 96 well plates, 50000 cells per well) were treated for 24 hours with pioglitazone (5 and 10 µM) at 37°C under 5% CO_2. Microglial proliferation was determined by the crystal violet assay as described in Wosikowski et al. (Wosikowski, et al., 1993)

3.7. Model of middle cerebral artery occlusion

Mice were anesthetized for induction with 1.5% isoflurane and maintained in 1.0% isoflurane in 69% N_2O and 30% O_2 using a vaporizer. Ischemia experiments were essentially performed as described previously (Endres, et al., 1999, Endres, et al., 2000). In brief, brain ischemia was induced with an 8.0 nylon monofilament coated with a silicone resin/hardener mixture (Xantopren M Mucosa and Activator NF Optosil Xantopren, Haereus Kulzer, Germany). The filament was introduced into the left common carotid artery up to the anterior cerebral artery. Thereby the middle cerebral artery and anterior choroidal arteries were occluded (Figure 10). Filaments were withdrawn after 30 min to allow reperfusion. Core temperature during the experiment was maintained at 36.5°C ± 0.5°C with a feed-back temperature control unit and the same temperature was kept until 2 h after reperfusion using a heating pad. After a survival time ranging from 3 days to 6 weeks, animals were killed for histological analysis.

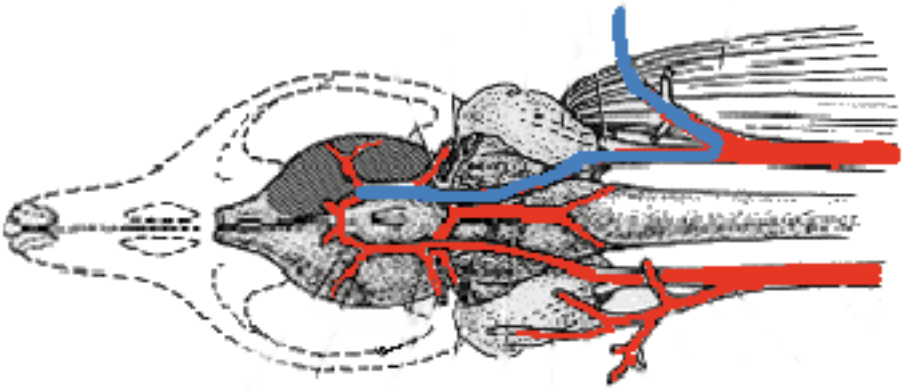


Figure 10. A basal view of mice brain vessels.
Red indicates arteries and blue indicates microfilament.

3.8. Physiologic parameters

In randomly selected animals, the left femoral artery was cannulated with a PE 10 catheter for blood pressure, heart rate and blood gas determination as described previously (Endres, et al., 1998, Endres, et al., 2004). Arterial blood samples were analyzed for pH, arterial oxygen pressures, and partial pressures of carbon dioxide.

3.9. Determination of lesion size

At 3 days or 6 weeks after brain ischemia, animals were deeply anesthetized by isoflurane anesthesia (4%) and decapitated. Brains were snap-frozen in isopentane on dry ice and stored at -80°C until further use. Cerebral infarct sizes were determined on hematoxylin and eosin-stained (H&E staining) 20 µm cryostat sections with an image analysis system (SigmaScan Pro 4.0; Jandel Scientific, Corte Madera, CA) and calculated by summing the infarct volumes of each section directly or indirectly using the following formula: contralateral hemisphere (mm^3) - undamaged ipsilateral hemisphere (mm^3) (Swanson, et al., 1990). Differences between direct and indirect infarct size are likely to be accounted for by brain swelling.

3.10. Regional absolute cerebral blood flow (rCBF) measurements

rCBF was measured with the ^{14}C-iodoantipyrine technique under etomidate anesthesia (0.03 mg/kg body wt/min; Etomidate Lipuro®, Braun, Melsungen, Germany) as previously described (Jay, et al., 1988, Yamada, et al., 2000). Mice were infused with ^{14}C-iodoantipyrine (125 µCi/kg body wt in 100µl saline) through the left femoral vein via a pump at a progressively increasing infusion rate during a 1-minute period. Arterial blood samples from the catheterized femoral artery were collected onto the pre-weighed filter paper disks. After 1 min of infusion, animals were decapitated, brains quickly removed and snap frozen in -45°C cold 2-methylbutane. The concentration of radioactivity in the timed blood samples was maintained by liquid scintillation counting and calculated after the weight of the samples to volume was converted (density of the blood set as 1.05 g/ml). Frozen brains were cut into 10-µm coronal cryostat sections, thaw-mounted on glass cover-slips, immediately dried on a hot plate (60°C) and exposed to X-ray film (Kodak Min-R MRDM1) for 14 days together with a set of calibrated ^{14}C-methyl metacrylate standards (Amersham). Optical densities were measured with an image analyzing system (MCID Elite™, Imaging Research, Canada) and the set of co-exposed calibrated radioactive standards were used to convert to tissue concentrations. CBF calculations were performed using the Sakurada equation including corrections for catheter sampling time lag and catheter washout. A brain-blood partition coefficient of 0.7 was used for the final calculations of CBF.

3.11. Immunohistochemistry

Mice were killed with an overdose of pentobarbital and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Brains were stored in the fixative for 48 h and then transferred into 30% sucrose in 0.1 mol/L phosphate buffer for 24 h. Coronal sections of 40 µm thickness were cut from a dry ice-cooled block on a sliding microtome (Leica, Bensheim, Germany). Sections were stored at -20°C in cryoprotectant solution containing 25% ethylene glycol, 25% glycerin, and 0.05 mol/L phosphate buffer. Sections were stained free floating with antibodies diluted in Tris-buffered saline containing 3% donkey serum and 0.1% Triton X-100 (Gertz, et al., 2006, Katchanov, et al., 2001). Primary antibodies were rabbit polyclonal against Iba1 (1:500; Wako, Neuss, Germany), goat anti-Doublecortin (1:200; Abcam), mouse anti-NeuN (Chemicon) and rat anti-BrdU (1:500, Harlan Seralab). Brain tissue was prepared for BrdU immunohistochemistry as described previously (Kronenberg, et al., 2003). Immunohistochemistry followed the peroxidase method with biotinylated secondary antibodies raised in donkey (Jackson ImmunoResearch Laboratories, West Grove, PA), 1-500, ABC Elite reagent (Vector Laboratories, Burlingame, CA), and diaminobenzidine (Sigma) as chromogen.

3.12. Cell counting

Densities of Iba1+ and Brdu+ cells in ischemic striatum were quantified by circling the ischemic striatum in a reference section at approximately bregma 1.32 and bregma 0.14 using StereoInvestigator Software (Microbrightfield Europe, Magdeburg, Germany). All cells within the ischemic striatum were marked and counted and this cell count was then divided by the corresponding volume of tissue. StereoInvestigator was also used to quantify Brdu+/DCX cells on cryostat sections in SVZ at approximately bregma 1.32 and bregma 0.14 and SGZ at approximately bregma -1.90.

3.13. Behavior tests

Wire hanging

Balance and grip strength were assessed on the Wire Hanging test. The test apparatus consisted of a steel wire (1mm) that was stretched between two posts 60 cm above a foam pillow as described (Figure 11A) (Gertz, et al., 2006). For two days prior to actual testing, mice were trained to cling to the wire with their forepaws. On the actual day of testing, latency to fall was tested twice (maximum: 3 minutes) and results averaged.

Pole test

The pole test was adapted from a previously published protocol (Matsuura, et al., 1997) with minor modifications. A vertical steel pole was covered with tape (Durapore) to create a rough surface. The animal was placed head upward near the top of the pole. The time taken to turn completely downward (t turn) and the total time to reach the floor with all four paws (t floor) were recorded (Figure 11B). If the animal was unable to turn completely, the time to reach the floor was also attributed to t turn. Each animal was tested on 5 trials and the average score was taken as the final pole test score.

Morris water maze test

Experiments were performed as described with minor modifications (Bert, et al., 2002, Harker and Whishaw, 2002, Winter, et al., 2004). A 120-cm-diameter, 60-cm-high circular swimming pool was filled to a depth of 32 cm with 20°C opaque water. Visible cues were placed on the walls of the pool and remained on their fixed position throughout the whole experiment. A clear Plexiglas platform with a diameter of 11cm was submerged 1cm below the water level. A full experiment had two phases; a place task (learning period) with 3 trials per day for 7 consecutive days and a probe trial (spatial probe) on day 8. For the place task the platform was placed near the centre of a quadrant and mice were released into the water from 1 of the 3 remaining quadrants to search for the platform. If after 90 seconds an animal did not reach the platform, it was guided to the platform. After reaching the platform, animals were allowed to remain there for 30 seconds. The intertrial interval was 1.5 minutes, and mice were dried with a towel and put under a heating lamp between each trial to avoid hypothermia. Latencies, path lengths, and swim speeds for a single day were averaged to a come up with a daily mean. In the probe trial, mice were allowed to swim freely for 90 seconds in the absence of the platform. The time spent in

each quadrant and crosses through the location of the former platform were measured. A computer-based system (TSE Systems) was used to track the swimming performance of the animals (Figure 11C).

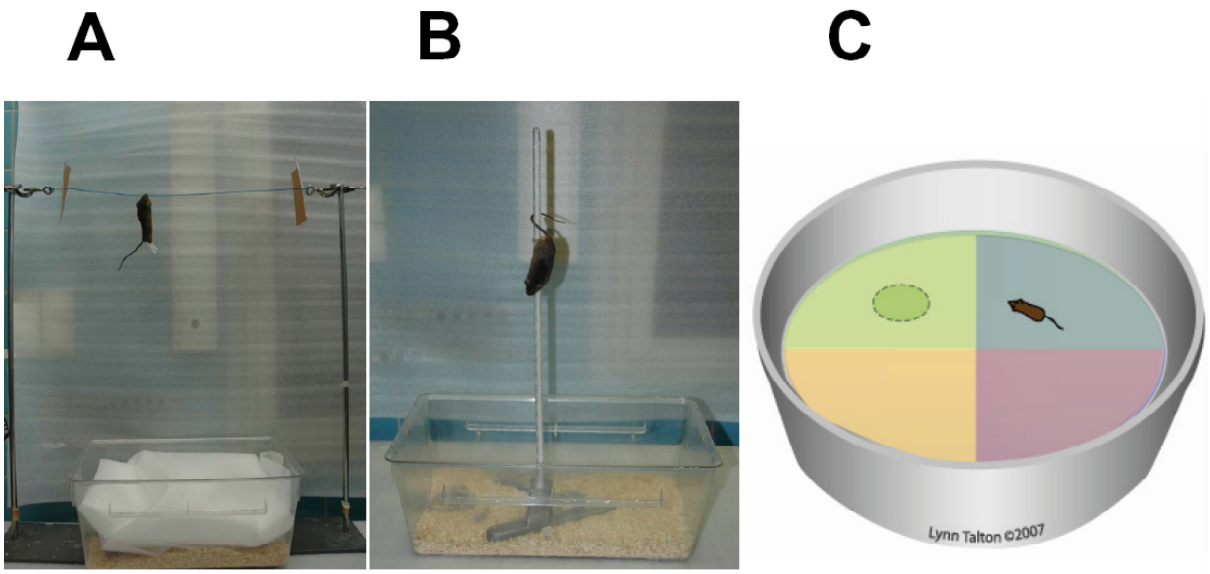


Figure 11. Behavior tests pictures
A. wire hanging. B. pole test. C. water maze.

3.14. Statistical Analysis

Data are presented as mean ± SEM. Differences between groups were evaluated by one-way ANOVA followed either by post hoc testing as indicated or by Student´s t-test. N>=7 animals per group. P-values of <0.05 were considered statistically significant.

4. Results

4.1. Acute neuroprotection by pioglitazone after mild brain ischemia without effect on long-term outcome

4.1.1. Pioglitazone attenuates ATP induced microglial migration

To analyze the effect of pioglitazone on microglial migration, we used the Boyden Chamber microchemotaxis model. Here, we stimulated primary mouse microglial cells with pioglitazone (1, 10 µM) alone and in combination with the chemoattractant agent ATP (300 µM). Pioglitazone did not exert a significant effect on constitutive microglial migration either at 1 µM (to 93.89 ± 9.01 %; n=5) or at 10 µM (to 112.15 ± 12.29 %; n=5) as compared to the control (medium only, 100 %). Interestingly, pioglitazone attenuated the ATP-induced migration rate significantly at 10 µM (to 222.52 ± 49.23 %; n=6), but not at 1 µM (289.22 % ± 61.52; n=6) relative to ATP alone (356.44 ± 55.44 %; n=6) (Figure 12 A and B).

4.1.2. Pioglitazone attenuates microglial proliferation

To analyze the functional relevance of pioglitazone on microglial proliferation, we stimulated primary microglial cells with pioglitazone (1 and 10 µM) for 24 hours and analyzed the proliferation rate by using cresyl violet staining. We found that pioglitazone attenuates microglial proliferation slightly but significantly at 10 µM (to 91.42 % ± 1.34; n = 3), but not at 1 µM (99.03 % ± 1.78, n = 3) as compared to control (DMEM/10% FCS, 100 %) (Figure 12C).

4.1.3. Pioglitazone protects against mild transient brain ischemia/reperfusion

Adult male 129/SV mice were subjected to 30 min occlusion of the left middle cerebral artery (MCAo) followed by reperfusion and treated with pioglitazone (20 mg/kg body weight) or vehicle. Treatment was continued for an additional 3 days or 6 weeks after MCAo. At three days after MCAo/reperfusion, lesion volume as assessed by H&E staining was significantly reduced by 28% in the acutely pioglitazone-treated mice as compared to vehicle injected animals ($F_{2,21}$=5.4, p=0.01, 1-β=0.79; post hoc vehicle versus acute treatment with pioglitazone: p=0.004; post hoc vehicle versus delayed treatment with pioglitazone: p=0.06) (Figures 13 A and B). At 6 weeks, lesion size was evaluated

immunohistochemically using NeuN staining. Long-term pioglitazone treatment did not result in significant differences in lesion size between controls and the pioglitazone-treated groups ($F_{2,25}$=1.8, p=0.19, 1-β=0.33) (Figures 14 A and B).

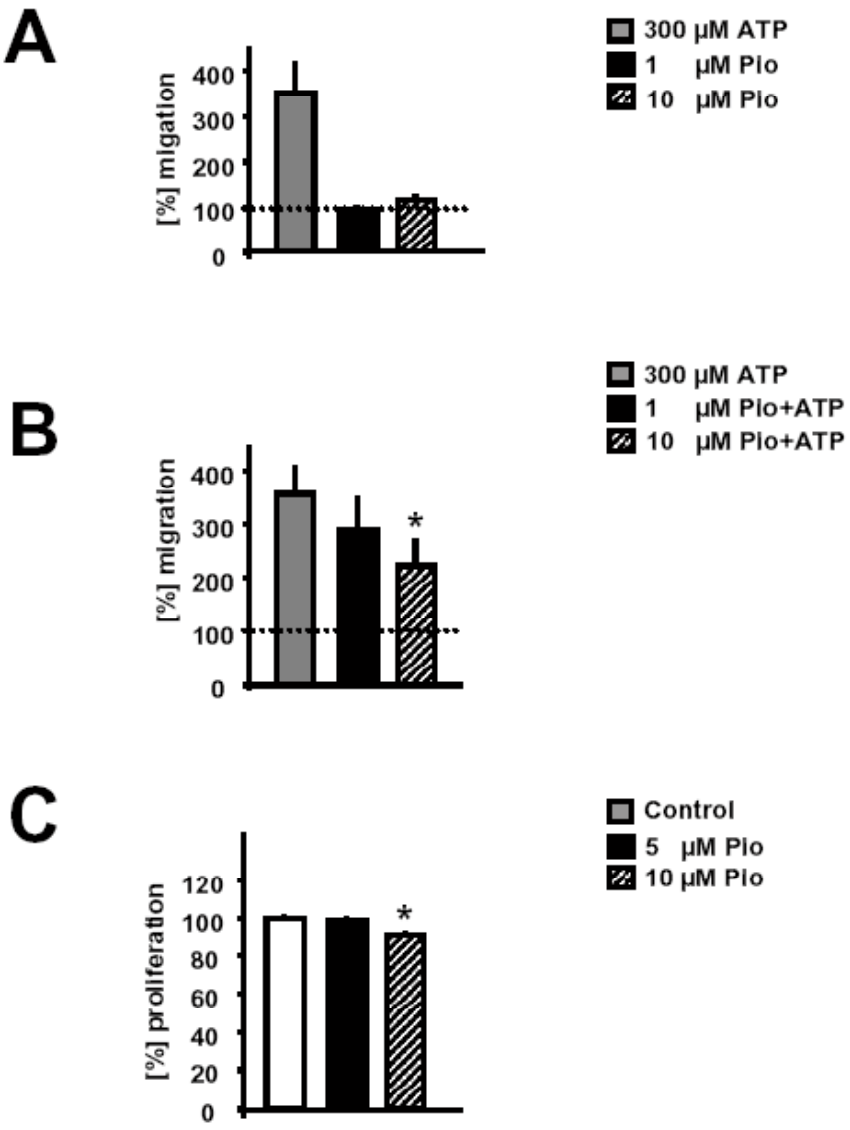


Figure 12. Pioglitazone attenuates migration and proliferation of primary mouse microglia

Microglial migration was measured in a Boyden chamber chemotaxis assay in response to increasing concentrations of pioglitazone alone (1 and 10 µM) (A) and in combination with ATP (300 µM) (B) over a time period of 3 hours. The migration rate was normalized to basal migration (DMEM only, 100 %) as indicated by the dotted line. The graph shows the mean migration rate ± SEM summarized from 5-6 independent experiments. C. Microglia were exposed to pioglitazone (5 and 10 µM) for 24 hours. Proliferation was determined by using cresyl violet assay. The proliferation rate was standardized on DMEM/10 % FCS treated cells (ctl; 100 %). Data are mean + SEM summarized from 3 independent experiments. *p<0.05 versus control.

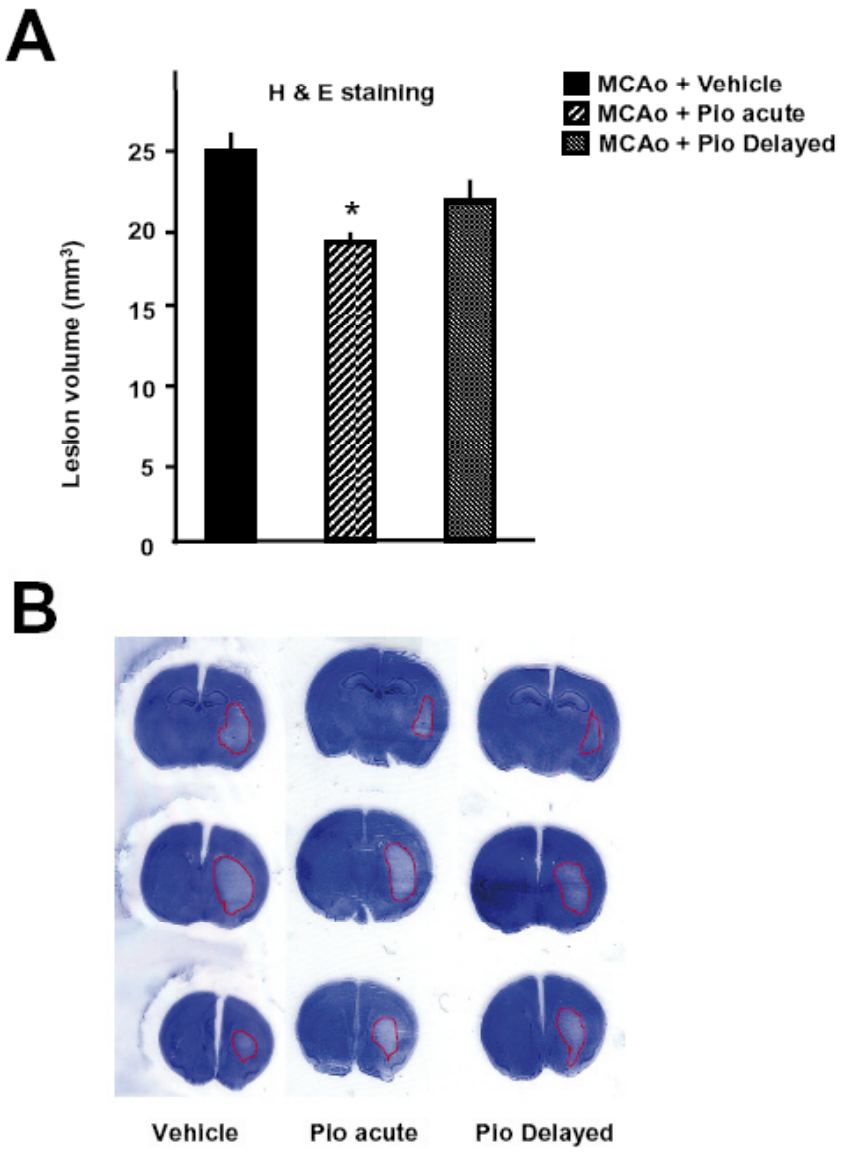


Figure 13. Pioglitazone confers acute neuroprotection after mild transient brain ischemia.

129/SV mice were subjected to 30 min filamentous MCAo/reperfusion or sham operation. A. In the short-term survival paradigm (3 days), cerebral lesion volumes were determined on five 20µm coronal brain sections by computer-assisted volumetry. The 'pioglitazone acute' treated group showed significantly decreased lesion volumes as compared to controls. *p<0.05 versus control. B. Representative examples of H&E-stained 20 µm coronal brain sections in the short-term survival paradigm.

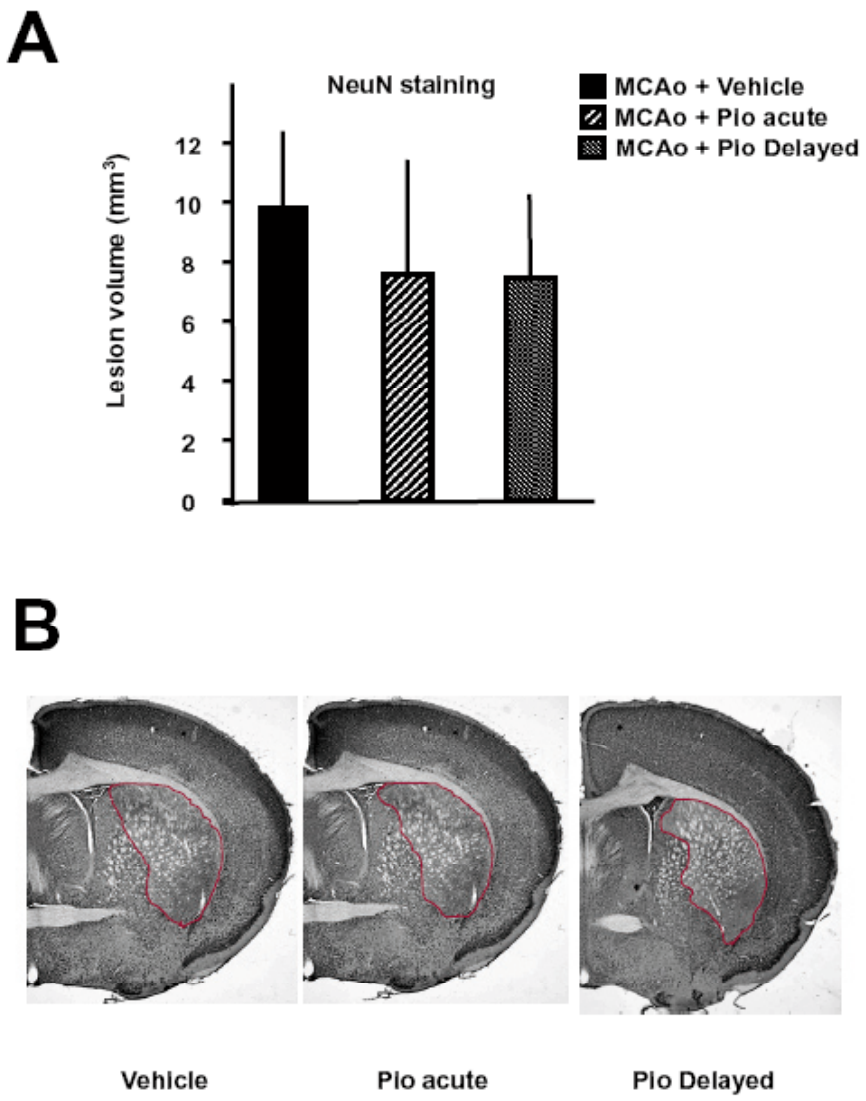


Figure 14. Pioglitazone does not lead to reduced lesion size at 6 weeks after mild transient brain ischemia

129/SV mice were subjected to 30 min filamentous MCAo/reperfusion. A. In the 6-weeks survival paradigm, cerebral lesion volumes were determined on 40 µm coronal brain sections by NeuN immunohistochemistry. There was no significant effect of pioglitazone treatment. B. Representative examples of 40 µm coronal brain sections stained for neuronal marker NeuN in the 6-week survival paradigm.

4.1.4. Effects of pioglitazone treatment on histological outcome after transient brain ischemia

Iba1+ cells in striatum

Iba1 staining was used to label activated microglia. At 3 days after MCAo/reperfusion, both pioglitazone groups (acutely treated animals and animals, whose treatment was begun at 24 hours after MCAo/reperfusion) displayed significantly decreased Iba1+ cell counts in the ischemic striatum as compared to vehicle-treated animals

($F_{4,33}$=38.2, p<0.0001, 1-β=1; post hoc vehicle versus acute treatment with pioglitazone after MCAo: p=0.008; post hoc vehicle versus delayed treatment with pioglitazone after MCAo: p=0.0001) (Figure 15A). By contrast, in the long-term groups, we only detected a difference between sham and MCAo groups ($F_{4,40}$=9.6, p<0.0001, 1-β=1). Pioglitazone did not exert a significant effect within the MCAo groups (post hoc vehicle versus acute treatment with pioglitazone: p=0.29; post hoc vehicle versus delayed treatment with pioglitazone: p=1.0) (Figure 15B).

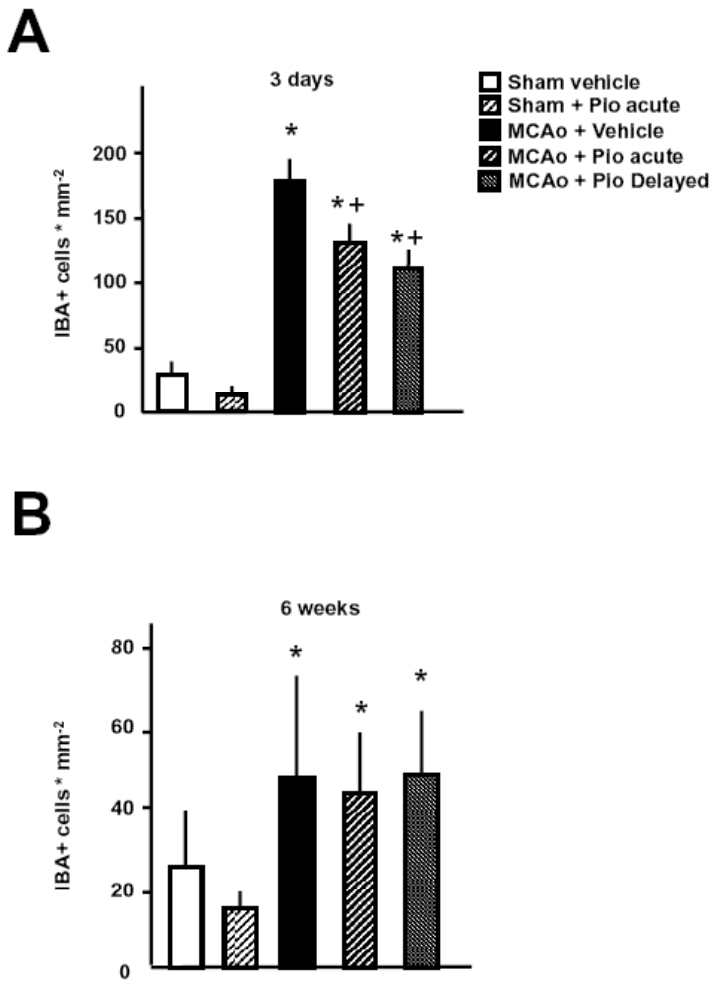


Figure 15. Pioglitazone treatment and Iba1+ cells in striatum

129/SV mice were subjected to 30 min MCAo/reperfusion or sham-operation. Iba1+ cells were identified by Iba1-immunohistochemistry. A. In the 3 days survival paradigm, Iba1+ cells in the ischemic striatum were quantified on 20 µm coronal brain sections in two reference sections at approximately bregma 1.32 and bregma 0.14, respectively, using StereoInvestigator® Software. Sham groups showed significantly reduced Iba1 counts as compared to all MCAo groups. Within the MCAo groups, pioglitazone-treated animals showed significantly decreased numbers of Iba1+ cells as compared to the vehicle group. +p<0.05 compared to 'MCAo vehicle'; *p<0.05 compared to 'sham vehicle'. B. In the 6-weeks long-term survival paradigm, Iba1+ cells in ischemic striatum were quantified in 40 µm coronal brain sections in reference sections at approximately bregma 1.32 and bregma 0.14, respectively, using StereoInvestigator® Software. *p<0.05 compared to sham vehicle.

BrdU+ cells in striatum

We labelled newly generated cells with intraperitoneal BrdU twice daily following cerebral ischemia. At 3 days, as compared to vehicle-treated animals, the number of newly-generated cells in the stroke area was significantly increased in acutely pioglitazone-treated mice (Figure 16A). In the long-term survival groups, BrdU was administered for a period of 7 days. We again detected robust differences between sham and MCAo groups. Within the MCAo groups, both the pioglitazone acute and pioglitazone delayed treatment conditions showed significantly decreased BrdU+ cell densitities in the ischemic striatum relative to the vehicle group (Figure 16B).

4.1.5. Behavioral outcome

In the long-term survival groups we assessed complex behavioral outcomes with tests for overall motor function, spatial learning and memory.

In the pole test, mice from the ‘MCAo vehicle’ group needed more time to completely turn (t turn) and to reach the floor (t floor) as compared to mice from the sham-operated groups. Pioglitazone treatment did not have any significant effects on these parameters either in the MCAo or in the sham groups (time to turn: $F_{4,41}$=3.1, p=0.02, 1-β=0.77; post hoc ‘sham vehicle’ versus ‘MCAo vehicle’ p=0.009; post hoc ‘MCAo vehicle’ versus acute pioglitazone treatment after MCAo: p=0.057; ‘MCAo vehicle’ versus delayed pioglitazone treatment after MCAo: p=0.22; time to reach the floor: $F_{4,41}$=3.3, p=0.02, 1-β=0.79; post hoc ‘sham vehicle’ versus ‘MCAo vehicle’: p=0.01; post hoc ‘MCAo vehicle’ versus acute pioglitazone treatment after MCAo: p=0.22; ‘MCAo vehicle’ versus delayed pioglitazone treatment after MCAo: p=0.89) (Figure 17 A and B). Similarly, the wire hanging test also revealed significant differences between MCAo and sham groups with no additional effects of pioglitazone treatment ($F_{4,41}$=4.0, p=0.009, 1-β=0.88; post hoc ‘sham vehicle’ versus ‘MCAo vehicle’: p=0.008; ‘MCAo vehicle’ versus acute pioglitazone treatment after MCAo: p=0.85; ‘MCAo vehicle’ versus delayed pioglitazone treatment after MCAo: p=0.61) (Figure 17C).

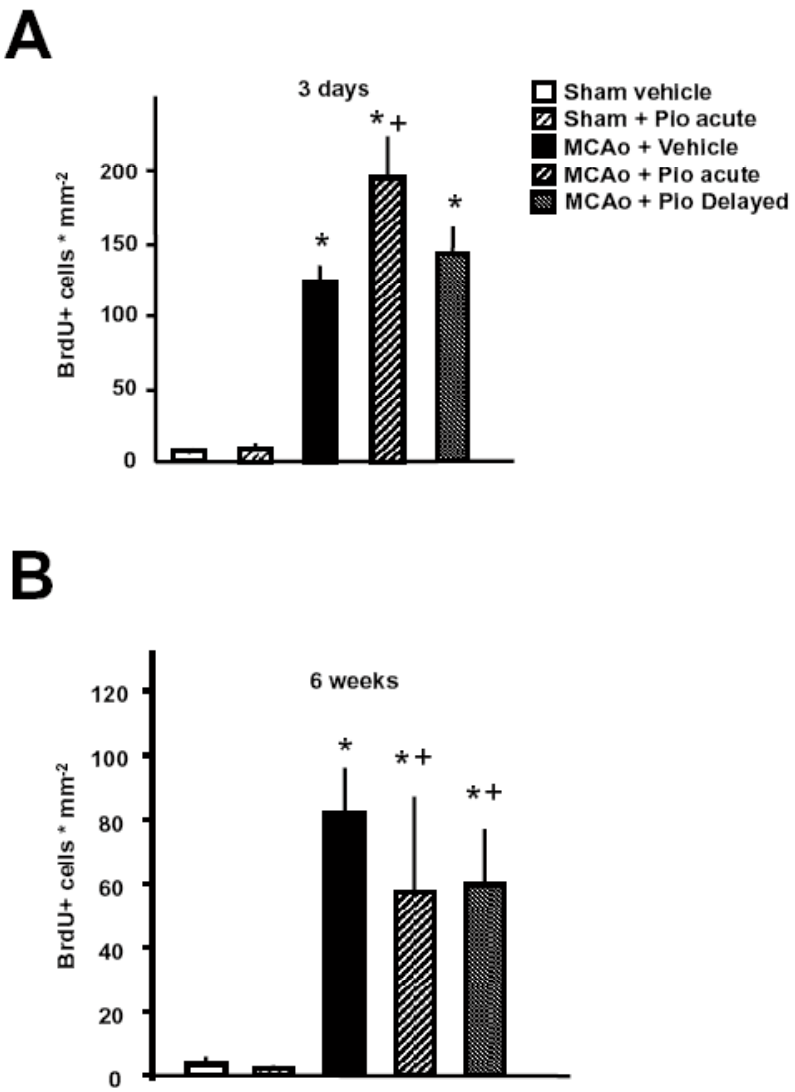


Figure 16. Pioglitazone treatment and BrdU+ cells in striatum

129/SV mice were subjected to 30 min MCAo/reperfusion or sham-operation. BrdU+ cells were identified by BrdU-immunohistochemistry. A. In the 3 days survival paradigm, BrdU+ cells in the ischemic striatum were quantified on 20 µm coronal brain sections in two reference sections at approximately bregma 1.32 and bregma 0.14, respectively, using StereoInvestigator® Software. Sham groups showed significantly reduced BrdU counts as compared to all MCAo groups. Within the MCAo groups, pioglitazone acutely treated animals showed significantly increased density of BrdU+ cells as compared to the vehicle group. +p<0.05 compared to 'MCAo vehicle'; *p<0.05 compared to 'sham vehicle'. B. In the 6-weeks long-term survival paradigm, BrdU+ cells in ischemic striatum were quantified in 40 µm coronal brain sections in reference sections at approximately bregma 1.32 and bregma 0.14, respectively, using StereoInvestigator® Software. Sham groups showed significantly reduced BrdU counts as compared to all MCAo groups. Within the MCAo groups, pioglitazone-treated animals showed significantly decreased numbers of BrdU+ cells as compared to the vehicle group. +p<0.05 compared to 'MCAo vehicle'; *p<0.05 compared to 'sham vehicle'.

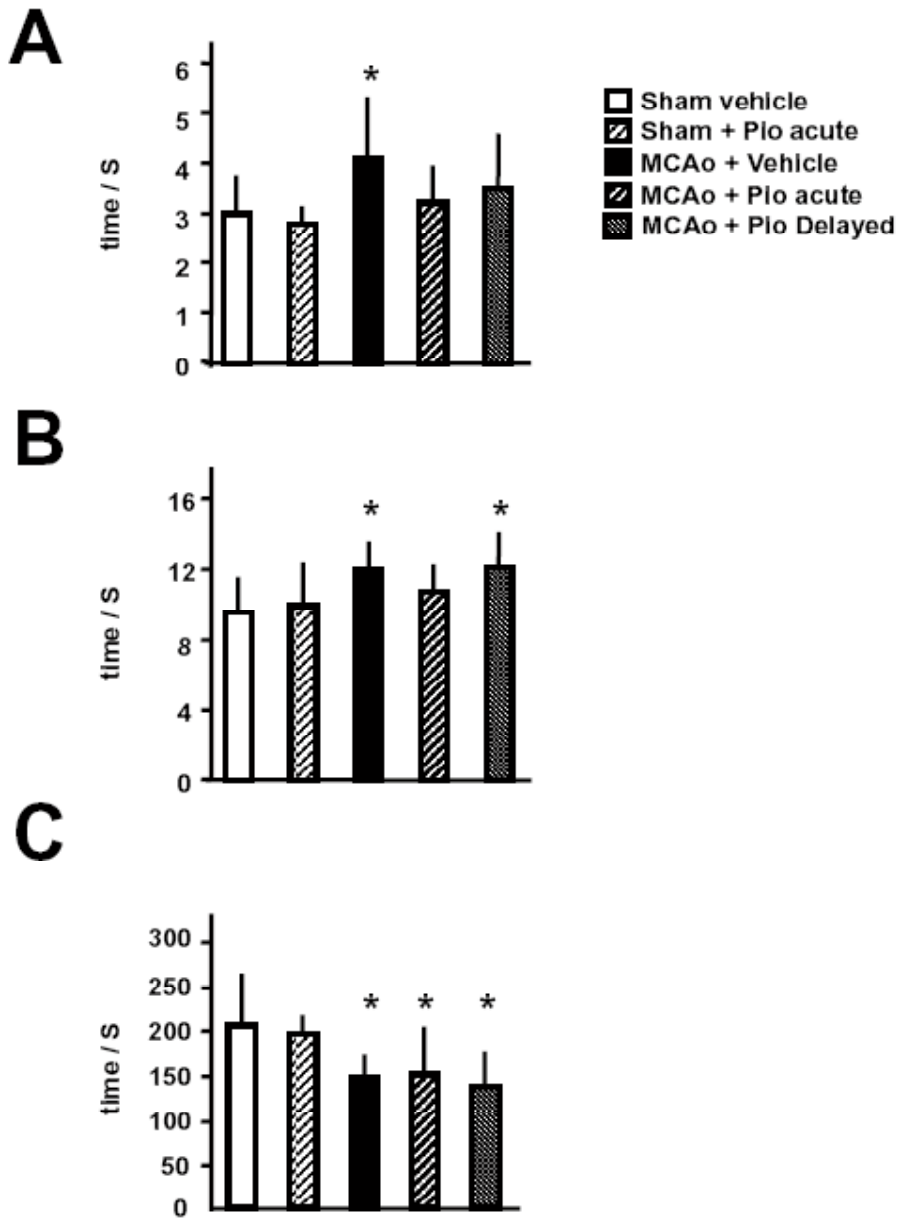


Figure 17. Pole test and wire hanging test

A. Time to turn the head downwards (s): MCAo animals took significantly more time to turn their head downwards on the pole (*p<0.05 versus sham vehicle) as compared to the sham-operated groups (t turn). No significant effect of treatment with pioglitazone. B. Time to reach the ground (s). No significant effect of pioglitazone treatment was observed. (*p<0.05 versus 'sham vehicle') C. Time to stay hanging on the wire (s): MCAo significantly decreased the amount of time that animals clung to the wire (*p<0.05 as compared to 'sham vehicle'). Pioglitazone treatment did not cause any significant difference among MCAo groups.

In the place task of the Morris Water maze, we observed a significant effect of time (i.e. day of testing) on escape latency ($F_{6,234}$=143.4, p<0.0001, 1-β=1) and distance ($F_{6,234}$=158.8, p<0.0001, 1-β=1). However, there was no significant difference between animals that had undergone MCAo as compared to sham-operated animals (repeated measures ANOVA for escape latency: $F_{1,42}$=0.8, p=0.37, 1-β=0.14; for distance to find the hidden platform: $F_{1,42}$=0.11; p=0.74; 1-β=0.06). Similarly, we did not detect significant differences between the five experimental groups (repeated measures ANOVA for escape latency: $F_{4,39}$=1.8, p=0.16, 1-β=0.48; distance to find the hidden platform: $F_{4,39}$=1.1, p=0.39, 1-β=0.3) (Figures 18A and B).

In the probe trial, there was also no difference between groups in terms of escape latency ($F_{4,39}$=0.6, p=0.64, 1-β=0.2) or quadrant preference (Figures 19A). Finally, pioglitazone treatment did not affect the number of platform crosses in the probe trial ($F_{4,39}$=2.8, p=0.04, 1-β=0.7; post hoc ‘sham vehicle’ versus ‘MCAo vehicle’: p=0.02; ‘MCAo vehicle’ versus acute treatment with pioglitazone after MCAo: p=0.70; ‘MCAo vehicle’ versus delayed pioglitazone treatment after MCAo: p=0.68; Figure 19B).

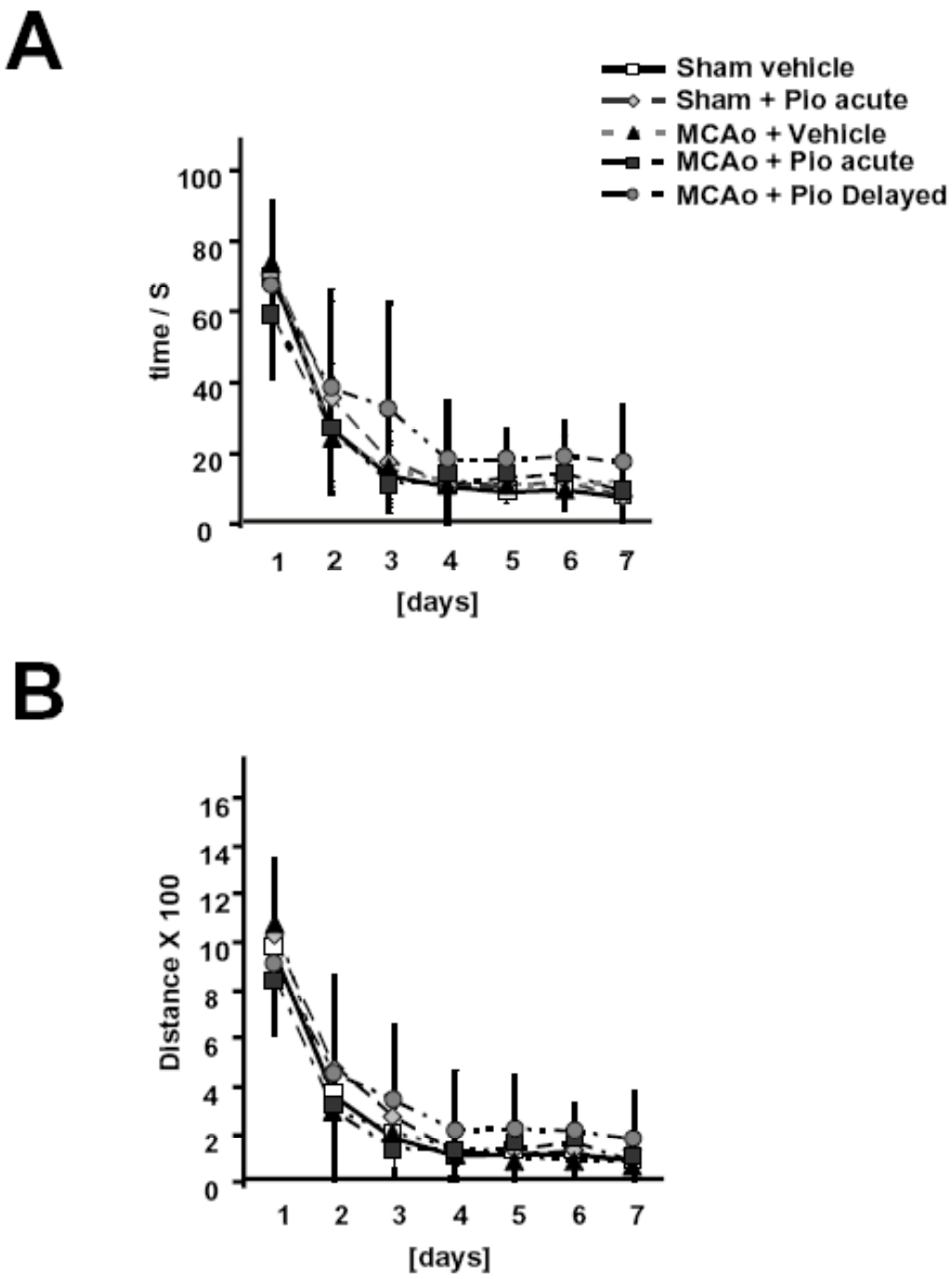


Figure 18. Place task of Morris water maze testing.

A. Latency (s) to find the hidden platform. There was no significant effect of MCAo or pioglitazone treatment in any of the groups. B. Distance (cm) travelled to reach the hidden platform. No significant effect of either MCAo or pioglitazone was observed in any of the groups.

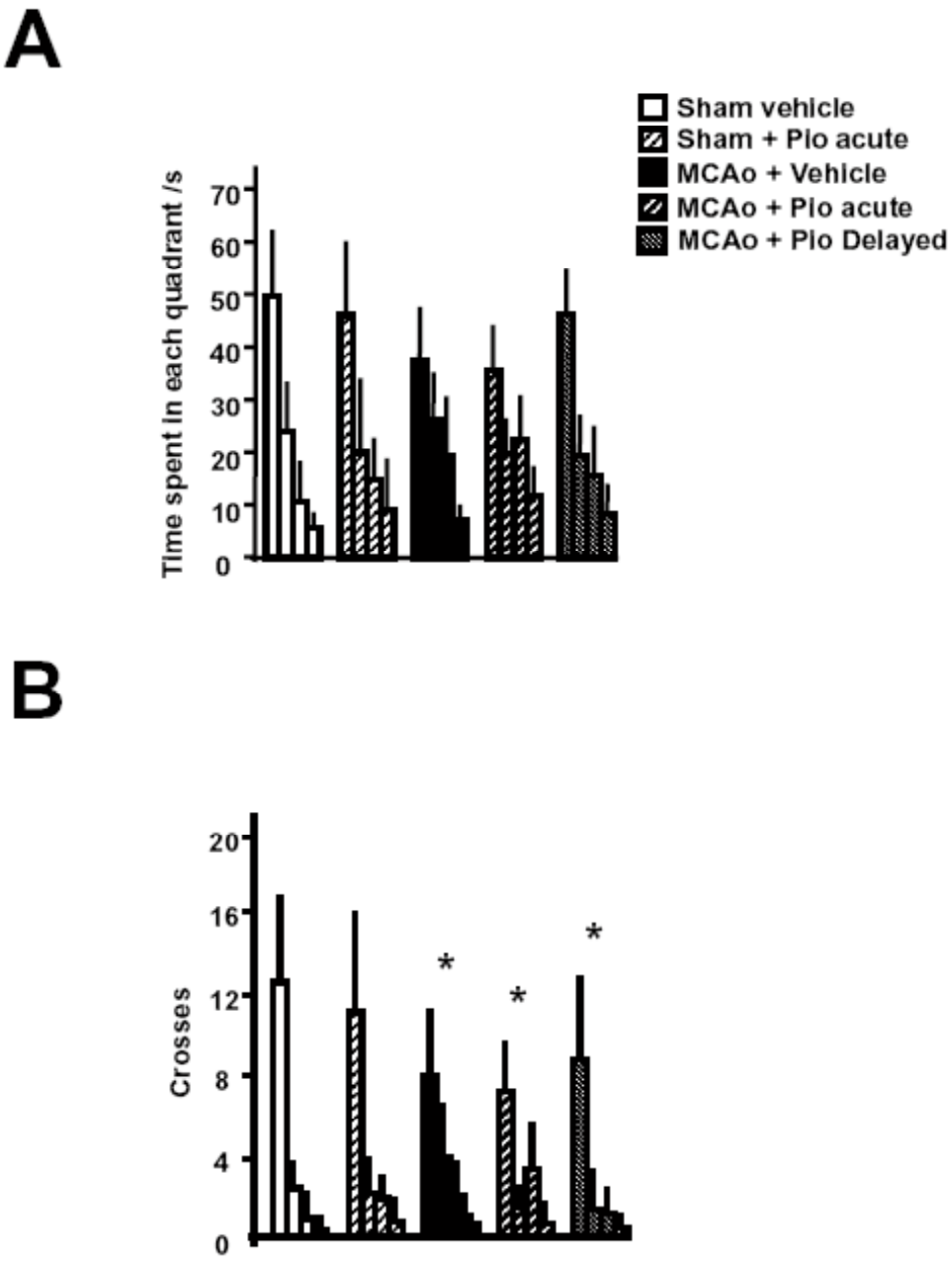


Figure 19. Probe trial of Morris water maze testing.

A. Average time (s) spent in each quadrant. There was no significant effect of MCAo or pioglitazone treatment across experimental groups. B. Number of crosses through the area of the former platform. MCAo animals made significantly fewer crosses (*$p<0.05$ versus sham vehicle). Pioglitazone treatment did not affect the number of platform crosses.

4.2. Physical activity protects from the late sequelae of stroke via up regulation of endothelial nitric oxide synthase and enhanced angiogenesis

4.2.1. Physical activity confers long-term protection after mild stroke, both NOS inhibitor L-NAME and angiogenesis inhibitor endostatin abolish the stroke-protective effects of exercise

Adult male 129/SV wild-type mice were subjected to 3 weeks of physical exercise in cages equipped with running wheels while sedentary life style mice were put in standard cages as controls. After 3 weeks, animals were exposed to a 30 min occlusion of the left middle cerebral artery (MCA) followed by reperfusion and put back to their respective home cages. Four weeks after MCAo/reperfusion, cerebral lesion size was reduced by 38% in the exercised group compared to the sedentary mice. To test our hypothesis that eNOS mediates the stroke protective effects of physical activity, similar experiments were performed on mice chronically treated with a NOS inhibitor. L-NAME, an inhibitor of NO synthase, was dissolved in the drinking water. Another group of animals received the anti-angiogenic agent, endostatin (10 µg), once daily for 14 days starting from day 1 after MCAo. Both L-NAME and endostatin completely abolished the protective effect of physical activity at 4 weeks (Figure 20).

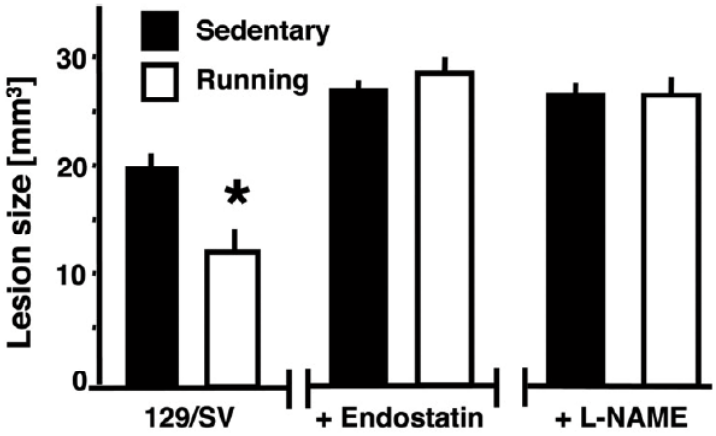


Figure 20. Physical activity confers long-term protection after mild stroke, both NOS inhibitor L-NAME and angiogenesis inhibitor endostatin abolish the stroke-protective effects of exercise

After 3 weeks of voluntary running or sedentary lifestyle, 129/SV mice were exposed to 30 minutes of MCAo/reperfusion or sham operation. Some animals received the angiogenesis inhibitor endostatin or the NOS inhibitor L-NAME for 14 days after MCAo. Four weeks later, cerebral lesion volumes were determined by computer-assisted volumetry. Mean±SEM; **P*<0.05 vs sedentary, Student's *t* test. n=8 to 10 animals per group for all experiments.

Physiologic parameters (i.e. blood pressure, blood gases) were measured in randomly selected animals at 4 weeks after the insult. No relevant differences were observed between 'exercise' and sedentary animals (Table 1).

PARAMETER	Sedentary	Exercise
MABP (mmHg)	123 ± 12	109 ± 5
Heart rate (n)	430 ± 11	426 ± 11
PH	7.31 ± 0.02	7.27 ± 0.1
$PaCO_2$ (mmHg)	50 ± 1	49 ± 3
PaO_2 (mmHg)	87 ± 9	88 ± 2

Table 1. Physiological parameters 4 weeks after MCAo/reperfusion in 129/SV mice: MABP (mean arterial blood pressure in mmHg) and heart rate (beats per min) were measured at four weeks after 30 min filamentous MCAo. Mean±SEM; n = 6 animals per group.

4.2.2. Physical activity augments absolute cerebral blood flow weeks after MCAo

Regular physical activity preserves regional absolute cerebral blood flow (rCBF) at the time of MCA occlusion compared to sedentary animals (Endres, et al., 2003). To test

(i) whether CBF is chronically compromised following mild stroke

(ii) whether physical exercise preserves or augments CBF levels compared to sedentary controls we measured absolute regional CBF (rCBF) using ^{14}C-iodoantipyrine tissue equilibration technique (Endres, et al., 2003, Jay, et al., 1988, Yamada, et al., 2000).

The first finding was that four weeks after MCAo absolute rCBF was significantly lower in the ischemic striatum compared to the contralateral side in sedentary mice. However, in exercised mice rCBF was significantly higher both in the ischemic and contralateral striatum compared to sedentary mice, and in exercised mice there were no statistically significant differences between the ischemic and contralateral striatum (Figure 21). In sham-operated mice, we detected no significant CBF alterations (data not shown).

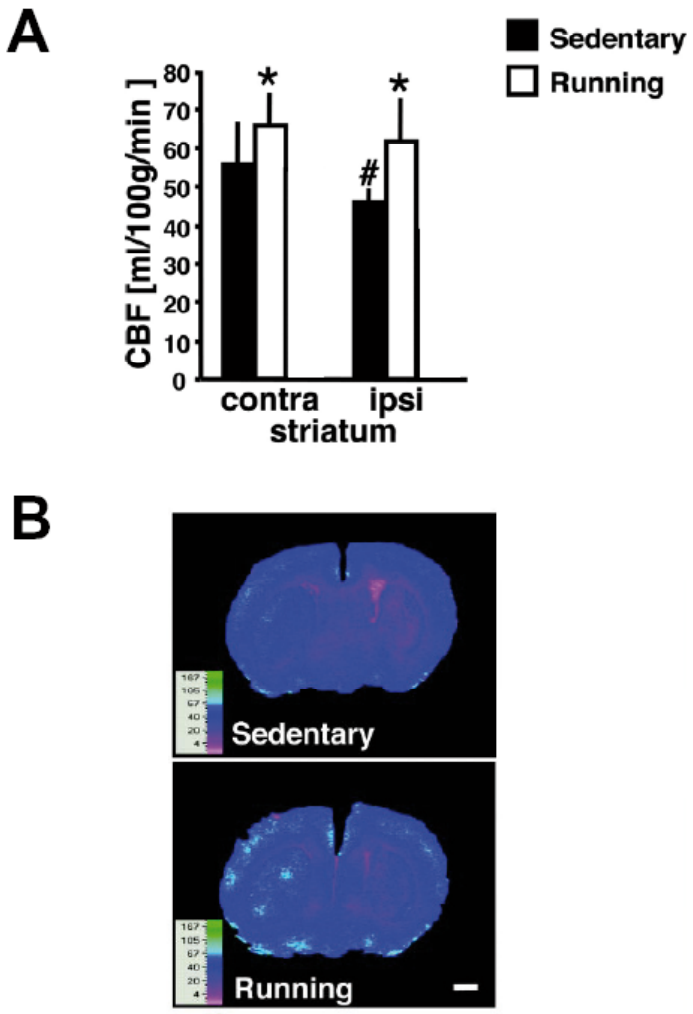


Figure 21. Physical exercise augments regional absolute cerebral blood flow weeks after ischemic stroke.
After 3 weeks of physical exercise or sedentary lifestyle, 129/SV mice were exposed to 30 minutes of MCAo/reperfusion or sham operation. Four weeks later, absolute CBF was determined in the striata of contralateral and ischemic hemispheres using ^{14}C-iodoantipyrine tissue equilibration technique. A. CBF levels (milliliters per 100 g per minute) and B. representative pseudocolored autoradiographic images of coronal brain sections. n=10 animals per group; *P<0.05 vs sedentary, #P<0.05 vs contralateral hemisphere. No significant CBF changes were detected in sham-operated animals (n=4). Scale bar=1 mm.

4.3. Effects of plant sterols on ischemic brain injury in wild-type mice

The sterol composition of the plant sterol–supplemented chow and commercially available spreads as quantitated by gas liquid chromatography are depicted in Table 2. SV/129 mice treated with normal chow (NC) or NC + plant sterol esters (PSE) for 4 weeks were subjected to 30 min of left filamentous MCA occlusion and 72 h of reperfusion. Figures

22 A and 22 B demonstrate that cerebral lesion size was significantly larger in the NC + PSE group compared with NC-fed group ($p < 0.05$) determined by an indirect method, which corrected for brain swelling. In addition, bigger lesion areas (direct measurement) were shown in standardized coronal brain sections (8 mm from frontal pole, $p < 0.01$) (Figure 22C). Mean arterial blood pressure, blood gas analysis (pH, PaO_2, $PaCO_2$) and rectal core temperature were not different between mice treated with NC or NC + PSE before or during cerebral ischemia (Table 3). The C57/Bl6 mouse strain weas subjected to ischemia/reperfusion with similar results.

Plant Sterol	Experimental Diet	Becel Pro-Activ
Sitosterol	46.2	46.7
Sitostanol*	2.3	2.2
Campesterol	25.3	26.2
Campestanol	0.6	0.6
Stigmasterol	19.1	19.4
Brassicasterol	1.2	2.4
Other plant sterols	4.9	2.5

Table 2. Sterol composition of plant sterol ester supplements compared with sterol composition of commercially available spread

Percent of total sterols.

* Sitostanol + Delta-5-avanasterol.

PARAMETER	NC	NC+PSE
MABP (mmHg)		
Before	86 ± 3	89 ± 7
during	103± 7	108± 9
PH		
Before	7.36 ± 0.03	7.33 ± 0.05
during	7.29 ± 0.06	7.28 ± 0.03
$PaCO_2$ (mmHg)		
Before	46 ± 5	46 ± 8
during	46 ± 2	47 ± 7
PaO_2 (mmHg)		
Before	99 ± 7	103 ± 8
during	93 ± 10	95 ± 3

Table 3. Physiologic Parameters During Cerebral Ischemia

129/SV male wildtype mice were fed with normal chow (NC) or with NC supplemented with 2% plant sterol esters (PSE). Animals were then subjected to 30 min MCAo followed by reperfusion. Mean arterial blood pressure (MABP) was measured both before and during cerebral ischemia. Fifty microliters of blood were withdrawn twice before and during cerebral ischemia, respectively, for blood gas determination (pH, PaO_2, $PaCO_2$). Core temperature was controlled and kept constant by means of a feedback temperature-control unit, n = 5 animals/group. Values are mean ± SD.

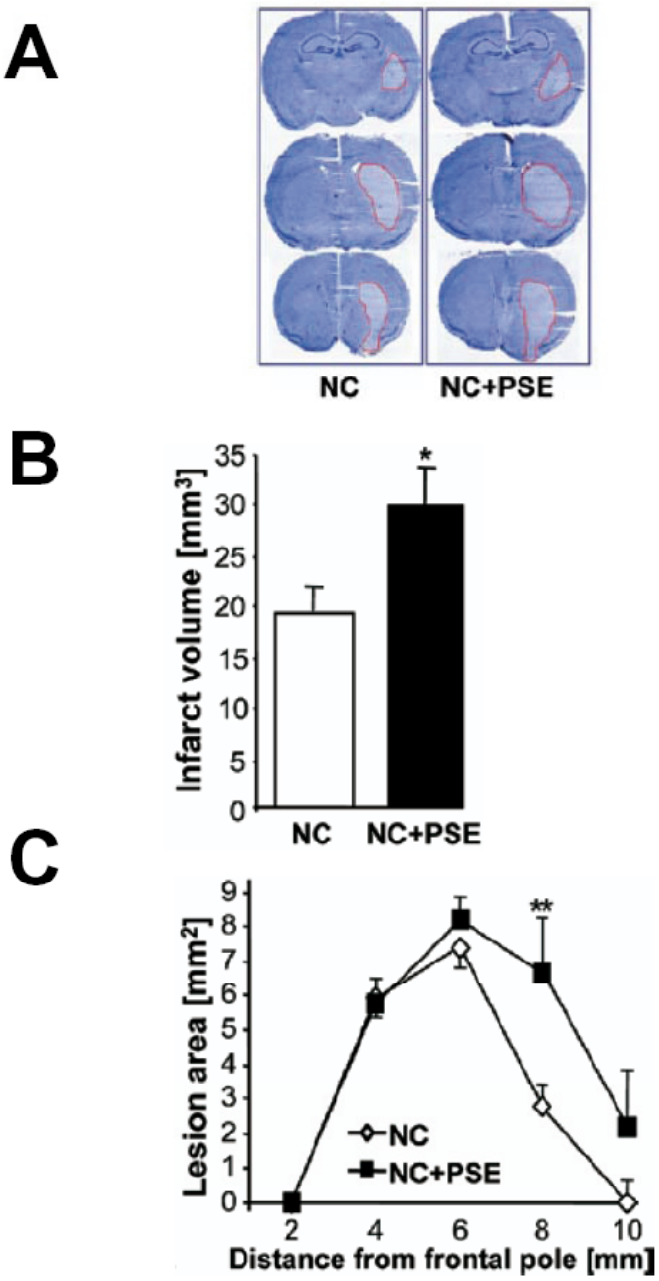


Figure 22. Effects of Plant Sterols on Cerebral Ischemia

Effects of normal chow (NC) + plant sterol ester (PSE) compared with NC on ischemic stroke after 4 weeks treatment. Mice were subjected to 30 min filamentous MCAo and 72 h reperfusion. A. Representative examples of 20-µm coronal brain cryostat sections. B. indirect cerebral lesion volumes, and C. direct cerebral lesion areas determined on 5 coronal brain sections (2 mm distance from frontal pole) by computer-assisted volumetry; n = 8 animals per group. Values are mean ± SEM; *p < 0.05, **p < 0.01.

5. Discussion

The overall goal of my doctoral thesis was to assess mechanisms of neuroprotection and neuroregeneration impacting longer-term stroke outcome. Therefore, I performed the following experiments:
1. I analyzed the effects of PPAR-γ agonist pioglitazone on short- and longer-term stroke outcome after mild transient brain ischemia both *in vitro* and *in vivo*. 129/SV mice were subjected to 30 min filamentous MCAo, followed by reperfusion. Post event, animals were treated with daily intraperitoneal (i.p.) pioglitazone or vehicle. Regarding functional longer-term outcome, motor function including pole test, wire hanging test and learning and memory in the Morris water maze were measured. 2. To test the hypothesis that the long-term stroke-protective effects of regular physical activity are mediated via up regulation of eNOS and enhanced neovascularization in a chronic stroke model, I used L-NAME or endostatin on groups of animals subjected to voluntary exercise vs. a sedentary lifestyle. After 3 weeks of physical training animals were exposed to mild cerebral ischemia induced by 30 min occlusion of the left MCA followed by reperfusion. Then animals were put back to their home cages and treatment was continued as before. Neuroprotection in 'exercise' animals was abolished by cotreatment with either L-NAME or endostatin. These experiments allowed me to approach my overall question of mechanisms impacting longer-term stroke outcome from different angles.

This study has the following major findings: 1. *In vitro*, pioglitazone attenuates microglial migration and proliferation. 2. At an early time point, pioglitazone, given systemically at a dose of 20mg/kg directly post event, protects from mild focal brain ischemia. 3. By contrast, assessment of long-term stroke outcome at six weeks did not yield evidence for a beneficial effect of pioglitazone treatment either on lesion size or on longer-term functional outcome (pole test, wire hanging, Morris water maze). 4. *In vivo*, pioglitazone given acutely after transient brain ischemia / reperfusion exerts anti-inflammatory effects at three days as evidenced by a reduced number of Iba1+ cells in the ischemic striatum. However, analysis at 6 weeks after MCAo/reperfusion yields no effect of pioglitazone on Iba1+ cell counts. 5. Relative to vehicle-treated animals, the number of newly-generated cells in the ischemic striatum was significantly increased in pioglitazone-treated animals at three days. However, the number of BrdU-positive cells was significantly decreased in the ischemic striatum of pioglitazone- as compared to vehicle-treated animals at 6

weeks. 6. Regular physical activity and exercise improve short-term stroke outcome via mechanisms related to NO-dependent CBF augmentation in an acute ischemia model (Endres, et al., 2003). In fact, neuroprotection is not observed in animals lacking eNOS (Endres, et al., 2003). 7. Supplementation of chow with PSE significantly increased lesion size after cerebral ischemia compared with the normal chow group.

5.1. Pioglitazone and mild ischemia

Inflammation is the basic response to injury by multi-cellular organisms. Experimental studies have shown that activated macrophages increase expression of PPAR-γ (Ricote, et al., 1998) and that pioglitazone potently downregulates three pro-inflammatory cytokines released by these cells, namely tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β) and IL-6 (Jiang, et al., 1998, Lee, et al., 2000, Niino, et al., 2001, Pineau and Lacroix, 2007, Streit, et al., 1998, Wang, et al., 1996). Anti-inflammatory effects are achieved partly by blocking the action of the transcription factors AP-1 and NF-kB, inducing inhibition of iNOS and COX-2 (Dehmer, et al., 2004, Feinstein, et al., 2002, Landreth and Heneka, 2001, McTigue, et al., 2007). PPAR-γ agonists attenuate the expression of ICAM-1, matrix metalloproteinase (MMP)-9 and many other inflammatory cytokines following brain ischemia (Culman, et al., 2007, Luo, et al., 2006, Pereira, et al., 2005).

Several *in vitro* studies have provided mechanisms that underlie the acute neuroprotective actions of PPAR-γ agonists following cerebral ischemia. In primary cortical neurons, pioglitazone reduces the induction of cyclooxygenase-2 (COX-2) and even in the absence of microglia prevents neuronal death after oxidative injury (Zhao, et al., 2006). Similarly, thiazolidinediones attenuate inducible nitric oxide synthase (iNOS) expression and protect cerebellar granule cells from cytokine-induced apoptosis (Culman, et al., 2007, Heneka, et al., 1999). Our report demonstrates a direct attenuating effect of pioglitazone both on microglia migration and on microglia proliferation.

The mechanisms underlying short-term neuroprotection by pioglitazone *in vivo* may include several pathways. First, a direct effect on neurons and microglia is likely. When applied systemically, ~18% of pioglitazone enter the CNS within 6 hours of administration (Maeshiba, et al., 1997). Since penetration into the brain is substantially facilitated by disruption of the blood-brain barrier – a key pathophysiological feature of cerebral ischemia

– access of pioglitazone to the CNS parenchyma is massively enhanced following brain injury (McTigue, et al., 2007). Importantly, CNS parenchymal cells (neurons and microglia) express PPAR-γ (Lin, et al., 2006, Ou, et al., 2006, Victor, et al., 2006, Zhao, et al., 2006) and display activity changes after PPAR-γ activation (Bernardo, et al., 2005, Cimini, et al., 2005, Moreno, et al., 2004, Roth, et al., 2003). Although oral administration is used for human patients, in our study administration by intraperitoneal injection was chosen since it is more reliable (after MCAo mice tend to show reduced food intake for 1–2 days). The dosage used in this study is comparable to similar previous studies which have reported advantageous results of pioglitazone (administered by intraperitoneal injection) in focal ischemia models (Collino, et al., 2006, Shimazu, et al., 2005, Victor, et al., 2006) and is also within the range of those studies using oral administration (Breidert, et al., 2002, Feinstein, et al., 2002, McTigue, et al., 2007, Niino, et al., 2001, Schutz, et al., 2005).

At three days after MCAo/reperfusion, lesion volume was significantly reduced by 28% in the acutely pioglitazone-treated mice as compared to vehicle-injected animals. Previous studies have shown that neuroprotection by PPAR-γ activation following ischemia may involve:

(a) inhibition of excitotoxicity: PPAR-γ agonism showed direct effect on cultured neurons protecting them against excitotoxicity (McTigue, et al., 2007, Zhao, et al., 2006).
(b) reduction of the number of inflammatory cells (Sundararajan, et al., 2005)
(c) prevention of harmful neuronal responses to the ischemic insult such as enhanced COX-2 expression (Culman, et al., 2007).

Also, a recent report showed a two-fold increase in neoangiogenesis and persistent upregulation of endothelial precursor cells in the blood and bone marrow of pioglitazone-treated animals (Gensch, et al., 2007). Thus, multiple mechanisms may have contributed to the improved outcome in the short term analysis of our study.

So far, studies have mainly focussed on acute stroke outcome and the long-term effects of pioglitazone after cerebral ischemia have been neglected. Therefore, we here performed a 6-week survival experiment on pioglitazone-treated mice. Descriptively, the pioglitazone-treated groups showed a small reduction in lesion size as compared to control animals that had received vehicle. However, differences in lesion size between controls and the pioglitazone-treated groups at 6 weeks were not statistically significant. By contrast, across groups lesion sizes decreased massively between assessment at 3 days and 6 weeks after MCAo.

Lesion size at 72 hours was analyzed using hematoxylin and eosin (H&E) staining. Lesioned neurons shrink, show eosinophilic staining due to mitochondrial condensation and display nuclear pyknosis. H&E staining permits identification of the infarct as a well-defined pale area, because apoptotic and necrotic cells do not stain with hematoxylin. H&E staining of cryostat sections is used routinely to assess short-term stroke outcome (Endres, et al., 1998, Endres, et al., 2004).

Antibodies directed against neuronal nuclear antigen NeuN are widely used to label neurons. However, NeuN may also stain neurons in the process of dying (Katchanov, et al., 2001, Kuan, et al., 2004, Semmler, et al., 2005). NeuN staining is therefore especially suited to assess the longer-term outcome after an insult (Ieraci and Herrera, 2006, Winter, et al., 2005).

A number of factors likely contribute to the marked regression of lesion size over time. Direct lesion size after three days may be increased by edematous brain swelling, which will resolve over time. Furthermore, after degeneration of injured neurons, lesion maturation may involve removal of injured tissue by microglia, phagocytosis, and finally exofocal, transsynaptic neurodegeneration.

The variabality of all parameters was clearly higher after 6 weeks than after 3 days. The statistical power (1-β) for the comparison of lesion sizes at three days was ~0.8. By contrast, in the long-term experiments, 1-β for the comparison of infarct sizes had decreased to 0.3. However, the number of animals analyzed was actually higher in the long-term experiment (n=28 versus n=24). A post hoc power calculation for the chronic experiments yielded 69 animals (23 animals per group) to achieve .80 power at the .05 criterion level.

Taken together, lesion maturation involves marked shrinkage of damaged/lesioned tissue over time. In comparison to this confounding effect any possible influence of pioglitazone on long-term lesion volume appears relatively minor. However, our data does not permit us to firmly exclude the possibility that such an influence on long-term infarct volume may exist. Additional studies will have to explain this apparent lack of action of pioglitazone in the chronic phase post-ischemia.

Functional outcome regarding motor and memory performance was assessed in a battery of behavioral tests in the long-term experiments. No significant differences regarding neurological outcome were observed between pioglitazone and vehicle treated groups, which is consistent with histological outcome (infarct area) at 6 weeks. On the other hand, we did detect a significant deterioration of motor skills in MCAo animals in both the pole test and the wire hanging test at six weeks. In line with earlier studies from our group using our transient mild ischemia model (Winter, et al., 2005), we did not detect differences between animals that had undergone MCAo and sham animals in the place task of the Morris water maze. However, in the probe trial, we observed significant differences in the number of platform crosses between sham animals and all MCAo groups irrespective of pioglitazone treatment.

In summary, we show that pioglitazone exerts anti-inflammatory effects and improves stroke outcome in acutely treated animals in a short-term survival paradigm. It is essential to use not only acute treatment results in order to assess an intervention but to also consider longer-term outcome. Here, analysis of longer-term stroke outcome did not yield a beneficial effect of pioglitazone treatment on lesion size or functional recovery. Our study thus underscores the importance of studying longer-term outcome in experimental stroke studies.

5.2. Long-term neuropreotective effect of exercise after mild ischemia

We looked for long-term effects of physical exercise after mild stroke and could link reduced infarct size to CBF augmentation and angiogenesis even weeks after ischemia. eNOS is found to be essential for the functional activity of progenitor cells and the formation of new vessels (Aicher, et al., 2003, Murohara, et al., 1998). Additionally, physical exercise increases endothelial precursor cells (EPCs) and improves neo-vascularization via NO-dependent mechanisms (Laufs, et al., 2004). Therefore, we postulated that long-term neuroprotective effects of exercise are achieved by eNOS upregulation. In exercised animals EPC levels in bone marrow, blood and spleen were significantly increased compared to sedentary mice. Remodeling of microvascular structures occurs after cerebral ischemia with enlargement of perfused vessels: However, density of capillaries

was increased following physical exercise with normal morphology compared to the control group. Also, we found significantly augmented CBF within the ischemic lesion of 'exercise' as compared to sedentary control animals (Gertz, et al., 2006).

Importantly, in our study, these beneficial effects of regular physical activity on endothelium function, progenitor cell regulation and neo-vascularization toward long-term neuroprotection could be directly linked to eNOS and further to angiogenesis. In L-NAME treated mice, we found no significant difference on infarct sizes between the control and the exercised group, indicating that NOS inhibition abolished neuroprotection. Using anti-angiogenic agent endostatin, we obtained the following lesion sizes: control (27.1 mm^3) vs exercise group (28.9 mm^3) ($p<0.05$). We concluded that the long-term neuroprotective effects of exercise were NOS dependent and related to angiogenesis (Figure 23).

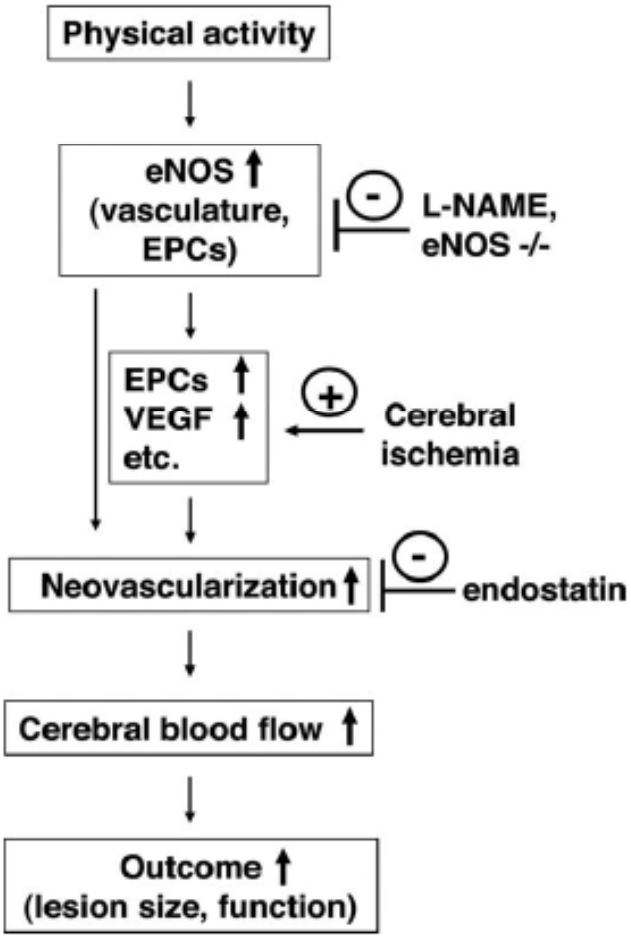


Figure 23. Schematic representation of long-term stroke protection via eNOS-dependent pathways after physical activity

Furchgott and Zawadszki in 1980 discovered the classic vasodilator, endothelium-derived NO, which is produced by the enzyme eNOS through oxidative conversion of L-arginine to L-citrulline (Figure 24). NO activates soluble guanylate cyclase when formed by the vascular endothelium. Guanylate cyclase in turn mediates many beneficial effects of NO (Endres, et al., 2004). In vascular smooth muscle, NO is a potent vasodilator and regulates regional blood flow (Iadecola, 1997, Loscalzo, 1995, Rudic and Sessa, 1999, Samdani, et al., 1997). In addition, NO has antithrombotic, anti-inflammatory and antiproliferative properties. By contrast, its loss contributes to impaired vascular relaxation, platelet aggregation, increased proliferation of vascular smooth muscle, enhanced leukocyte adhesion to the endothelium and increased blood pressure (Huang, et al., 1995). Taken together, endothelium-derived NO has a protective function in the vascular wall (Endres, et al., 2004). The non-selective NOS inhibitor L-NAME was used in this study, which exerts inhibitory effects also on nNOS and iNOS after ischemic stroke. But because of systemic oral administration of L-NAME, pharmacological effects on eNOS should predominate.

Figure 24. NO is produced by the enzyme eNOS through oxidative conversion of L-arginine to L-citrulline.

Physiologic parameters (i.e. blood pressure, blood gases) showed no significant differences between 'exercise' and sedentary animals during MCAo/reperfusion in an acute ischemia mice model (Endres, et al., 2003). In addition, we measured these parameters in randomly selected animals at 4 weeks after the ischemic injury and also did not observe relevant differences. Arterial blood pressure levels were somewhat lower in the exercise group. However, it is unlikely that this may have contributed to long-term protection.

A healthy mind in a healthy body, is a well-known Roman aphorism: *mens sana in corpore sano*. Protective effects of physical exercise on brain function are already well established. Regular physical activity may potentially contribute to neuroprotection via a plethora of molecular, cellular, and systemic effects (Carro, et al., 2000, Endres, et al., 2003, Neeper, et al., 1995, Stummer, et al., 1995, Tong, et al., 2001).

In addition, a large amount of evidence shows the beneficial role of physical activity and enriched environment on neurogenesis, which may contribute to some aspects of brain

function, such as long-term potentiation and learning (Kempermann, et al., 1997, van Praag, et al., 1999). The generation of new neurons in the adult is largely restricted to two regions: the subventricular zone (SVZ), which locates on the lateral wall of the lateral ventricles (LV) and generates new interneurons that reach the olfactory bulb via the rostral migratory stream (RMS), and the subgranular zone (SGZ) of the dentate gyrus (DG), which gives rise to new granule cells (Gage, 2000, Kokaia and Lindvall, 2003). In the transient focal cerebral ischemia model, evidence has shown that neurogenesis occurs (Arvidsson, et al., 2002, Nakatomi, et al., 2002). However, it was shown that more than 80% of newly formed neurons die after a transient cerebral ischemia even when the integrity of the microcirculation is maintained. This phenomenon is most possible due to unfavorable environmental conditions including a lack of trophic support and exposing to toxins from injured tissue. These considerations may explain the observation that only 0.2% of nonviable ischemic neurons are replaced via neurogenesis (Arvidsson, et al., 2002). Consequently, to enhance endogenous neurogenesis and improve functional recovery, activated neovessel formation seems to be essential. In our study, we did not assess neurogenesis at the level of Brdu/NeuN positive cells, which could, however, be addressed in future studies. Endostatin-mediated suppression of endothelial proliferation and the direct negative effects on EPCs which may lead to diminished angiogenesis abrogated the beneficial effect of regular physical activity on neuroprotection.

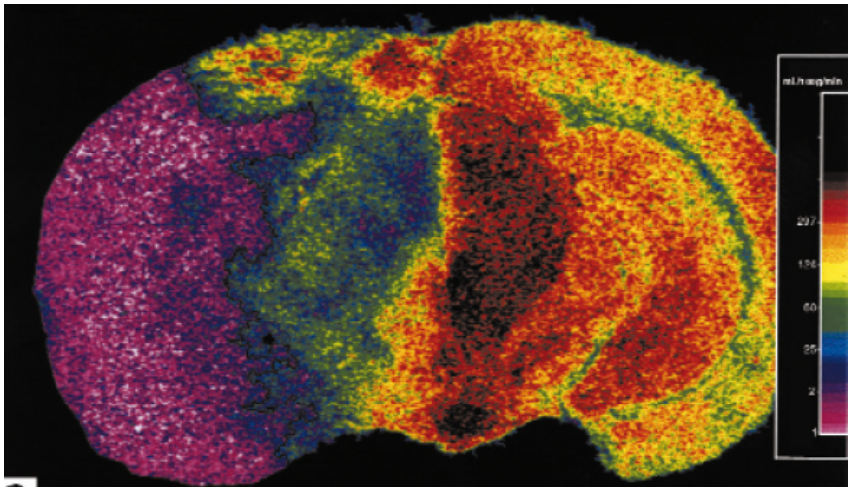
Angiogenesis is a multi-step process. New capillaries sprout from pre-existing blood vessels. Under normal conditions, angiogenesis is modulated via a balance between endogenous angiogenic inducers and endogenous angiogenic inhibitors that keep the angiogenic process in check and prevent inappropriate vascularization of tissues. Angiogenesis is activated following cerebral ischemia and modifies the capillary network. Regular physical exercise upregulates EPCs and contributes to neovascularization. Endostatin inhibits endothelial cell migration *in vivo* and *in vitro* and induces endothelial cell apoptosis. Our study demonstrates that treatment with endostatin completely abrogates the stroke-protective effects of regular physical activity providing a direct link between therapeutic neovascularization after stroke and neuroprotection.

Our observations support the notion that neovessel formation, achieved through regular physical activity before and after stroke, plays a crucial role in long-term outcome. Thus, enhanced angiogenesis may have a great potential for the treatment of neurodegenerative disorders and stroke (Taguchi, et al., 2004).

5.3. Food supplementation with PSE aggravates ischemic brain injury

Plant sterols decreased atherosclerotic plaque formation in a model of lipid-driven atherogenesis and cholesterol levels (Weingartner, et al., 2008); however, surprisingly, feeding with plant sterols impaired endothelium-dependent vasodilatation while endothelium-independent vasodilator function remained unchanged. Endothelial dysfunction predicts and precedes cardio- and cerebrovascular events (Weingartner, et al., 2008). These findings seem to indicate a potential harmfulness of increased plasma plant sterol concentrations.

Previous studies had shown that endothelial function is the determinator of the survival of the penumbra area. After MCA occlusion, there was little or no blood flow to the core infarct zone of the parietal lobe (Figure 25). The blood flow in the penumbra, adjacent peri-ischemic region, was slightly higher than that of the core infarct zone, but nevertheless this was substantially lower than that of the non-ischemic contralateral hemisphere. Impaired endothelial function induces reduced blood flow to the core infarct zone and the penumbra, which resulted in bigger infarct lesion size in the animals fed with the special chow containing plant sterols (Endres, et al., 2003, Laufs, et al., 2000). Importantly, mice fed with sterol-enriched chow did not differ from the control mice regarding physiological parameters such as blood pressure or heart rate, but exhibited negative vascular effects.



Laufs et al., 2000

Figure 25. Cerebral blood flow following MCA occlusion in mice

Regional variations have been shown on pseudo-color coronal brain sections (mid-thalamic level) from [^{14}C]-iodoantipyrine autoradiography in cerebral blood flow following MCA occlusion in mice. The level of radioactivity is color-coded to indicate lower (blue-purple) and higher (yellow-red) regional blood flow.

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Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen

in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Selbständigkeitserklärung

„Ich, Shengbo Ji, erkläre, dass ich die vorgelegte Dissertation mit dem Thema:

Effects of peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists and physical activity on long-term stroke outcome in a mouse model

selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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