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# PPAR Agonism as New Pharmacological Approach to the Management of Acute Ischemic Stroke

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## 1. Introduction

Despite increasing knowledge of the biochemical mechanisms that occur in the brain following an ischemic insult and the availability of several diverse animal models of stroke, there are still no drugs that can be given to stroke patients soon after the onset of symptoms to minimize the subsequent neurological damage. To date, the thrombolytic compound recombinant tissue Plasminogen Activator (rt-PA) remains the only approved drug for the treatment of stroke. At present, intravenous administration of rt-PA is the only proven effective treatment to re-establish cerebral blood flow in the case of acute vessel occlusion, but unfortunately, only few patients with acute ischemic stroke are qualified to receive this drug. The failure of rt-PA to achieve rapid reperfusion in many patients and its bleeding risk have prompted the development of fibrinolytic agents with greater fibrin specificity and better risk-benefit profiles, such as tenecteplase or desmoteplase, which are now under active investigation. Early restoration of blood flow remains the treatment of choice for limiting brain injury following stroke, but a second fundamental goal of intervention is to protect neurons by interrupting or slowing the ischemic cascade. Current research is being done to develop neuroprotective agents that are able to block amino acid pathways and decrease neurotransmitter activity of injured tissue. Drugs blocking voltage-dependent calcium channels were effective in stroke rodent models but the results of clinical trials have been often discouraging. Overactivation of the N-methyl-D-aspartate receptor (NMDAR) is crucial for neuronal death after stroke. Several compounds that interfere with glutamate receptor activation have been developed and tested, in particular noncompetitive NMDA antagonists. However, their clinical use is limited by intolerable side effects, including some psychomimetic symptoms, as these blockers may also impair some key brain functions mediated by the same receptor. Accumulating evidence strongly suggests that apoptosis contributes to neuronal cell death in stroke injury and currently several caspase inhibitors are under investigation, but to date the efficacy of antiapoptotic agents in human stroke patients has not yet been tested. Anti-inflammatory approaches to stroke treatment intended

to block cell-mediated inflammation with different strategies such as humanized antibodies against ICAM-1, inhibitors of interleukin-1 beta or a interleukin-1 receptor antagonist. However, there have been no successful clinical trials of these anti-inflammatory agents so far.

The complexity of events in cerebral ischemia and the disappointing results from human clinical stroke trials using a single agent suggest that perhaps to treat the stroke a new pleiotropic approach is required. In the pharmacological perspective, the evaluation of drugs with multiple effects on the ischemic cascade may be more effective in reducing infarct size and improving outcome in respect to single target strategy, because the ischemic cascade is diverse and it is likely that many different mechanisms of ischemia induced cell death occur simultaneously. Therefore, the development of neuroprotective drugs with multiple effects on the ischemic cascade is potentially more appealing than drugs acting on only one component of the cascade, if the safety profile is reasonable and the preclinical assessment package fulfils recent recommendations. Most recent discoveries portray Peroxisome Proliferator-Activated Receptors (PPARs) as promising pharmacological targets for the treatment of acute ischemic stroke, thanks to their ability to simultaneously interfere with several mechanisms that underlie the pathophysiology of brain ischemia, thus leading to an interesting protective strategy to counteract the multiple deleterious effects of ischemic injury.

## 2. PPAR

Peroxisome Proliferator-Activated Receptors (PPARs) are members of the nuclear hormone receptor (NHR) superfamily of ligand-activated transcription factors. There are three PPAR subtypes:  $\alpha$ ,  $\beta/\delta$  and  $\gamma$ , named also NR1C1, NR1C2 and NR1C3, respectively, according to the unified nomenclature of nuclear receptors (Nuclear Receptors Nomenclature Committee, 1999). The three isoforms are the products of distinct genes: the human PPAR $\alpha$  gene was mapped on chromosome 22 in the general region 22q12-q13.1, the PPAR $\gamma$  gene is located on chromosome 3 at position 3p25, whereas PPAR $\beta/\delta$  has been assigned to chromosome 6, at position 6p21.1-p21.2 (Sher, Yi et al. 1993; Greene, Blumberg et al. 1995; Yoshikawa, Brkanac et al. 1996). PPARs were originally identified by Isseman and Green (Isseman and Green 1990) after screening the rat liver cDNA library with a cDNA sequence located in the highly conserved C domain of NHRs. The name PPAR is derived from the fact that activation of PPAR $\alpha$ , the first member of the PPAR family to be cloned, results in peroxisome proliferation in rodent hepatocytes (Desvergne and Wahli 1999). Activation of neither PPAR $\beta/\delta$  nor PPAR $\gamma$ , however, elicits this response and, interestingly, the phenomenon of peroxisome proliferation does not occur in humans. The molecular basis for this difference between species is not yet clear. With respect to the PPAR $\gamma$  isotype, alternative splicing and promoter use results in the formation of two further isoforms: PPAR $\gamma$ 1 and PPAR $\gamma$ 2. In particular, differential promoter usage and alternate splicing of the gene generates three mRNA isoforms. PPAR $\gamma$ 1 and PPAR $\gamma$ 3 mRNA both encode the PPAR $\gamma$ 1 protein product which is expressed in most tissues, whereas PPAR $\gamma$ 2 mRNA encodes the PPAR $\gamma$ 2 protein, which contains an additional 28 amino acids at the amino terminus and is specific to adipocytes (Gurnell 2003). PPAR $\beta/\delta$  was initially reported as PPAR $\beta$  in *Xenopus laevis* and NUC1 in humans (Schmidt, Endo et al. 1992). Subsequently, a similar transcript was cloned from mice and termed PPAR $\delta$  (Amri, Bonino et al. 1995). Though now

recognised as homologues for each other, it was not originally certain whether PPAR $\beta$  from *Xenopus* was identical to murine PPAR $\delta$ , hence the terminology PPAR $\beta/\delta$ . All members of this superfamily share the typical domain organization of nuclear receptors (Figure 1). The N-terminal A/B domain contains a ligand-independent transactivation function. In the  $\alpha$  and  $\gamma$  isotypes, the activity of this domain can be regulated by Mitogen-Activated Protein Kinase (MAPK) phosphorylation (Hu, Kim et al. 1996). The C domain is the DNA binding domain with its typical two zinc-finger-like motifs, as previously described for the steroid receptors, and the D domain is the co-factor docking domain (Schwabe, Neuhaus et al. 1990). The E/F domain is the ligand binding domain, it contains a ligand-dependent trans-activation function (AF)-2 (Fajas, Auboeuf et al. 1997), and is able to interact with transcriptional coactivators such as steroid receptor coactivator (SRC)-1 (Onate, Tsai et al. 1995) and CREB-binding protein (CBP) (Amri, Bonino et al. 1995).

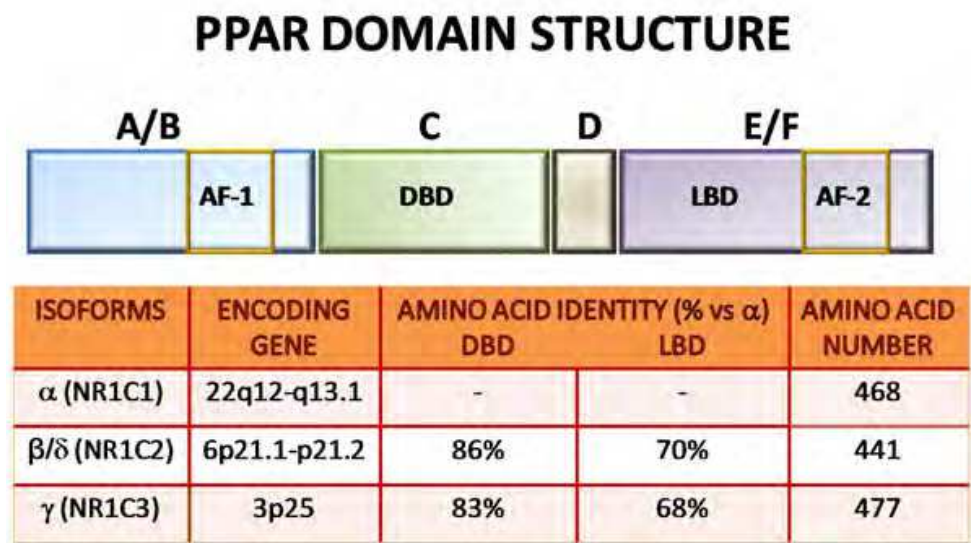


Fig. 1. Schematic representation of the domain organization of human PPAR isoforms. The A/B domain contains the Activation Function 1 (AF-1) which has a ligand-independent transcriptional activity. The C domain corresponds to the DNA Binding Domain (DBD). The D domain is the co-factor docking domain. The E/F domain contains the Ligand Binding Domain (LBD) and carries the Activation Function 2 (AF-2), which has a ligand-dependent transcriptional activity. The human chromosome regions in which disting genes encoding for PPAR isoforms are mapped, the percentage of amino acid sequence identity (in comparison with PPAR $\alpha$ ) and the amino acid number of different isoforms are reported in the Table.

The highest PPAR $\alpha$  expression has been found in the liver and in tissues with high fatty acid catabolism, such as the kidney, heart, skeletal muscle, and brown fat (Lefebvre, Chinetti et al. 2006). PPAR $\alpha$  mainly regulates energy homeostasis, activating fatty acid catabolism and stimulating gluconeogenesis (Kersten, Seydoux et al. 1999). This increased fatty acid oxidation in response to PPAR $\alpha$  activation with a selective agonist, WY14643, results in lower circulating triglyceride levels and reduction of lipid storage in liver, muscle, and adipose tissue (Chou, Haluzik et al. 2002), which is associated with improved insulin sensitivity (Kim, Haluzik et al. 2003). Consequently, fibrates (fenofibrate, bezafibrate,

gemfibrozil), which are synthetic agonists for PPAR $\alpha$ , are in wide clinical use for the treatment of dyslipidaemias.

PPAR $\gamma$  is expressed in white and brown adipose tissue, gut, and immune cells (Feige, Gelman et al. 2006). It is involved in adipocyte differentiation and lipid storage in white adipose tissue (Rosen, Sarraf et al. 1999). Furthermore, PPAR $\gamma$  is involved in glucose metabolism via an improvement of insulin sensitivity (Hevener, He et al. 2003). Therefore, synthetic PPAR $\gamma$  agonists (thiazolidinediones) are in clinical use as insulin sensitizers to treat patients with type-2 diabetes.

PPAR $\beta/\delta$  remained an enigma for almost a decade after its cloning in 1992. It has been reported to be ubiquitously expressed in almost every tissue and, in the past, this widespread tissue expression has suggested a possible “general housekeeping” role for PPAR $\beta/\delta$  (Kliwer, Forman et al. 1994). More recently, the use of transgenic mouse models and the availability of high-affinity synthetic ligands has led researchers to a better understanding of its physiological role. Specifically, increasing evidence has shown a particular role for PPAR $\beta/\delta$  in insulin sensitivity regulation, lipid metabolism and the inflammation response. However, in contrast to PPAR $\alpha$  and  $\gamma$ , PPAR $\beta/\delta$  agonists are not yet in clinical use.

## 2.1 Endogenous and synthetic PPAR ligands

Although many fatty acids are capable of activating all three PPAR isoforms, some fatty acids are also specific for a particular PPAR isoform. X-ray crystallography studies of PPAR $\beta/\delta$  revealed an exceptionally large ligand-binding pocket of approximately 1,300 Å<sup>3</sup>, similar to that of PPAR $\gamma$  but much larger than the pockets of other nuclear receptors (Xu, Lambert et al. 1999). The increased dimension is believed to accommodate the binding of various fatty acids or other amphipathic acids to PPAR $\beta/\delta$  via hydrogen bonds and hydrophobic interactions. The long-chain polyunsaturated fatty acids and their oxidized derivatives, especially eicosanoids such as 8-S-hydroxyeicosatetraenoic acid (8-S-HETE), leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and arachidonate monooxygenase metabolite epoxyeicosatrienoic acids have been shown to potently activate PPAR $\alpha$  with high affinity (Theocharisa, Margeli et al. 2003; Feige, Gelman et al. 2006). PPAR $\gamma$  can be activated by several prostanoids, such as 15-deoxy- $\Delta$ 12,14-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) and 12- and 15-hydroxy-eicosatetraenoic acid (12- and 15-HETE), which are derivatives of arachidonic acid synthesized through the lipoxygenase pathway, as well as modified oxidised lipids, 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODE) (Willson, Brown et al. 2000; Theocharisa, Margeli et al. 2003). PPAR $\beta/\delta$  agonists include linoleic acid, oleic acid, arachidonic acid and eicosapentaenoic acid (EPA), which have been shown to co-crystallize within the ligand binding domain of this nuclear receptor (Xu, Lambert et al. 1999). A number of eicosanoids, including prostaglandin (PG)A<sub>1</sub> and PGD<sub>2</sub>, and carbaprostacyclin, a semi-synthetic prostaglandin, have micromolar affinities for PPAR $\beta/\delta$  (Forman, Chen et al. 1997). Recently, cows milk, ice cream, butter, and yoghurt were described as activators of PPAR $\beta/\delta$  in reporter assays, but a specific common compound was not identified (Suhara, Koide et al. 2009).

With respect to the synthetic ligands, fibrates (e.g. fenofibrate, clofibrate), which are hypolipidaemic drugs, are well-known ligands for PPAR $\alpha$  (Willson, Brown et al. 2000). Fibrates are capable of activating PPAR $\alpha$  at pharmacological doses leading to increased expression of lipid metabolizing enzymes that effectively lower serum lipid levels in



humans. In contrast to the well-documented therapeutic effect, there is also evidence of liver toxicity induced by activation of PPAR $\alpha$ , mainly hepatocarcinogenesis. The most serious safety risk associated with fibrates, although rare, is myopathy and rhabdomyolysis. Studies suggest that the mechanism of myotoxicity through fibrates is not entirely clear, because complex and multifactorial mechanisms are involved, including genetic predisposition, pharmacokinetics, drug interactions, and dose. It is of interest to note that increased expression of lipoprotein lipase, which is a known PPAR $\alpha$  target gene, in skeletal muscle leads to severe myopathy in mice.

The most widely used PPAR $\gamma$  agonists belong to the thiazolidinedione (TZD) or glitazone class of anti-diabetic drugs used in the treatment of type-2 diabetes. Troglitazone, the first TZD approved for this use, was withdrawn from the market in March 2000 following the emergence of a serious hepatotoxicity in some patients. Since troglitazone induces CYP3A4, it has been hypothesized that potentially toxic quinones derived from CYP3A4-dependent metabolism could cause liver damage (Yamamoto, Yamazaki et al. 2002). Rosiglitazone and pioglitazone are the only available thiazolidinediones in North America, but meta-analyses of randomised controlled trials have suggested an increased risk of ischaemic cardiovascular events with rosiglitazone (Nissen and Wolski ; Singh, Loke et al. 2007). In contrast, meta-analysis of trials of pioglitazone indicates the possibility of an ischaemic cardiovascular benefit (Lincoff, Wolski et al. 2007). Robust evidence also shows that both drugs increase the risk of congestive heart failure and fractures, but whether any meaningful difference exists in the magnitude of risk between the two thiazolidinediones is not known (Singh, Loke et al. 2007; Loke, Singh et al. 2009). The European Medicines Agency has recommended the suspension of marketing authorisation for rosiglitazone, whereas the US Food and Drug Administration has allowed the continued marketing of rosiglitazone with additional restrictions.

On the contrary, there are no PPAR $\beta/\delta$  drugs in clinical use yet. However several selective PPAR $\beta/\delta$  ligands have been recently designed, including GW0742, GW2433, GW9578, L-783483, L-165041, or GW501516 (Berger, Leibowitz et al. 1999; Lim and Dey 2000; Martens, Visseren et al. 2002). As yet only one selective PPAR $\beta/\delta$  antagonist has been described GSK0660. In skeletal muscle myoblast cells in culture, GSK0660 inhibited GW0742 induction of established PPAR $\beta/\delta$  target genes (carnitine palmitoyltransferase 1A, angiopoietin-like 4 protein and pyruvate dehydrogenase kinase-4)(Shearer, Steger et al. 2008).

## 2.2 Molecular mechanisms of PPAR activation

There are at least three primary mechanisms by which PPARs can regulate biological functions: transcriptional transactivation, transcriptional transrepression and ligand-independent transrepression (Figure 2).

### 2.2.1 Mechanism of transcriptional transactivation

PPARs function as heterodimers with their obligatory partner the Retinoid X Receptor (RXR). Like other NHRs, the PPAR/RXR heterodimer most likely recruits co-factor complexes - either co-activators or co-repressors - that modulate its transcriptional activity (Shi, Hon et al. 2002). The PPAR/RXR heterodimer then binds to sequence specific PPAR Response Elements (PPREs), located in the 5'-flanking region of target genes, thereby acting as a transcriptional regulator (Palmer, Hsu et al. 1995). The PPRE consists of two direct repeats of the consensus sequence AGGTCA separated by a single nucleotide, which constitutes a DR-1 motif. PPAR binds 5' of RXR on the DR-1 motif and the 5'-flanking

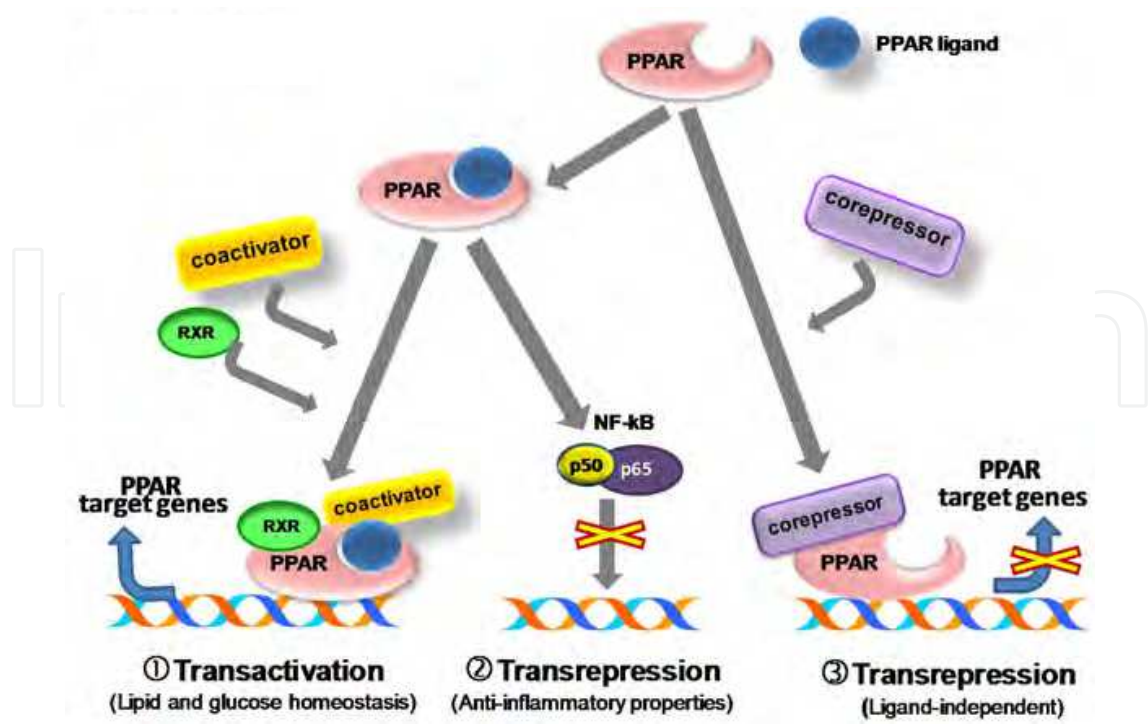


Fig. 2. Molecular mechanisms of PPAR activation. After ligand binding, PPAR undergoes conformational changes, which lead to recruitment of Retinoid X Receptor (RXR) and coactivators. The resultant heterodimer binds to specific DNA response elements called PPAR response elements, causing target gene transcription (Transactivation). A second mechanism (Transrepression) involves interfering with other transcription-factor pathways by negatively regulating the expression of pro-inflammatory genes. Lastly, PPAR may repress the transcription of direct target genes in the absence of ligands (ligand-independent Transrepression) recruiting corepressor complexes that mediate active repression.

sequence conveys the selectivity of binding between different PPAR isotypes (Juge-Aubry, Pernin et al. 1997). In the absence of a ligand, to prevent PPAR/RXR binding to DNA, high-affinity complexes are formed between the inactive PPAR/RXR heterodimers and co-repressor molecules, such as nuclear receptor co-repressor or silencing mediator for retinoic receptors. In response to ligand binding, PPAR undergoes a conformational change, leading to release of auxiliary proteins and co-repressors and recruitment of co-activators that contain histone acetylase activity. Acetylation of histones by co-activators bound to the ligand-PPAR complex leads to nucleosome remodelling, allowing for recruitment of RNA polymerase II causing target gene transcription. The search for PPAR target genes with identified PPREs has led to the identification of several genes involved in lipid metabolism, oxidative stress and inflammatory response, as widely documented in the literature.

2.2.2 Mechanism of transcriptional transrepression

PPARs can also negatively regulate gene expression in a ligand-dependent manner by inhibiting the activities of other transcription factors, such as Activated Protein-1 (AP-1), Nuclear Factor-κB (NF-κB) and Nuclear Factor of Activated T cells (NFAT) (ligand-dependent transrepression). In contrast to transcriptional activation, which usually involves the binding of PPARs to specific response elements in the promoter or enhancer regions of

target genes, transrepression does not involve binding to typical receptor specific response elements (Pascual and Glass 2006). Several lines of evidence suggest that PPARs may exert anti-inflammatory effects by negatively regulating the expression of pro-inflammatory genes. To date, several mechanisms have been suggested to account for this activity, but despite intensive investigation, unifying principles remain to be elucidated.

Firstly, competition for limited amounts of essential, shared transcriptional co-activators may play a role in transrepression. The activated PPAR/RXR heterodimer reduces the availability of co-activators required for gene induction by other transcriptional factors. Thus, without distinct co-factors, transcription factors cannot cause gene expression.

Secondly, PPAR/RXR complexes may cause a functional inhibition by directly binding to transcription factors, preventing them from inducing gene transcription or inducing the expression of inhibitory proteins, such as the protein inhibitor of kappa B (I $\kappa$ B) $\alpha$ , which sequesters the NF- $\kappa$ B subunits in the cytoplasm and consequently reduces their DNA binding activity (Delerive, Martin-Nizard et al. 1999).

Thirdly, PPAR/RXR heterodimers may also inhibit phosphorylation and activation of several members of the MAPK family. In general very little is known about the molecular mechanisms by which PPARs and their ligands modulate kinase activities.

Recent studies have suggested another mechanism based on co-repressor-dependent transrepression by PPARs. Evidence has been presented in which PPAR $\beta/\delta$  controls the inflammatory status of macrophages based on its association with the transcriptional repressor BCL-6 (Lee, Chawla et al. 2003). Free BCL-6 suppresses the expression of multiple proinflammatory cytokines and chemokines. PPAR $\beta/\delta$ , but not PPAR $\alpha$  and PPAR $\gamma$ , exhibits BCL-6 binding ability (Barish, Atkins et al. 2008; Takata, Liu et al. 2008). In the absence of a ligand, PPAR $\beta/\delta$  sequesters BCL-6 from inflammatory response genes. In contrast, in the presence of a ligand, PPAR $\beta/\delta$  releases the repressor, which now distributes to NF- $\kappa$ B-dependent promoters and exerts anti-inflammatory effects by repressing transcription from these genes.

### 2.2.3 Mechanism of ligand-independent transrepression

PPARs may repress the transcription of direct target genes in the absence of ligands (ligand-independent repression). PPARs bind to response elements in the absence of any ligand and recruit co-repressor complexes that mediate active repression. The co-repressors are capable of fully repressing PPAR-mediated transactivation induced either by ligands or by cAMP-regulated signalling pathways. This suggests co-repressors as general antagonists of the various stimuli inducing PPAR-mediated transactivation. Co-repressors can display different ligand selectivity: the nuclear receptor co-repressor NCoR interacted strongly with the ligand-binding domain of PPAR $\beta/\delta$ , whereas interactions with the ligand-binding domains of PPAR $\gamma$  and PPAR $\alpha$  were significantly weaker (Krogsdam, Nielsen et al. 2002).

Very recently, a team of Harvard Medical School researchers has shown that PPAR $\gamma$  is phosphorylated at Ser273 by cyclin dependent kinase 5 (CDK5) during obesity which results in deregulation of a subset of genes; including a number of key metabolic regulators, such as adiponectin, a central regulator of insulin sensitivity *in vivo* (Choi, Banks et al.). Ser273 phosphorylation did not alter the chromatin occupancy of PPAR $\gamma$ , suggesting that other mechanisms, such as differential recruitment of co-regulators, may cause these differences in target gene expression. PPAR $\gamma$  ligands inhibited Ser273 phosphorylation and reversed



associated changes in gene expression. Critically, the extent to which PPAR $\gamma$  ligands inhibit CDK5-mediated phosphorylation of PPAR $\gamma$  is not correlated with the extent to which they exert PPAR agonism, suggesting that these compounds have two distinct and separable activities. Whether or not similar mechanisms of receptor phosphorylation lead to changes in gene expression also in the other two PPAR isoforms - $\alpha$  and  $\beta/\delta$  is a very important question, so far not yet addressed.

### 3. PPAR in the brain

All three PPAR isotypes are co-expressed in the nervous system during late rat embryogenesis. Their expression peaks in the central nervous system at mid-gestation. Whereas PPAR $\beta/\delta$  remains highly expressed in this tissue, the expression of PPAR $\alpha$  and PPAR $\gamma$  decreases postnatally in the brain (Braissant, Foulle et al. 1996). While PPAR $\beta/\delta$  has been found in neurons of numerous brain areas of adult rodents, PPAR $\alpha$  and PPAR $\gamma$  have been localized to more restricted areas of the brain (Moreno, Farioli-Vecchioli et al. 2004). The localization of PPARs has also been investigated in purified cultures of neural cells. PPAR $\beta/\delta$  is expressed in immature oligodendrocytes where its activation promotes differentiation, myelin maturation and turnover. The PPAR $\gamma$  isotype is the dominant isoform in microglia. Astrocytes possess all three PPAR isotypes, although to different degrees depending on the brain area and animal age (Cristiano, Bernardo et al. 2001). The role of PPARs in the CNS is mainly related to lipid metabolism; however, these receptors have been implicated in neural cell differentiation and death as well as in inflammation and neurodegeneration. The expression of PPAR $\gamma$  in the brain has been extensively studied in relation to inflammation and neurodegeneration. PPAR $\alpha$  has been suggested to be involved in acetylcholine metabolism, excitatory amino acid neurotransmission and oxidative stress defence. PPAR $\beta/\delta$  seems to play a critical role in regulating myelinogenesis and differentiation of cells within the CNS (Peters, Lee et al. 2000).

### 4. PPARs and cerebral ischemia

#### 4.1 Experimental data on the effects of PPAR ligands in ischemic stroke

Although the relevance of animal models to the development of therapies for acute stroke has been often questioned, evidence demonstrates that animal models of stroke do have clinical relevance and are useful in the development of drugs that attenuate the ischemic damage. The characteristics of brain injury depends on the severity and the duration of cerebral blood flow reduction but it can be significantly exacerbated by the following phase of reperfusion; for this reason several animal models of the so-called "cerebral ischemia/reperfusion injury (IRI)" have been developed, demonstrating that often reperfusion after a long ischemic period may cause a larger infarct than that associated with permanent vessel occlusion. In general, the role of neuroprotective agents is to interfere with one or more of the mechanisms involved in the "IRI cascade" and thereby limit the resultant tissue damage. It seems reasonable to assume that drugs that work on a specific biochemical mechanism must be given at the time that the mechanism is active, mainly during ischemia and/or reperfusion. Accordingly, in general, two different experimental paradigms can be identified: prophylactic administration, aimed to evaluate drug effects on stroke prevention, and therapeutic administration, when the drug is administered during reperfusion to test its potential beneficial effects on IRI after stroke had occurred. A role for PPARs in reducing IRI

has been first established in animal models of acute myocardial infarction (Yue TL, Chen et al. 2001). More recently, good evidence supporting the beneficial role of PPAR in stroke has been provided by several *in vivo* experimental models of cerebral IRI, evaluating the effects of both prophylactic and therapeutic administration of PPAR agonists. It has been demonstrated that a 14-day preventive treatment with fenofibrate reduced susceptibility to stroke in apolipoprotein E-deficient mice as well as decreased cerebral infarct volume in wild-type littermates (Deplanque, Gele et al. 2003). The authors demonstrated that fenofibrate administration was associated with a decrease in cerebral oxidative stress depending on the increase in activity of several anti-oxidant enzymes and with a reduced expression of adhesion molecules. In another study, it was confirmed that two different PPAR $\alpha$  agonists, fenofibrate and WY14643, provided similar brain protection when administered 3 or 7 days, respectively, before the induction of cerebral ischemia (Inoue, Jiang et al. 2003). More recently, we have found that PPAR $\alpha$  agonists may also reduce cerebral I/R injury when administered just before ischemia or during reperfusion (Collino, Aragno et al. 2006). We showed that the potential neuroprotective effects of PPAR $\alpha$  agonists is manifested by modulation of protein S100B levels in the rat CNS. S100B is a calcium-binding protein, mainly expressed in the brain and recent preclinical and clinical studies indicate that increased S100B levels is a reliable indicator of infarct size in acute ischemic stroke (Buyukuysal 2005; Foerch, Singer et al. 2005). Pre-treatment of rats with the selective PPAR $\alpha$  agonist, WY14643, prior to cerebral ischemia causes a marked reduction of S100B levels in the rat hippocampus. This protective effect is reversed by administration of the PPAR $\alpha$  antagonist, MK886, thus confirming the involvement of PPAR $\alpha$  activation in neuroprotection. Similarly, fenofibrate pretreatment for 14 days significantly reduced the cerebral infarct volume in an experimental model of Middle Cerebral Artery Occlusion (MCAO), although its withdrawal 3 days before induction of cerebral ischemia decreased the neuroprotective effect (Ouk, Laprais et al. 2009). Also prophylactic administration of gemfibrozil resulted in reduction of infarct size 24 h after MCAO and increased cortical blood flow in the ischemic hemisphere (Guo, Wang et al. 2009). However, the principal focus of studies of PPAR agonists has been on agonists of the PPAR $\gamma$  isoform. Emerging studies have reported the protective effects of PPAR $\gamma$  agonist administration in animal models of cerebral IRI (Sundararajan, Gamboa et al. 2005; Collino, Aragno et al. 2006; Allahtavakoli, Shabanzadeh et al. 2007) and in models of permanent ischemia (Sayan-Ozacmak, Ozacmak et al.; Zhang, Xu et al.). The effect of delayed post ischemia administration of a PPAR $\gamma$  agonist, rosiglitazone, has been recently evaluated, demonstrating that post-treatment with rosiglitazone, 24 h after stroke induction, may reduce ischemic injury, improve neurological outcome, and prevent neutrophilia, thus supporting an extended therapeutic window for the treatment of ischemic stroke (Allahtavakoli, Moloudi et al. 2009). Recent experimental data confirmed that PPAR $\gamma$  agonists are protective at clinically relevant doses, independent of any effects on systemic blood pressure or cerebral blood flow and, most notably, the timing of reperfusion relative to drug administration, may significantly influence the ability of PPAR $\gamma$  agonists to reduce infarction volume and improve neurologic function following ischemic injury (Gamboa, Blankenship et al.). The relevance of PPAR $\gamma$  as an endogenous protective factor was also shown by the fact that treatment with a PPAR $\gamma$  antagonist increased infarct size (Victor, Wanderi et al. 2006). Moreover, it was demonstrated that in primary cortical neurons of PPAR $\gamma$  KO mice exposed to ischemia there was a reduced expression of numerous key gene products (including superoxide dismutase-1, catalase, and glutathione S-transferase) along

with an increased damage. PPAR $\gamma$  mRNA is up-regulated in ischemic brain, especially in the peri-infarct area. Increased PPAR $\gamma$  mRNA was detected in the infarcted brain as early as 6 h following focal ischemia (Ou, Zhao et al. 2006), and PPAR $\gamma$  immunopositive neurons were detected between 4 h and 14 days, whereas in neurons and microglia only transiently at 12 h in the post-ischemic brain (Zhao, Patzer et al. 2005; Victor, Wanderi et al. 2006). The beneficial role of PPAR $\beta/\delta$  in stroke has been demonstrated by two different studies in which PPAR $\beta/\delta$  knockout mice subjected to cerebral IRI showed significantly larger infarct size than wild-type littermates (Pialat, Cho et al. 2007). This finding is confirmed by another study demonstrating that intracerebroventricular administration of high affinity PPAR $\beta/\delta$  agonists such as L-165041 and GW501516 significantly decreased the infarct volume at 24 h of reperfusion after cerebral ischemia in rats (Iwashita, Muramatsu et al. 2007).

#### 4.2 Clinical evidence of beneficial effects of PPAR ligands in ischemic stroke

Although various PPAR agonists applied before the onset of ischemia can effectively protect the brain in animal models of acute IRI, these treatments are seldom possible in the clinical setting of stroke because patients with stroke present after onset of the ischemic attack. Neuroprotective interventions applied after the onset of ischemia would thus seem to have greater clinical potential. Although some preclinical data provide evidence that administration of PPAR agonists during reperfusion decreases cerebral IRI, to date, there are no clinical data on the therapeutic efficacy of PPAR agonists administration after the onset of the ischemic event. Nevertheless, it must be noted that there may be subgroups of patients at high risk for stroke that could benefit from taking neuroprotective agents as prophylactic treatment. As already mentioned, pioglitazone and rosiglitazone (the TZD class of PPAR $\gamma$  agonists) have proven to be beneficial in type-2 diabetes mellitus patients. Diabetics are at an increased risk of stroke incidence and stroke causes more damage in diabetics compared to normoglycemic individuals. For this reason, such patients might benefit from taking an antidiabetic medication with neuroprotective properties, which might lessen the incidence and/or the severity of acute ischemic stroke. However, it's important to assess whether the potential benefits of taking an oral neuroprotective drug chronically outweighs the risks, including potential side effects. The use of a PPAR $\gamma$  agonist, specifically pioglitazone, as a preventive approach to ischemic brain injury has been recently addressed by two large clinical trials: the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive) and the Insulin Resistance Intervention after Stroke Trial (IRIS trial). The PROactive study has demonstrated that pioglitazone significantly reduces the combined risk of heart attacks, strokes and death by 16% in high risk patients with type-2 diabetes (Dormandy, Charbonnel et al. 2005). Enhanced functional recovery was also reported in a small group of stroke patients with type-2 diabetes treated with pioglitazone (Lee, Olson et al. 2006). However, it remain unclear whether the suggested beneficial effects of pioglitazone are mediated by insulin sensitization or by additional observed reductions in risk factors, such as hyperthension and dyslipidemia. This question and that related to the potential beneficial effects of pioglitazone in non-diabetic patients with stroke will be addressed by the IRIS trial, a randomized, double-blind, placebo-controlled trial on more than 3000 non-diabetic subjects who are insulin resistant and have had a recent transient ischemic attack or ischemic stroke. The IRIS study (ClinicalTrials.gov Identifier: NCT00091949) began on February 2005 and it is still recruiting patients. Interestingly, high

plasma levels of 15d-PGJ2 (the natural ligand for PPAR $\gamma$ ) have been associated with good neurological outcome and smaller infarct volume in patients with an acute atherothrombotic stroke (Blanco, Moro et al. 2005). Moreover, a recent report suggests that the Pro12Ala polymorphism of PPAR $\gamma$ 2 is associated with a reduced risk for ischemic stroke (Lee, Olson et al. 2006), further supporting the importance of PPARs in cerebral ischemia. Nevertheless, as TZDs are hampered by adverse effects related to increased weight gain, fluid overload, and congestive heart failure, the risks associated with chronic TZD administration needs to be better elucidated.

Abnormal levels of serum lipids, including triglycerides, low density lipoprotein (LDL) and high density lipoprotein (HDL), are regarded as other important risk factors for cerebrovascular disease, including stroke. The association between hypercholesterolemia and stroke has become more apparent because of data from prospective cohort studies that show higher risks of ischemic stroke with increasing levels of total cholesterol in both men and women. Increased HDL cholesterol levels have a protective effect against the occurrence of ischemic stroke and elevated triglyceride levels have also been reported as a risk factor for stroke. Overall, elevated total cholesterol confers an approximately two-fold relative increase in stroke risk for men and women. As fibrates are used as lipid-lowering agents, it has been supposed that these PPAR $\alpha$  agonists could also protect the brain against noxious biological reactions induced by cerebral IRI. A recent systematic meta-analysis of randomized clinical trials shows that fibrates do not significantly reduce the odds of stroke (Saha, Kizhakepunnur et al. 2007). However, data from large trials specifically investigating the role of fibrates in stroke event reduction are needed to conclusively elucidate their potential neuroprotective role. For instance, a large clinical trial, named Action to Control Cardiovascular Risk in Diabetes (ACCORD) is currently testing the ability of fenofibrate to decrease stroke incidence in high-risk patients with type-2 diabetes (ACCORD study group 2007).

## 5. Molecular mechanisms of beneficial effects of PPARs against cerebral ischemia

Cerebral IRI is known to induce generation of ROS, as well as the expression of cytokines, adhesion molecules and enzymes involved in the inflammatory response, and is known to be regulated by oxygen- or redox-sensitive mechanisms. Recent studies have confirmed the pivotal role of both oxidative stress and inflammatory response in the pathogenesis of acute ischemic stroke. Through various mechanisms PPARs can regulate both inflammatory and oxidative pathways and PPAR agonist-induced neuroprotection seems to be specific for injuries in which inflammation or free radical generation are the main causes of cell damage. For instance, PPAR $\alpha$  activation can induce expression and activation of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH). We have demonstrated that administration of a highly selective PPAR $\alpha$  agonist, WY14643, 30 min prior to IRI, decreased ROS production and lipid peroxidation in rats subjected to IRI and, at the same time, offered protection against GSH depletion (Collino, Aragno et al. 2006). Similar results on oxidative stress modulation have been reported when another PPAR $\alpha$  agonist, fenofibrate, was tested in a mouse model of middle cerebral artery occlusion (Deplanque, Gele et al. 2003). Interestingly, PPAR $\gamma$  KO mice have been found to exhibit significant increases in oxidative stress and lipid peroxidation much earlier in their life than



wild-type littermates (Poynter and Daynes 1998). The PPAR-induced protective effect on oxidative stress could be related to a direct effect on antioxidant enzyme expression, as the catalase and SOD gene promoters contain the PPRE. In fact, rats that have been treated with a diet containing PPAR $\alpha$  ligands, WY14643 or fenofibrate, have demonstrated an enhanced expression of antioxidant enzymes such as SOD and catalase (Toyama, Nakamura et al. 2004). Based on gene expression microarray experiments, Coleman and colleagues (Coleman, Prabhu et al. 2007) have demonstrated that PPAR $\beta/\delta$  activation increased mRNA for aldehyde dehydrogenase and glutathione-S-transferase, thus protecting the cell from oxidative damage. In normotensive and hypertensive animals treated with rosiglitazone, ischemic hemispheres showed increased catalase and Cu/Zn-SOD activity in the peri-infarct region (Tureyen, Kapadia et al. 2007) and the level of Cu/Zn-SOD was demonstrated to increase in the ischemic cortex of animals treated with pioglitazone for 4 days prior to focal cerebral ischemia (Shimazu, Inoue et al. 2005). As we have recently shown, treatment of rats with either pioglitazone or rosiglitazone before occlusion of the common carotid artery decreased the production of ROS and nitrite, decreased lipid peroxidation and reversed the depleted stores of glutathione in the hippocampus (Collino, Aragno et al. 2006). These findings are supported by data from an *in vitro* model demonstrating that pre-treatment with PPAR $\gamma$  agonists protected an immortalized mouse hippocampal cell line against oxidative stress induced by glutamate or hydrogen peroxide (Aoun, Watson et al. 2003). Moreover, PPAR $\gamma$  agonists attenuate the expression of iNOS in inflammatory cells, which is an important source of nitric oxide (NO). NO may react with ROS to produce peroxynitrites, with deleterious effects on neuronal survival. Thus, iNOS inhibition may represent a further mechanism for neuroprotection by PPAR agonists. Mitochondria are the major source of ROS, which are mainly generated at complexes I and III of the respiratory chain. There is now evidence indicating that rosiglitazone and pioglitazone exert direct and rapid effects on mitochondrial respiration, inhibiting complex I and complex III activity (Brunmair, Lest et al. 2004). As PPAR $\gamma$  agonists partially disrupt the mitochondrial respiratory chain, both electron transport and superoxide anion generation are affected. Moreover, a novel mitochondrial target protein for PPAR $\gamma$  agonists ("mitoNEET") has recently been identified (Colca, McDonald et al. 2004). MitoNEET was found associated with components of complex III, suggesting how binding of PPAR $\gamma$  agonists to mitoNEET could selectively block different mitochondrial targets. The ability of PPAR $\gamma$  agonists to influence mitochondrial function might contribute to their inhibitory effects on ROS generation that is evoked by IRI.

Another mechanism through which PPAR agonists may provide neuroprotection is by down-regulating the inflammatory response associated with IRI. Depending on the affected tissue and which PPAR isoforms are involved, PPAR agonists can differently modulate the intensity, duration and consequences of inflammatory events. For instance, ischemia-induced COX-2 overexpression is prevented by PPAR $\gamma$  agonists but not by PPAR $\alpha$  agonists (Sundararajan, Gamboa et al. 2005; Collino, Aragno et al. 2006; Collino, Aragno et al. 2006). Activation of PPAR $\gamma$  attenuates the expression of matrix metalloproteinase (MMP)-9 and various inflammatory cytokines in ischemic brain tissue (Pereira, Hurtado et al. 2005). PPAR $\gamma$  is constitutively expressed in macrophages and microglial cells and the systemic treatment of rodents with rosiglitazone reduces the infiltration of these cells into peri-infarct brain regions. Both chronic and acute administration of PPAR $\gamma$  agonists has been demonstrated to prevent cerebral IRI-induced expression of vascular cell adhesion

molecule-1 (VCAM-1) and ICAM-1 in two independent studies (Deplanque, Gele et al. 2003; Collino, Aragno et al. 2006). In the brain, the decreased expression of these adhesion molecules might contribute to inhibit the infiltration of the brain ischemic area by neutrophils. Studies addressing the molecular mechanisms of these anti-inflammatory actions demonstrated that the involvement of PPARs in the control of IRI-induced inflammation is mediated mainly through their transrepression capabilities. PPARs can suppress the activities of many distinct families of transcription factors. The range of transcription factors affected and the mechanisms involved may be different for each PPAR isotype, although a common mechanism of PPAR $\alpha$  and PPAR $\gamma$  neuroprotection appears to involve inhibition of p38 MAPK activation and NF- $\kappa$ B nuclear translocation. A recent study confirms that PPAR $\gamma$  activation prevents the post-ischemic cerebral expression of pro-inflammatory transcription factors, such as Egr1, C/EBP and NF- $\kappa$ B, possibly by decreasing DNA binding (Tureyen, Kapadia et al. 2007). The inhibitory protein I $\kappa$ B $\alpha$ , which is an indicator of NF- $\kappa$ B transcriptional activity, is remarkably increased in the brain of rats that underwent cerebral ischemia and completely blocked by rosiglitazone and 15d-PGJ2 administration, thus further confirming that both endogenous and synthetic PPAR $\gamma$  ligands inhibit NF- $\kappa$ B signalling (Pereira, Hurtado et al. 2006). Similarly, p38 MAPK and NF- $\kappa$ B activation by cerebral IRI has been demonstrated to be inhibited by pre-treatment with the PPAR $\alpha$  agonist WY14643 or the PPAR $\gamma$  agonist pioglitazone. However, as MAPK and NF- $\kappa$ B are functionally interconnected and do not act independently, we cannot rule out the possibility that PPARs affect NF- $\kappa$ B activation by interfering with the MAPK signalling cascade or vice versa.

The generation of ROS is known to be associated with the induction of apoptosis and, in neurons, inhibition of cell death is an important factor to prevent during IRI. PPAR activation may decrease the IRI-induced activation of apoptotic pathways depending on the increase in activity and expression of numerous anti-oxidant enzymes. Moreover, by their anti-inflammatory action on microglia and astrocytes, PPAR agonists prevent the release of neurotoxic agents, which induce neuronal apoptosis. PPAR $\gamma$  agonists may attenuate ischemia-induced reactive oxygen species and subsequently alleviate the post-ischemic degradation of Bcl-2, Bcl-xl, and Akt, by increasing SOD/catalase and decreasing nicotinamide adenine dinucleotide phosphate oxidase levels (Fong, Tsai et al.). Chu and colleagues (Chu, Lee et al. 2006) have demonstrated that rosiglitazone-fed rats had better neurological scores and reduced number of TUNEL-positive cells following transient focal ischemia. Interestingly, these authors also reported an increased vasculature in the rosiglitazone-treated group with increased number of endothelial cells positive for BrdU, suggesting there may be enhanced angiogenesis following PPAR $\gamma$  activation. Administration of a selective PPAR $\gamma$  agonist (L-796449) 10 min prior to permanent cerebral artery occlusion, resulted in decreased apoptosis, measured as reduction of caspase-3 activity (Pereira, Hurtado et al. 2005). Another study confirmed inhibition on caspase-3 activity by both exogenous and endogenous PPAR $\gamma$  agonists, rosiglitazone and 15d-PGJ2, in the ischemic cortex (Lin, Cheung et al. 2006). The same authors observed that rosiglitazone and 15d-PGJ2 exhibit a concentration-dependent paradoxical effect on cytotoxicity, when tested in an *in vitro* model of hydrogen peroxide induced neuronal apoptosis. The drugs induced pro-apoptotic effects when used at concentrations higher than 5  $\mu$ mol/L but protect neurons from necrosis and apoptosis at concentrations lower than 1  $\mu$ mol/L. The reason for

this paradoxical action is unclear and further studies are needed to better clarify the effects of PPARs in IRI induced-apoptosis and necrosis.

Recently published data suggest that an increased uptake of cerebral extracellular glutamate levels after ischemia may represent an additional mechanism for the neuroprotection exerted by PPAR $\gamma$  activation (Romera, Hurtado et al. 2007). Both *in vivo* and *in vitro* experiments showed that rosiglitazone administration increased the expression of the GLT1/EAAT2 glutamate transporter in the brain, thus preventing the extracellular glutamate levels from rising to neurotoxic values.

## 6. Conclusion

Although clinical data are limited, a wide array of evidence obtained in animal models now shows that PPAR activation may be a rational and effective strategy against ischemic brain damage. The beneficial effects of PPAR agonists in experimental models of stroke are mediated by different mechanisms, as expected based on their pleiotropic pharmacological profile. The neuroprotective actions appear to be mainly related to the reduction in oxidative damage as well as anti-inflammatory and anti-apoptotic effects. These results have been essentially obtained with PPAR $\alpha$  and PPAR $\gamma$  agonists, while the PPAR $\beta/\delta$  pathway remains largely unexplored, despite a significant interest in this target. Selective activation of different isoforms of PPARs may account for the difference in molecular pathways underlying neuroprotection and these different features still remain far from being completely understood. In conclusion, currently available management protocols for patients with stroke may benefit from the use of PPAR agonists that target detrimental processes associated with IRI. However, several critical issues still need to be resolved. For instance, well-structured clinical trials aimed at evaluating the effects of PPAR ligands on stroke recovery are needed before firm conclusions are drawn about their therapeutic efficacy. A more stringent approach regarding the concentration range of PPAR agonists, especially within the CNS, and the duration of exposure should be applied. Also acceptable water solubility with satisfactory blood-brain barrier penetrability is an important aspect of PPAR agonists that needs to be optimized.

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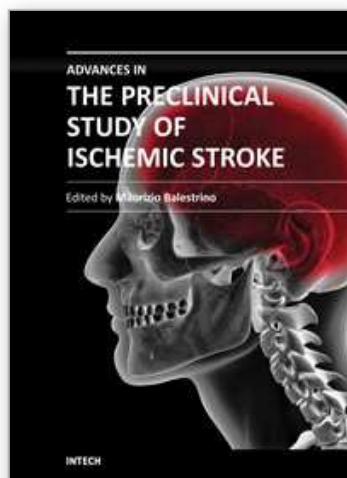
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## **Advances in the Preclinical Study of Ischemic Stroke**

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This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

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