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Anti-apoptotic Actions of PPAR-γ Against Ischemic Stroke

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Abstract Stroke is a leading cause of adult disability and mortality. Diabetes is a major risk factor for stroke. Patients with diabetes have a higher incidence of stroke and a poorer prognosis after stroke. Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a ligand-modulated transcriptional factor and a therapeutic target for treating type II diabetes. It is well-documented that activation of PPAR-γ can also attenuate postischemic inflammation and damage. In this review, we focus on the newly revealed anti-apoptotic actions of PPAR-γ against cerebral ischemia. PPAR-γ, by increasing superoxide dismutase/catalase and decreasing nicotinamide adenine dinucleotide phosphate oxidase levels, attenuated ischemia-induced reactive oxygen species and subsequently alleviated the postischemic degradation of Bcl-2, Bcl-xl, and Akt. The preserved Akt phosphorylated Bad. Meanwhile, PPAR-γ also promotes the transcription of 14-3-3ε. Elevated 14-3-3ε binds and sequesters p-Bad and prevents Bad translocation to neutralize the anti-apoptotic function of Bcl-2. This review further supports the notion that PPAR-γ may serve as a potential therapeutic target for treating ischemic stroke.

Keywords Fatty acids · 15d-PGJ$_2$ · Rosiglitazone · ROS · Bcl-2 · Bad · 14-3-3ε

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-modulated transcriptional factors and belong to the nuclear hormone receptor superfamily. PPARs are also named NR1Cs [1]. PPAR was originally cloned from liver peroxisomes, organelles that participate in fatty acid metabolism [2]. To date, three novel members have been cloned, namely PPAR-α (NR1C1), PPAR-β/δ (NR1C2), and PPAR-γ (NR1C3), which are encoded by three distinct genes located on human chromosomes 22, 6, and 3, respectively [3, 4]. Aside from mediating peroxisome proliferation, PPARs have been shown to involve in insulin sensitivity, glucose homeostasis, fatty acid oxidation, cytokine production, and vasculoprotection [5].

PPARs form heterodimers with the retinoid X receptor (RXR), which is activated by 9-*cis* retinoid acid. There is no specific role for RXR within the PPAR/RXR complex; however, synthetic RXR agonists can also activate this complex, leading to antidiabetic outcome as PPAR agonist [6]. Heterodimers then bind to specific DNA sequences called peroxisome proliferator response elements (PPREs) in the promoter of target genes. In the absence of ligand, heterodimers associate with corepressor complexes which repress target genes. While in the presence of ligand, PPAR/RXR binds to coactivator complexes and initiates transcription of target genes [7, 8].

The three PPAR isoforms share a high degree of structural similarity, despite exhibiting considerable tissue specific expression. PPAR-α is highly expressed in tissues that catabolize fatty acids. PPAR-β/δ ubiquitously expressed in all cell types with higher levels in proliferating and differentiating cells. PPAR-γ is abundantly expressed in the adipose tissue. All three isoforms are co-expressed in the nervous system during embryo genesis, although PPAR-

β/δ seems to be the predominant one. While PPAR-β/δ remains highly expressed, the expression of PPAR-α and PPAR-γ decreases postnatally in the developing brain [9]. All three isoforms have been indicated in ischemic brain injury [10, 11]. However, their actions remain to be fully elucidated. In the present review, we will focus on PPAR-γ because it is the most extensively studied among these three isoforms in stroke.

PPAR-γ Gene

Human PPAR-γ gene was mapped to chromosome 3 at position 3p25 while the mouse gene is found in chromosome 6. The PPAR-γ gene consists of exons 1–6 in the open reading frame. Exons 2 and 3 encode the DNA-binding domain, and exons 5 and 6 encode the ligand-binding domain (LBD). Located in the C terminus of the LBD is the ligand-dependent activation domain, AF-2. This region is intimately involved in binding of the receptors' coactivator. A ligand-independent activation function, AF-1, is found in close proximity to the N terminus of the receptor [9].

The 5′ terminal is the most variable and is the determinate of PPAR-γ transcript isoforms. Up-to-date, two highly conserved transcripts, PPAR-γ1 and PPAR-γ2, were identified in mouse [12]; seven transcripts, PPAR-γ1~7, were identified in monkeys [13]; and six transcripts, PPAR-γ1~5 and 7, were identified in human [14]. The reason for existing different transcripts among species is not known, and their specific functions remain to be studied. These seven different transcripts encode for only three different proteins: (1) a 54.5-kD PPAR-γ1 protein for PPAR-γ1, 3, 5, and 7 transcripts; (2) a 57.6-kD PPAR-γ2 protein for PPAR-γ2 transcript, and (3) a 55.3-kD PPAR-γ4 protein for PPAR-γ4 and 6 transcripts. Again, the physiological role for multiple protein isoforms is largely unknown. Intriguingly, a study by Chen et al. [14] further indicated that a PPAR-γ-specific ligand thiazolidinediones (TZD) could induce the expression of PPAR-γ5 and 7 transcripts but inhibit the expression of PPAR-γ1 and 2 transcripts. Ironically, TZD induces the expression of PPAR-γ1 and 2 in rodent, and PPAR-γ2 is the most well-studied isoform in mice. The differential regulation of PPAR-γ transcripts among species deserves further studies.

PPAR-γ Ligands

A broad spectrum of PPAR-γ ligands has been identified and can be divided in to three major groups [3, 15] (Table 1):

(a) Natural (endogenous) agonists which can be further divided into four subgroups: (1) unsaturated fatty acids, (2) eicosanoids, (3) oxidized phospholipids, and (4) nitroalkenes. Among these compounds, the cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15d-PGJ_2) not only is the most potent ligand but is also by far the most commonly used naturally occurring ligand for PPAR-γ. In studies in vitro, it takes micromolar range of 15d-PGJ_2 to activate PPAR-γ; however, the physiological level of 15d-PGJ_2 is at low picomolar concentrations [16]. Whether 15d-PGJ_2 is the physiological ligand for PPAR-γ in adipogenesis is still unclear. Nitroalkenes (nitric oxide (NO)-derived unsaturated fatty acids) is a newly identified potent endogenous PPAR-γ ligand. In human blood, the level of nitrolinoleic acid (LNO_2) is around 0.5 μM representing an abundant pool [17]. Whether nitroalkenes can sever as the true physiological ligand for PPAR-γ also deserves further study.

Table 1 PPAR-γ ligands

Natural (endogenous) agonists	
Unsaturated FAs	DHA, linoleic acid…
Eicosanoids	15d-PGJ_2, 15d-PGD_2, PGA_1…
Oxidized lipids	azPC, LPA…
Nitroalkenes	LNO_2, OA-NO_2…
Synthetic agonists	
TZDs	Rosiglitazone, pioglitazone…
Non-TZDs	GW-347845, NSAIDs, indenones…
Dual-α/γ	Muraglitazar, tesaglitazar, resveratrol…
Pan-α/δ/γ	Benzafibrate, GW-677954…
SPPAR-γM	Telmisartan, halofenate…
Synthetic antagonists	
T0070907	IC_{50}=1 nM
GW9662	IC_{50}=3.3 nM
PD068235	IC_{50}=0.82 μM
LG100641	IC_{50}=0.9 μM
G3335(1a)	IC_{50}=8.67 μM
BADGE	IC_{50}=100 μM
SR-202	IC_{50}=140 μM

FAs fatty acids, *DHA* docosahexaenoic acid, *azPC* 1-*O*-hexadecyl-2-azelaoyl-sn-glycero-3-phosphocholine, *LPA* lysophosphatidic acid, *TZDs* thiazolidinediones, *Nitroalkenes* NO-derived unsaturated FAs, *LNO_2* 9, 10, 12, 13-nitro-9, 12-*cis*-octadecadienoic acid, *OA-NO_2* 9-, 10-nitro-9-*cis*-octadecenoic acids, *NSAIDs* nonsteroidal anti-inflammatory drugs, *15d-PGJ_2* 15-deoxy-Δ-12,14-prostaglandin J_2, *SPPAR-γM* selective PPAR-γ modulator, *BADGE* bisphenol A diglyceryl ether

Cerebral ischemia is caused by obstruction of blood flow to the brain, resulting in energy failure and subsequently initiating a series of metabolic events. One such critical metabolic event is the activation of

phospholipase A_2, which led to membrane phospholipids hydrolysis and subsequent releasing of free fatty acids including arachidonic acid, a metabolic precursor of eicosanoids [18–21]. It is very interesting to note that all the above natural ligands are metabolites of ischemic stroke. PPAR-α and PPAR-β/δ also use fatty acid metabolites as their natural ligands, however, with different affinity/specificity [5]. Genetic deletion or decreasing the level of individual PPARs can lead to an increase in ischemic brain injury, indicating the pivotal role of all three PPARs in the pathogenesis of ischemic stroke [10, 22] (Fig. 1).

(b) Synthetic agonists may be further divided into five subgroups: (1) TZDs (also called glitazones), (2) non-TZDs, (3) dual-α/γ agonist (also called glitazars), (4) pan-α/δ/γ agonist, and (5) selective PPAR-γ modulator (SPPAR-γM). Among them, TZD, a class of antidiabetic drugs known to improve insulin sensitivity, was the first ligand discovered to bind to and activate PPAR-γ [23]. Troglitazone was the first Food and Drug Administration-approved TZD used in the clinical setting, which was later discontinued due to hepatotoxicity. Rosiglitazone (Avandia) and pioglitazone (Actos) are newer TZD derivatives with low liver toxicity. However, these compounds are associated with adverse effects including weight gain, peripheral edema, cardiac load, and hemodilution [24]. Consequently, the adverse effects encouraged more compounds to be developed. For example, SPPAR-γM was developed in the hope to moderately stimulate the PPAR-γ and reduce its detrimental side effects. The Dual-α/γ and Pan-α/δ/γ agonists have been developed to promote synergistic effects since all three PPARs attenuated ischemic injury as described above [10, 11]. Nonetheless, one should keep in mind that not all the adverse effects of TZD are PPAR-γ dependent. Specificity always is an inherited problem for using agonists [25, 26].

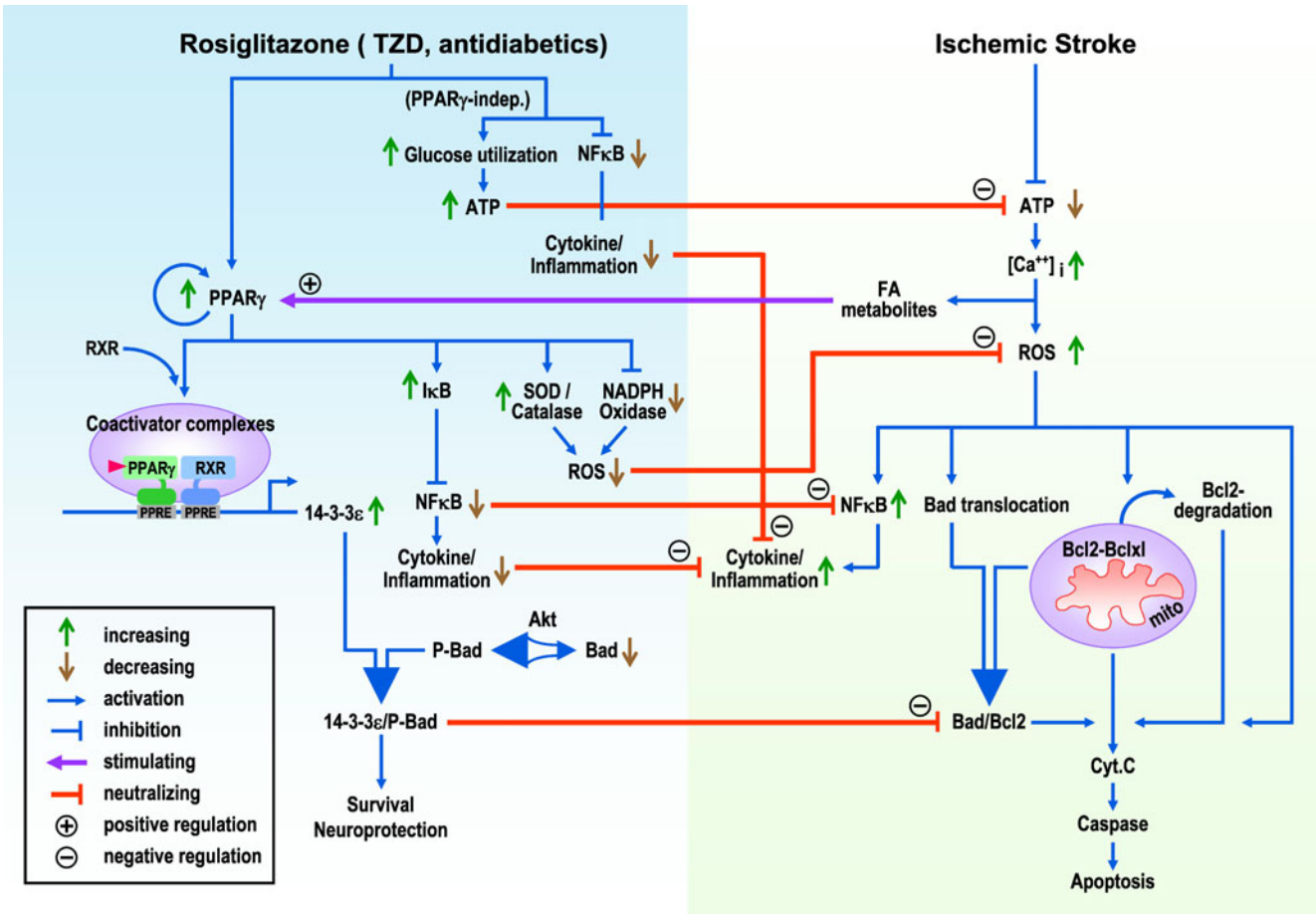

Fig. 1 Schematic drawing elucidates the actions of rosiglitazone and PPAR-γ against ischemic stroke. Rosiglitazone by (1) activates PPAR-γ, (2) increases glucose utilization and ATP level, and (3) inhibits NF-κB and inflammation to attenuate stroke injury. Furthermore, PPAR-γ by (1) increases 14-3-3ε level, which then sequestering p-Bad and preventing its translocation to neutralize Bcl-2's anti-apoptotic function; (2) increases IκB, which then downregulating NF-κB and inflammation; and (3) increase SOD/catalase as well as decrease NADPH oxidase activities, which then reducing ROS production, to counteract ischemic-induced cell death signaling

(c) Synthetic antagonists which include bisphenol A diglyceryl ether, GW9662, LG100641, PD068235, and SR-202. Antagonists are important tools to decipher the signaling and functions of PPAR-γ. So far, no endogenous antagonist for PPAR-γ has been reported.

PPAR-γ agonists can also initiate PPAR-γ-independent (or nonreceptor) effects. For example, 15d-PGJ_2 (natural) may engage distinct PGD_2 receptors (DP_1, DP_2) to activate protein kinase A (anti-inflammatory) and stimulate Ca^{2+} influx (inflammatory). In the cytosol, PGJ_2 may directly activate the Ras/Erk mitogen-activated protein kinase pathway and bind to and inhibit IκB kinase, the activator of NF-κB. Additionally, PGJ_2 may inhibit NF-κB activity by (1) directly blocking NF-κB DNA binding sites in the nucleus and (2) activating PPAR-γ to stimulate synthesis of the NF-κB inhibitory subunit IκB [26]. TZD's (synthetic) receptor-independent effects include suppression of inflammatory gene expression, modification of energy and fuel metabolism, suppression of cell proliferation and induction of cytotoxicity, perturbation of mitochondrial function, and others [25, 27].

PPAR-γ Agonists and Anti-inflammatory Effect

TZDs and 15d-PGJ_2 have been reported to be effective in attenuating tissue damage caused by ischemia–reperfusion (I/R) in animal models [28–32]. The mechanism by which they protect tissues against I/R injury is not entirely clear. Since inflammation is a crucial pathophysiological process that accompanies tissue injury and ligand-activated PPAR-γ represses the expression of pro-inflammatory genes [33–35], the protective effect of PPAR-γ ligands is attributed to control of inflammation. The anti-inflammatory actions of PPAR-γ agonists in ischemic stroke have been extensively reviewed recently [3, 36–39]. However, it is not clear whether receptor-independent anti-inflammatory action also play a role in this event [25, 26] (Fig. 1).

PPAR-γ Directly Attenuates Cerebral Infarction

It is well established that PPAR-γ ligands reduce ischemic infarct by activation of PPAR-γ. However, there are possibilities for the involvement of receptor-independent signals. In fact, there is no definitive evidence indicating that activated PPAR-γ pathways are critical for the beneficial effects of TZDs. Therefore, the extent stimulation of PPAR-γ contributes to the neuroprotective effect of PPAR-γ agonists, and the underlying molecular mechanisms remain to be elucidated.

Lacking the direct proof of PPAR-γ attenuated ischemic injury is due largely to embryonic lethal upon disruption of the PPAR-γ gene [40]. A recent study indicated that the heterozygote PPAR-γ P465L mutant mice are viable, which possess only half of the PPAR-γ activity as compared to their wild-type control [41]. Interestingly, infarct volumes induced by transient focal ischemia in these mutant mice were twice as large as their wild-type litter mates control [22]. Furthermore, conditional neuron-specific PPAR-γ knockout mice also showed significantly more brain damage and oxidative stress in response to middle cerebral artery occlusion [42]. These mice are viable and appeared to be normal with respect to their gross behavior and brain anatomy. Moreover, the neuroprotective effect of PPAR-γ was wiped out in CaMKIIalphaCre/LMO4loxP mice with LMO4, a PPAR-γ coactivator, ablated in neurons of the forebrain [43]. On the other hand, intraventricular infusion of PPAR-γ recombinant protein led to smaller infarct volume [11]. Similarly infusion of adenovirus carrying PPAR-γ gene also attenuated infarct volume (unpublished data). The above studies provide direct evidences and support for the pivotal role of PPAR-γ in ischemic stroke.

PPAR-γ and Post-ischemic Apoptosis

The term "programmed cell death (apoptosis)" was first reported in 1972. It is characterized by a series of well-defined and distinct morphological and biochemical changes. The Bcl-2 family proteins are located at outer mitochondria membranes, and it controls the activation of downstream caspases, thus representing a critical proximal intracellular checkpoint in the mitochondria-mediated apoptosis pathway [44–46]. Apoptosis is triggered following cerebral ischemia by a variety of death signals, such as production of free radicals, cytochrome C release during mitochondrial injury, and caspases activation, etc. This chain of event ultimately leads to DNA damage and breakdown of cellular infrastructure. Importantly, such events are likely to occur in the penumbra, where cellular integrity is still preserved and sufficient energy is available. Furthermore, severity of the insult also determines the development and extension of infarction in the penumbra [47].

Recent studies suggest that ligand-activated PPAR-γ controls apoptosis and contributes to tissue protection. Several laboratories, including ours, have provided experimental evidence to support the anti-apoptotic actions of 15d-PGJ_2 and rosiglitazone [28, 48–50]. However, others have reported pro-apoptotic actions of PPAR-γ ligands [51–53]. The reason for the opposite action of TZDs and 15d-PGJ_2 is unclear. Nonetheless, we and others have reported that in culture cells, 15d-PGJ_2 and TZDs at

relatively low concentrations are anti-apoptotic, while at high concentrations, they are pro-apoptotic [28, 50]. Intriguingly, high concentrations of PPAR-γ ligands were used in all the studies that reported pro-apoptotic actions. The occurrence of low-dose stimulation and high-dose inhibition or "a U-shaped dose–response relationships" is often termed hormesis, has been known for a long time and documented in numerous pharmacological investigations [54]. Nevertheless, the underlying cellular and molecular mechanisms are not clearly understood. Over 30 agents with neuroprotective properties were found acting via U-shaped dose responses, suggesting that this type of dose response can be a general phenomenon in animal stroke models and an important phenomenon in clinical practice [55]. Nevertheless, other sources of variations such as cell types and experimental conditions may also contribute to the opposite results.

In vitro study revealed that rosiglitazone at concentrations up to 5 μM protected against oxygen-glucose deprivation (OGD)-induced cytotoxicity and apoptosis. Rosiglitazone suppressed H_2O_2 generation, maintained mitochondrial membrane potential (MMP), attenuated cytochrome C release, inhibited caspase 9 and caspase 3 activation, and suppressed cleavage of poly(ADP-ribose) polymerase. PPAR-γ overexpression alone had a comparable anti-apoptotic effect. The anti-apoptotic effects of rosiglitazone or PPAR-γ overexpression were abrogated by GW9662, a selective PPAR-γ antagonist or by PPAR-γ small interference RNA (siRNA). Thus, rosiglitazone–PPAR-γ protects against hypoxia- and oxidant-induced apoptosis by attenuating reactive oxygen species (ROS) production and thereby controls premitochondrial apoptotic targets [11]. Furthermore, PPAR-γ agonists mediate the transcription of superoxide dismutase (SOD) and catalase genes, PPRE sites were found at the promoter region of these two genes, and their upregulation is reversed by the PPAR-γ antagonist GW9662, while PPAR-γ agonists downregulate the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [56–59]. SOD and catalase are known free radical scavengers, while NADPH oxidase is a ROS generator.

Mitochondrial potential is maintained by a balance between anti-apoptotic Bcl-2 family proteins, notably Bcl-2 and Bcl-xl, and pro-apoptotic family members, such as Bad and Bax [44, 46]. Bcl-2 and Bcl-xl are localized to mitochondrial membrane. Upon injury signals, the pro-apoptotic members, such as Bad and Bax, translocate to mitochondria where they bind Bcl-2 and Bcl-xl, neutralize their anti-apoptotic properties, and induce changes in MMP. OGD caused a significant reduction of Bcl-2 and Bcl-xl protein levels, which could be restored by pretreatment with rosiglitazone or PPAR-γ overexpression. Restoration of Bcl-2 and Bcl-xl proteins were abrogated by PPAR-γ siRNA or GW9662, indicating that this effect of rosiglitazone is PPAR-γ dependent [11, 48]. Similarly, p-Bad, p-Akt, and PDK-1 levels were suppressed by OGD but were restored by rosiglitazone or PPAR-γ overexpression. Since Bad is phosphorylated by Akt, it is very likely that rosiglitazone maintains p-Bad via the PI-3K/PDK-1/Akt pathway, which then attenuates Bad translocation and MMP breakdown [11, 60–62]. However, how does PPAR-γ preserve Bcl-2/Bcl-xl and PI-3K/PDK-1/Akt remain to be studied.

Proteomic analysis further revealed that brain 14-3-3ε was highly upregulated in ischemic rats treated with rosiglitazone. Upregulation of 14-3-3ε was abrogated by PPAR-γ siRNA or antagonist. 14-3-3ε promoter region harbors three PPAR response elements (PPREs). Promoter analysis and chromatin immunoprecipitation assay revealed that rosiglitazone induced PPAR-γ binding to specific regulatory elements on the 14-3-3ε promoter and thereby increased 14-3-3ε transcription. 14-3-3ε siRNA abrogated the anti-apoptotic actions of rosiglitazone or PPAR-γ overexpression, whereas 14-3-3ε recombinant proteins rescued brain tissues and N2-A cells from ischemia-induced damage and apoptosis. 14-3-3ε upregulation enhances sequestration of p-Bad and prevents Bad translocation to neutralize the anti-apoptotic functions of Bcl-2 and ultimately suppresses apoptosis. These results provide strong evidence for an essential role of PPAR-γ-mediated 14-3-3ε upregulation in neuroprotection [22].

Intriguingly, 14-3-3ε expression was also upregulated by prostacyclin, a PPAR-δ/β ligand, in cultured endothelial cells (human umbilical vein endothelial cells). Elevated 14-3-3ε protein level also enhanced sequestration of Bad and inhibited endothelial cells apoptosis [63]. These results indicate that 14-3-3ε upregulation represents a novel protective way for both neurons and endothelial cells, by ligand-restricted PPAR-γ or PPAR-δ/β activation. Therefore, 14-3-3ε may serve as a target for treating diseases such as ischemic stroke, in which both neuron and endothelial were injured. Since none of the existing dual-PPAR-α/γ agonists had reached market expectations, dual-PPAR-δ/γ agonist may be a good alternative.

Summary

PPAR-γ, by increasing SOD/catalase and decreasing NADPH oxidase levels, attenuated ischemia-induced ROS and subsequently alleviated the postischemic degradation of Bcl-2, Bcl-xl, and Akt. The preserved Akt phosphorylated Bad to prevent Bad binding to Bcl-2 and membrane potential breakdown. Meanwhile, PPAR-γ also promotes the transcription of 14-3-3ε. Elevated 14-3-3ε can bind and sequester p-Bad and prevents Bad translocation to

neutralize Bcl-2's anti-apoptotic function, thereby suppresses apoptosis. In short, PPAR-γ-mediated 14-3-3ε upregulation plays a pivotal role in the beneficial effects of rosiglitazone against ischemic stroke.

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Conflict of interest Authors declare no conflict of interest.

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