Hydroxysafflor Yellow A Shows Protection against PPARγ Inactivation in Nitrosative Neurons

Li Sun, Yan-Wei Xu, Jing Han, Chen Xiao, Shan-Shan Cao, Hao Liang, and Yan Cheng

Tianjin Medical University General Hospital, Tianjin Neurological Institute, Key Laboratory of Post-trauma Neuro-repair and Regeneration in Central Nervous System, Ministry of Education and Tianjin City, Tianjin 300052, China

Correspondence should be addressed to Li Sun; lisun@tmu.edu.cn

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Peroxynitrite-mediated nitrosative stress in the brain has been associated with various neurodegenerative disorders. Recent evidence highlights peroxisome proliferator-activated receptor γ (PPARγ) as a critical neuroprotective factor in neurodegenerative diseases. Here, we observed the effect of the herb hydroxysafflor yellow A (HSYA) during nitrosative stress in neurons and investigated the mechanism based on PPARγ protection. We found that a single exposure of primary neurons to peroxynitrite donor SIN-1 caused neuronal injury, which was accompanied by the increase of PPARγ nitration status and lack of activation of the receptor, as measured by PPARγ DNA-binding activity, by agonist (15d-PGJ2 or rosiglitazone) stimulation. The crucial role of PPARγ in neuronal defense against nitrosative stress was verified by showing that pretreatment with 15d-PGJ2 or rosiglitazone attenuated SIN-1-induced neuronal injury but pretreatment with GW9662, a PPARγ antagonist, aggravated SIN-1-induced neuronal injury. The addition of HSYA not only inhibited SIN-1-induced neuronal damage but prevented PPARγ nitrative modification and resumed PPARγ activity stimulated by either 15d-PGJ2 or rosiglitazone when the agonists were coincubated with SIN-1. Furthermore, HSYA also showed the ability to rescue the neuroprotective effect of 15d-PGJ2 or rosiglitazone when the agonists were coincubated with SIN-1. Finally, in vivo experiments demonstrated that the administration of HSYA also efficiently blocked PPARγ nitration and loss of activity in the SIN-1-injected hippocampus and reversed the increased neuronal susceptibility which was supported by the inhibition of Bcl-2 protein downregulation induced by SIN-1. The results suggest that HSYA protects neurons from nitrosative stress through keeping PPARγ as a functional receptor, allowing a more effective activation of this neuroprotective factor by the endogenous or exogenous agonist. Our findings provide new clues in understanding the role of the neuroprotective potential of the herbal HSYA.

1. Introduction

Excessively produced nitric oxide (NO) and superoxide lead to the generation of peroxynitrite (ONOO−). Peroxynitrite-mediated nitrosative stress causes severe damage to proteins, lipids, and DNA, resulting in cell apoptosis or death. 3-Nitrotyrosine (3-NT) formation has been used extensively as a footprint for the nitrosative stress induced by peroxynitrite [1]. The concentration of 3-NT has been reported to increase in a wide range of neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, and traumatic or ischemic brain injury [2–5]. In the ischemic brain, the formation of 3-NT was elevated markedly and the significantly elevated 3-NT was positively correlated with infarct volume in ischemic animals [2]. Also, 3-NT accumulation has been proven to associate with cognitive decline in the AD brain [5]. Furthermore, the inhibition of 3-NT formation protects against brain injury in these disorders [2–5]. Thus, peroxynitrite-mediated nitrosative stress represents an important pathogenic mechanism of neurodegenerative diseases.

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor that regulates lipid metabolism and glucose homoeostasis. 15-Deoxy-delta prostaglandin J2 (15d-PGJ2), unsaturated fatty acids, and oxidized phospholipids are PPARγ natural ligands. Its synthetic ligands include the thiazolidinedione (TZD) class of insulin-sensitizing agents (troglitazone, pioglitazone,
ciglitazone, and rosiglitazone) and a few of nonsteroidal anti-inflammatory drugs (NSAIDs). Recent studies have shown that, in addition to its classical role, PPARγ activation is neuroprotective against inflammatory reaction and oxidative stress in models of neurodegenerative conditions [6–8]. For example, PPARγ agonist troglitazone or pioglitazone reduced inflammation and infarct volume and improved neurological function following middle cerebral artery occlusion in rats [7]. In cultured hippocampal neurons, rosiglitazone was of protection against mitochondrial damage, oxidative stress, and apoptosis induced by β-amyloid (Aβ) [8].

Hydroxysafflor yellow A (HSYA) (C27H32O16, MW 612.53), as presented in Figure 1(a), is a water-soluble monomer extracted from the safflower plant (Carthamus tinctorius L.). HSYA has been reported to be a natural antioxidant used in traditional Chinese medicine. The antioxidant properties of HSYA in the brain are of particular interest because of the fundamental role that oxidative damage plays in numerous forms of brain diseases. It has been reported that HSYA is able to provide neuroprotective effects via decreasing the level of lipid peroxidation products [9, 10] and inhibiting ROS generation [11]. Recently, HSYA was also demonstrated to modulate endogenous antioxidant defenses of the brain by increasing the activity of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), as well as the ratio of glutathione (GSH)/glutathione disulfide (GSSG) [12]. However, few studies have investigated the action of HSYA on nitrosative stress of neurons and the underlying mechanism. In this study, we hypothesize that HSYA rescues neurons from nitrosative injury through inhibition PPARγ nitrosative modification and inactivation.

2. Materials and Methods

2.1. Chemicals and Reagents. HSYA was generously provided by Zhejiang Yongning Pharmaceutical Co. Ltd. (Zhejiang, China). The chemical structure of HSYA is shown in Figure 1(a).

Figure 1: Protective effects of HSYA against SIN-1-induced cytotoxicity in primary neuron cultures. (a) Structural formula of hydroxysafflor yellow A (HSYA). (b–d) The primary neurons were incubated with SIN-1, HSYA, or their combinations as described in Materials and Methods. LDH release assay for cytotoxicity and MTT assay for cell viability (b), Hoechst staining for apoptotic cells (c), and Western blotting for 3-nitrotyrosine (3-NT) expression (d) were carried out after 24 h incubation. Data are expressed as mean ± SEM (n = 6). *P < 0.05 compared to control (untreated) and #P < 0.05 compared to SIN-1 alone.
Cultures were maintained at 37 °C in a 5% CO₂/95% room air, humidified incubator. On day 3 of culture, cells were treated for 48 h with 0.5 μM cytosine arabinoside to prevent glial growth. On day 9 in culture, the cells formed extensive axonal and dendritic networks and were ready for the experiments. Neuron purity was determined using MAP2 labeling, a cell marker for neurons, which showed >95% purity in cultures. To expose the cells to various agents, culture medium was replaced by MEM supplemented with 5.5 mg/ml D-glucose, 2 mM glutamine, 5% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. In some studies, cells were incubated with increasing concentrations of SIN-1 (0.05–2 mM, in PBS) for 24 h. In a different set of experiments, cells were exposed to HSYA (0.01–1 mM, in PBS) 10 min prior to the addition of SIN-1 (1 mM) and then coincubated for 24 h. Both SIN-1 and HSYA were prepared from embryonic day 17 Sprague-Dawley rats as previously described [8, 13, 14]. Briefly, cells were dissociated from the hippocampus and maintained in serum-free, B27 neurobasal media (Invitrogen) on poly-D-lysine-coated dishes. After 1 d in vitro, the medium was changed to MEM (Invitrogen) supplemented with 5.5 g/ml D-glucose, 2 mM glutamine, 10% fetal bovine serum (FBS) (Invitrogen), 1 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. This medium change was required to reduce oxidative stress from the hippocampus and maintain in serum-free, media 10 min prior to PPARγ activation, neurons were incubated with SIN-1 (1 mM) alone or in combination with HSYA (1 mM) for 24 h and then treated with 15d-PGJ2 (5 μM, in PBS) or rosiglitazone (1 μM, in DMSO) for 6 h. To test the effect of PPARγ agonist or antagonist by pretreatment regimen, cells were pretreated for 24 h with the PPARγ agonist (5 μM 15d-PGJ2 or 1 μM rosiglitazone) or PPARγ antagonist (5 μM GW9662, in DMSO) and then exposed to SIN-1 (1 mM) for further 24 h. In the experiments with both PPARγ agonist and PPARγ antagonist, GW9662 was added to the media 10 min prior to PPARγ agonist. To test the effect of PPARγ agonist by cotreatment regimen, cells were exposed to PPARγ agonist with or without HSYA (0.1 mM), 10 min prior to the addition of SIN-1 (1 mM), and then coincubated for 24 h. The concentration of 15d-PGJ2, rosiglitazone, and GW9662 is based on our preliminary concentration-response experiments and the previously published data [8, 16]. In each study, the experimental conditions contained identical concentrations of DMSO which never exceeded 0.1%.

2.3. Determination of Lactate Dehydrogenase Activity. Cytotoxicity was quantified by measuring the percentage of total lactate dehydrogenase (LDH) release from cells into the media using the LDH Cytotoxicity Assay Kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer’s instructions. Cells were treated with SIN-1 alone or in various combinations with other agents. 24 h after the initiation of SIN-1 treatment, the supernatant (100 μl) was transferred to a 96-well plate for the measurement of LDH activity. The percentage of LDH released into the media was calculated by the following formula: (LDH activity in the media/total LDH activity) × 100, where total LDH activity represents LDH activity in cells and media. Total LDH was determined in cells treated with 0.1% Triton X-100.

2.4. Cell Viability Assay. To assess neuronal viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed. The principle of the assay is based on the cleavage of tetrazolium salts by mitochondrial succinate reductase in viable cells to form formazan dye. Briefly, MTT solution (0.5 mg/ml) was added to the culture well 24 h after SIN-1 treatment. Following incubation for 4 h at 37°C, the formed formazan crystals were dissolved in DMSO. The absorbance of each well was measured at 570 nm using an automatic plate reader, and the cell viability was expressed as percent of control.

2.5. Hoechst 33258 Staining. Cell apoptosis was measured by the procedure described previously using Hoechst 33258 stain [8, 17]. Changes in nuclear morphology characteristics of apoptosis were observed in cells labeled with Hoechst 33258. The nuclei in normal cells presented uniformly hypochromatic blue color, and the nuclei in apoptotic cells presented fragmented and condensed staining. The number of apoptotic nuclei in at least 10 randomly chosen fields was counted and expressed as percent of total cells.

The cell loss in the hippocampus of rats was measured by counting the numbers of the cell nuclei stained with Hoechst 33258. Six preselected areas of the hippocampus were counted per each animal. Six animals from each group were used for the analyses. Each area subjected to the cell nuclei counting was set as 300 μm × 300 μm.

2.6. Western Blot Analysis. Samples were separated by SDS-PAGE and then transferred onto the nitrocellulose membrane. After blocking for 1 h in 0.1% Tween 20/PBS containing 5% fat-free milk, the blot was then incubated with anti-PPARγ antibody, anti-nitrotyrosine antibody, or anti-Bcl-2 antibody at 4°C overnight. After incubation with the appropriate HRP-conjugated secondary antibodies, the blot was visualized by chemiluminescence. The density of the bands was evaluated densitometrically using the program Quantity One 4.6.2 (Bio-Rad Laboratories, Hercules, CA). The specificity of the bands for nitrated tyrosine was confirmed in pilot experiments of Western blot. The SDS-PAGE-transferred membrane was incubated...
with anti-nitrotyrosine antibody that was preabsorbed for 4 h with an excess of free nitrotyrosine (10 mM), aminotyrosine (10 mM), phosphotyrosine (10 mM), methlytyrosine (10 mM), or tyrosine (10 mM). The nitrated protein bands were abolished by preabsorption of the antibody with nitrotyrosine but not aminotyrosine, phosphotyrosine, methlytyrosine, or tyrosine. This method is also used by others to verify the specificity of the assay for protein tyrosine nitration [18].

2.7. Immunoprecipitation Analysis. For immunoprecipitation assay, samples were precleared with protein A/G agarose bead slurry on a shaker at 4°C for 10 min to remove the non-specific binding protein. The protein A/G beads were removed by spin at 14,000 g at 4°C for 10 min. The supernatant (500 μg protein in 0.5 mg/ml) was incubated with 2 μg mouse anti-PPARγ or anti-IgG (control) antibodies and rotated at 4°C for 3 h. The Ag/Ab immunocomplexes were captured by adding protein A/G agarose beads and rocked at 4°C overnight. Agarose beads were collected by centrifugation at 14,000 g at 4°C for 10 min and then washed three times in PBS. Finally, immunocomplexes were dissociated from agarose beads by boiling with SDS-PAGE sample buffer for 5 min and Western blotting was performed with rabbit anti-3-nitrotyrosine antibody to detect the nitrated PPARγ. A HRP-conjugated VeriBlot for IP detection reagent was used to exclude interference from the antibody heavy and light chains.

2.8. PPARγ DNA-Binding Assay. PPARγ activity was quantified by PPARγ DNA-binding assay using a sensitive and specific TransAM PPARγ transcription factor assay kit (Active Motif, Carlsbad, CA, USA), as we described [13]. This assay measures the capacity of PPARγ binding to an oligonucleotide probe that contains the specific peroxisome proliferator response element (PPRE), immobilized on a 96-well plate. Nuclear proteins were isolated with a nuclear protein extraction kit (Active Motif) at 6 h following the initiation of the treatment with PPARγ agonist. 10 μg of nuclear extract protein was applied to the wells and allowed to bind to the PPRE. Bound PPARγ was then detected by adding the specific anti-PPARγ primary antibody, an HRP-conjugated secondary antibody, and HRP substrate solution and spectrophotometer reading (450 nm). The specificity of the assay was confirmed by the addition of wild-type and mutated consensus oligonucleotides. The wild-type consensus oligonucleotide can prevent PPARγ binding to the probe, whereas the mutated consensus oligonucleotide has little effect on PPARγ binding.

2.9. Immunohistochemistry. Fresh-frozen sections were stained for 3-NT as we described [13]. Briefly, sections were permeabilized with 0.3% (v/v) Triton X-100 in PBS for 30 min and blocked with 1% (w/v) BSA in PBS for 1 h and then incubated with mouse monoclonal antibody for 3-NT (1:200) at 4°C overnight. After rinsing with PBS, sections were incubated with rhodamine isothiocyanate (TRITC) labeled goat anti-mouse antibody for 1 hr at 37°C in the dark. The fluorescent images were observed under a fluorescent microscope. For double labeling, sections were incubated first with antibody of mouse anti-nitrotyrosine (1:200) followed by a specific neuron marker antibody of rabbit anti-NeuN (1:200) or an antibody of rabbit anti-PPARγ (1:200). Following three washes in PBS, immune complexes were visualized with Texas Red conjugated anti-mouse IgG (1:500) and FITC conjugated anti-rabbit IgG (1:500). The specificity of staining was confirmed by replacement of the primary antibody with nonimmune control IgG or by elimination of the primary antibody.

2.10. Hippocampus Injection and Treatments. All animal experiments were carried out according to an institutionally approved protocol, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University. Male Sprague-Dawley (SD) rats (Academy of Military Medical Sciences, Beijing, China) weighing from 280 to 330 g were housed and cared for in the Animal Resource Center under 12 h light-dark cycles and allowed free access to food and water. All animal manipulations were conducted during the lights-on phase (0700–1900 h). Briefly, anesthetized rats were placed in a stereotaxic apparatus and 3 μl SIN-1 (25 mM) in PBS was infused into the right hippocampus using the following coordinates: 4.0 mm posterior to the bregma, 2.0 mm lateral from midline, and 4.0 mm below the dural surface. The above procedures were completed under sterile conditions, and penicillin (200,000 U, intramuscularly) was injected to prevent infection. The dosage of SIN-1 was chosen according to the previously published study, in which the dose 25 mM was found to be the most effective in inducing protein nitration by hippocampus injection [17]. The control group was injected with the same volume of vehicle. Body temperature was maintained at 37°C with the use of a heating pad throughout the surgery procedure and until animals regained consciousness. Thereafter, animals were returned to their home cages and allowed free access to food and water. HSYA dissolved in PBS was administered intravenously through the caudal vein at a dose of 1, 5, or 10 mg/kg 30 min before SIN-1 treatment. Our previous experiments have shown the neuroprotective effects of HSYA injected within this range of dosage in ischemia/reperfusion rats [2]. The ability of HSYA to cross the blood-brain barrier (BBB) following intravenous administration has been confirmed previously [19]. At 24 h after hippocampal injection with SIN-1, 10 μg of 15d-PGJ2 in 10 μl of PBS was administered intracerebroventricularly (ICV) at a rate of 1 μl/min using a syringe pump as described in our previous study [13]. The effect of vehicle without any drug was tested in pilot experiments, and no effects were observed.

2.11. Statistical Analysis. The experimental data are expressed as mean ± SEM, and SPSS 11.0 software package was used for data processing. One-way ANOVA was used to compare the means of different groups. Comparisons between two groups were conducted by t-test. A P value less than 0.05 was considered as statistically significant.
3. Results

3.1. HSYA Protected Neurons from SIN-1-Induced Cytotoxicity. To model nitrosative damage, primary hippocampal neurons (d 9) were exposed to SIN-1, a well-known peroxynitrite donor, for 24 h. As expected, neurons treated with SIN-1 exhibited cytotoxic damage, as determined by LDH released into the media and MTT assay for cell viability (Figure 1(b)), the Hoechst 33258 staining assay for apoptotic nuclei (Figure 1(c)), or protein nitrative modification based on 3-NT formation (Figure 1(d)).

To observe the effect of HSYA on SIN-1-induced cytotoxicity, varying concentrations of HSYA were added to the media together with a toxic level of SIN-1. As illustrated in Figure 1(b), inclusion of HSYA resulted in decreases in LDH release induced by SIN-1. Similar conclusions demonstrating a neuroprotective effect of HSYA were generated through measurement of MTT assay (Figure 1(b)), the Hoechst 33258 staining (Figure 1(c)), and 3-NT accumulation (Figure 1(d)).

3.2. HSYA Inhibited SIN-1-Induced PPARγ Nitration and Inactivation. Searching for a possible mechanism to explain the beneficial effect of HSYA, we considered PPARγ which is an important factor in the neuronal defense mechanisms against oxidative injuries [8, 20]. PPARγ has been described to be modified and inactivated by nitration of tyrosine residues in nonneuronal cells [21]. To determine neuronal PPARγ sensitivity to nitration, the level of PPARγ in the nitrated form was detected at 24 h following the exposure to SIN-1 with or without HSYA. The presence of nitrated PPARγ (nitr-PPARγ) was examined by immunoprecipitating proteins from cellular extract with anti-PPARγ antibody, and then the PPARγ precipitating proteins from cellular extract with anti-PPARγ antibody followed by WB with nitrotyrosine Ab. The bar graph illustrates the densitometric analysis of the related bands. Data are expressed as mean ± SEM (n = 3). *P < 0.05 compared to control (untreated), #P < 0.05 compared to SIN-1 alone. (d) Restoration of agonist-dependent PPARγ activation by HSYA in SIN-1-treated neurons. Primary neuron cultures were incubated with 1 mM HSYA, 10 min before the addition of 1 mM SIN-1. After 24 h coincubation, cells were treated for an additional 6 h in the absence (filled bars) or presence (open bars) of PPARγ agonist 15d-PGJ2 (5 μM) or rosiglitazone (Ros) (1 μM). Nuclear proteins were extracted, and activated PPARγ was quantified by PPARγ DNA-binding activity utilizing the PPARγ transcription factor assay kit. Data are expressed as mean ± SEM (n = 3). *P < 0.05 compared to control (untreated), #P < 0.05 compared to PPARγ agonist (15d-PGJ2 or Ros) alone, and ##P < 0.05 compared to SIN-1 plus agonist (15d-PGJ2 or Ros).

Figure 2: Protection against SIN-1-induced PPARγ nitration and inactivation by HSYA in primary neurons. (a–c) Inhibition of PPARγ nitration by HSYA in SIN-1-treated neurons. The neurons were incubated with increasing concentrations of HSYA (0.01, 0.1, and 1 mM), 10 min before the addition of SIN-1 (1 mM). After 24 h coincubation, neurons were harvested for analysis of PPARγ nitration and total PPARγ accumulation. GAPDH expression was shown as a loading control. (a) The cell extracts were immunoprecipitated (IP) with antibody specific to nitrotyrosine (Nitrotyr.). The nitrotyrosine immunoprecipitates were successively immunoblotted (WB) with PPARγ antibody followed by WB with nitrotyrosine Ab. The bar graph illustrates the densitometric analysis of the related bands. Data are expressed as mean ± SEM (n = 3). *P < 0.05 compared to control (untreated) and #P < 0.05 compared to SIN-1 alone. (b) The cell extracts were IP with anti-PPARγ antibody followed by WB with nitrotyrosine Ab. The bar graph illustrates the densitometric analysis of the related bands. Data are expressed as mean ± SEM (n = 3). *P < 0.05 compared to control (untreated) and #P < 0.05 compared to SIN-1 alone. (c) Inhibition of PPARγ nitration by HSYA in SIN-1-treated neurons. Primary neuron cultures were incubated with 1 mM HSYA, 10 min before the addition of 1 mM SIN-1. After 24 h coincubation, cells were treated for an additional 6 h in the absence (filled bars) or presence (open bars) of PPARγ agonist 15d-PGJ2 (5 μM) or rosiglitazone (Ros) (1 μM). Nuclear proteins were extracted, and activated PPARγ was quantified by PPARγ DNA-binding activity utilizing the PPARγ transcription factor assay kit. Data are expressed as mean ± SEM (n = 3). *P < 0.05 compared to control (untreated), #P < 0.05 compared to PPARγ agonist (15d-PGJ2 or Ros) alone, and ##P < 0.05 compared to SIN-1 plus agonist (15d-PGJ2 or Ros).
the reciprocal experiment was carried out by immunoprecipitating proteins with 3-NT antibody first and then immunoblotting with PPARγ antibody (Figure 2(b)). In the results of both experiments, SIN-1 treatment resulted in an increase of PPARγ nitrification, which was reversed by the cotreatment of HSYA in a concentration-dependent manner. However, the abundance of PPARγ protein was not affected by either SIN-1 alone or in combination with HSYA.

Nitritative modification could leave PPARγ to become refractory to the activation by its activating agents [21]. We then evaluated whether SIN-1 exposure affected the response of PPARγ to its ligand stimulation. The neurons in culture were incubated with 15d-PGJ2, a natural ligand for PPARγ, for 6 h following the 24 h exposure to SIN-1 alone or in combination with HSYA. PPARγ DNA-binding activity was increased about 2-fold by the exposure to 15d-PGJ2 alone, indicating the activation of PPARγ (Figure 2(d), A). SIN-1 treatment inhibited 15d-PGJ2-induced elevation in PPARγ DNA-binding activity, which was restored by the presence of HSYA (Figure 2(d), A). In analogy to results with 15d-PGJ2, HSYA also resumed PPARγ activation by rosiglitazone, a synthetic agonist for PPARγ, in SIN-1-treated neuron cultures (Figure 2(d), B). Notably, a significant reduction in PPARγ activity was detected following SIN-1 exposure alone, suggesting the loss of PPARγ response to endogenous ligands, whereas treatment of HSYA with SIN-1 fully compensated for this SIN-1-induced dysfunction (Figure 2(d), filled bars). Overall, the HSYA-mediated protection of PPARγ activity was consistent with improved neuronal damage.

Finally, the effect of HSYA on PPARγ was also observed in normal neurons. No significant alterations in either PPARγ protein expression or DNA-binding activity were detected (data not shown), suggesting that HSYA itself did not emerged as a direct inducer of PPARγ activity.

3.3. HSYA Resumed the Protective Effect of PPARγ Agonists against SIN-1-Induced Cytotoxicity. To determine whether PPARγ activity plays a crucial role in the defense against SIN-1-induced nitrosative stress, the PPARγ-specific agonist and/or antagonist was added to the cultures 24 h prior to the treatment with a toxic level of SIN-1. As demonstrated in Figure 3(a), PPARγ agonist (15d-PGJ2 or rosiglitazone) pretreatment significantly attenuated SIN-1-induced LDH release, which was reversed by the copretreatment of PPARγ antagonist GW9662. Alternatively, pretreatment with GW9662 alone aggravated SIN-1-induced neuronal injury (Figure 3(a)). These results suggested that PPARγ activation could increase resistance to SIN-1 cytotoxicity whereas PPARγ inactivation caused neurons to be more sensitive to SIN-1-induced insult.

In another experiment, cotreatment of PPARγ agonist (15d-PGJ2 or rosiglitazone) with SIN-1, however, failed to either activate PPARγ (Figure 3(b)) or protect neurons against SIN-1-induced cytotoxicity (Figure 3(c)), indicating that a preactivation of PPARγ is required to inhibit neuronal insult by SIN-1. Alternatively, the nitration of PPARγ induced by SIN-1 could prevent PPARγ activation and thus the neuroprotection by PPARγ agonist. To verify this last hypothesis, neurons were exposed to HSYA, at a submaximal concentration, together with PPARγ agonist plus SIN-1. As demonstrated in Figures 3(b) and 3(c), the combined treatment of HSYA and PPARγ agonist not only rescued PPARγ response to its activating agents (Figure 3(b)) but afforded additional protection against SIN-1-induced cell insult when compared with the HSYA plus SIN group (Figure 3(c)). These findings suggested that HSYA not only itself has neuroprotective capacity but could help to resume PPARγ agonist-based protection against SIN-1-induced insult.

3.4. HSYA Inhibited PPARγ Nitrification and Loss of Activity in the SIN-1-Injected Hippocampus of Rats. To determine whether HSYA has similar effects on PPARγ in vivo, we employed an animal model of nitrosative stress based on hippocampus injection of SIN-1. To confirm the production of peroxynitrite in the SIN-1-injected hippocampus, 3-NT expression was measured at 24 h following SIN-1 injection. Figure 4(a) displayed that SIN-1 induced a time-dependent increase in 3-NT abundance, which was more than 3-fold higher than the one observed in control rats, for 24-hour-treated rats. Administration of HSYA significantly ameliorated 3-NT expression and immunoreactivity in the SIN-1-injected hippocampus (Figures 4(b) and 4(c)).

We then examined the effect of HSYA on nitro-PPARγ expression and PPARγ activity. As shown in Figure 5(a), HSYA inhibited nitro-PPARγ generation induced by SIN-1 injection in a dose-dependent manner (Figure 5(a)). PPARγ protein expression was not affected by either SIN-1 alone or coinjection with HSYA (Figure 5(a)). Consistently, a reduced PPARγ DNA-binding activity was found in the SIN-1-injected hippocampus, which was reversed by HSYA treatment (Figure 5(b)). HSYA also resumed PPARγ response to its ligand 15d-PGJ2 in the SIN-1-injected hippocampus (Figure 5(b)). The cellular distribution of nitro-PPARγ in the hippocampus was also characterized. As shown in Figures 5(c) and 5(d), the immunoreactivity of 3-NT was observed in the cytoplasm of most 3-NT-positive cells, implicating that nitro-PPARγ also preferentially occurred in neurons of the hippocampus.

In contrast to SIN-1-induced insults in cultured neurons, no obvious cell loss or apoptosis-like morphology was observed in rats injected with SIN-1 alone or coinjected with HSYA, as assessed by Hoechst 33258 staining (Figure 5(e)), indicating that the single injection of SIN-1 was not sufficient to cause cell loss and cell apoptosis. Furthermore, no significant difference in spatial memory retention, a process associated with the hippocampus, could be detected in the Morris water maze test (data not included). These results were consistent with the previous report of an SIN-1-injected hippocampus [17]. PPARγ loss of function in neurons, however, has been proven to be associated with increased susceptibility to oxidative stress, which is reflected in downregulation of the Bcl-2 antiapoptotic protein [8].
Accordingly, Bcl-2 protein expression was determined in the hippocampus. As the changes occurred in PPARγ activity, similar downregulation and upregulation of Bcl-2 protein expression were observed in SIN-1-injected and HSYA-coinjected rats, respectively (Figure 5(f)), suggesting the increased vulnerability to damage in the SIN-1-injected hippocampus and the potential properties of HSYA to decrease this predisposition.

Administration of HSYA alone to normal rats had no significant effect on any of the measured indices (data not included).

4. Discussion

Our experiments demonstrated that SIN-1-induced neuronal damage or increased vulnerability was notably reduced by the herb HSYA. This neuroprotective effect was established in both neurons in culture and animal models of nitrosative stress. We further demonstrated that the neuroprotective effect of HSYA may be associated with inhibition of PPARγ nitration and inactivation induced by SIN-1. Next, in support of the above statement, the crucial role of PPARγ in neuronal defense against nitrosative stress was verified by showing the
evidence that the PPARγ agonists attenuated SIN-1-induced neuronal injury but the PPARγ antagonist aggravated SIN-1-induced neuronal injury. Finally, we postulated that HSYA may potentiate the PPARγ-mediated neuroprotective effects by inhibition of PPARγ inactivation since the combined treatment of HSYA with PPARγ agonist rescued the effects of agonist on both PPARγ activation and PPARγ protection against SIN-1-induced cytotoxicity.

Evidence has proven that PPARγ is important in neuronal self-defense against oxidative injuries. For example, in PC12 neuronal cell, PPARγ loss of function increased susceptibility to H$_2$O$_2$- or β-amyloid- (Aβ-) induced oxidative toxicity, whereas PPARγ overexpression could prevent H$_2$O$_2$- or Aβ-induced ROS production and cell insult [8]. Consistently, increased brain damage and oxidative stress were observed in neuronal PPARγ knockout (N-PPARγ-KO) mice in response to middle cerebral artery occlusion [20]. Also, the primary neurons from N-PPARγ-KO mice were significantly more vulnerable to oxidative injury, albeit deficiency of PPARγ did not affect the baseline neuronal health [20]. In support of this notion, our study demonstrated that PPARγ may also contribute to the defensive mechanism against nitrosative stress in neurons by showing that PPARγ agonist attenuated SIN-1-induced cytotoxicity but PPARγ antagonist enhanced SIN-1-induced cytotoxicity. Indeed, in our study, a certain level of PPARγ activity was demonstrated in the control neuron cells and the hippocampus, suggesting the activation of PPARγ by endogenous natural agonists, such as 15d-PGJ2 or oxidized lipids, in the normal settings of the brain. The SIN-1-induced decrease in PPARγ activity in our study indicated the loss of PPARγ function, which may also become refractory to the stimulation of exogenous PPARγ agonists after inactivation by SIN-1 [22].

In addition to the endogenously produced agonists, PPARγ also became refractory to the stimulation of exogenously added agonists after inactivation by SIN-1. This could help explaining some findings in PPARγ agonist-based therapy against nitrosative stress, which show that PPARγ agonists have actions without PPARγ activation. It has been shown in a MPP+/MPTP model of Parkinson’s disease that...
Figure 5: The inhibitory effects of HSYA on PPARγ nitration and inactivation in the SIN-1-injected rat hippocampus. (a) Inhibition of SIN-1-induced PPARγ nitration by HSYA in the hippocampus of rats. Rats were treated with increasing doses of HSYA (1, 5, or 10 mg/kg), 30 min before SIN-1 (25 mM) injection. The effects of HSYA were evaluated at 24 h after SIN-1 injection. The protein levels of nitro-PPARγ and PPARγ were characterized by immunoprecipitation or Western blotting. The bar graphs illustrate the densitometric analysis of the related bands. Data are expressed as mean ± SEM (n = 3). *P < 0.05 compared to control (untreated) and #P < 0.05 compared to SIN-1 alone. (b) Prevention of PPARγ inactivation by HSYA in the SIN-1-injected hippocampus. Rats were injected with HSYA (10 mg/kg), SIN-1 (25 mM), or their combination as described in Materials and Methods. At 24 h postinjection, rats were treated intracerebroventricularly (ICV) with either vehicle (filled bars) or 10 μg 15d-PGJ2 (open bars). Hippocampal nuclear extracts were prepared for the detection of PPARγ DNA-binding activity 6 h after ICV administration. Data are expressed as mean ± SEM (n = 3). *P < 0.05 compared to control (untreated) and #P < 0.05 compared to SIN-1 plus 15d-PGJ2. (c, d) Localization of nitrated PPARγ in the SIN-1-injected hippocampus. The rat hippocampus was injected with SIN-1 (25 mM). The neuronal distribution of nitro-PPARγ in the hippocampus was detected by immunofluorescent double labeling at 24 h after SIN-1 injection. (c) Photomicrographs show the colocalization of 3-NT (red) with NeuN (green) in the CA1 and dentate gyrus (DG) areas of the hippocampus. Scale bar, 20 μm. (d) Photomicrographs with increased magnification show the colocalization of PPARγ (green) with 3-NT (red) in the cytoplasm of most 3-NT-positive cells. (e) Rats were treated with HSYA (10 mg/kg), 30 min before SIN-1 (25 mM) injection. At 24 h postinjection, the cell loss was measured by counting the numbers of Hoechst 33258-stained nuclei. Summary bar graph illustrates cell counts in the hippocampus. Data are expressed as mean ± SEM (n = 6 fields counted in 6 animals). (f) Inhibition of SIN-1-induced Bcl2 expression by HSYA in the hippocampus of rats. Rats received HSYA treatment of 1, 5, or 10 mg/kg 30 min before SIN-1 (25 mM) injection. The effects of HSYA on the protein levels of Bcl-2, a downstream target of PPARγ signaling, were evaluated by Western blotting at 24 h after SIN-1 injection. The bar graphs illustrate the densitometric analysis of the related bands. Data are expressed as mean ± SEM (n = 3). *P < 0.05 compared to control (untreated) and #P < 0.05 compared to SIN-1 alone.
PPARγ activity was important for protecting against MPTP toxicity. However, only non-PPARγ-mediated neuroprotective effects of rosiglitazone were observed in MPTP-treated mice, as these actions of rosiglitazone were not associated with the upregulation of PPARγ target gene and could not be reversed by cotreatment with PPARγ antagonist [23]. Consistently, in a model of brain trauma, whose pathogenesis also involves nitrosative stress, PPARγ agonist pioglitazone demonstrated beneficial functions through mechanisms not related to PPARγ activation [24]. In agreement with these findings, NCX 2216, the NSAID class of PPARγ agonist, controls microglial activation through PPARγ-dependent and PPARγ-independent actions. Prolonged treatment of microglial cultures with NCX 2216 can induce PPARγ nitrilation. Following nitrilation, NCX 2216 can no longer demonstrate the effects associated with PPARγ activation; however, its PPARγ-independent effects were still being observed in microglial cultures [25]. In our study, the PPARγ agonist significantly inhibited SIN-1-induced cytotoxicity in neurons whereas the specific PPARγ antagonist prevented such inhibition, suggesting that the effect was mediated by PPARγ. However, the PPARγ-mediated effect was observed in pre-treatment regimen of PPARγ agonist but not in cotreatment regimen. We speculated that the ineffectiveness of cotreatment may be due to PPARγ nitrilation and subsequent inactivation, since the cotreatment of HSYA in combination with PPARγ agonist demonstrated synergistic effects on PPARγ activation and cytoprotection. Further investigations are required to confirm whether this synergistic action of HSYA is associated with the inhibition of PPARγ nitrative modification, thus allowing a more effective activation of PPARγ by the agonists.

In our in vivo study, the SIN-1-induced PPARγ nitrilation and inactivation were suggested to be mainly occurring in neurons of the hippocampus. Concurring with that of our result, the tau protein has been identified as one of the targets of peroxynitrite and the nitrated tau protein was also suggested to have a strong neuronal signature [17]. Similarly, the peroxynitrite-induced 3-NT expression was also proved to be primarily accumulated in neurons in the acute phase of cerebral ischemia/reperfusion injury [26], albeit no target protein for nitrilation was evaluated. The reason why proteins in neurons are prone to nitrative modification by peroxynitrite may be related with the lower concentrations of antioxidants in neurons than in glial cells [27]. Of particular note is the low concentration of reduced glutathione (GSH). The susceptibility of cells to peroxynitrite toxicity has been proven to largely depend on the amount of intracellular GSH [28]. The GSH concentration in neurons is one-half of that of astrocytes, and the activity of γ-glutamylcysteine synthetase, a key enzyme in glutathione synthesis, is approximately several fold lower in neurons than that of astrocytes. [27]. This low antioxidative potential may be predisposed to neuronal PPARγ nitrilation and inactivation in response to peroxynitrite injury. On the other hand, neurons rely heavily on their metabolic coupling with astrocytes to combat oxidative stress. Astrocytes produce and secret GSH to protect neurons as well as provide the precursors for neuronal GSH synthesis [14, 29]. Without the antioxidant support from astrocytes, neurons are of high susceptibility to the oxidative damage [14, 29]. This may provide a likely explanation for the observation in our study showing that there is SIN-1-induced significant cell injury in the neuron-enriched culture system whereas no toxicity (apoptosis or cell loss) to cells in the SIN-1-injected hippocampus was observed.

The Bcl-2 antiapoptotic protein has been shown to be a key downstream target of PPARγ signaling in neurons for protection against oxidative stress [8, 30, 31]. PPARγ loss of function results in downregulation of Bcl-2 protein in neurons and thereby renders cells vulnerable to oxidative insult [8]. PPARγ agonists protected neurons against oxidative damage by enhancing Bcl-2 expression [8, 30]. Moreover, a putative PPARγ response element (PPRE) has been reported in the 3′-untranslated region of the bcl-2 gene [32], suggesting the dependence of PPARγ. Consistently, in our study, PPARγ nitrilation and inactivation were accompanied by a parallel decrease in Bcl-2 expression in the SIN-1-injected hippocampus. This data may provide an additional link to our speculation that SIN-1-induced PPARγ nitrilation and inactivation in vivo may be reflected in the increased vulnerability to brain damage. Concomitantly, the inhibition of Bcl-2 downregulation induced by SIN-1 supported the protective effect of HSYA. Further study is warranted to confirm the association between PPARγ and Bcl-2 in our study.

In the nervous system, protein tyrosine nitration represents a major cytotoxic pathway during peroxynitrite-mediated nitrative stress. However, other covalent modifications of PPARγ cannot be excluded to explain some of our observations. For example, PPARγ is known to be modified by phosphorylation at serine residue [33]. Studies have demonstrated that peroxynitrite, acting as a signaling molecule, regulates mitogen-activated protein kinase- (MAPK-) mediated signal transduction pathways. PPARγ contains a MAPK site, and phosphorylation by extracellular signal-regulated kinase- (ERK-) 1/2 leads to inhibition of PPARγ activity [33]. It was reported that peroxynitrite potently activated ERK1/2 in a wide variety of cell types, including neural cells [34, 35]. Thus, PPARγ is possibly susceptible to the modification of phosphorylation in nitrosative conditions. We assumed that increased tyrosine nitration after SIN-1 treatment was the primary reason for the inactivation of PPARγ. However, to be sure of this assumption, the degree of PPARγ nitration without alterations of other amino acids will have to be determined in future studies. Additionally, the specific tyrosine residue in PPARγ that is nitrated is also required to be determined. Actually, many additional transcription factors exhibit sensitivity to both reactive oxygen species and nitric oxide-related species, e.g., NF-κB, AP-1, and p53. Posttranslational modification plays an important role in regulating the activity of transcription factors during oxidative and nitrosative stresses. For example, studies have demonstrated that NF-κB resides in the cytoplasm in an inactive complex with the inhibitor IκB and oxidizing conditions in the cytoplasm promote NF-κB activation. Phosphorylation of IκB proteins represents a convergence point for most signal transduction pathways under the oxidizing conditions leading to NF-κB
activation [36]. Recent data also disclosed that peroxynitrite stimulates NF-xB activation via nitration of tyrosine in IkB, thereby increasing IkB degradation [37]. It has been speculated that posttranslational modification of transcription factors is a mechanism by which cells sense the redox changes [38]. Accordingly, PPARγ nitration and subsequent inactivation may provide a signal for neurons sensing nitrosative condition in neurons.

Although HSYA is a hydrophilic drug with low oral bioavailability, the ability of HSYA to cross the blood-brain barrier has been confirmed previously following intravenous administration of HSYA [19]. Furthermore, since the blood-brain barrier is disrupted in varying degrees in numerous pathological conditions of the brain, like stroke or traumatic brain injury, it should not pose a significant obstacle to HSYA delivery in these settings. Indeed, evidence has proven that HSYA is absorbed in the brain tissues of the TBI rats after being orally administered with HSYA [12]. These data validated that the action of HSYA may result from its central effect in our study.

In our previous study, we have demonstrated that HSYA profoundly protected against tyrosine nitration elicited by authentic peroxynitrite in a cell-free system, indicating the role of HSYA as a peroxynitrite scavenger [2]. Accordingly, we speculated that HSYA may act through directly scavenging peroxynitrite and/or its derived radicals to inhibit nitro-PPARγ formation in SIN-1-induced neurons. The structure of HSYA is quinochalcone c-glycoside [39]. Chalcones, a group of aromatic ketones, have been linked with antioxidant activity. Moreover, chalcones have been expected to directly react with peroxynitrite [40]. It is possible that HSYA could function as a competing substrate for peroxynitrite-triggered reaction and therefore protects free tyrosine or tyrosine residue of protein from nitritative modification. Whether HSYA specifically scavenges peroxynitrite, but not superoxide or NO, needs to be determined. Recently, HSYA was demonstrated to modulate endogenous antioxidant defenses of the brain by increasing the activity of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), as well as the ratio of glutathione (GSH)/glutathione disulfide (GSSG) [12]. This effect may be also involved in its mechanism underlying the inhibition of PPARγ nitration.

Our present data and others have shown that PPARγ is crucial to the defensive mechanism of neurons against nitrosative stress and oxidative stress, both of which playing a role in the pathogenesis of many neurodegenerative diseases. Thus, keeping PPARγ function active could become particularly important for the neurons in degenerative diseases. HSYA’s protection against SIN-1-induced negative regulation of PPARγ activity may help in potentiating the control of nitrosative stress and offer new therapeutic opportunities for treating neurodegenerative diseases.

Data Availability
The data used to support the findings of this study are included within the article.

Conflicts of Interest
The authors have no conflict of interest.

Authors’ Contributions
Li Sun and Yan-Wei Xu contributed equally to this work.

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