Rosiglitazone enhances the proliferation of neural progenitor cells and inhibits inflammation response after spinal cord injury

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It has been previously shown that peroxisome proliferators-activated receptor gamma (PPAR-\gamma) is beneficial for nervous system injury. In present study, we examined the effect of rosiglitazone, a PPAR-\gamma agonist, on spinal cord injury (SCI) in rats. SCI was induced by dropping a 10 g weight rod at a height of 25 mm. The animals were randomly divided into vehicle group, rosiglitazone treated group, and G3335 treated group. Locomotor function recovery was evaluated by the Basso–Beattie–Bresnahan locomotor rating scale (BBB scale). NF-\kappa B expression and endogenous neural progenitor cells (NPCs) proliferation and differentiation was assessed by flow cytometry and immunohistochemistry. Compared with the vehicle groups, we found that the rosiglitazone could significantly ameliorate locomotor recovery, reduce NF-\kappa B expression, and increase the proliferation of endogenous NPCs. When the PPAR-\gamma antagonist was used, these effects were abolished. However, neurons differentiating from endogenous NPCs were inhibited when PPAR-\gamma was activated. Our results suggest that the activation of PPAR-\gamma may be a potential alternative treatment for spinal cord injury.

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Spinal cord injury (SCI) induces a series of deficits including paralysis (Para or tetraplegia), sensory disturbances and autonomic dysfunctions [10]. Besides progressive neural cell loss, the inflammation-induced inhibition of proliferation of endogenous neural progenitor cells (NPCs) may contribute considerably for the recovery of deficits. In 1996, NPCs have been discovered in adult spinal cord [21]. The neurogenesis continues in some regions of the adult brains, including humans. Many researchers suggested that modulation of adult endogenous NPCs may be potentially beneficial for SCI treatment [16].

According to results of previous studies, anti-inflammation improves functional recovery after SCI [6]. More recently, some studies showed that adult NPCs transplantation could promote functional recovery and plasticity in SCI [12,15].

Peroxisome proliferators-activated receptor-gamma (PPAR-\gamma) is a ligand-activated transcription factor [11]. Its ligands include two groups: the natural ligands and the synthesis ligands (thiazolidinediones, TZD) [7]. Besides effects on metabolic diseases, TZD exhibits protection of central nervous system injury [14].

Several researchers have pointed out that TZD can improve anatomical and locomotor recovery. Moreover, TZD could also decrease neuronal damage, myelin loss and inflammation after SCI [19]. The mechanism is still unclear. It has been recently shown that PPAR-\gamma plays an important role in controlling the proliferation of NPCs and SOX1 and SOX3 transcription, which are known to be involved in neural stem cell (NSC) proliferation [3,20]. However, its effects on proliferation of NPCs after SCI remain elusive.

Therefore, in this study in order to examine whether PPAR-\gamma stimulation could be a potential treatment for SCI, we used rosiglitazone (PPAR-\gamma synthesis ligand) and G3335 (PPAR-\gamma special antagonist) to agonist and antagonist PPAR-\gamma, and then analyzed in vivo the change of motor function, inflammation, endogenous NPCs proliferation and differentiation after SCI.

Under halothane anesthesia [induction, 4%; maintenance, 2% in an oxygen and nitrous oxide (50:50) mixture], SCI was induced in adult female Sprague-Dawley rats by dropping an impactor (10 g weight rod, 2.5 mm in diameter) at a height of 25 mm as previous report and T9–T10 laminectomy was performed [8]. Rats were housed in a temperature-controlled room at 27 °C. During this period, injured rats underwent manual bladder twice a day. SCI rats were randomly divided into three groups: vehicle group (n = 38), rosiglitazone group (n = 38) and G3335 group (n = 38). Rosiglitazone

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(3 mg/kg, Cayman Chemicals, Ann Arbor, MI) or G3335 (2 mg/kg; PPAR-γ antagonist; Sigma Chemicals, St. Louis, MO, USA) or vehicle (0.01 M PBS) was injected i.p., at 5 min, 6 h, 24 h.

In all experiments, drug administrations were randomized and blinded. All procedures were carried out follow the Sun Yat-sen University Institutional Animal Care and Use Committee (IACUC) guidelines.

BrdU (Sigma, St. Louis, MO, USA) was given to rats (50 mg/kg/d, i.p.) for 6 days as described by Ke et al. [13]. SCI was induced in rats at the last day of BrdU administration, and euthanized at 1st, 3rd, 7th and 28th day after injury.

Motor function recovery after SCI was studied with the Basso-Beattie-Bresnahan locomotor rating scale [BBB scale] [1]. If there was no spontaneous hind limb movement, the score would be 0, and the score of 21 suggested normal locomotion. To ensure that all animals began with a score of 21, all rats were tested prior to injury. Then on 8h, 1st, 3rd, 7th, 14th, 21st, 28th day post-injury (or up to the day the animal was euthanized) each rat was scored for 4 min by two observers blinded to the study groups.

The T8–T10 segmental (1-cm length) of spinal cord (centered on the lesion point) was harvested. The injury epicenter area was chosen to section. Sections spanning the lesion site were immunolabeled with the neuronal marker NeuN, NPCs marker Nestin, the NF-κB for detecting inflammation. The tissue sections were incubated in blocking buffer (0.1% triton X-100 in 0.01 M PBS, pH 7.5) for 30 min and then incubated with 3% H2O2 for 20 min at room temperature. Primary antibody Nestin (rabbit anti-rat; Sigma; St. Louis, MO, USA; 1:400), mature neurons (NeuN, mouse anti-rat; Millipore; USA; 1:200), NF-κB (rabbit anti-rat; Thermo, UK; 1:100) were applied and incubated overnight at 4°C. The following day, sections were washed 3 times (5 min each) in 0.01 M PBS containing 0.1% triton X-100 and then incubated with Horseradish Peroxidase anti-rabbit IgG for 30 min at room temperature. Then the sections were washed 3 times (5 min each) in 0.01 M PBS and incubated with DAB substrate. The sections were counterstained by hematoxylin. For negative control staining, sections were incubated with omission of primary antibodies.

Spinal cord tissues (T8–T10) were harvested and dissociated in trypsin (0.5 mg/ml) and collagenase (0.5 mg/ml). We separated the debris from cells as described in previous studies [18]. Then the spinal cord cells were incubated 20 min in 0.5 ml 2M HCl and 0.5%IFS; 2 min in 0.1 M Na2B4O7; 5 min in TritonX-100 solution; 30 min in 100% normal rabbit, goat serum; 2 h with a rabbit-anti-NF-κB antibody and goat anti-rabbit Tritc conjugated IgG, or 1 h an FITC conjugated BrdU antibody, a Tritc conjugated NeuN or a Tritc conjugated Nestin antibody. All flow cytometric gates were set using control IgG isotype labeled spinal cord cells. The mean values of cells positive for BrdU, Nestin, NeuN and NF-κB determined by flow cytometry were expressed as percentage (±S.D.) relative to control IgG isotype labeled spinal cord cells.

All images of the posterior funiculi or the ventral horn of rat spinal cord within the lesion of the sections were analyzed by a blinded observer (sections were spaced 100 μm apart), beginning 2.4 mm rostral to epicenter and spanning to 2.4 mm caudal to epicenter. And then examined with a Nikon fluorescent microscope E800 equipped with the Spot digital. Quantifications of Nestin, NeuN, NF-κB staining optical intensities or positive cell numbers in rat spinal cords were performed with the NIH software Image-Pro Plus 6.0. Five sections/rat and 4 rats were analyzed.

The scores of BBB for each animal were averaged and used to create the group mean each day. The data of BBB scores, immunohistochemistry and flow cytometry are expressed as means ± S.D. Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by a Tukey–Kramer multiple comparisons post hoc test.

All rats had a BBB score of 21 before SCI and lost locomotor function 2 h after SCI. At the 24 h, the BBB scores of all the groups were still not more than 1. At the 3rd day to 28th day, compared with vehicle group and G3335 group, the rosiglitazone group showed significant higher BBB scores (P<0.05; n=6) (Fig. 1). At 28th day, all the groups had highest BBB scores respectively in their group (BBB score: 13.33±0.52, 9.5±0.55 and 8.67±0.82 respectively for rosiglitazone group, vehicle group and G3335 group; P<0.05; n=6).

The expressions of NF-κB were assessed by flow cytometry. At the 3rd day to 28th day, the values of NF-κB-positive cells in rosiglitazone group were higher than the other two groups. And at the 7th day the values were highest were observed in all the groups respectively. At 28th day after SCI, all the groups had the lowest values of NF-κB-positive cells respectively in their own group. Compared with vehicle group and G3335 group, the rosiglitazone group had a lower value of NF-κB expression at all time points (Fig. 2). The expression of NF-κB in G3335 animals had higher values than vehicles, though there were no significant differences (Fig. 2).

The expression of NF-κB in posterior funiculus of rats’ spinal cord was detected by immunohistochemistry. The expression of NF-κB was significantly lower in rosiglitazone animals at the 1st, 3rd, 7th, 28th day than that in the other two groups. In contrast, the G3335 group showed a significantly higher expression at 1st, 3rd day than the vehicles (Table 1 and Fig. 4).

The NPCs proliferation was assessed by flow cytometry. At 1st day, and at the 7th day to 28th day, compared with vehicle group and G3335 group, rosiglitazone group had significant higher values of Nestin/BrdU-positive expression. At 3rd day, no significant differences were found between the rosiglitazone animals and vehicle animals; but the G3335 animals still had a significantly lower value than the rosiglitazone and vehicle animals (Fig. 3). The highest values were observed at the 7th day and this trend is same with the expression of NF-κB.

The expressions of Nestin in the rats’ posterior funiculus of spinal cord were detected by immunohistochemistry. The
Table 1
NF-κB immunohistochemistry IOD result.

<table>
<thead>
<tr>
<th>Time group</th>
<th>1st day</th>
<th>3rd day</th>
<th>7th day</th>
<th>28th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>38,248 ± 2087.2b</td>
<td>41,244 ± 1764.0b</td>
<td>46,554 ± 1412.7</td>
<td>21,522 ± 1674.1</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>21,497 ± 1937.6a,b</td>
<td>18,963 ± 1479.5a,b</td>
<td>21,588 ± 1479.6a,b</td>
<td>17,541 ± 1415.1a,b</td>
</tr>
<tr>
<td>G3335</td>
<td>42,50 ± 2100.6a</td>
<td>44,429 ± 1927.5a</td>
<td>47,553 ± 1366.1</td>
<td>20,985 ± 1558.2</td>
</tr>
</tbody>
</table>

The values of figure are means ± S.D. (n=4 rats/group; 5 sections/rat). (a) P<0.05, compared with the respective vehicle and (b) P<0.05, compared with the respective G3335 control by ANOVA followed by a Tukey–Kramer multiple comparisons post-test.

expression of Nestin optical density in rosiglitazone group was higher than in vehicle and G3335 groups at 1st, 7th 28th day after SCI (Table 2 and Fig. 4).

The cells labeled with BrdU and NeuN were detected by flow cytometry. At 1st to 28th day, compared with rosiglitazone group and G3335 group, the vehicle group showed higher ratio of labeled cells. At the 1st day, all the three groups had the lowest value of BrdU/NeuN expression (28.25 ± 2.98; 35.38 ± 2.31; 9.43 ± 1.59 respectively for rosiglitazone group, vehicle group and G3335 group; P<0.05; n=4). At the 7th day after SCI, all the three groups showed highest results respectively (51.09 ± 2.43; 56.58 ± 2.14%; 42.50 ± 13.02% respectively for rosiglitazone group, vehicle group and G3335 group; P<0.05; n=4).

The expressions of NeuN in ventral horns of rats’ spinal cord were detected by immunohistochemistry. There were higher expression of NeuN in rosiglitazone group compared with vehicle at 1, 7, 28 days. The G3335 groups show the lowest numbers at all time points after SCI (Table 3 and Fig. 4).

Brambilla demonstrated that inhibition of NF-κB activity exerts neuroprotection in CNS injuries [2]. Our results showed that rosiglitazone significantly decreased the expression of NF-κB after SCI. When we antagonise the PPAR-γ pathway by using G3335, NF-κB expression was enhanced, and we found a better locomotor function at the same time. This is concordant with previous report that PPAR-γ could inhibit NF-κB activation [4].

Traumatic SCI results in severe inflammation and decreased cellular regeneration which lead to difficult functional recovery [9]. Some researchers showed that SCI could induce the proliferation of NPCs [13]. In our study, we demonstrated that rosiglitazone could improve endogenous NPCs proliferation up to the 28th day and the proliferation peak at the 7th day after SCI by double-labeled endogenous NPCs with the anti-Nestin and BrdU. According to the statistical analysis, the significant differences of NPCs proliferation have been found between rosiglitazone and vehicle groups. When PPAR-γ antagonist G3335 was used, the expression of endogenous NPCs was inhibited. Our data was concordant with previous reports [17]. Although the mechanism is still unclear, our results indicated rosiglitazone’s role in regulation of endogenous NPCs after SCI.

In our study, we found that expression of NF-κB from endogenous NPCs in rosiglitazone group was lower compared with the vehicle group. Neither agonizing nor antagonizing PPAR-γ

Table 2
Nestin staining IOD results.

<table>
<thead>
<tr>
<th>Time group</th>
<th>1st day</th>
<th>3rd day</th>
<th>7th day</th>
<th>28th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>33,368 ± 2898.3b</td>
<td>46,882 ± 1398.4b</td>
<td>50,365 ± 2476.5b</td>
<td>47,860 ± 1764.5b</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>51,733 ± 2879.3a,b</td>
<td>51,370 ± 2037.3b</td>
<td>62,972 ± 2462.3a,b</td>
<td>53,846 ± 2366.3a,b</td>
</tr>
<tr>
<td>G3335</td>
<td>24,298 ± 2449.2a</td>
<td>25,300 ± 2344.4a</td>
<td>45,707 ± 2145.1a</td>
<td>32,796 ± 2048.6a</td>
</tr>
</tbody>
</table>

The values of figure are means ± S.D. (n=4 rats/group; 5 sections/rat). (a) P<0.05, compared with the respective vehicle and (b) P<0.05, compared with the respective G3335 control by ANOVA followed by a Tukey–Kramer multiple comparisons post-test.
Table 3
Numbers of NeuN.

<table>
<thead>
<tr>
<th>Time group</th>
<th>1st day</th>
<th>3rd day</th>
<th>7th day</th>
<th>28th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>23.7 ± 2.452a,b</td>
<td>27.05 ± 3.649b</td>
<td>33.3 ± 3.045a,b</td>
<td>26.5 ± 2.565a,b</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>27.7 ± 3.496b</td>
<td>28.2 ± 3.651b</td>
<td>37.1 ± 2.789a</td>
<td>31.3 ± 3.42b</td>
</tr>
<tr>
<td>G3335</td>
<td>15.35 ± 2.007a</td>
<td>15.7 ± 2.273a</td>
<td>27.15 ± 2.7a</td>
<td>16.25 ± 2.425a</td>
</tr>
</tbody>
</table>

The values of figure are means ± s.d. (n=4 rats/group; 5 sections/rat). (a) P<0.05, compared with the respective vehicle and (b) P<0.05, compared with the respective G3335 control by ANOVA followed by a Tukey-Kramer multiple comparisons post-test.

pathway improves the neuronal differentiation of endogenous NPCs. Our results were concordant with some researchers before [20]. There is a possible mechanism to explain this phenomenon: the activation of PPAR-γ inhibits the expression of NF-κB which is necessary for NPCs differentiation [5]. However, inhibiting the PPAR-γ pathway induces a loss of neurons in the secondary spinal cord injury which is the same as we have found so we concluded that rosiglitazone could promote the neuronal survival [19].

Collectively, our study showed the important role of PPAR-γ after SCI. PPAR-γ activated by rosiglitazone could induce neuroprotection by inhibiting the inflammation and improving the proliferation of endogenous NPCs. PPAR-γ agonists are under researched currently, their effects on endogenous NPCs differentiation into oligodendrocytes or astrocytes need more studies. And further evaluation in the transcription level of potential protection of SCI needs more studies.

Fig. 4. Spinal tissue slide is stained with DAB and counterstained with hematoxylin (the positive area were brown and the nucleus were blue). (A) The representative NF-κB staining in the posterior funiculus 1 mm caudal areas of rats’ spinal cord were counted (ob. 40×). The positive staining mainly localized in the cytoplasm. (B) The representative Nestin staining in the posterior funiculus of rats spinal cord (ob. 40×). The positive staining mainly localized in the cytoplasm. (C) The representative NeuN staining in the ventral horn of rat spinal cord (ob. 20×). The positive staining localized in the cytoplasm and nuclei. All the pictures were taken from day 1 after SCI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Acknowledgments

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References


