PPARγ Plays an Important Role in Acute Hepatic Ischemia-Reperfusion Injury via AMPK/mTOR Pathway

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Background. Hepatic ischemia-reperfusion (IR) injury is one of the severe complications associated with liver surgery and leads to liver dysfunction. PPARγ is always linked with various physiologic pathways, and it can alleviate liver damage in IR injury. Aim. In this study, we explored the potential mechanism of PPARγ in the pathogenesis of hepatic IR injury by mice model. Methods. After treated with si-PPARγ or rosiglitazone, mice were subjected to hepatic ischemia-reperfusion. Liver tissue and blood samples were collected to evaluate liver injury and detected relative mRNA and protein expressions. Results. The expression of PPARγ was increased after reperfusion. And the alleviation of PPARγ aggravated the liver damage in IR; at the same time, upregulation of the expression of PPARγ released the liver damage. And these effects of PPARγ in IR were related to the AMPK/mTOR/autophagy signaling pathway. Conclusion. PPARγ plays an important role in hepatic IR injury at least partly via the AMPK/mTOR/autophagy pathway.

1. Introduction

Ischemia-reperfusion (IR) is a phenomenon occurring after the restoration of arterial blood flow to a specific organ or tissue [1]. The pathophysiology of IR injury is mainly the induction of oxidative stress and inflammatory cascade reaction. Thus, the reperfusion of blood flow may result in oxidative damage and inflammation rather than recovery. Hepatic IR injury is one of the severe complications associated with liver surgery and leads to liver failure or primary nonfunction, thus, increasing morbidity and mortality after liver surgery [2–4]. Since hepatectomy or liver transplantation is the most effective method for the treatment of end-stage liver diseases, it is essential to detect the possible preoperative and perioperative interventions for minimizing IR-induced hepatocellular damage, especially in patients with cirrhosis.

As a member of the family of nuclear receptors, peroxisome proliferator-activated receptor-γ (PPARγ) acts as heterodimers with DNA response elements and can regulate various metabolism responses [5, 6]. PPARγ has an important role in regulating the inflammatory response and oxidative stress in hepatic IR injury [7–9]. The protective effects of PPARγ agonists, such as telmisartan [10], irbesartan [11], darglitazone [12], rosiglitazone [13], and pioglitazone [14], in IR injury have been reported [15]. And these evidences suggested that PPARγ agonists can modulate inflammatory responses, oxidative stress, and metabolism in IR. The adenosine monophosphate-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) signaling pathway has been confirmed that it is a critical regulator of cellular processes, including cell growth, viability, differentiation, survival, and metabolism. And PPARγ can modulate the mTOR pathway. In this study, we downregulated and upregulated the expression of PPARγ and explored the function of PPARγ in hepatic IR injury, and we treated mice with mTOR inhibitor, rapamycin (Rapa), to make sure PPARγ showed its effects in hepatic IR injury via AMPK/mTOR pathway.
2. Methods

2.1. Animal Preparation. This project was approved by the Animal Care and Use Committee of Shanghai Tongji University and Shanghai Tenth People's Hospital (SHDSYY-2021-4990), China. And all animal experiments complied with the guidelines of the China National Institutes of Health. Six-week-old male Balb/c mice (Shanghai SLAC Laboratory Animal, Shanghai, China) were used in our experiments. All mice weighed 23-28 g and housed in a standard environment. All efforts have been done to minimize the suffering of mice in this research.

The animals underwent either sham surgery or ischemia-reperfusion (IR) operation. Partial warm hepatic ischemia was induced as described previously [20]. After anesthetized with 1.25% sodium pentobarbital (Nembutal, St. Louis, MO, USA), the blood supply to the left lateral and median lobes of the liver was interrupted, causing 70% ischemia. After 45 minutes of hepatic ischemia, we restored blood supply and initiated reperfusion. We performed the same operation protocol in sham control mice without vascular occlusion. Mice were sacrificed after 2, 6, 12, and 24 hours of ischemia-reperfusion, and blood and liver were collected for further analysis.

Rapamycin (S1039, Selleck) was dissolved in dimethyl sulfoxide (DMSO) at 25 mg/ml before administration. In the rapamycin-treated groups, animals have received rapamycin at a dose of 1.5 mg/kg per day before injury through the tail vein with control siRNA once a day for 2 weeks without operation.

2.2. In Vivo Transfection with PPARy Short Interfering RNA (siRNA). Firstly, siRNA PPARy (guide: 5’UCAGCUCCUGGGAUCCUCUGUAA, passenger: 5’AUUACGAGAGAUCCACGGGAGCU’) or siRNA control (Genepharma, Suzhou, China) was bought from Genema. Then, according to the producer instruction, siRNA PPARy or siRNA control was dissolved in RNase-free water to the concentration of 1 μg/μL. Then, 5 μL PPARy siRNA or 5 μL control siRNA and 5 μL in vivo transfection reagent (18668-11, Engreen, Co., Beijing, China) were, respectively, diluted with 5 μL 10% glucose. Finally, the mixtures were injected into the tail vein of mice.

2.3. Animal Experiment Design. According to our experiment plan, mice were distributed into the following groups:

1. Natural group (n = 3): mice without any treatment
2. Sham group (n = 5): mice underwent sham surgery
3. Vehicle group (n = 5): mice were treated with methylcellulose orally once a day for 5 days without operation
4. Drug group (n = 5): mice were treated with 10 mg/kg rosiglitazone orally once a day for 5 days without operation
5. Si-control group (n = 5): mice were injected from the tail vein with control siRNA once a day for 2 weeks without operation
6. Si-PPARγ group (n = 5): mice were injected from the tail vein with PPARγ siRNA once a day for 2 weeks without operation
7. IR groups (n = 20): mice underwent IR operation and sacrificed at 2, 6, 12, and 24 hours after reperfusion
8. IR + Rosi groups (n = 20): after treated with 10 mg/kg rosiglitazone orally once a day for 5 days, mice underwent IR operation and sacrificed at 2, 6, 12, and 24 hours after reperfusion
9. IR + si-PPARγ groups (n = 20): after injected with PPARγ siRNA once a day for 2 weeks, mice underwent IR operation and sacrificed at 2, 6, 12, and 24 hours after reperfusion
10. IR + si-PPARγ + Rosi groups (n = 20): after treated with both PPARγ siRNA and 10 mg/kg rosiglitazone, mice underwent IR operation and sacrificed at 2, 6, 12, and 24 hours after reperfusion
11. IR + Rapa (n = 5): after intraperitoneal injected with the mTOR inhibitor rapamycin for 5 days, mice underwent IR operation and sacrificed at 12 hours after reperfusion
12. IR + si-PPARγ + Rapa (n = 5): after treated with both PPARγ siRNA and rapamycin, mice underwent IR operation and sacrificed at 12 hours after reperfusion

2.4. Serum Enzyme Analysis. Serum was separated by centrifugation at 1,500 g for 10 min and stored at -80°C. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by kits bought from Jiancheng Co. (Nanjing, China).

2.5. Histology, Immunohistochemical(IHC) Staining, and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay. Liver tissue samples were fixed and embedded in the following standard steps. Liver sections were cut and stained with hematoxylin and eosin. For IHC, the slices were dewaxed and rehydrated; and after an antigen retrieval process and blocking, slices were incubated with primary antibodies described in western blot analysis part overnight. For the TUNEL, the slices were treated according to the instruction and then incubated in the TUNEL reaction mixture (Roche, Mannheim, Germany) at room temperature for 1 h.

2.6. Western Blot Analysis. Western blotting was performed as standard protocol. Protein was extracted from frozen liver samples. A total of 80 ng protein was loaded onto 6%, 10%, and 12.5% SDS-polyacrylamide gels, and the separated proteins were transferred to PVDF membranes. The membranes were incubated overnight at 4°C with primary antibodies followed by incubation with a secondary antibody (1: 2,000). Finally, the blots were scanned using an Odyssey two-colour infrared laser imaging system (Li-Cor, Lincoln, NE, USA). Western blots were performed using the following
antibodies; PPARγ (Cell Signaling Technology), mTOR (Cell Signaling Technology), p-mTOR (Cell Signaling Technology, Ser2448), AMPKα (Cell Signaling Technology), p-AMPKα (Cell Signaling Technology, Thr172), TNF-α (Cell Signaling Technology), LC3 (Proteintech), and β-actin (Abcam).

3. Results

3.1. The Expression of PPARγ in IR Injury. To confirm the activation of PPARγ during the hepatic ischemia-reperfusion injury, we detected the expression of PPARγ by western blot and qRT-PCR. In Figures 1(a) and 1(b), both the protein and mRNA expression of PPARγ were increased after reperfusion, especially after 6 hours. Following, we did a histopathological analysis for IR injury (Figure 1(c)). Obvious necrosis could be seen after 6 hours, and the rate of necrosis was over 50% after 12 hours after reperfusion. Figure 1(d) exhibited the immunohistochemical staining of PPARγ in collected liver tissues, and the number of positive cells changed almost in parallel with the above results. We hypothesized that this change was due to the protective mechanism of PPARγ on damaged hepatocytes. And we compared natural group, sham group, vehicle group, drug group, and siRNA-control groups to exclude their influence on the following results (Supplementary Figure 1).

3.2. Alleviation the Expression of PPARγ Aggravated the Liver Damage in IR. Si-PPARγ was injected into mice via the tail vein to downregulate its expression, and the pathological alteration after reperfusion was compared with that in normal mice. Serum levels of ALT and AST were measured after reperfusion (Figure 2(a)), and we found that the downregulation of PPARγ aggravated the damage of hepatocytes. And then we evaluated the damage in terms of inflammation and apoptosis.

We detected the level of inflammation through proinflammatory cytokine TNF-α and IL-1β. The circulating levels of them were indeed upregulated by PPARγ downregulation (Figure 2(b)). Consistent with the former, the expression of TNF-α and IL-1β was higher in the si-PPARγ group (Figures 2(c) and 2(e)). Apoptosis is a prominent feature of IR injury, and its participation was confirmed. Bax is a famous proapoptotic family member, and we detected its mRNA expression firstly. The graph in Figure 2(d) showed that with the increase of Bax expression during IR injury, PPARγ downregulation made this increase more obvious. Then, we used western blot to measure the protein expression of Bax and caspase9, whose results (Figure 2(e)) exhibited that the injection of si-PPARγ increased the occurrence of apoptosis during IR injury. Thus, the alleviation of PPARγ aggravated the liver damage in IR.

3.3. Upregulation of the Expression of PPARγ Released the Liver Damage in IR. Rosiglitazone is a typical PPARγ agonist and is widely used in clinics. A group of mice were treated with 10 mg/kg rosiglitazone orally for 5 days before IR, and we also compared their pathological alteration after reperfusion with that in normal mice. Figure 3(a) showed the serum levels of ALT and AST, and we found that the upregulation of PPARγ reduced the damage of hepatocytes. And then we evaluated the damage in the same way we used it before.

We detected the level of TNF-α and IL-1β. The circulating levels of them were downregulated by rosiglitazone (Figure 3(b)). Consistent with the former, the expression of TNF-α and IL-1β was lower in the rosiglitazone treatment group (Figures 3(c) and 3(e)). The graph in Figures 3(d) and 3(e) showed that the increased expression of Bax and caspase9 during IR injury was relieved by the treatment of rosiglitazone, that is, the occurrence of apoptosis during IR injury was reduced. Thus, the upregulation of PPARγ mitigated liver damage in IR.

3.4. The Influence of PPARγ in IR May Be Linked with AMPK/mTOR/Autophagy Signaling Pathway. To make sure the effect of PPARγ in IR injury, those mice, which were injected with si-PPARγ, were treated with Rosi. And we detected the index of apoptosis and inflammation in these mice after reperfusion for 12 hours, which were exhibited
in Figures 4(a)–4(d). These results showed that liver damage during IR injury, including hepatocyte apoptosis and inflammation responses, was definitely related to the expression of PPARγ. Besides, we also detected the changes of pyroptosis in our study, which were exhibited in Supplementary Figure 2.

As an important kinase in energy hemostasis, AMPK is an upstream target and negative regulator of mTOR, which is a major negative regulator of autophagy. Autophagy plays a vital role in various liver damage. Combined with previous reports, we speculated that the effects of PPARγ during IR injury may be related to the AMPK/mTOR/autophagy...
Figure 2: Continued.
signaling pathway. Thus, we measured the protein expression of p-AMPK, p-mTOR, and autophagy-related proteins, LC3, P62, and Beclin1, whose expressions were changed along with PPARγ (Figures 4(e) and 4(f)). The treatment of Rosi obviously promoted AMPK phosphorylation and reduced the phosphorylated form of mTOR, which contributed to the suppression of autophagy in mouse livers. And si-PPARγ leads to opposite effects. Furthermore, we detected the above changes in mice treated with both si-PPARγ and Rosi, and the results were in agreement with those mentioned above. Thus, the effects of PPARγ in IR injury were linked with the AMPK/mTOR/autophagy signaling pathway.

To make sure our conclusion, we treated mice with rapamycin to inhibit the function of mTOR and detected changes in inflammation response and apoptosis, whose results were shown in Figure 5. We measured the expression of TNF-α, Bax, and Beclin1, and we found that effects caused by si-PPARγ were blocked by rapamycin. Accordingly, we confirmed the relationship between PPARγ and AMPK/mTOR.

4. Discussion

In the liver, IR injury can occur in several clinical situations, for example, liver trauma, resection, and transplantation. The pathogenesis of IR is closely related to oxidative stress, energy metabolism disorders, inflammatory response, and cell apoptosis and autophagy [21]. As is known to all, PPARγ demonstrated significant functions in the tissue protection and repair [22–24]. And advances in PPAR ligands and agonists renew opportunities for drug development [25]. Here, in our present study, we found that the activation of PPARγ could confer hepatoprotective effects against hepatic IR injury and also investigated the therapeutic potential of PPARγ agonists for the protection of hepatic injury. The major findings of this study include (1) the expression of PPARγ were increased after reperfusion, which hinted the protective role of PPARγ in hepatic IR injury; (2) alleviation the expression of PPARγ could aggravate the liver damage in IR; otherwise, the upregulation released liver damage; (3) the protective effects of PPARγ may involve anti-inflammatory and antiapoptosis activity as demonstrated in vivo; (4) the underlying mechanism of PPARγ in IR injury may be linked with AMPK/mTOR/autophagy signaling pathway.

PPARγ is a ligand-activated transcription factor of the nuclear hormone receptor superfamily known to modulate target genes involved in the regulation of various inflammatory responses, cell growth and apoptosis, metabolism, fibrosis, and tissue repair [26, 27]. Several studies have indicated...
Figure 3: Continued.
that the activation of PPARγ is a therapeutic target for acute hepatic IR injury [7, 15, 28]. Additionally, agonists of PPARγ exhibited anti-inflammatory and antiapoptosis properties both in vitro and in vivo and could impart protection from IR in mice models [29, 30]. In the present experiment, our results demonstrated that the expression of PPARγ was increased during reperfusion. Combined with the previous researches, we hypothesized that this change was due to the spontaneous protective mechanism of PPARγ on damaged hepatocytes.

To clarify our hypothesis, we regulated the expression of PPARγ by injection of si-PPARγ or administration of rosiglitazone, a typical PPARγ agonist. After the above treatment, serum liver enzymes ALT and AST showed the same changes as expected. And then we detected the liver damage in terms of inflammation and apoptosis. Excessive inflammatory response and hepatocyte apoptosis are recognized as key mechanism of liver IR injury. We detected the level of inflammation through proinflammatory cytokines TNF-α and IL-1β. The circulating levels of them were obviously upregulated by si-PPARγ and downregulated by rosiglitazone. Results of qRT-PCR and western blot exhibited that when compared with the IR group, the expression of TNF-α and IL-1β was higher in the si-PPARγ group and lower in the rosiglitazone group. Apoptosis is a prominent feature of IR injury, and its participation was confirmed. Bax and caspase9 are famous proapoptotic indices, and we detected their mRNA and protein expression. With the increase of Bax and caspase9 expression during IR injury, PPARγ down-regulation exacerbated this increase; however, the treatment of rosiglitazone relieved it. Thus, the alleviation of PPARγ aggravated the liver damage in IR; and at the same time, the upregulation of PPARγ mitigated the liver damage. Further, it has been reported that rosiglitazone is protective on a variety of injuries, including IR injury of many organs. Our results indicated that rosiglitazone may reduce, although do not ablate, hepatic damage after IR injury.

When further elucidating the potential mechanism of the effects of PPAR in IR, we focused on the pathway, AMPK/mTOR mediated autophagy. AMPK exists in all eukaryotic cells as a highly conserved protein kinase. It is a major regulator of energy homeostasis that balances energy supply and demand and ultimately modulates cellular and organ growth [31, 32]. AMPK can be activated by a variety of stresses including poisonous metabolites and pathological precursors such as starvation, ischemia, and hypoxia [33]. mTOR, a 289
Figure 4: Continued.
**Figure 4: Continued.**
kDa serine/threonine kinase, is a master negative regulator of autophagy, modulating cell growth, cell proliferation, cell cycle, and cell motility [16]. Autophagy plays a key role in the modulation of inflammation, cellular homeostasis and dysregulation, and cell death or survival. It has been proved associated with various liver disorders, including hepatitis, liver fibrosis, fatty liver, and acute IR injury [34–37]. It has been accepted that AMPK inhibits mTORC1 through phosphorylation, thus, inducing autophagy in response to cellular stress cues.

The relationship between PPARγ and AMPK/mTOR pathway has been discussed before [17, 38]. Jimenez-Flores et al. [39] and Zhong et al. [40] reported that PPAR-γ expression levels are significantly reduced in diabetic mouse livers and the increase of the expression of alleviated liver damage. Zhong et al. [41] found db/db mice showed significantly decreased PPAR-γ and p-AMPK expression levels and increased p-mTOR expression, and the expression of Atg7, Beclin-1, and LC3 was also decreased. Micheliolide reversed these effects and alleviated the

Figure 4: The influence of PPARγ in IR may be linked with the AMPK/mTOR/autophagy signaling pathway. Notes: (a) the levels of serum ALT and AST for IR after 12 h (n = 5, # means p < 0.05 for si-PPARγ versus IR, + means p < 0.05 for si-PPARγ+Rosi versus si-PPARγ); (b) the levels of serum TNF-α and IL-1β for IR after 12 h (n = 5, # means p < 0.05 for si-PPARγ versus IR, + means p < 0.05 for si-PPARγ+Rosi versus si-PPARγ); (c) protein expression of TNF-α, IL-1β, Bax, and cleaved caspase9 for IR after 12 h (n = 3, # means p < 0.05 for si-PPARγ versus IR, + means p < 0.05 for si-PPARγ+Rosi versus si-PPARγ); (d) TUNEL staining (×200) showed apoptotic cells in mice liver for IR after 12 h; (e) protein expression of AMPK, p-AMPK, mTOR, p-mTOR, LC3, and Beclin1 for IR after 12 h (n = 3, # means p < 0.05 for IR versus control, + means p < 0.05 for IR ± si-PPARγ±Rosi versus IR); (f) immunohistochemistry staining (×200) showing the expression of p-AMPK, p-mTOR, and LC3 for IR after 12 h (n = 3, # means p < 0.05 for IR versus control, + means p < 0.05 for IR ± si-PPARγ±Rosi versus IR).
Figure 5: The involvement of AMPK/mTOR in the effects of PPARγ in IR. Notes: (a) relative mRNA expression of TNF-α, Bax, and Beclin1 ($n = 3$, # means $p < 0.05$ for IR versus control, + means $p < 0.05$ for IR ± si-PPARγ ± Rapa versus IR); (b) protein expression of mTOR, p-mTOR, TNF-α, Bax, and Beclin1 ($n = 5$, # means $p < 0.05$ for si-PPARγ versus IR, + means $p < 0.05$ for si-PPARγ+Rosi versus si-PPARγ).
inflammatory response and lipotoxicity in hepatocytes. Besides, the link of PPARγ and AMPK/mTOR/autophagy pathway was explored in other disease models [42–48]. To make sure the mechanism, mice were treated with at least one of the si-PPARγ and Rosi. The diminished expression of PPARγ caused by si-PPARγ leads to obvious inhibition of AMPK phosphorylation and thus promoted the phosphorylation of mTOR, inducing autophagy in mouse livers. The treatment of Rosi leads to opposite effects. Further, we treated mice with both si-PPARγ and rosiglitazone and got the same result as expected. In addition, we used rapamycin to confirm the involvement of mTOR and found that inflammation response and apoptosis caused by rapamycin in the IR injury were changed opposite to si-PPARγ. Therefore, we believed that the activation of PPARγ can not only relieve the inflammatory response and hepatocyte apoptosis but also exert its hepatoprotective effect via the AMPK/mTOR/autophagy pathway (Figure 6).

5. Conclusion

In summary, we found that PPARγ is continuously activated in hepatocytes during hepatic IR injury. Mice with significantly diminished expression of PPARγ got more grievous liver injury after hepatic ischemia-reperfusion injury. Conversely, activation of PPARγ caused by rosiglitazone resulted in attenuated liver injury. Through the use of si-PPARγ and rosiglitazone, we confirmed that one possible mechanism by which PPARγ activation results in protection against IR-related liver injury is through AMPK/mTOR-mediated autophagy. These results suggested that PPARγ may be a vital regulator and potential therapeutic target in the liver ischemic injury. And our results provided confidence for the follow-up development of PPARγ-related drugs for IR injury.

**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>IHC</td>
<td>Histology, immunohistochemical staining</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling assay</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR analysis</td>
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<td>IR</td>
<td>Ischemia-reperfusion</td>
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<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor-γ</td>
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<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>Rosi</td>
<td>Rosiglitazone</td>
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**Data Availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Conflicts of Interest**

The authors declare that there is no conflict of interest regarding the publication of this article.

**Acknowledgments**

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**Supplementary Materials**

Supplementary Materials 1: comparison of control groups. We compared natural group, sham group, vehicle group, drug group, and siRNA-control groups to exclude their influence on the results. We detected serum ALT and AST levels of those groups and observed pathological changes of their liver tissues. Results were exhibited as follows. We found that there is no significant difference among these groups, which meant that they would not cause obvious influence in our study. 2: PPARγ could affect pyroptosis in the IR injury. NOD-like receptor protein 3 (NLRP3) inflammasome plays a big role in the development of pyroptosis and can lead to the activation of caspase1. In the supplementary experiments, we measured the circulating level of IL-18 (a) and the mRNA and protein expression of caspase1 and NLRP3 among different groups of mice (b and c) and detected that relationship among IR, PPARγ, and pyroptosis. We found that pyroptosis was increased in the IR, and the alleviation of PPARγ aggravated pyroptosis, but the upregulation of PPARγ released it. (Supplementary Materials)
References


