Rosiglitazone enhances neovascularization in diabetic rat ischemic hindlimb model

Majid Khazaei, Ensieh Salehi

Background. There is increasing evidence that peroxisome proliferator–activated receptors (PPARs) may be involved in the regulation of angiogenesis. In this study, we examined whether rosiglitazone, a PPARγ agonist, can restore angiogenesis in a rat hindlimb ischemia model of diabetes.

Methods. Male wistar rats were divided into four groups (n=6 each): control, diabetic and control and diabetic rats who received rosiglitazone (8 mg/kg/day). Diabetes was induced by streptozotocin (55 mg/kg; ip). After 21 days, serum concentrations of nitric oxide (NO), vascular endothelial growth factor (VEGF) and soluble VEGF receptor-2 (VEGFR-2) were measured and neovascularization in ischemic legs was evaluated by immunohistochemistry.

Results. Capillary density and capillary/fiber ratio in hindlimb ischemia of diabetic animals were significantly lower than the control group (P<0.05). Rosiglitazone significantly restored neovascularization in diabetic animals (P<0.05).

Conclusions. Rosiglitazone enhances neovascularization in diabetic ischemic skeletal muscle and could be considered for treatment of peripheral artery disease in diabetic subjects.

Key words: diabetes, rosiglitazone, angiogenesis, hindlimb ischemia

INTRODUCTION

Type 2 diabetes is a major cause of morbidity and mortality in advanced societies. Cardiovascular disease is responsible for up to 80% of death in diabetic subjects. Some of the long-term complications of diabetes are associated with impaired angiogenesis which can result in severe organ damage. Angiogenesis is defined as sprouting of blood vessels from preexisting ones and is considered a physiological response to tissue ischemia. Hypoxia is the main stimulus for angiogenesis. Thus, angiogenic therapy is a novel approach for improving tissue perfusion in diabetic patients with reduced regional organ perfusion.

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors that have three nuclear receptor isoforms, PPARα, PPARδ and PPARγ (ref. 7). Rosiglitazone is a PPARγ agonist that belongs to a new class of insulin sensitizers, used clinically in the management of diabetes. PPARγ is expressed in endothelial and vascular smooth muscle cells. It is indicated that PPARγ ligands not only have beneficial effects on endothelial function, the amelioration of hyperlipidemia and hyperglycemia, but also, upregulate angiogenic factors such as endothelial nitric oxide (NO) synthase and vascular endothelial growth factor (VEGF) expression in vascular smooth muscle cells. In recent years, there is increasing evidence that PPARs might be involved in regulation of physiological and pathological angiogenesis.

Since peripheral artery disease is a major complication of diabetes, in this study, we test the hypothesis that rosiglitazone can improve skeletal muscle angiogenesis in diabetic and control rats in hindlimb ischemia model.

MATERIALS AND METHODS

Animals

Ten week old male wistar rats weighing between 180-230 g were provided by the Pasteur Institute of Iran. The animals were randomly divided into two groups: diabetic and control. Experimental diabetes was induced by a single intraperitoneal injection of streptozotocin (55 mg/kg) dissolved in 0.9% saline. Control rats received the same volume of 0.9% saline. After 48 h, blood samples were taken and the animals with blood glucose concentration higher than 16.7 mmol/l were considered diabetic. Then, all rats were randomly divided into 4 groups as follows:

- Group1: control rats received vehicle.
- Group2: control rats received rosiglitazone (8 mg/kg/day) by gavage.
- Group3: diabetic rats received vehicle.
- Group4: diabetic rats received rosiglitazone (8 mg/kg/day) by gavage.

The treatments were started on day one after induction of hindlimb ischemia and lasted for 21 days. All experimental procedures were approved by the ethics committee of the authors’ institution.
Rat hind limb ischemic model

All rats were anesthetized with ketamine (75 mg/kg) and xylazine (7.5 mg/kg), intraperitoneally. Unilateral hindlimb ischemia was induced as previously described. In brief, the left legs were shaved and locally disinfected. Through a small incision, the left femoral artery was isolated. The proximal and distal portions of the femoral artery and distal portion of saphenous artery with side branches were ligated and excised. Subsequently, the skin was closed with 3-0 silk surgical suture. Then, the animals were returned to their cages.

Capillary density analysis

For capillary density measurement, the ischemic gastrocnemius muscles were dissected. After overnight fixation at 10% formalin, they were embedded in paraffin and cut with 5µm thickness. Then, the sections were deparaffinized and incubated with a rat-monoclonal antibody directed against mouse CD31 (Abcam Co.). Finally, capillary density was measured at 400× in ten different fields from each tissue preparation and determined as the number of CD31 positive cells per mm². To avoid overestimate or underestimate of capillary density because of muscle atrophy or interstitial edema, capillary/muscle fiber ratio was also expressed.

Measurement of plasma parameters

After 12h fasting, blood samples were taken from retroorbital space before and at the end of experiment. Blood samples were centrifuged with 10000 rpm for 15 min to obtain serum triglycerides (TG), High-density lipoprotein cholesterol (HDL-C), Total cholesterol (TC) and Low-density lipoprotein cholesterol (LDL-C), glucose and insulin concentrations with commercially available kits.

Measurement of serum NO, VEGF and VEGFR-2 concentrations

Serum NO concentrations were measured using Griess reagent method (Promega Corp, USA). In this method, serum nitrite, the main metabolite of NO, was measured. The limit of detection is 2.5µM. Serum VEGF and VEGFR-2 concentrations were measured by Enzyme-linked Immunosorbent assay using available reagents and recombinant standards (R&D systems, Minneapolis, USA). The minimum sensitivity of VEGF and VEGFR-2 assays are 3.9 pg/ml and 0.027 ng/ml, respectively.

RESULTS

Plasma parameters

As shown in Table 1, the plasma level of TG was significantly reduced and serum HDL-C increased in control rats treated with rosiglitazone (P<0.05). In diabetic groups, rosiglitazone also reduced serum TG and increased serum HDL-C concentrations (P<0.05). Blood glucose levels were higher than 16.7 mmol/l in diabetic rats throughout the study and rosiglitazone administration did not alter the blood glucose level or serum insulin concentrations compared to control (P>0.05) (data not shown).

Evaluation of serum angiogenic factors

Fig. 1. illustrates serum nitrite concentrations on day 21 after operation. Diabetic animals had lower serum nitrite concentration than control (P=0.08). Rosiglitazone did not change serum nitrite concentration in control or diabetic animals (P>0.05). Serum VEGF and VEGFR-2 concentrations were not different between control and diabetic animals (P>0.05). There were no significant differences in serum VEGF and VEGFR-2 concentrations between rosiglitazone-treated and non-treated groups (P>0.05) (Fig. 2A and B).

Evaluation of neovascularization

Neovascularization was evaluated as capillary density (CD31-positive cells) per mm² and number of capillaries per muscle fiber ratio. Neovascularization was significantly impaired in hindlimb ischemia of diabetic animals com-

Table 1. Serum lipid profile before and after study in experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Cholesterol (mg/dl) Before</th>
<th>Total Cholesterol (mg/dl) After</th>
<th>Triglyceride (mg/dl) Before</th>
<th>Triglyceride (mg/dl) After</th>
<th>HDL-C (mg/dl) Before</th>
<th>HDL-C (mg/dl) After</th>
<th>LDL-C (mg/dl) Before</th>
<th>LDL-C (mg/dl) After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>66.00±6.79</td>
<td>84.40±8.74</td>
<td>93.16±6.72</td>
<td>73.40±5.88</td>
<td>28.33±4.19</td>
<td>44.66±5.01*</td>
<td>25.56±1.94</td>
<td>28.52±3.66</td>
</tr>
<tr>
<td>Control+ Rosiglitazone</td>
<td>63.8±5.16</td>
<td>53.6±6.93</td>
<td>86.8±6.56</td>
<td>45.6±6.12*</td>
<td>24.8±5.13</td>
<td>47.00±3.06*</td>
<td>19.64±2.29</td>
<td>10.20±0.01</td>
</tr>
<tr>
<td>Diabetic</td>
<td>70.33±5.44</td>
<td>72.25±7.06</td>
<td>81.80±15.02</td>
<td>71.25±12.37</td>
<td>26.00±3.55</td>
<td>33.40±2.76*</td>
<td>19.63±2.44</td>
<td>20.76±2.56</td>
</tr>
<tr>
<td>Diabetic+ Rosiglitazone</td>
<td>66.6±4.85</td>
<td>62.2±19.33</td>
<td>91.8±4.59</td>
<td>68.50±4.74*</td>
<td>28.40±3.84</td>
<td>42.20±8.56</td>
<td>21.24±1.90</td>
<td>29.60±14.8</td>
</tr>
</tbody>
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Data are expressed as Mean ± SE; *: P<0.05 compare to before experiment.
Fig. 1. Effect of rosiglitazone (R) on serum nitrite concentration (µmol/lit.) in experimental groups. Data are shown as mean ±SE, n=6 per group.

Fig. 2. Effect of rosiglitazone (R) on serum VEGF (A) and VEGFR-2 (B) concentrations. Data are shown as mean ±SE, n=6 per group.

Fig. 3. Capillary density (A) and capillary/fiber ratio (B) in hindlimb ischemia. Data are shown as Mean±SE, n=6 per group. *: P<0.05 compare to other groups.

DISCUSSION

In this study, we investigated the role of rosiglitazone, a PPARγ agonist, on angiogenesis in hindlimb ischemia in diabetic and control rats. Our data illustrated that diabetes is associated with reduced angiogenesis in ischemic skeletal muscle and rosiglitazone administration restored neovascularization in hindlimb ischemia of diabetic animals. Rosiglitazone is a drug from thiazolidinediones (TZDs) that is not only used for improvement of insulin resistance in diabetic patients but also safeguards diabetic patients from cardiovascular events. In the present study, we found that rosiglitazone improved serum HDL and lowered TG concentration in control and diabetic rats, however, it did not change serum insulin or glucose concentrations. In this study, we used normal rodent chow, not high-fat diet, however, our findings are in agreement with previous studies which showed that activation of PPARγ lowered plasma triglyceride levels and increased plasma HDL. However, a study on cholesterol-fed rabbits revealed that rosiglitazone significantly reduced aortic atherosclerosis without modifying the plasma levels of glucose, insulin or lipid profile.

It is believed that PPARs are involved during the angiogenesis process. In this study, we found that angiogenesis in hindlimb ischemia of diabetic animals was
impaired compared to control. In addition, serum NO concentration in diabetic animals was lower than control. Enhanced angiogenesis has an important role in some complications of diabetes including diabetic retinopathy and nephropathy, on the other hand, reduced angiogenesis which is related to lower arteriogenesis and poor growth of collateral artery has an important role in cardiovascular diseases in diabetes. NO not only enhances angiogenesis, but also, other angiogenic growth factors exert their angiogenic response through increasing NO production. Reduced NO bioavailability in diabetic subjects has been reported in several studies. Suppression of endothelial NO synthase (eNOS) expression and its activity, overproduction of superoxide and activation of protein kinase C (ref) during high-glucose concentration are possible mechanisms for lower NO availability in diabetic subjects. VEGF is another angiogenic factor in a variety of in vivo models. VEGFR-2 is also an effector of proangiogenic signaling in the angiogenesis process.

In the present study, we found no significant differences in serum VEGF and VEGFR-2 concentrations between control and diabetic animals. It is suggested that without considering the serum VEGF level, the VEGF signaling pathway is impaired during diabetes which is considered as VEGF resistance. Therefore, it is possible that reduced serum NO concentration may be responsible for lower neovascularization in hindlimb ischemic tissue of diabetic animals.

We also found that rosiglitazone restored neovascularization in the ischemic leg of diabetic animals. The angiogenic abilities of PPARγ agonists have been broadly examined; however, the results are contradictory. Studies in different angiogenesis models revealed that activation of PPARγ upregulates receptor of antiangiogenic factor thrombospondin in chorioallantoic membrane, inhibits bFGF- and VEGF-mediated angiogenesis, suppresses VEGF-induced angiogenesis in rat cornea model, and inhibits tumor growth angiogenesis and metastasis.

Fig. 4. Representative images of immunohistochemical staining (×400) with anti-CD31 monoclonal antibody in ischemic skeletal muscles. Arrows indicate CD31-positive cells. R: rosiglitazone.
In agreement with our results, a recent study on KKAy mice indicated that pioglitazone administration restored ischemia-induced angiogenesis. In that study, Huang et al. used pioglitazone as the PPARγ agonist. It was shown that pioglitazone is a partial PPARγ agonist and considered as a less potent ligand, whereas, rosiglitazone appears to be a pure PPARγ agonist with high affinity to receptor. Another study demonstrated that PPARγ agonists increase angiogenesis after focal cerebral ischemia. Rosiglitazone also has a myocardial protective role during ischemia/reperfusion injury. Several mechanisms have been suggested for the angiogenic role of PPARγ agonists. Huang et al. suggested that activation of eNOS is the main mechanism for enhanced angiogenesis. In the present study, we found that serum NO, VEGF and VEGFR-2 concentrations did not alter after rosiglitazone treatment. Increase in number of endothelial progenitor cells is suggested as another mechanism for enhanced angiogenesis of PPARγ activation. Therefore, it is possible that the effect of PPARγ agonists on angiogenesis in pathological or ischemic condition is different.

In conclusions, diabetes is associated with impaired angiogenesis in ischemic skeletal muscle and rosiglitazone restored neovascularization in diabetic animals. Since diabetes is one of the most important risk factors for the development of peripheral vascular disease, it seems that rosiglitazone can be considered for treatment of peripheral artery disease in diabetic subjects. Further studies are needed to clarify the exact role and mechanisms of PPARγ agonists on physiological and pathological angiogenesis.

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REFERENCES

Rosiglitazone belongs to the thiazolidinedione class of compounds that exhibit agonist activity on PPAR-gamma (peroxisome proliferator-activated receptor gamma). These drugs enhance the sensitivity of tissues to the effects of endogenous insulin. Rosiglitazone was registered in the European Union as Avandia in July 2000. As an oral antidiabetic drug, it was used in the treatment of patients with type 2 diabetes mellitus. Rosiglitazone was served as a second-line drug where other treatments had failed. In June 2010, studies were published describing the adverse effects of this antidiabetic drug especially on the cardiovascular system\(^1,2\). Based on this information, the European Medicines Agency recommended the withdrawal of rosiglitazone in the EU member states.

The paper, entitled “Rosiglitazone enhances neovascularization in diabetic rat ischemic hind limb model” describes a new effect of rosiglitazone on angiogenesis in an animal model (rat). The results of this communication are interesting and may contribute to the future use of rosiglitazone in for new indications. This is of course a question for further experiments and studies.

REFERENCES
