

Original Article

Decreased PPAR- γ expression after internal carotid artery stenting is associated with vascular lesions induced by smooth muscle cell proliferation and systemic inflammation in a minipig model

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Abstract: Vascular restenosis after stenting is known to be largely mediated by proliferation of vascular smooth muscle cells. Recently, peroxisome proliferator-activated receptor gamma (PPAR- γ) has been implicated as a regulator of cellular inflammatory responses, and the PPAR- γ agonist rosiglitazone (ROSI) has been shown to attenuate atherosclerosis formation. However, whether ROSI can inhibit neointimal formation by regulating the inflammatory response and inhibiting vascular smooth muscle hyperplasia after stenting-induced injury remains to be clarified. Accordingly, in this study, 10 minipigs were randomly divided into two groups: the stenting group (n = 5) and the ROSI group (n = 5). Morphometric analysis was conducted for the stented arteries. The protein expressions of PPAR- γ and smooth muscle 22-alpha (SM22 α) were analyzed by immunohistochemistry and western blotting, and the serum interferon- γ and interleukin-10 levels were measured by enzyme-linked immunosorbent assay. Three months after implantation, morphometric analysis revealed that administration of ROSI (0.5 mg/kg/d, continuous administration for 90 days) resulted in significant reductions of luminal stenosis, the neointimal area, and neointimal thickness, as compared to the stenting groups. The expression of PPAR- γ and the PPAR- γ /SM22 α ratio in the ROSI group were higher than in the stenting group. Furthermore, the serum interferon- γ and interleukin-10 levels were found to be increased and to reach peak levels at 4 h and 7 days after stenting, respectively, after which both declined. However, ROSI treatment resulted in decreased interferon- γ and increased interleukin-10 levels after stenting. In both groups, the cytokine levels returned to the baseline levels on day 56 after stenting. Taken together, these results suggest that ROSI can reduce neointimal formation after stenting by inhibiting the local and systemic inflammatory responses as well as vascular smooth muscle hyperplasia.

Keywords: Stent implantation, peroxisome proliferator-activated receptor- γ , interferon- γ , interleukin-10

Introduction

Permanent brain damage secondary to stroke has become a leading cause of disability in adults, and, worldwide, cerebral apoplexy is now the third leading cause of death following cardiovascular disease and cancer. Carotid artery disease is responsible for 25% of all ischemic strokes in the adult population, and between 60% and 70% of these events are caused by extracranial arterial stenosis [1, 2]. With the advent of endovascular technology, internal carotid artery stenting has become a recognized alternative to carotid endarterectomy [3-5]. Carotid artery stenting is currently

widely used in clinical practice; however, its short-term stent restenosis (in-stent restenosis) rate is relatively high.

Vascular restenosis after stent placement is a complex process that includes intimal hyperplasia and vascular remodeling, which may cause postoperative thrombosis. Prior research on preventing restenosis has focused mainly on the endometrium, with the proposed strategies including drug interventions and new supportive technologies focusing on facilitating vascular intimal endothelialization [6, 7]. In recent years, transformation of the vascular smooth muscle cell (VSMC) membrane phenotype has

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become the focus of research efforts on the vascular remodeling process [8], and the results of these previous studies suggest that VSMC membrane proliferation and migration are the main causes of restenosis. In addition, during the deployment of the stent, it may be difficult to avoid touching of the plaque, which results in damage to the endothelial cells and vascular wall, consequently leading to a series of inflammatory reactions. Lastly, it is important to keep in mind that the stent itself is a foreign body, which can also cause inflammatory and/or immunological responses.

Peroxisome proliferator-activated receptor gamma (PPAR- γ) is a nuclear transcription factor that is activated by various ligands and that has many biological effects, including regulation of the transcription of genes related to lipid and glucose metabolism, as well as to inflammation and cell development. It is known to play an important role in the development of obesity, diabetes, insulin resistance, ischemia-reperfusion injury, cancer, and atherosclerotic processes. Additionally, brain PPAR- γ may inhibit central nervous system inflammation, and, recently, some studies have moreover reported that PPAR- γ plays an important role in regulating cell inflammation and protecting cells from ischemia-reperfusion injury [9, 10]. Rosiglitazone (ROSI) is the most potent activator of PPAR- γ , and is currently used clinically to treat diabetes, along with metformin and sulfonylureas [11]. As a PPAR- γ agonist, ROSI has been shown to have anti-inflammatory effects in the presence of ischemia/reperfusion injury [12-14]. However, only very limited data on the effects of ROSI on the inflammatory response after stent injury are currently available.

Thus, this study aimed to investigate the impacts of PPAR- γ expression on the prevention of restenosis of the internal carotid artery stent using a minipig model. We analyzed the changes in the phenotypic expression of VSMCs and the degree of inflammation post-stent implantation in minipigs treated with or without ROSI.

Materials and methods

Vascular injury model

Ten minipigs (3-4 months, 18-22 kg, Animal Center, Third Military Medical University, Chongqing, China) were fasted for 12 hours be-

fore surgery and injected with atropine (0.04 mg/kg) and ketamine (12 mg/kg) intramuscularly. Subsequently, venous channels were established in the ear vein of the minipigs, and 3% pentobarbital sodium (25 mg/kg) was injected intravenously. The body temperature, arterial blood pressure, and heart rate were assessed, and the blood gases and acid-base balances were monitored throughout the whole procedure by arterial blood sampling.

Right femoral artery incision was performed under aseptic conditions, and a 6-Fr introducer sheath was inserted. Next, a 6-Fr guiding catheter was advanced through the aortic arch into the carotid artery after administration of 200 U/kg of unfractionated heparin. Left and right carotid angiograms were obtained, and the sizes of the arteries were measured by quantitative angiography [15]. A bare-metal stent ([BMS], 3.0-3.5 mm in diameter and 20 mm in length) was implanted in the left carotid artery of each minipig at high pressure (12-14 atm) for > 30 s. The stent-to-artery ratio was maintained at 1.5:1. Heparin (400 U/h) was continually infused during the whole procedure. Before the procedure, all minipigs were pretreated with aspirin (300 mg loading dose) and clopidogrel (150 mg loading dose) for three days.

After establishment of the vascular injury model, the minipigs were randomly divided into the stenting (n = 5; injection with 0.9% sodium chloride as a placebo) and ROSI groups (n = 5; rosiglitazone, GlaxoSmithKline, USA, 0.5 mg/kg/d, continuous administration for 90 days). Arterial blood was collected 24 h before the procedure and at 4 h and 7, 14, 28, and 56 days after the operation. The serum was separated and stored for further analysis at -70°C.

The study protocol was approved by and all experiments were performed according to the guidelines set by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University, Chongqing, China.

Quantitative internal carotid artery assessment

Angiograms were obtained on day 90. Medical digital subtraction angiography (Artis Floor, SIEMENS, Germany) was performed to quantitatively assess the internal carotid artery by two experienced neurologists who were blinded to the treatment protocol. Any discrepancies were

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resolved through mutual consultation and discussion between the two neurologists.

Quantitative angiography

The pre-stent, post-stent, and follow-up quantitative angiography parameters were analyzed by a computer-assisted quantitative arteriographic edge detection algorithm. The minimal lumen diameter (MLD) and reference diameter were calculated at each time point.

Tissue harvest and processing for histology

The minipigs were injected with atropine (0.04 mg/kg) and ketamine (12 mg/kg) intramuscularly and sacrificed by intravenous injection of potassium chloride after 90 days [15-17]. Their carotid arteries were excised and placed in 0.9% heparinized saline followed by 4% neutral formalin for fixation. The stent-containing vessels from each group were sliced into six 2-mm long pieces, fixed in 4% formalin, and embedded in paraffin or glycol methacrylate. Cross-sections from the distal, medial, and proximal pieces were stained with hematoxylin and eosin for morphometric analysis. The remaining paraffin-embedded pieces were used for immunohistochemical analyses. An independent observer analyzed the morphometric neointima from the histology slides, and the neointimal thickness and intimal area were measured using digital morphometry.

Immunohistochemistry and western blotting

After deparaffinization and hydration, the specimens were blocked with 0.3% H₂O₂ at room temperature for 15 min. A rabbit polyclonal antibody against pig-PPAR- γ (2.5 μ g/ml, ABD Serotec, USA) was used for immunohistochemical staining. After blocking with 5% bovine serum albumin for 30 min, the slides were incubated with the primary antibody in a moisture chamber overnight at 4°C. Subsequently, the slides were rinsed in Tris-buffered saline (TBS) and incubated with the secondary biotinylated antibody for 30 min at 37°C. After rinsing in TBS, the slides were next incubated with streptavidin for 30 min at 37°C and visualized using 3,3'-diaminobenzidine. The immunohistochemical staining results were judged by the method described by Krajewska [18], by rating each slide in a double-blinded fashion under a microscope.

For western blot analysis, the tissues from the carotid artery area were harvested and minced. The following primary antibodies were used: anti-PPAR- γ (1:1000, ABD Serotec, USA), anti-smooth muscle 22-alpha (SM22 α) (1:300, Santa Cruz, USA), and anti- β -actin (1:2000, GeneTex, USA). After extensive rinsing, horseradish peroxidase-conjugated secondary antibodies were used to detect the immunocomplexes by enhanced chemiluminescence. The relative expressions of PPAR- γ and SM22 α were analyzed by ImageJ analysis software.

Enzyme-linked immunosorbent assay (ELISA)

The levels of interferon (IFN)- γ and interleukin (IL)-10 were determined by using a double-antibody sandwich ELISA kit (4 Abio, Beijing, China). The samples were homogenized using a mortar, dissolved in 500 ml of extraction buffer, and centrifuged at 10,000 rpm for 12 min. The supernatants of all specimens were analyzed using a multi-detection microplate reader in accordance with the manufacturer's protocols. The concentrations of IFN- γ and IL-10 were normalized to the content of total protein in the samples.

Statistical analysis

All statistics were calculated using GraphPad Prism v5.0 software. The data were analyzed using independent-samples t-test and are presented as means \pm standard deviations. A value of $P < 0.05$ was considered significant.

Results

Animal health

Ten minipigs underwent successful implantation of 20 BMSs (2 BMCs for each) in their internal carotid arteries. All animals survived stent implantation and the follow-up procedures without complications until they were sacrificed on day 90 post-implantation.

Damage caused by artery stents and drugs can be effectively improved by ROSI treatment

The vascular stent was visible throughout the entire vasculature and the vessel was angiographically patent without stenosis (**Figure 1A**). In the stenting group, three months after stent implantation, the implantation site showed sig-

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Figure 1. Internal carotid arteries 90 days after stent placement. Arteriotomy was conducted on the right femoral arteries of 10 minipigs, and the stents were implanted under sterile conditions. The stent-to-artery ratio was maintained at 1.5:1. The animals were randomly assigned to either the control or rosiglitazone (ROSI) groups after injury. A: Normal control group, B: Stenting group, C: Stenting and ROSI treatment group.

Table 1. Quantitative internal carotid artery results

Parameter	Stent group (n = 5)	ROSI group (n = 5)	P value
Reference diameter (mm)	4.20±0.38	4.07±0.23	0.55
Immediate MLD (mm)	4.95±0.18	5.05±0.19	0.40
Follow-up MLD (mm)	3.73±0.38	4.40±0.20	< 0.01
Late lumen loss (mm)	1.21±0.40	0.65±0.28	< 0.05

Data are presented as mean \pm SD. $P < 0.05$ indicates a significant difference. ROSI: rosiglitazone, MLD: minimal lumen diameter.

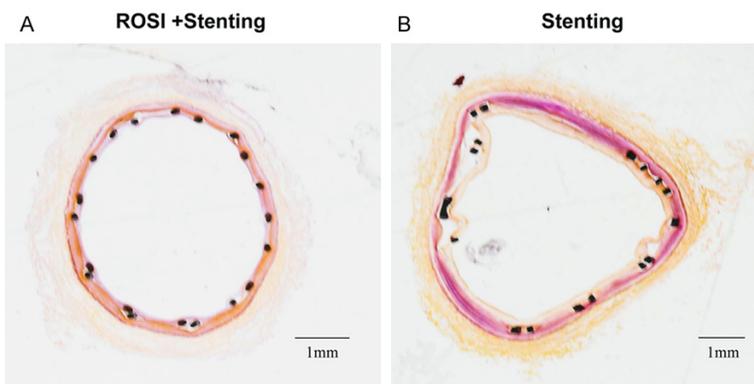


Figure 2. Intimal hyperplasia 90 days after stenting injury (aldehyde-fuchsin staining). The intima of the stenting plus rosiglitazone (ROSI) treatment group (A) was thinner than that of the stenting group (B).

nificantly higher levels of stenosis compared to the normal controls (**Figure 1B**). Conversely, in the ROSI group, the site of stent implantation

(**Figure 1C**, arrow) showed improved contrast filling and no significant stenosis. During the follow-up, the reduction in the luminal diameter in the ROSI group was significantly lower than that in the stenting group (**Figure 1B, 1C**).

Table 1 summarizes the results of the quantitative internal carotid artery analysis. There were no differences between the groups in terms of the reference diameters or immediate MLD.

In the stenting group, the mean intimal area and intimal thickness were $4.35 \pm 0.57 \text{ mm}^2$ and $0.33 \pm 0.11 \text{ mm}$, respectively, and ROSI treatment resulted in decreases in the neointimal area and intimal thickness by 23% ($3.36 \pm 0.64 \text{ mm}^2$) and 33% ($0.22 \pm 0.09 \text{ mm}$), respectively (**Figure 2**, $P < 0.05$ for both).

PPAR- γ and SM22 α expressions

The protein expressions of PPAR- γ in the three groups (stenting, ROSI, and normal control groups), as determined by immunohistochemical analysis, are shown in **Figure 3**. Quantification of PPAR- γ staining in the intima of the stented arteries at the follow-up showed that the mean PPAR- γ expressions scores were 5.85 ± 0.29 and 0.90 ± 0.22 in the normal control and stented groups, respectively ($P < 0.05$). On the other hand, in the ROSI group, the mean PPAR- γ expression score was 5.04 ± 0.46 in the stented arteries, which was significantly increased compared to in the stenting group

($P < 0.05$). However, no significant differences were observed between the ROSI and normal control groups.

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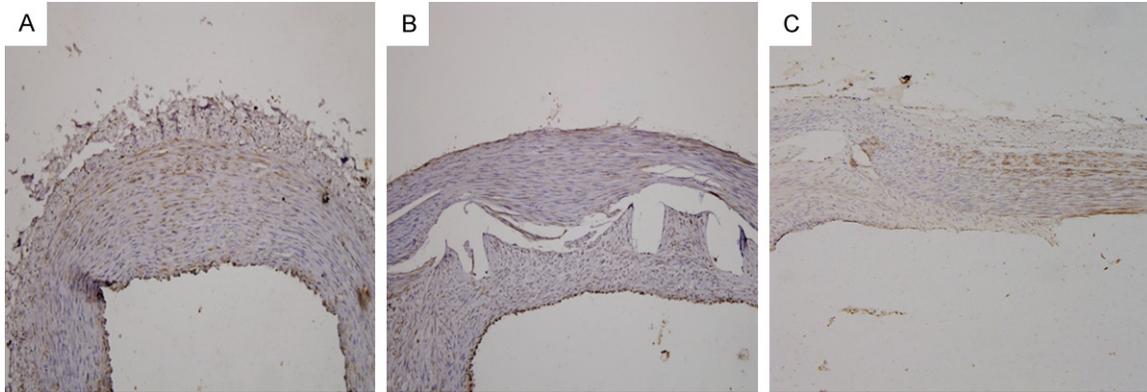


Figure 3. Immunohistochemical staining for peroxisome proliferator-activated receptor gamma (PPAR-γ) expression in the vascular smooth muscle cells (VSMCs) in each group. The immunohistochemical staining was conducted 90 days after stenting. A: Normal control group, B: Stenting group, C: Stenting plus rosiglitazone (ROSI) treatment group. PPAR-γ-positive VSMCs are shown by their nuclear brown staining ($\times 100$).

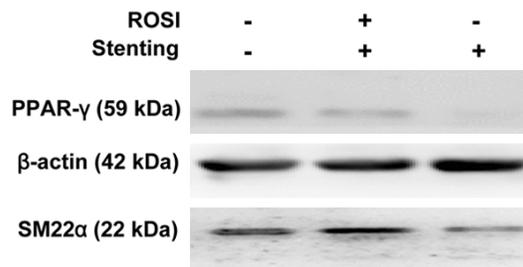


Figure 4. Western blot assay for the expression of minipig carotid artery peroxisome proliferator-activated receptor gamma (PPAR-γ) and smooth muscle 22-alpha (SM22α). The carotid artery tissues were harvested and the total protein was extracted for the western blot analysis by using anti-PPAR-γ, anti-SM22α, and anti-β-actin primary antibodies. The immunocomplexes were detected using horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence. The relative molecular masses of the target proteins are indicated.

The western blot assay showed that the PPAR-γ/SM22α ratios of the normal control, stenting, and ROSI groups were $19.87 \pm 5.33 / 68.85 \pm 9.73$, $3.12 \pm 1.42 / 16.72 \pm 5.34$, and $23.17 \pm 4.23 / 58.63 \pm 12.81$, respectively (**Figure 4**). The PPAR-γ/SM22α ratio was significantly lower in the stenting group than in the normal control group ($P < 0.05$). Moreover, changes in the VSMC phenotype from contractile to synthetic transformation, as determined by the PPAR-γ/SM22α expression, were significantly more commonly observed in the ROSI group than in the stenting group ($P < 0.05$), whereas no significant differences were observed between the ROSI and control groups (**Figure 4**).

Serum IFN-γ and IL-10 levels

Table 2 shows the serum IFN-γ and IL-10 levels in the groups. The serum IFN-γ levels were the highest four hours after stenting, after which they began to gradually decline. Treatment with ROSI resulted in decreased levels of IFN-γ after stenting. On the other hand, the serum IL-10 levels were the highest seven days after stenting, and treatment with ROSI resulted in significantly increased IL-10 levels 2 days after stenting compared with the stenting group ($P < 0.05$). On day 56, all cytokine levels had returned almost to the baseline levels.

Discussion

Inflammation and VSMC proliferation and migration are two of the most important stages of restenosis [8, 19-22]. After stenting, inflammatory cells release a large quantity of cytokines and growth factors, thereby promoting the proliferation and migration of VSMCs, consequently resulting in restenosis. In recent years, the application of drug-eluting stents has significantly reduced the incidence of restenosis [23]; however, their long-term effects remain to be determined [24].

In this study, we successfully constructed a minipig carotid artery stent implantation model and investigated the effects of ROSI on intimal hyperplasia within 3 months after stent implantation. Our results confirmed that ROSI treatment can effectively inhibit the protein expression of PPAR-γ/SM22α and that it resulted in significantly reduced neointimal hyperplasia as compared to stent implantation only.

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Table 2. Serum IFN- γ and IL-10 levels (pg/mL)

Time (after stenting)	IFN- γ		P value	IL-10		P value
	Stent group	ROSI group		Stent group	ROSI group	
Pre-stenting	101.75 \pm 11.86	97.83 \pm 17.30	0.76	10.86 \pm 2.48	10.78 \pm 2.91	0.97
4 h	249.20 \pm 29.15	264.70 \pm 6.17	0.33	11.81 \pm 1.92	14.81 \pm 2.92	0.21
2 d	214.09 \pm 41.98	157.12 \pm 35.31	0.08	9.34 \pm 1.91	31.31 \pm 11.49	0.03
7 d	181.91 \pm 11.05	151.39 \pm 15.31	0.01	33.90 \pm 12.82	87.01 \pm 23.01	0.02
14 d	158.02 \pm 33.06	104.86 \pm 20.74	0.03	14.75 \pm 4.90	61.24 \pm 25.23	0.03
28 d	113.46 \pm 16.65	97.17 \pm 11.89	0.24	11.90 \pm 2.91	20.94 \pm 6.24	0.08
56 d	102.00 \pm 55.82	96.89 \pm 17.73	0.88	11.87 \pm 5.42	13.17 \pm 5.10	0.78

Data are presented as mean \pm SD. $P < 0.05$ indicates a significant difference. IFN- γ : interferon- γ , IL-10: interleukin-10, ROSI: rosiglitazone.

Cerebral vascular restenosis after stent implantation is closely related to vascular remodeling. A variety of cellular and extracellular processes have been demonstrated to be involved in vascular remodeling, including (1) endothelial injury resulting from stripped vascular endothelial cells, damaged endometrium, and activation of the clotting enzyme system, which consequently leads to platelet adhesion, aggregation, and thrombosis; (2) VSMC proliferation and migration due to release of proinflammatory factors and biological active substances from the damaged endothelial cells; (3) stimulation of DNA synthesis due to stent stimulation, balloon expansion, and other factors related to mechanical tension-release, which in turn promotes cell division, proliferation, and migration, and eventually endometrial and outer membrane hyperplasia; and (4) hyperplasia of the outer membrane of fibroblasts.

As mentioned above, endothelial cell injury causes pathological smooth muscle cell proliferation and migration, resulting in wall thickening, luminal stenosis, and, most importantly, vascular remodeling [25]. PPAR- γ has been demonstrated to improve endothelial function and inhibit vascular inflammation and VSMC proliferation and migration, and recent studies have moreover confirmed that PPAR- γ expression in the cerebral arteries plays a clear role in inhibiting vascular remodeling [9, 33, 35]. Benkirane et al. demonstrated that PPAR- γ can regulate VSMC phenotype transformation by a variety of molecular interactions [26]. Angiotensin II is the main factor promoting the transformation of VSMCs to the synthetic phenotype, which is a characteristic feature of hypertension, and which is mediated through the angiotensin II1 (AT1) receptor, and ROSI has

been shown to inhibit the effects of angiotensin II on the expression of the AT1 receptor in the mesenteric artery [26].

In recent years, many studies have focused on the role of inflammation in post-carotid artery stent implantation and stenosis [27-32]. Carotid artery stenting is a potent inflammatory stimulus and has been demonstrated to generate local inflammation. In general, the levels of white blood cells change within 15 minutes of coronary vessel stent implantation. Moreover, stent implantation can also cause systemic inflammatory responses. Systemic inflammation results from secretion of tumor necrosis factor- α from the activated immune cells, which in turn causes the liver to produce C-reactive protein, an acute phase reaction protein.

ROSI is a thiazolidinedione that can increase insulin sensitivity, in addition to inhibiting VSMC proliferation and migration. PPAR- γ not only regulates the synthesis of proinflammatory mediators, but can also affect the anti-inflammatory mediators, and thereby maintains the balance between the proinflammatory and anti-inflammatory responses of the cells. PPAR- γ agonists such as ROSI can enhance the anti-inflammatory gene transcription and expression through a variety of mechanisms, and have hence also been hypothesized to play a role in neuroprotection [33, 34]. PPAR- γ is activated upon ligand binding, and subsequently binds to the peroxisome proliferator response element of the target gene DNA. This results in increased gene transcription and expression, and consequently in increased protein expressions of different adhesion molecules, thereby alleviating the inflammatory infiltration and reducing the toxic effects of the activated inflammatory cells. Re-

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cent experiments have confirmed that PPAR- γ ligands can reduce the inflammatory reaction of ischemic tissues [35]. Tureyen et al. reported that, after administering PPAR- γ agonists, PPAR- γ was activated and directly blocked the cytokine signal transduction pathways, inhibited microglial activation, reduced the levels of neutrophil and macrophage infiltration, decreased the expression of inflammatory genes, enhanced the activity of antioxidant enzymes, and increased the expression of heat shock protein [36]. In this condition, it acted as a neuroprotective agent. Furthermore, Yi et al. confirmed the effectiveness of the PPAR- γ agonist ROSI in the treatment of traumatic brain injury in mice [37]. They found that ROSI significantly reduced the area of cerebral contusion. Similarly, Park et al. showed that the use of PPAR- γ agonists in rats with spinal cord injury resulted in significantly reduced spinal cord contusion [38], and, in experimental models of cerebral ischemia, injection of exogenous ROSI reduced the levels of caspase-3 and H₂O₂, inhibited apoptosis and necrosis of nerve cells, and reduced the area of cerebral infarction [39].

In the present study, we found that the serum levels of IFN- γ and IL-10 were increased after porcine carotid artery stent implantation, indicating vascular injury. Four hours after the vascular injury, the inflammatory response, as assessed by the IFN- γ level, was significantly increased, and the level did not return to normal until 56 days after the stent implantation. Similarly, two days after injury, the IL-10 serum levels were also significantly increased, and these had also returned to normal after 56 days. When comparing the ROSI group to the stenting group, the IFN- γ level was found to be lower, whereas the IL-10 level was higher in the ROSI group. IFN- γ is a typical early proinflammatory mediator mainly produced by activated T cells, which plays a key role in vascular inflammation after vascular stent implantation [40, 41]. IFN- γ can activate macrophages, vascular endothelial cells, neutrophils, and natural killer cells via binding to its receptor, and continuous monitoring of the IFN- γ level can hence reflect the development of in-stent stenosis. On the other hand, IL-10 has inhibitory effects on inflammation and immune responses, and therefore helps to improve the control of the systemic inflammatory responses [42]. Thus, while monitoring of IFN- γ can detect the proinflam-

matory response intensity, monitoring of IL-10 can conversely help assess the anti-inflammatory responses.

As mentioned above, in the present study, by creating a pig model of carotid artery stenting and administering the PPAR- γ agonist ROSI, we found that, compared to the stenting group, the ROSI group showed lower IFN- γ levels and increased vascular smooth muscle SM22 α expression. Accordingly, our findings confirmed that ROSI plays an anti-inflammatory role post-stenting, likely by activating PPAR- γ pathways through the carotid artery VSMC phenotype transformation process. ROSI moreover plays important roles in vascular remodeling and IFN- γ expression inhibition, and, hence, in reducing vascular inflammation. However, the specific mechanisms behind these observations remain to be further elucidated.

In conclusion, we here found that ROSI treatment significantly inhibited restenosis after stent implantation. The mechanisms behind this phenomenon may be related to the activation of PPAR- γ , stabilization of vascular smooth muscle phenotype conversion, and inhibition of local and systemic inflammatory responses.

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Disclosure of conflict of interest

None.

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