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Protective effects of rosiglitazone on the endothelial cells in a rabbit iliac artery balloon injury model

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To investigate the protective effects of rosiglitazone on the endothelial cells in a rabbit iliac artery balloon injury, thirty male New Zealand white rabbits were randomly divided into control group (hypercholesterol diet), balloon injury group (hypercholesterol diet plus balloon endothelial denudation) and rosiglitazone group (balloon endothelial denudation plus hypercholesterol diet and rosiglitazone). Rabbits were fed for eight weeks, and balloon endothelial denudation was performed. Rosiglitazone was administrated at a dose of 0.5 mg·kg^{-1}·d^{-1} from three days before surgery, and lasted for 4 weeks. Four weeks later, blood was collected and serum lipids, glucose, hs-CRP, NO, NOS and ET-1 levels were measured. Then, local iliac arteries were obtained for morphological examination and immunohistochemistry for PCNA and apoptosis. When compared with balloon injury group, the levels of serum TC, TG, LDL-C, ET-1 were significantly decreased in the rosiglitazone group. Morphological examination showed the intimal area (IA), medial area (MA) and intimal proliferation index in rosiglitazone group were significantly lower than those in the balloon injury group. The PCNA expression was markedly decreased and the apoptosis rate significantly increased in rosiglitazone group when compared with the balloon injury group. Rosiglitazone could exert protective effects on the endothelial cells after balloon injury in which the lipid- lowering, inhibited proliferation and increased apoptosis of endothelial cells, and compromised NOS activity and secretion of NO and ET-1 played important roles.

Key words: Rosiglitazone, apoptosis, nitric oxide synthase, endothelin, rabbit.

INTRODUCTION

Percutaneous transluminal coronary angioplasty (PTCA) has been an ideal strategy in the treatment of coronary artery stenosis. But the incidence of restenosis is still at a high level after PTCA, which limits the long term efficacy. The mechanism underlying the restenosis after PTCA is still poorly understood. Increasing studies show endothelial injury and endothelial dysfunction play critical roles in the post-PTCA restenosis (Kipshidze et al., 2004). Rosiglitazone is an anti-diabetic drug in the thiazolidinedione class of drugs. It works as an insulin sensitizer, by binding to the PPAR receptor (PPAR) in fat cells and making the cells more responsive to insulin

(Edvardsson et al., 1999). In addition, rosiglitazone can improve lipid metabolism and exert anti-oxidative effects which play pivotal roles in the cardiovascular protection of rosiglitazone (Ceriello, 2008). The present study was to investigate the effects of rosiglitazone on the endothelial functions, intimal cell proliferation and apoptosis in a rabbit iliac artery balloon injury model aiming to explore the possible mechanism underlying the protective effects of rosiglitazone.

MATERIALS AND METHODS

Drug and instruments

Rosiglitazone maleate (GSK China, lot number: H20020475), balloon and pressure pump for 2.5F PTCA (Boston Scientific, USA),

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HPIAS2000 color image analysis system, Unicel Dxc 800 automatic biochemical analyzer (BECKMAN COULTER, USA), NO detection kit and ET- 1 radioimmunoassay kit (Nanjing Jiancheng Biotech, China), and immunohistochemistry kit for proliferating cell nuclear antigen (PCNA) and TUNEL kit (Wuhan Boster Biotech, China) were used in the present study.

Modeling and grouping

Male New Zealand rabbits (n=30) weighing 2.6±0.2 kg were purchased from the Animal Center of Wuhan General Hospital of Guangzhou Military Region. All animal experiments were approved by the Administrative Committee of Experimental Animal Care and Use of Wuhan General Hospital of Guangzhou Military Region (WHJ2009-0001), and conformed to the National Institute of Health guidelines on the ethical use of animals. Animals were housed for 1 week for accommodation and then divided into 3 groups (n=10 per group): (1) control group: rabbits were fed with normal chow; (2) balloon injury group: rabbits were fed with high-fat chow and underwent iliac artery balloon endothelial denudation (Nuthakki et al., 2004); (3) Rosiglitazone group: rabbits were fed with high-fat chow, underwent iliac artery balloon endothelial denudation and then treated with rosiglitazone. The high fat diet is the chow supplemented with 1% cholesterol, 7.5% egg yolk powder and 8% pork fat which was administered to animals for 8 weeks followed by balloon endothelial denudation of iliac artery.

Animals were intramuscularly anesthetized with SUMAAN α followed by sterilization. A longitudinal incision was made at the site where the pulse of femoral artery is the most obvious. Then, 2~3 cm femoral artery was separated and the distal end was ligated. The proximal end was clamped and an incision of about 45° was made in the femoral artery between the distal end and proximal end. A 2.5F balloon angioplasty catheter was introduced through the right carotid artery and advanced over a 0.014-in guide wire into the iliac artery (12-15 cm in depth). The balloon was inflated in the distal portion of the external iliac artery to 6~8 atm. The inflated balloon was then pulled back to the proximal end of femoral artery, and an additional three inflations were performed. This procedure has been shown to cause complete endothelial denudation. Then, the balloon was emptied and removed. The vessel was ligated followed by wound closure. Antibiotics (800000 U of penicillin; i.v.) were given for consecutive 3 days for prophylaxis of infection. Normal chow was administered for consecutive 4 weeks. In rosiglitazone group, rosiglitazone was orally administered once daily at 0.5 mg/kg/d 3 days before surgery for consecutive 4 weeks.

Serum parameters

Four weeks after surgery, animals were fasted for 8 h and blood was collected from the ear vein. The detection of serum parameters including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and fasting blood glucose (FPG) was performed with an automatic biochemical analyzer. Colorimetry was used to detect the NO level, NOS detection kit to determine NOS expression and radioimmunoassay to detect the ET-1 level. Procedures were carried out according to manufacturers' instructions.

Histopathological examinations

Animals were sacrificed by air embolism 4 weeks after surgery and laparotomy was performed. The abdominal aorta was separated to iliac artery and femoral artery followed by catherization and subsequent flushing of iliac artery and abdominal aorta. Then, 1~2 cm of arteries were obtained downward the bifurcation of left and

right iliac artery, fixed in 10% formalin and embedded in paraffin followed by HE staining and immunohistochemistry. Five fields were randomly selected and the areas of newly generated intima (IA) and tunica media (MA) were analyzed with the image analysis system. The intimal proliferation index (IPI) was calculated as follow:

IPI=IA/(IA+MA).

Detection of apoptosis by TUNEL staining

The paraffin embedded sections were deparaffinized and hydrated. Endogenous peroxidase was inactivated by hydrogen peroxide and antigen retrieval was performed in citrate buffer. After washing with PBS and digestion with protease K, sections were incubated with a solution containing terminal deoxynucleotidyl transferase followed by washing with PBS and then treated with Streptavidin/Peroxidase conjugated horseradish peroxidase. Development was performed with DAB followed by counter staining with hematoxylin. The sections were observed under light microscope. Positive cells showed brown. Five fields were randomly selected at a magnification of x400 and the total number of cells and the number of positive cells were determined. The apoptosis rate (AI) was calculated as follow:

AI= (number of positive cells/total number of cells) x 100%.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 statistic software package and data were expressed as means±standard deviation ($\overline{X} \pm S$). T test was used to compare the difference between two groups and comparisons between multiple groups were performed with analysis of variance. Comparisons of rates were done with chi square test. A value of P<0.05 was considered statistically significant.

RESULTS

Serum parameters

In the experiment, 1 rabbit died and the remaining 29 animals survived. The levels of serum lipids and FPG at baseline were not different between 3 groups (P>0.05). At the end of experiment, the levels of serum TC, TG, LDL-C, NOS and ET-1 in balloon injury group and rosiglitazone group were significantly higher than those in normal control group (P<0.01). Furthermore, the levels of these parameters were decreased to different extents in rosiglitazone group when compared with balloon injury group. However, there were no significant differences in the HDL-C and FPG between different groups (Tables 1 and 2).

Pathological examination and immunohistochemistry

H&E staining showed, in control group, the intima was smooth and the smooth muscle cells in the tunica media were spindle-shaped with regular arrangement around

**Table 1.** FPG, serum lipid and hs-CRP in different groups ($\bar{x}$±s,mmol/L).

Group	n	FPG	TC	TG	HDL-C	LDL-C	hs-CRP
Control	10	8.26±0.45	1.41±0.38	0.74±0.31	0.88±0.41	0.74±0.51	1.75±0.43
Balloon injury	9	8.31±0.19	27.62±0.23[b]	1.21±0.11[b]	0.90±0.14	19.27±0.41[b]	6.39±0.18[b]
Rosiglitazone	10	8.37±0.26	18.54±0.32[b,d]	0.84±0.18[c]	0.87±0.28	7.58±0.26[b,d]	4.32±0.31[b,d]

a, P<0.05, b, P<0.01 vs. control group; c, P<0.05, d, P<0.01 vs. balloon injury group, IA: intima; MA: tunica media; IPI: intimal proliferation index; IPI=IA/(IA+MA).

Table 2. Levels of NO, NOS and ET-1 in different groups ($\bar{x}$±s).

Group (mol /L)	n	NO (U /ml)	NOS (U /ml)	i NOS (U /ml)	c NOS (U /ml)	ET- 1 (ng/L)
Control	10	92.17 ±20.35	13. 98 ± 2. 32	5.76 ± 0. 12	9.14 ± 0. 06	263.57± 26.45
Balloon injury	9	50.83 ± 14.52[b]	63.26 ± 0. 14[b]	48.14 ± 0. 33[b]	15.64 ± 2.67[b]	498.64± 22.31[b]
Rosiglitazone	10	68.81±13.46[b,d]	47. 53±0. 28[b,d]	25.68 ± 0.19[b,d]	23.76 ± 0.45[b,d]	343.19±19.86[b,d]

a, P<0.05, b, P<0.01 vs. control group; c, P<0.05, d, P<0.01 vs. balloon injury group.
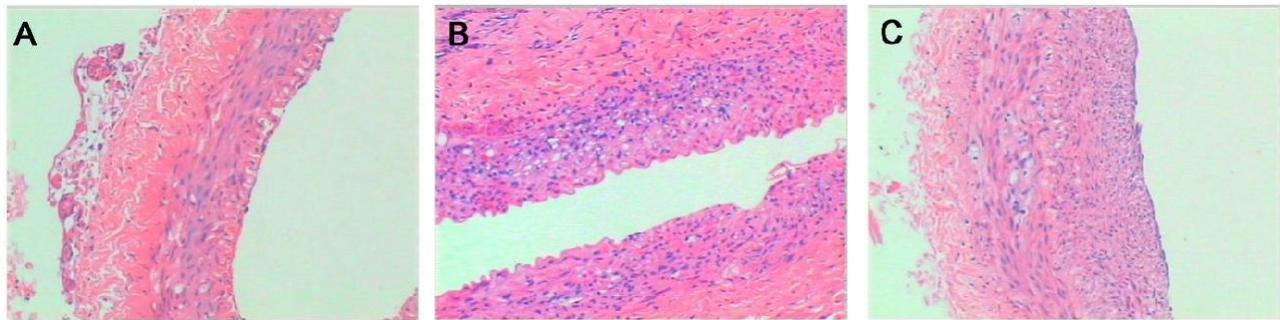
Figure 1. Intimal proliferation in different groups at 12 weeks after surgery (H&E, 100x). (A) control group, (B) balloon injury group, (C) rosiglitazone group.

**Table 3.** Results of pathological examination and immunohistochemistry($\bar{x}$±s).

Group	n	IA (mm^2)	MA (mm^2)	IPI	PCNA expression
Control	10	0.10±0.07	0.41±0.17	----	----
Balloon injury	9	0.36±0.07[b]	0.63±0.09[b]	0.36±0.07	58.76±5.44
Rosiglitazone	10	0.19±0.04[a,d]	0.47±0.03[c]	0.29±0.05[d]	21.36±3.89[d]

a, P<0.05, b, P<0.01 vs. control group; c, P<0.05, d, P<0.01 vs. balloon injury group.

the vessel lumen. In balloon injury group, the intima was thickened and the stenosis of vessel lumen was observed. Proliferation of smooth muscle cells was found in the subintima with irregular arrangement. In addition, the elastic fiber layer was thickened and disordered with evident protrusion of plaque, and a lot of foam cells were noted. In rosiglitazone group, the intima and the tunica media were thickened to different extents and the

stenosis of vessel lumen to a certain extent was found. Proliferation of smooth muscle cells was also noted in the subintima. But the changes in balloon group were more obvious than in rosiglitazone group (Figure 1 and Table 3).

The PCNA positive cells were brown and mainly found in the newly generated intima. In control group, PCNA positive cells were less noted in the intima and PCNA
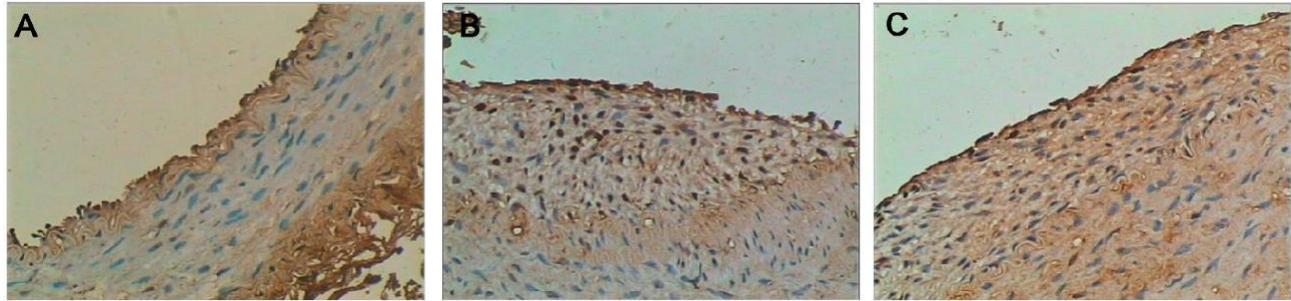
Figure 2. PCNA expression in different groups at 12 weeks after surgery (TUNEL, 100x). (A) control group, (B) balloon injury group, (C) rosiglitazone group.

was expressed to different extents in balloon injury group and rosiglitazone group. When compared with balloon group, the PCNA expression in rosiglitazone group was markedly decreased (P<0.01) (Figure 2).

TUNEL assay

In control group, only a few apoptotic cells were observed. The apoptotic cells were characterized by yellow nucleus and mainly found in the newly generated intima and tunica media. When compared with balloon injury group, the number of apoptotic cells was increased in rosiglitazone group. Image analysis showed the number of apoptotic cells in balloon injury group and in rosiglitazone group was 21.84±1.75 and 37.35±2.46, respectively, showing significant difference (P<0.05).

DISCUSSION

Endothelial dysfunction is an initiator and a key factor of arteriosclerosis and vascular complications of diabetes and increasing attention has been paid to it. In addition, high blood glucose, high serum lipid, insulin resistance and production of pro-inflammatory cytokines are the critical factors in the pathogenesis of arteriosclerosis and vascular complications of diabetes. Studies have demonstrated that proliferation of intima and vascular remodeling play important roles in the restenosis after PTCA, in which vascular dysfunction is involved (Weintraub, 2007). In the present study, rabbits were fed with food rich in cholesterol for 8 weeks and then iliac artery balloon endothelial denudation was carried out. Four weeks after surgery, the levels of serum lipid and hs-CRP, an indicator of inflammation, were significantly higher than those in control group. Furthermore, histology showed evident proliferation of intima, disordered generation of NO/ET-1 and increased apoptosis of smooth muscle cells.

Thiazolidinediones (TZDs) are highly selective agonist of PPAR and have been used in the treatment of diabetes. TZDs are insulin sensitizers and rosiglitazone is a representative of TZDs. TZDs can bind to the endogenous receptors resulting in activation, which may improve the insulin resistance in type 2 diabetes, hyperinsulinemia, hyperglycemia, etc and then maintain the long term glycemic control. In addition, increasing studies have revealed TZDs also play roles in the blood pressure lowing, regulation of lipid metabolism, inhibition of inflammation, anti-atherosclerosis and renoprotection (Campbell, 2005; Ryan et al., 2004; Pistrosch et al., 2004). Our results showed, after 4 weeks of rosiglitazone treatment, the blood glucose, TC, TG and LDL-C levels were decreased to different extents when compared with balloon injury group. Furthermore, the intima proliferation, ET-1 level and apoptosis of endothelial cells were also reduced. These findings suggest rosiglitazone can exert anti- atherosclerotic effect and endothelial protection. NO and ET-1 are vasoactive substances secreted by endo-thelial cells and confer antagonistic effects. Both of them play an important role in the regulation and maintenance of endothelial function (Matsumoto et al., 2008). NO is a product of L- arginine in the presence of NOS and involves in the maintenance of stable circulation and the regulation of cardiovascular activity. NOS is a key enzyme in the synthesis of NO and its activity determines the amount of NO and the biological effects of NO. cNOS and iNOS are two subtypes of NOS. In cNOS, eNOS can continuously catalyze L-arginine producing a small amount of NO, which results in relaxation of vascular smooth-muscle cells, inhibition of endothelial cell prolife-ration and platelet aggregation exerting protective effects. However, activation of iNOS may lead to production of a large amount of NO, which may damage the DNA in endothelial cells exerting cytotoxic effects. In addition, NO derived from iNOS can up-regulate the expressions of adherence molecules and chemotactic proteins resulting in damage to endothelial cells.

ET-1 is a potent vasoconstrictor and can stimulate the proliferation of endothelial cells and vascular smooth muscle cells and promote platelet aggregation. Evidence

has shown the ET-1 level is significantly increased in diabetic vascular diseases, hypertension, and vascular diseases of heart, brain and kidney, and can be used as an indicator of endothelial injury. In the present study, the iNOS level was decreased after rosiglitazone treatment and eNOS level was increased. But the total NOS level was remained unchanged. These results were consistent with the study of Blaschke et al. (2006). These findings suggest rosiglitazone can activate eNOS and inhibit iNOS, exerting protective effects. Therefore, we speculate rosiglitazone can exert protective effects on endothelial function partially through increasing production of NO by endothelial cells.

In recent years, a lot of experiments and trials have demonstrated, after endothelial denudation, the proliferation and apoptosis of smooth muscle cells can be observed in the newly generated intima and tunica media, which is the pathological basis of restenosis after PTCA. Law et al. (1996) for the first time showed TZDs could regulate the proliferation and migration of vascular smooth muscle cells after injury. Our results also confirmed rosiglitazone treatment for 4 weeks can inhibit the proliferation of intima and vascular smooth muscle cells and promote the apoptosis of these cells. Our results revealed rosiglitazone could suppress the over-proliferation of vascular smooth muscle cells and facilitate the apoptosis of these cells. In addition, rosiglitazone could affect the activity of NOS, and influence the secretion of NO and ET-1 exerting protective effects on endothelial cells. Due to the limitations of small sample size and short duration, further experiment and clinical trials are needed.

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