Role of peroxisome proliferator-activated receptor α in atherosclerosis

HENG CAO*, GAO WEN* and HONGLI LI

Department of Cardiology, Shanghai First People's Hospital, College of Medicine, Shanghai Jiaotong University, Shanghai 200080, P.R. China

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Abstract. Atherosclerosis is an inflammatory disease involving the immune response. In addition to lowering the cholesterol level, the peroxisome proliferator-activated receptor α (PPAR-α) can prevent atherosclerosis via its pleiotropic anti-inflammatory effects. However, the role of PPAR-α in modulating inflammatory progression of atherosclerosis has rarely been studied. Thus, we aimed to investigate the role of PPAR-α in atherosclerosis by evaluating the expression of inflammatory cytokines induced by PPAR-α in an *in vivo* rabbit model. New Zealand White rabbits were randomly divided into 5 groups: control, high-fat diet + balloon injury, high-fat diet + balloon injury + placebo, high-fat diet + balloon injury + fenofibrate, and high-fat diet + balloon injury + WY-14643. The femoral arteries of rabbits were balloon-injured after initiation of the high-fat diet and before administration of fenofibrate, WY-14643 or placebo solution. Atherosclerosis was induced by high-fat diet and balloon angioplasty, and the vessel wall lumen occlusion was determined by measuring the stenosis rate. PPAR-α gene expression was examined by quantitative polymerase chain reaction analysis. The cellular localization and distribution of PPAR-α was observed by immunohistochemistry, and its protein level was assessed by western blot analysis. The production of interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α) and P-selectin, which are major inflammatory factors involved in atherosclerosis, was monitored by an enzyme-linked immunosorbent assay (ELISA). Treatment with PPAR-α agonists (fenofibrate or WY-14643) reduced the vascular occlusion rate, as compared to the high-fat diet + balloon injury and the placebo groups. Furthermore, the expression of PPAR-α at both the protein and the mRNA level was increased in the fenofibrate and WY-14643 groups. According to the results, the TNF-α and P-selectin levels were reduced in the fenofibrate and WY-14643 groups. These results suggest that PPAR-α activation can attenuate the effects of atherosclerosis by inhibiting the expression of major inflammatory factors in a rabbit atherosclerosis model.

Correspondence to: Dr Hongli Li, Department of Cardiology, Shanghai First People's Hospital, College of Medicine, Shanghai Jiaotong University, 100 Haining Road, Shanghai 200080, P.R. China
E-mail: drhonglili@126.com

*Contributed equally

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Introduction

Atherosclerosis is a major pathological process related to a number of important adverse vascular events, including coronary artery disease, stroke, and peripheral arterial disease (1). Atherosclerosis is a chronic inflammatory disease of the arterial wall, characterized by the formation of lipid-laden lesions (2). Reducing the inflammatory reaction at these sites may ameliorate atherosclerosis.

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that act as ligand-activated transcription factors to transactivate or transrepress target genes via distinct mechanisms (3). Three subtypes, PPAR-α, PPARβ/δ and PPARγ, have been identified and are differentially expressed in distinct tissues. Numerous studies provided evidence that peroxisome proliferator-activated receptor α (PPAR-α), mainly expressed in high energy-requiring tissues such as skeletal muscle, heart and liver (4), can limit inflammation and regulate lipid metabolism (5,6). The beneficial effects of PPAR-α have been highlighted in acute pancreatitis (7). However, the role of PPAR-α in modulating inflammatory progression of atherosclerosis has been rarely reported. A number of studies have suggested that PPAR-α activation plays a pivotal role in regulating fatty acid oxidation, lipid metabolism, vascular responses, and various metabolic and intracellular signaling pathways that lead to microvascular complications (8). As a general modulator of the inflammatory response, PPAR-α was first reported to be involved in a prolonged inflammatory response to leukotriene B4 (LTB4) in a PPAR-α-null mice model (9). Moreover, increasing evidence suggests that the effects of PPAR-α in inflammation are associated with Alzheimer's disease (AD) and Crohn's disease (10,11). Inflammation in atherosclerosis is characterized by a sequential release of anti- and pro-inflammatory cytokines, including interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α) and P-selectin (12-14). In this study,

we investigated the role of PPAR-α in atherosclerosis and in the induction of expression of these inflammatory factors.

Materials and methods

Animal experiments. Adult male New Zealand White rabbits weighing 2.0-2.5 kg (n=25), were purchased from the Experimental Animal Breeding Co. (Shanghai, China). Following a one-week adaptation period, they were randomly divided into 5 groups of 5 rabbits each: Control, high-fat diet + balloon injury, high-fat diet + balloon injury + placebo, high-fat diet + balloon injury + fenofibrate, high-fat diet + balloon injury + WY-14643. Rabbits were housed in a temperature-, humidity- and light-controlled room with free access to water. The control group was fed with the standard diet; the high-fat + balloon injury group was fed with the high-fat diet (cholesterol 2%, lard 10%, and standard diet 88%) and was subjected to balloon injury. Rabbits in the placebo, fenofibrate and WY-14643 groups were subjected to balloon injury and then received placebo, fenofibrate or WY-14643, respectively. Specifically, the placebo group was fed with cholesterol diet and was intraperitoneally injected with saline (1 mg/kg/day). The fenofibrate group was fed with cholesterol diet containing fenofibrate pellets (130 mg/kg/day), as previously used by Jeanpierre *et al* (15). The WY-14643 group was fed with cholesterol diet and received an intraperitoneal injection of WY-14643 (1 mg/kg/day) (16). All experiments were performed according to the guidelines of the Experimental Animal Center of Shanghai First People's Hospital (SYXK, Shanghai, 2009-0086). The study was approved by the Ethics Committee of Shanghai Jiaotong University (Shanghai, China)

Angioplasty. Rabbits (n=20) were left to adapt to the environment for a week, were fed with high-fat diet for 4 weeks and were then subjected to femoral artery balloon injury. Animals were anesthetized with intravenous infusion of ketamine (0.5 ml/kg Ketalar; Pfizer Inc., New York, NY, USA) and xylazine (0.25 ml/kg Rompun, Bayer Healthcare, Whippany, NJ, USA). Femoral artery de-endothelialization was induced using a 3F Fogarty® balloon catheter (Baxter, Deerfield, IL, USA). Using ophthalmic scissors, a 'V'-shaped small hole was created in the artery wall, followed by insertion of a balloon catheter (1:15 diluted heparin saline infiltration) into the iliac artery (~15 cm). The balloon was connected to a 20 ml injector and ~10 ml air (~2 atm) was injected into the balloon. The balloon was slowly withdrawn from the femoral artery, causing injury, and reinserted. This stretching was repeated twice, 30 sec each time, with 1-min intervals to ensure intimal injury. The catheter was removed, the vascular proximal and distal ends were ligated, and the subcutaneous tissue and the skin were sutured. The wound was washed with penicillin sodium, with intramuscular injection of 400,000 units performed for 3 days.

Histological assessment of artery damage. The artery was transversally sliced and fixed in 10% formalin for 24 h prior to embedment in paraffin. Sections (3 mm) were prepared for histological assessment by staining with hematoxylin and eosin (H&E). The percentage of vessel wall lumen occlusion was calculated with the following formula, as previously described (17): 1 - [L area ÷ (I + L area x 100)], where L denotes the lumen and I the intima.

Immunohistochemistry. Paraffin sections of the artery were deparaffinized, and the endogenous peroxidase activity was inactivated with 3% H_2O_2 for 10 min. The primary antibody mouse anti-rabbit PPAR-α (no. NB300-537; Novus Biologicals, Cambridge, UK) or normal blocking serum was added and incubated overnight. Biotin-conjugated goat anti-mouse immunoglobulin G (IgG) (Novus Biologicals) was used as the secondary antibody and incubated for 30 min. An avidin-biotin enzyme reagent (Novus Biologicals) was sequentially added and incubated for 20 min. A peroxidase substrate was added and incubated until the desired stain intensity was reached. Finally, sections were covered with a glass coverslip and observed under a light microscope. The intensity of positive staining in the tissues was analyzed by integrated optical density (IOD) using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA). Briefly, four 20X TIF-format images from five individual rabbits in each group were analyzed. The IOD and area were calculated, as well as the lumen and the internal elastic lamina area. The PPAR-α expression was expressed as [(IOD/area) x 100] in accordance with a previous study (18).

Western blot analysis. These vascular tissue samples were washed with ice-cold phosphate-buffered saline, and lysed for 20 min on ice with the lysis buffer. The lysates were centrifuged for 4 min at 12,000 x g, and the supernatant was collected in a fresh tube kept on ice. Protein concentrations in each sample were determined using the bicinchoninic acid (BCA) assay. One hundred micrograms of total protein were mixed with loading buffer with the anionic denaturing detergent sodium dodecyl sulfate (SDS), were boiled for 5 min, and then resolved by 10% SDS polyacrylamide gel electrophoresis. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking the membrane in Tris-buffered saline with Tween 20 (TBST) containing non-fat milk for 1 h at 4˚C under agitation, the membrane was washed three times in TBST and incubated for 2 h with mouse anti-rabbit PPAR-α antibody (1:200 dilution) or GAPDH monoclonal antibody (1:200 dilution, no. 20028; Abmart, Shanghai, China). After washing three times in TBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:1,000) for 1 h and then washed three times with TBST. Immuno-stained proteins were detected using a streptavidin amplification reagent (no. WBKL SOO 50; Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

RNA extraction and quantitative PCR. Total RNA was extracted from arterial samples using the TRIzol® reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). One microgram of total RNA was used as template to synthesize cDNA using a reverse transcription kit from BioDev-Tech Co., Ltd (Beijing, China). Quantitative PCR was performed by monitoring the increase in fluorescence of the SYBR-Green dye using the GreenMaster mix (Genaxxon BioScience GmbH, Ulm, Germany) according to the manufacturer's instructions. The primer sets used to amplify the *PPAR-α* gene were: 5'-gttccg-

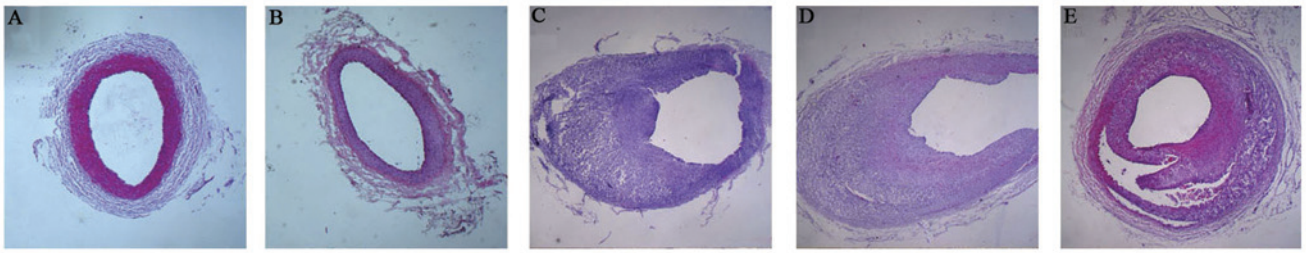

Figure 1. Microphotographs (magnification, x50) of vascular tissue stained with hematoxylin and eosin (H&E) in rabbits of the (A) high-fat diet + balloon injury; (B) control; (C) high-fat diet + balloon injury + placebo; (D) high-fat diet + balloon injury + fenofibrate; and (E) high-fat diet + balloon injury + WY-14643 groups.

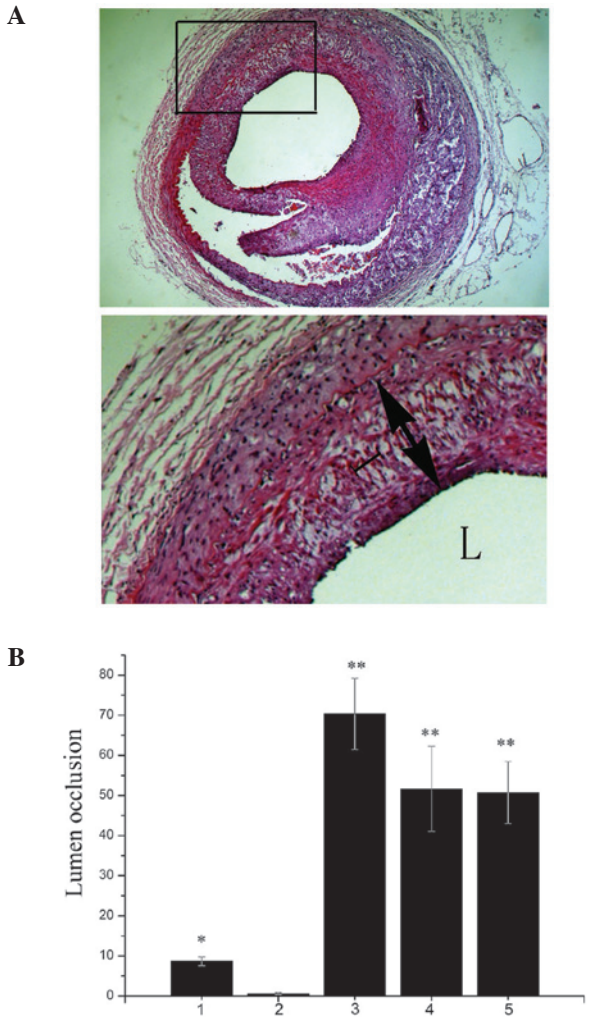

Figure 2. (A) Hematoxylin and eosin staining of the vessel wall. The inset box (lower panel) shows the lumen (L) and the intima (I; arrow). (B) Percentage of vessel wall lumen occlusion in the (1) high-fat diet + balloon injury; (2) control; (3) high-fat diet + balloon injury + placebo; (4) high-fat diet + balloon injury + fenofibrate; and (5) high-fat diet + balloon injury + WY-14643 groups. **P<0.05.

gtggcgttgat-3' (forward) and 5'-gcggtcgcatttgtc-3' (reverse). The primer sets used to amplify *GAPDH* were 5'-ccactttgtgaagctcatttcct-3' (forward) and 5'-tcgtcctcctctggtgctct-3' (reverse). PCR amplification was performed for 32 cycles using a *Taq* polymerase for the reverse transcription kit, with the following program: 95˚C for 45 sec, 62˚C for 30 sec, and 72˚C for 1 min. The $2^{-\Delta\Delta Ct}$ method was used to quantify the expression of the *PPAR-α* and *GAPDH* genes. The resulting values were used to express the relative quantity (RQ) of *PPAR-α* with regard to that of *GAPDH*.

Enzyme-linked immunosorbent assay (ELISA) for TNF-α, IL-10 and P-selectin. Fresh blood (3 ml) was extracted from all animals via the femoral vein and centrifuged at 3,000 x g for 10 min at 4˚C. The supernatant was transferred in a clean centrifuge tube, and frozen at -20˚C. Concentrations of plasma TNF-α, IL-10 and P-selectin were assayed with ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis. Data were analyzed using the SPSS 11.5 software for Windows (IBM, Armonk, NY, USA). Quantitative data were expressed as mean ± standard deviation. Comparisons between multiple groups were conducted with analysis of variance, and pairwise comparisons with the Student-Newman-Keuls test. P<0.05 was considered to indicate statistically significant differences.

Results

Histological changes. H&E-stained sections of the aorta were examined for signs of atheroma. High-fat diet and balloon injury induced a notable atherosclerotic lesion, whereas animals receiving standard diet had no visible atheroma. In the high-fat diet + balloon injury group, the lesion site showed foam cell formation, accumulation of smooth muscle cells in the intima, and fragmentation of the internal elastic lamina of the femoral artery. In the high-fat diet + balloon injury + placebo group, the lesion site showed smooth muscle cell migration, foam cell formation, and a lipid core in the intima. In the high-fat diet + balloon injury + fenofibrate/WY-14643 groups, the lesion also showed smooth muscle cell migration, foam cell formation, and a lipid core in the intima. The percentage of vessel lumen occlusion in the fenofibrate and WY-14643 groups was significantly decreased compared to the placebo group (Fig. 1).

The percentage of vessel wall lumen occlusion was calculated (Fig. 2). No obviously occluded lumen was observed in the control group, while the high-fat diet + balloon injury and high-fat diet + balloon injury + placebo groups showed lumen occlusion. Both fenofibrate and WY-14643 reduced the percentage of vessel wall lumen occlusion compared to the placebo group. There was no difference in the percentage of vessel wall lumen occlusion between the high-fat diet + balloon injury + fenofibrate and high-fat diet + balloon injury + WY-14643 groups.

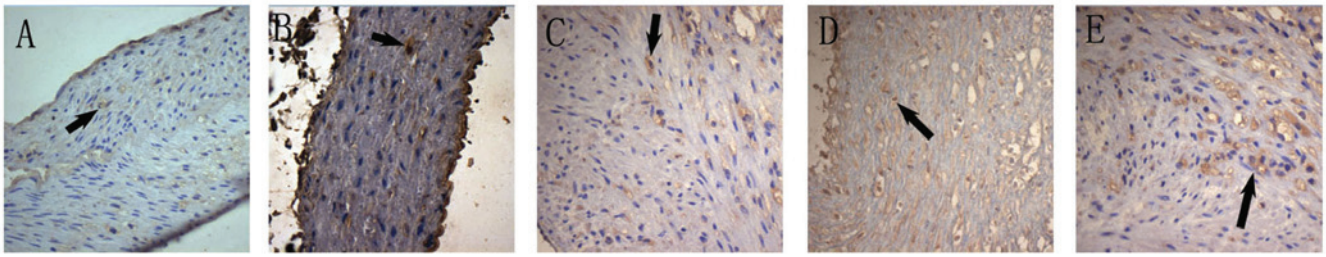

Figure 3. Immunohistochemical detection of peroxisome proliferator-activated receptor-α (PPAR-α; arrows) in vascular tissue of the (A) high-fat diet + balloon injury; (B) control; (C) high-fat diet + balloon injury + placebo; (D) high-fat diet + balloon injury + fenofibrate; and (E) high-fat diet + balloon injury + WY-14643 groups.

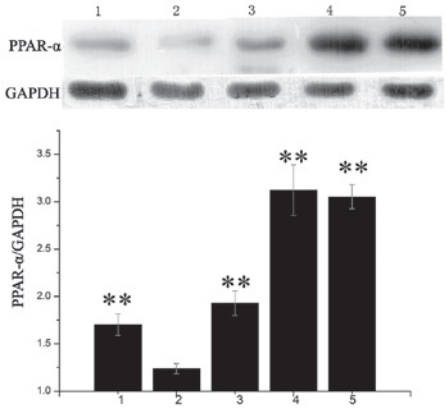

Figure 4. Protein expression of peroxisome proliferator-activated receptor-α (PPAR-α) relative to that of GAPDH in vascular tissue of rabbits from the (1) high-fat diet + balloon injury; (2) control; (3), high-fat diet + balloon injury + placebo; (4) high-fat diet + balloon injury + fenofibrate; and (5) high-fat diet + balloon injury + WY-14643 groups.**P<0.05.

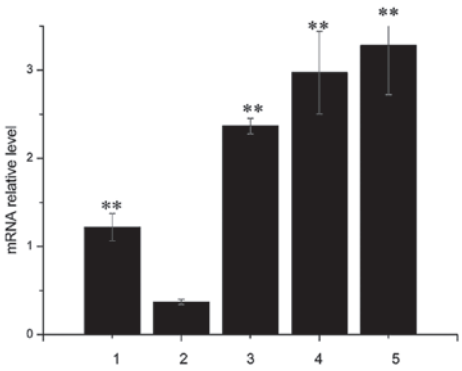

Figure 5. mRNA levels of the peroxisome proliferator-activated receptor-α (*PPAR-α*) gene relative to that of *GAPDH* in vascular tissue of rabbits from the (1) high-fat diet + balloon injury; (2) control; (3) high-fat diet + balloon injury + placebo; (4) high-fat diet + balloon injury + fenofibrate; and (5) high-fat diet + balloon injury + WY-14643 groups.**P<0.05.

Localization of PPAR-α protein. Immunohistochemical examination showed that the PPAR-α protein was rarely detected in the cytoplasm of the control group (Fig. 3). By contrast, PPAR-α was found at high staining intensities in the cytoplasm of macrophages, especially near the intima, in the high-fat diet + balloon injury, high-fat diet + balloon injury + placebo, high-fat diet + balloon injury + fenofibrate and high-fat diet + balloon-injury + WY-14643 groups (Fig. 3). Compared to the placebo group, the staining intensity of PPAR-α was higher in the fenofibrate and WY-14643 groups.

Expression of PPAR-α protein. Western blot analysis was performed to determine the protein level of the PPAR-α protein in femoral artery tissues. Western blot bands were quantified by densitometry. Compared to standard treatment, high-fat diet and balloon injury increased the PPAR-α level (Fig. 4). Pretreatment with fenofibrate and WY-14643 significantly increased the expression of the protein.

Expression of the PPAR-α gene. The mRNA level of *PPAR-α* was analyzed by quantitative PCR. Compared to the control group, high-fat diet + balloon injury increased *PPAR-α* expression (Fig. 5), and pretreatment with fenofibrate or WY-14643 significantly increased *PPAR-α* expression.

IL-10, TNF-α and P-selectin levels in the serum. The serum concentrations of IL-10, TNF-α and P-selectin were measured in order to assess the degree of inflammatory response. Compared to the control group, IL-10, TNF-α and P-selectin levels were all significantly increased in the high-fat diet + balloon injury group (Fig. 6). Fenofibriate or WY-14643 treatment markedly reduced the TNF-α and P-selectin level, while it did not change the IL-10 level induced by high-fat diet and balloon injury.

Discussion

Atherosclerosis is a major pathological process related to a number of important adverse vascular events, including coronary artery disease, stroke, and peripheral arterial disease (19). However, the exact mechanisms underlying this condition remain elusive. Inflammation is an important step in the progression of atherosclerosis. The prevention of inflammation is expected to delay the progression of atherosclerosis. PPAR-α is considered an inhibitor of inflammation during atherosclerosis, although the relevant mechanism is still unclear. In this study, we investigated the role of PPAR-α in atherosclerosis and in the induction of the expression of inflammation factors. We investigated the protective effects of PPAR-α activation by fenofibrate and WY-14643 in a high-fat diet and balloon injury-induced atherosclerotic model. High-fat diet and balloon injury induce the formation of vascular lesions, where inflammatory cytokines are activated. The harmful effects can be inhibited by fenofibrate or WY-14643 through activation of PPAR-α.

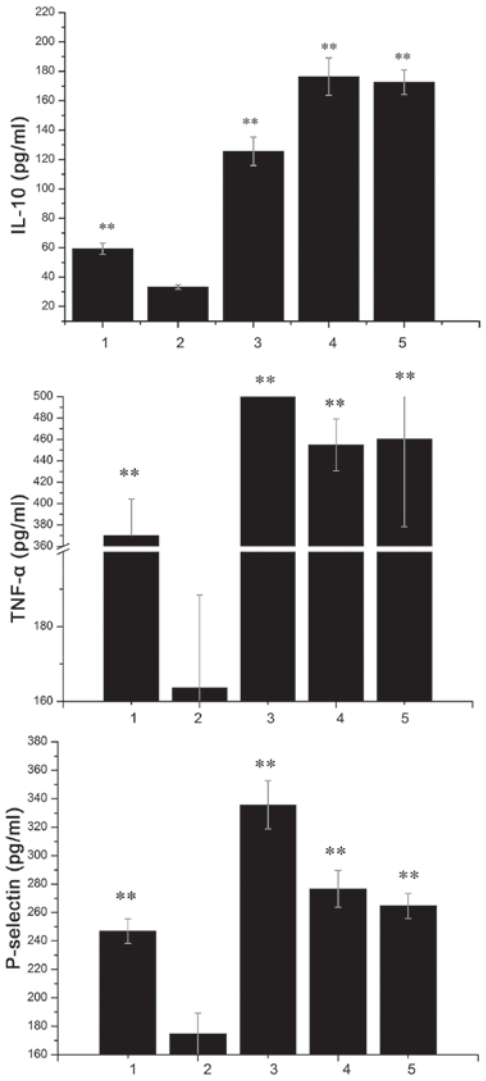

Figure 6. The levels of interleukin 10 (IL-10), tumor necrosis factor-α (TNF-α) and P-selectin in the serum of rabbits from the (1) high-fat diet + balloon injury; (2) control; (3) high-fat diet + balloon injury + placebo; (4) high-fat diet + balloon injury + fenofibrate; and (5) high-fat diet + balloon injury + WY-14643 groups. **P<0.05.

PPAR-α, as a member of a nuclear receptor family, plays an important role in regulating fatty acid oxidation, lipid and lipoprotein metabolism, and inflammatory and vascular responses, all of which are involved in atherosclerosis (5,20). The expression of inflammatory factors regulated by PPAR-α correlates to prostate cancer and benign prostatic hyperplasia (21). Expression of PPAR-α was also increased in our atherosclerotic model, in both the high-fat diet and the placebo groups compared to the control. Furthermore, PPAR-α levels in the high-fat diet group were lower than those in the control group. It was previously shown that overexpression of VEGF and TGF-β1 correlates with the progression of cervical intraepithelial neoplasia to cancer (22,23). Therefore, in the high-fat diet and balloon injury model, PPAR-α may be activated by lipoprotein lipolytic products, without mediation of exogenous agonists. These data indicate that PPAR-α associates with the progression of atherosclerosis, which could constitute a self-regulatory mechanism to prevent atherosclerotic lesion formation. Fenofibrate and WY-14643 are effective agonists of PPAR-α (24). Our results showed that fenofibrate and WY-14643 can enhance the anti-inflammatory response in the atherosclerotic model induced by high-fat diet and balloon injury. Notably, fenofibrate and WY-14643 minimized the vascular lesions induced by high-fat diet and balloon injury. The percentage of vessel wall lumen occlusion in the fenofibrate and the WY-14643 groups was considerably lower compared to that of the placebo group, and fenofibrate and WY-14643 induced an increase in the expression of PPAR-α. A previous study demonstrated that PPAR-α can inhibit vascular wall inflammation (6). Thus, PPAR-α agonists may modulate atherosclerosis by inhibiting the inflammatory response.

The major role played by the inflammatory response in the progression of atherosclerosis is an emerging concept (25). Activation of PPAR-α favorably modulates inflammation markers. IL-10 is an anti-inflammatory cytokine produced by a variety of immune cells with anti-atherogenic potential (26). By contrast, TNF-α and P-selectin are pro-inflammatory cytokines, involved in atherosclerosis and other metabolic and inflammatory conditions such as obesity and insulin resistance, which are additional risk factors for the development of cardiovascular diseases (27,28). Previous studies indicated that PPAR-α significantly reduces the level of pro-inflammatory cytokines such as TNF-α and P-selectin in ischemia-reperfusion injury (28,29), and enhances the expression of IL-10 in acute pancreatitis (7). As important inflammatory cytokines, IL-10, TNF-α and P-selectin are also involved in the development of atherosclerosis (30,31). Based on these observations, we aimed to determine whether the presence and/or the stimulation of PPAR-α can enhance the anti-inflammatory response in atherosclerosis, by measuring the serum levels of IL-10, TNF-α and P-selectin. Fenofibrate and WY-14643 induced the anti-inflammatory response in our experimental model of atherosclerosis. TNF-α and P-selectin levels were increased in the placebo group and were reduced by treatment with fenofibrate or WY-14643, together with reduced vascular lesions. The IL-10 level was higher in the fenofibrate and WY-14643 groups compared to the placebo group. These results are in agreement with previous studies indicating that increased expression of the *IL-10* gene might be relevant to a series of coronary and cerebrovascular events, and that low capacity to produce IL-10 entails an increased risk for stroke incidents (32,33). It was also shown that the increased concentration of IL-10 is associated with an increased risk for cardiovascular events (34). Taken together, these results highlight the complexity of roles played by pro- and anti-inflammatory molecules during the process of atherosclerosis. In parallel with elevated levels of PPAR-α in the high-fat diet and placebo groups, the increased expression of IL-10 may control the inflammatory response as a self-protective mechanism.

In conclusion, activated PPAR-α can prevent vascular lesions induced by high-fat diet and balloon injury in rabbits. Our findings suggest that the anti-atherosclerotic and anti-inflammatory effects of PPAR-α are associated with the increased expression of IL-10 and the reduced expression of TNF-α and P-selectin. Notably, IL-10, TNF-α and P-selectin, as well as PPAR-α expression were increased in the high-fat diet and the placebo groups compared with the control group. These results suggest that a mild activation of PPAR-α signaling may occur in atherosclerosis without the presence

of exogenous PPAR-α agonists, but it may not be sufficient to prevent the progression of atherosclerosis. To fully address the role(s) of PPAR-α in atherosclerosis, experiments using a PPAR-α inhibitor need to be carried out in the future.

Acknowledgements

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