PPARγ Gene Transfer Sustains Apoptosis, Inhibits Vascular Smooth Muscle Cell Proliferation, and Reduces Neointima Formation After Balloon Injury in Rats

Soo Lim, Cheng Ji Jin, Min Kim, Sung Soo Chung, Ho Seon Park, In Kyu Lee, Choon Taek Lee, Young Min Cho, Hong Kyu Lee, Kyong Soo Park

Objective—There is still debate as to whether antiatherosclerotic effect of PPARγ ligands is dependant on PPARγ gene itself or some other pathway.

Methods and Results—To investigate the effect of PPARγ gene modulation on neointima formation after balloon injury, we delivered adenoviral vectors expressing the wild-type (WT) dominant negative (DN) PPARγ, or a control gene (β-galactosidase [BG]) into carotid artery after balloon injury in rosiglitazone (a PPARγ ligand)-treated (R+) (3 mg/kg/d) and nontreated (R−) rats. Two weeks after gene delivery, in both R+ and R− animals, the PPARγ-WT gene transfer showed a significantly lower intima-media ratio (IMR) than control group. Moreover, the delivery of a PPARγ-DN form showed the highest IMR (in R+WT, 0.51±0.15; R+BG, 0.89±0.14; R+DN, 1.20±0.18, $P<0.05$ and in R−WT, 0.91±0.21; R−BG, 1.44±0.23; R−DN, 1.74±0.29, $P<0.05$). Proliferation and migration showed same result pattern as IMR. In addition, apoptotic indices were significantly higher in the PPARγ-WT gene transferred group than in the PPARγ-DN group.

Conclusions—In vivo transfer of the PPARγ-WT gene was found to inhibit smooth muscle proliferation, sustain apoptosis, and reduce neointima formation after balloon injury irrespective of rosiglitazone treatment. These results indicate that PPARγ overexpression itself has a protective role against restenosis after balloon injury. (***Arterioscler Thromb Vasc Biol*. 2006;26:808-813.)**

Key Words: PPARγ ■ vascular smooth muscle ■ neointima ■ proliferation ■ apoptosis

Peroxisome proliferator activated receptor gamma (PPARγ) is a crucial factor in many cellular signaling pathways and is known to regulate several transcription factors. Researches have provided many insights into the pleiotropic role that PPARγ plays in cell proliferation, migration, and differentiation and adipocyte differentiation. In fact, PPARγ activation inhibits vascular smooth muscle cell (VSMC) proliferation,[1,2] for which several mechanisms have been suggested; blocking the reentry of quiescent VSMCs into the cell cycle,[3] inhibiting VSMC migration by controlling the mitogen-activated protein kinase (MAPK) pathway and the production of matrix metalloproteinase (MMP),[4,5] and reducing inflammation by attenuating cytokine production and nuclear factor-κB transcription activity.[6,7]

In the field of endocrinology and metabolism, PPARγ has been identified as an insulin sensitizer and as an important regulator of glucose metabolism. In fact, the diabetic milieu is associated with endothelial dysfunction and several therapeutic interventions have been tested in this context.[8] Moreover, PPARγ is a good target for the treatment of restenosis because its expression is found in all cells composing blood vessels (ie, monocytes, macrophages, and endothelial and VSMCs).

It has recently been reported that the in vitro rosiglitazone treatment (a PPARγ ligand) results in a significant reduction in restenosis after coronary stent insertion.[9] Because PPARγ is located at a nodal point where multiple cell signals merge to control the proliferation and migration of VSMCs, it may represent a pharmacological target to treat restenosis after balloon injury.

Although PPARγ ligands have been found to have protective effect on atherosclerosis in vivo and in vitro,[10,11] there is some debate as to whether this protective effect is dependant on the PPARγ gene itself or some other pathway. As the expression of PPARγ increases when vascular damage occurs, it appears that it has an important role in protection against restenosis. However, the effects of modulating

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From the Seoul National University College of Medicine, Department of Internal Medicine (S.L., C.J.J., M.K., S.S.C., H.S.P., C.T.L., Y.M.C., H.K.L., K.S.P); and Kyungpook National University School of Medicine (I.K.L), Korea.

S.L. and C.J.J. contributed equally to this work.

Correspondence to Kyong Soo Park, MD, PhD, Seoul National University College of Medicine, Department of Internal Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul, Korea 110-744. E-mail kspark@snu.ac.kr

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PPARγ by gene transfer in animal models of angioplasty have not been studied previously. In the present study, we used an adenoviral vector containing wild-type (WT), or the dominant negative (DN) form of PPARγ, or β-galactosidase (BG) (as a control) to perform in vivo gene transfer into rat carotid artery segments injured by balloon catheter. The purpose of the study was to examine the role of the PPARγ gene in the protective effect conferred by rosiglitazone on vascular smooth muscle proliferation and survival during the process of neointimal formation.

Methods

Animals

Sixty adult male Sprague–Dawley rats weighing 300 to 400 g (Daehan Biolink Co, Seoul, Korea) were used for these experiments. Animals were handled in compliance with the Guide for Experimental Animal Research from the Laboratory for Experimental Animal Research, Clinical Research Institute, Seoul National University Hospital.

Rats (n=10 per group) were fed with regular food pellets containing rosiglitazone (3 mg/kg per day; GlaxoSmithKline) for 1 week before and 2 weeks after carotid injury. Rats were grouped into 2 groups with or without rosiglitazone treatment (R+ and R−). These 2 groups were further subdivided into 6 subgroups according to the type of transferred gene, ie, PPARγ WT, a DN form of PPARγ, or BG as a control (ie, PPARγ-WT into R+WT and R−WT, PPARγ-DN into R+DN and R−DN, β-galactosidase into R+BG and R−BG). PPARγ-WT and -DN form are both well documented their property in previous report.[11,12]

Rat Carotid Artery Balloon Denudation Injury and Adenoviral Vector–Mediated Gene Delivery

A previously well-established rat carotid artery balloon injury model was used in this study.[13] Adenoviral vectors expressing the PPARγ-WT, DN, and β-galactosidase were used for gene delivery. Rats were anesthetized with a combination anesthetic (ketamine, 70 mg/kg; xylazine, 7 mg/kg IP; Yuhan Corp, Bayer Korea). After the left external carotid artery had been exposed, heparin (35IU) was administered systemically via the external jugular vein. A 2F Fogarty embolectomy catheter (Baxter Healthcare Corp) was introduced into an external carotid arteriotomy incision, advanced to the common carotid artery, and inflated with 0.2 mL of saline and withdrawn 10 times with rotation. After clamping both the proximal common carotid artery and the proximal internal carotid artery, viral infusion mixtures with 5×10^8 pfu of virus containing either PPARγ-WT, PPARγ-DN, or control gene diluted to a total volume of 100 μL were instilled via the arterial segment between the two clamps. Perfusion was restored in the common carotid artery after 20 minutes of instillation.

Morphometric Analysis

Three days and 2 weeks after balloon injury, rats were euthanized with a lethal dose of pentobarbital, and carotid arteries were fixed by perfusion at 120 mm Hg with 4% formaldehyde via an 18G intravenous cannula placed retrograde in the abdominal aorta. Tissues were then embedded in paraffin, and sections were stained with H&E. The extent of neointimal formation in histologically stained sections was quantified by computed planimetry. The cross-sectional areas of the blood vessel layers, ie, the lumen, intimal, and medial areas, were quantified in 3 different sections (proximal, middle, and distal) using an Image Pro Plus Analyzer Version 4.5 (Media Cybernetics). The intima to media ratio (IMR) was calculated from the mean of these determinations.

Cell Culture System

Rat aortic smooth muscle cells, which were prepared from thoracic aortas of 2- to 3-month-old Sprague-Dawley rats using the explant technique, were used to investigate the effect of rosiglitazone or PPARγ gene transfer on proliferation, migration, and c-fos mRNA expression.

Thymidine Incorporation Assay for Proliferation

[^{3}H]-thymidine uptake was used to determine cell proliferation. Cells were seeded in triplicate in 24-well plates at a density of 5×10^4 cells per well and transfected with PPARγ-WT, DN, or β-galactosidase (at a multiplicity of infection of 10 in each cell) of 24 hours. Thereafter, cells were incubated with or without rosiglitazone 10 μmol/L for 24 hours. For tumor necrosis factor (TNF)-α stimulation, cells were incubated with 10 ng/mL of TNFα (R&D Systems) for another 24 hours. Subsequently, [^{3}H]-thymidine was added for a further 4 hours at 1 μCi per well. The incubation was terminated by removing the medium and adding 1 mL of 5% trichloroacetic acid (TCA) for 20 minutes. The fixed cells were then washed twice with 100% ethanol and treated with 0.3 NaOH/ 2% Na_2CO_3. The protein concentrations of cell lysates were measured using a Micro BCA Protein Assay kit (Pierce), and lysates were counted (LS6500 Multipurpose Scintillation Counter; Beckman) to determine tritium content. Thymidine index was defined as [total counts (cpm)]/[total protein present (μg)].

Migration

VSMCs (2×10^5) were seeded in triplicate in 6-well plates. Cells were then transfected with PPARγ-WT, PPARγ-DN, or β-galactosidase (at 10 multiplicity of infection) for 24 hours. Thereafter, cells were incubated with or without rosiglitazone at 10 μmol/L for 24 hours. For TNFα stimulation, cells were incubated with 10 ng/mL of TNFα (R&D systems) for another 24 hours. After removing exactly half of the cells in each well, the cells were incubated for another 24 hours. Cell migration distances were measured in 3 different sectors per well.

Northern Blot Analysis for c-fos mRNA Expression

For all experiments, primary cultured rat VSMCs were grown to 60% to 70% confluence. PPARγ or control genes were delivered for 48 hours, and cells were made quiescent by serum starvation (0.4% calf serum) for at least 12 hours. Then, rosiglitazone (10 μmol/L) was added for 24 hours. Finally basic fibroblast growth factor (bFGF; 20 ng/μL) was added 30 minutes before the harvest. RNA was harvested using Trizol and total RNA (20 μg) was electrophoresed through 1% agarose gels containing formaldehyde, transferred to charged nylon membranes (MSI), and cross-linked in a Stratlinker (Stratagene). A cDNA probe for c-fos, ^{32}P-labeled using a random primer method was used. GAPDH signal intensities were used as internal control to ensure equal loading.

Statistical Analysis

Results are reported as means±SEM. Mean values were compared for the active PPARγ gene transferred group and the control group by ANOVA with the post hoc test, and $P<0.05$ was considered statistically significant.

Immunohistochemical staining, immunofluorescence double staining, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining, Western blot analysis, and measurement for plasma adiponectin and CRP are described in the Methods section in the online data supplement (available at http://atvb.ahajournals.org).

Results

PPARγ Expression After Balloon Injury and Gene Expression In Vivo

Sustained PPARγ-positive cells were observed 2 weeks after PPARγ delivery by immunohistochemistry using anti-PPARγ antibody (Figure IA, available online at http://atvb.ahajournals.org). Fewer PPARγ-positive cells were

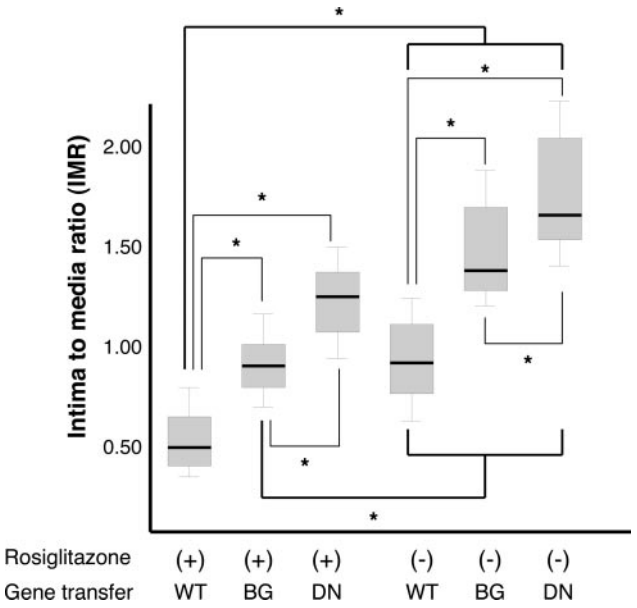

Figure 1. In vivo inhibition of neointimal formation. Intima to media ratios (IMRs) in the 6 subgroups, n=10 in each subgroup. In the R+ group, the WT gene transferred subgroup had the lowest IMR and the DN subgroup had the highest ($P<0.05$), and the same pattern was found in the R− group ($P<0.05$).

present in the R− group. Double-staining with the anti-GFP antibody (green) and anti-PPARγ antibody (red) showed that PPARγ-positive cells colocalized with the cells expressing adenovirus PPARγ gene (Figure IB). Immunoblot analysis performed after balloon injury showed a marked increase in the expression of the PPARγ gene in the PPARγ WT or DN transferred groups compared with the β-galactosidase group (Figure IC). In addition, rosiglitazone treatment was associated with an additional increase in PPARγ gene expression.

In Vivo Inhibition of Neointimal Formation

No differences were observed between WT (ie, R+WT and R−WT), DN (R+DN and R−DN) and BG (R+BG and R−BG) subgroups in terms of the extent of VSMC proliferation at 3 days after injury (data not shown); little or no intima was detected. However, 2 weeks after gene delivery, the R+ group showed a significant reduction in neointimal formation versus the R− group. The WT subgroup showed less neointimal formation than the BG control but the DN subgroup showed more neointimal formation (Figure IIA, available online at http://atvb.ahajournals.org). Immunofluorescence staining was done to localize GFP (exogenous PPARγ gene expression) by the use of anti-GFP antibody (Santa Cruz) and fluorescence-conjugated antibody (red) (Figure IIB). As shown in Figure 1, in the R+ group, the WT gene transferred subgroup had the lowest intima to media ratio (IMR) and the DN subgroup had the highest (in R+WT, 0.51±0.15; R+BG, 0.83±0.12; R+DN, 1.07±0.19; $P<0.05$), and the same pattern was found in the R− group (in R−WT, 0.91±0.21; R−BG, 1.27±0.12; R−DN, 1.52±0.20; $P<0.05$).

Inhibition of Vascular Smooth Muscle Proliferation In Vivo

As shown in Figure 2 (please see also Figure III, available online at http://atvb.ahajournals.org), the smooth muscle proliferative index (defined as the percentage of PCNA-positive cells versus total nucleated cells) was significantly lower in the R+ group than in the R− group (19.7±1.3% versus 32.9±1.2%, $P<0.001$). In both the R− and R+ groups, cell proliferation was markedly reduced in the WT subgroups versus the controls, and the DN subgroups showed the highest level of proliferation 2 weeks after gene delivery (P for trends <0.01 in R+ and 0.059 in R− group).

Sustained Apoptosis After PPARγ-WT Gene Transfer

TUNEL staining[14] was performed to examine the effects of WT gene transfer on apoptosis after balloon injury in vivo (please see Figure IV, available online at http://atvb.ahajournals.org). At 3 days after balloon injury/gene delivery, apoptotic levels were not significantly different among subgroups irrespective of rosiglitazone treatment or type of gene transfer (data not shown). However, at 2 weeks, the apoptosis index (calculated as the percentage of TUNEL-positive cells versus total nucleated cells) dropped substantially in the R− group versus the R+ group (17.2±2.5% versus 11.6±1.0%, $P<0.01$). In both R− and R+ groups, apoptosis was significantly higher in the WT subgroups that in their BG control groups, and the DN subgroups showed the lowest levels of apoptosis (P for trends <0.01 in R+ and 0.033 in R− group) (Figure 3)

Effect of PPARγ Gene Transfer or Rosiglitazone on VSMC Proliferation

Thymidine incorporation assays were performed to examine the effects of PPARγ-WT gene transfer on proliferation in a cultured cell system. As shown in Figure 4A, rosiglitazone treatment inhibited TNFα-induced DNA synthesis in rat

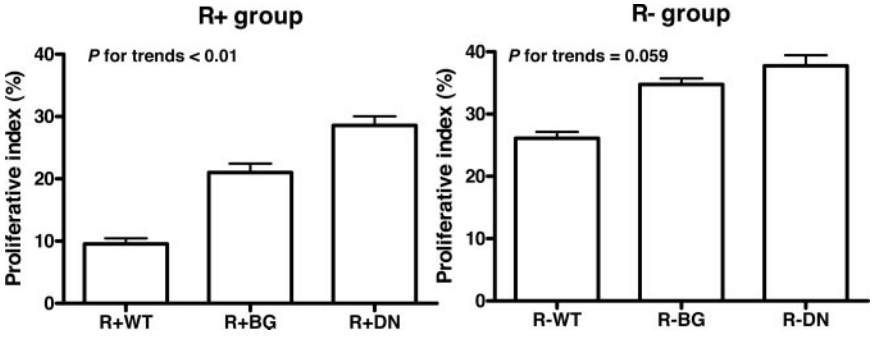

Figure 2. Effects on proliferation of in vivo PPARγ gene transfer (WT or DN) or β-galactosidase (BG) transfer as a control. The smooth muscle proliferative index was significantly lower in the R+ group than in the R− group at 2 weeks after balloon injury and gene delivery. In both the R− and R+ groups, cell proliferation was markedly reduced in the WT subgroups vs the controls, and the DN subgroups showed the highest level of proliferation 2 weeks after gene delivery.

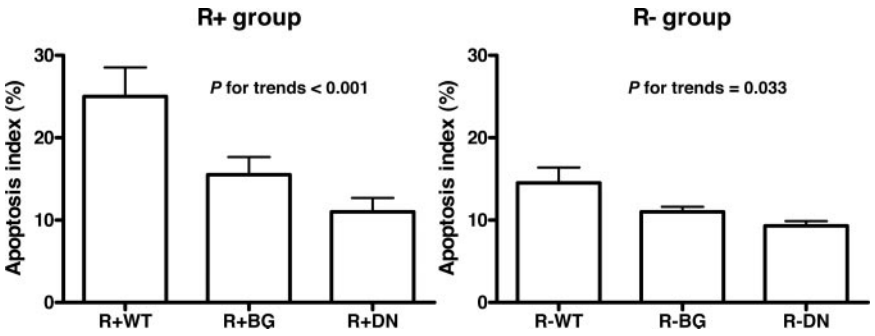

Figure 3. Apoptotic effects of in vivo PPARγ gene transfer (WT or DN) or β-galactosidase (BG) transfer as a control. Apoptosis index (%) at 2 weeks after balloon injury and gene delivery, which dropped substantially in the R− group vs the R+ group. In both R− and R+ groups, apoptosis was significantly higher in the WT subgroups that in their BG control groups, and the DN subgroups showed the lowest levels of apoptosis

VSMCs. Rosiglitazone also inhibited PDGF-stimulated VSMC proliferation (data not shown). PPARγ-WT gene transfer also reduced DNA synthesis in the R+ and R− groups. And, although PPARγ-DN transfer had no significant effect in the R− group, it almost blocked the inhibitory effect of rosiglitazone on DNA synthesis in the R+group.

Effect of PPARγ Gene Transfer or Rosiglitazone on the TNFα-Directed Migration of VSMCs

Rosiglitazone treatment inhibited TNFα-directed migration in rat VSMCs. In both the R+ and R− groups, PPARγ-WT gene transfer inhibited TNFα-directed migration (Figure 4B). In the PPARγ-DN gene transfer groups, the effect of rosiglitazone on migration was almost blocked, but no significant effect was observed in R− group.

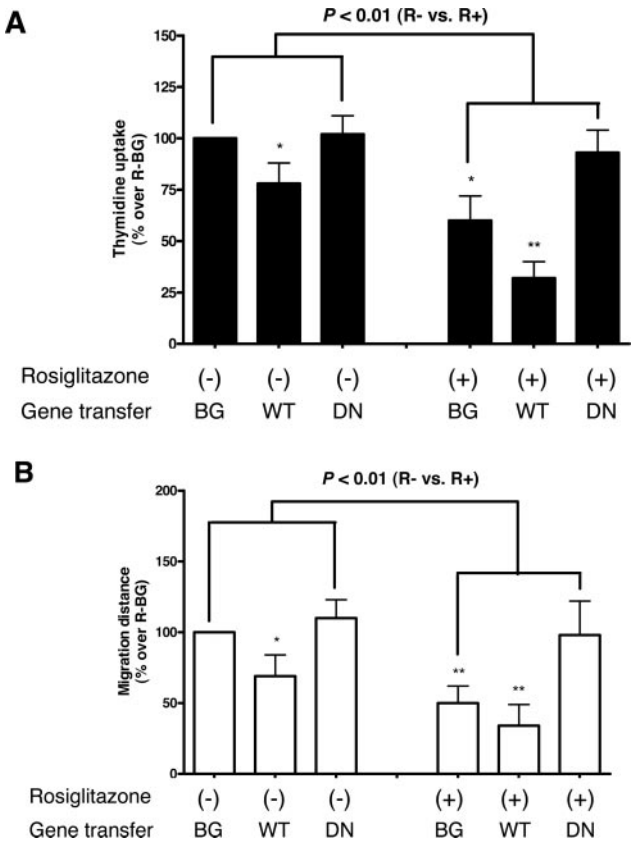

Figure 4. Effect of rosiglitazone and the PPARγ gene (WT or DN) transfer on TNFα-stimulated DNA synthesis (A) and migration (B) in rat VSMCs. Values are expressed as means±SEM (*$P<0.05$, ** $P<0.01$ compared with R−BG as a control). Rosiglitazone treatment inhibited TNFα-induced DNA synthesis and migration. PPARγ-WT gene transfer also reduced DNA synthesis in the R+ and R− groups. PPARγ-DN transfer, which had no significant effect in the R− group, almost blocked the inhibitory effect of rosiglitazone on DNA synthesis in the R+ group.

Effect of PPARγ Gene Transfer or Rosiglitazone on c-fos mRNA Expression

Rosiglitazone or PPARγ-WT gene transfer inhibited bFGF-induced c-fos mRNA expression, but PPARγ-DN transfer blocked the inhibitory effect of rosiglitazone on bFGF-induced c-fos mRNA expression (Figure 5).

Effect of PPARγ Gene Transfer or Rosiglitazone on Plasma Adiponectin and CRP Concentrations

Rosiglitazone treatment increased plasma adiponectin concentration and decreased CRP concentration. However, there was no difference in adiponectin or CRP level within subgroup divided by the type of gene transfer (BG, WT, or DN) (please see Figure V, available online at http://atvb.ahajournals.org).

Discussion

In this study, the PPARγ-WT gene transferred group showed significantly less neointimal formation (43%) than the control group did. This effect was observed regardless of rosiglita-

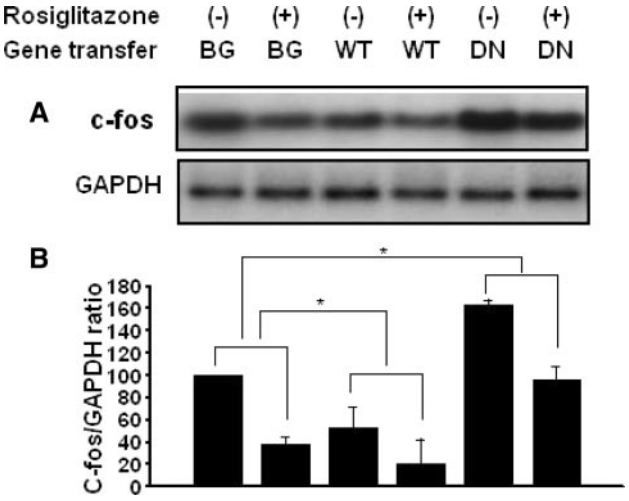

Figure 5. Effect of rosiglitazone and PPARγ gene transfer on c-fos mRNA induction by bFGF. A, Northern blot for c-fos mRNA, B, Quantitation of c-fos mRNA expression (*$P<0.05$). Rosiglitazone or PPARγ-WT gene transfer inhibited c-fos mRNA expression. However, the inhibitory effect of rosiglitazone on c-fos mRNA expression was almost blocked by PPARγ-DN transfer.

zone treatment. Interestingly, the transfer of the DN form of the PPARγ gene blocked the effect of rosiglitazone on neointimal formation, apoptosis, and VSMC proliferation and migration. Our study extends recent in vitro findings that PPARγ plays an important role in VSMC proliferation and apoptosis during the vascular remodeling that occurs after balloon injury.[1–4,10]

VSMC proliferation and migration are important contributors to neointima formation after balloon injury.[15,16] Therefore, prior efforts to reduce the extent of restenosis have focused on means of reducing the proliferation and migration of VSMCs or of increasing their apoptosis.[17] Of these trials, local gene delivery after balloon injury to rat carotid arteries is a well-established model for investigations of the mechanism of restenosis[13,18] and for testing the therapeutic implications of various genes.[19,20] Various genes have been examined with respect to their abilities to prevent restenosis,[21–23] and of these, PPARγ is a good candidate. PPARγ is highly expressed in human and murine atherosclerotic lesions,[5,24] and in the neointimal smooth muscle cells of rat arteries after balloon angioplasty.[25–27] Moreover, our previous work demonstrated that PPARγ plays a significant role in human endothelial cell proliferation.[11]

Investigations into the cellular role of PPARγ have shown that PPARγ is an important modulator of cell survival and apoptosis.[3] Accumulating data suggests that PPARγ is an important determinant of vascular structure and function.[28] PPARγ is expressed in endothelial cells,[29] VSMCs,[1,30] monocytes/macrophages,[31] and T lymphocytes.[32] Moreover, recent studies have documented that PPARγ ligands have proapoptotic,[32–35] antiproliferative,[36,37] and antiinflammatory[30] effects in VSMCs.

Thus, treatment with PPARγ ligand inhibits VSMC proliferation and promotes sustained apoptosis. However, there is some debate as to whether this effect is mediated by the PPARγ gene.[38–40] Even in the PPARγ dependent pathway, two different mechanisms could operate. First, PPARγ activation could induce gene expression via binding to peroxisome proliferator response element (PPRE) in the target gene promoter, the so-called classical genomic effect. Second, PPARγ activation could suppress or induce other gene expressions by antagonizing or stimulating transcriptional factors. Moreover, the existence of the genomic and nongenomic effects of PPARγ had been demonstrated in several studies.[5,41]

PPARγ has also been shown in vitro to function at the nodal point of various cell-signaling pathways.[40,42] In this study, we found that rosiglitazone (a PPARγ ligand) and PPARγ overexpression reduced bFGF-induced c-fos gene expression. In contrast, the transfer of PPARγ-DN blocked the inhibitory effect of rosiglitazone on c-fos mRNA expression. C-fos is involved in one of the final steps of the MAPK pathway, which is directly linked to cell proliferation. Moreover, the downregulation of c-fos is associated with protection against atherosclerosis.[43] Our findings showed that both PPARγ gene expression and activation by PPARγ ligand are important in regulating c-fos expression. However, exact mechanism of decreased c-fos expression by PPARγ WT gene transfer in currently unclear. Considering the results of the other studies showing PPARγ ligand reduced MAPK-dependent Elk-1 activity at the c-fos promoter[42] and our observation that effect of PPARγ ligand on c-fos expression is depend on PPARγ gene expression, the decreased expression of c-fos mRNA by PPARγ overexpression might be also resulted from a blockade of MAPK pathway.

Restenosis and atherosclerosis are also affected by circulating factors such as conventional risk factors (cholesterol, triglyceride, glucose, insulin, etc), molecules involved in inflammation (CRP, interleukin-1β, interleukin-6, MCP-1, etc) and adipokines (adiponectin, TNFα, resistin, FFA, etc), etc. In this study, rats treated with rosiglitazone had lower CRP and higher adiponectin level than those without R. This result suggests possibility that antirestenotic effect of PPARγ ligand is mediated by circulating factors. However, there was no difference in CRP or adiponectin level within subgroups divided by the type of gene transfer (BG, WT, or DN) in either group treated with or without rosiglitazone. Thus, we believe that the contribution of the gene transfer to restenosis was not systemic and confined to directly gene-transferred segment of carotid artery

In summary, this study demonstrates for the first time in vivo that PPARγ-WT gene transfer results in a significant reduction of neointima formation after balloon injury in rat carotid arteries by inhibiting the proliferation, migration, and sustained apoptosis of VSMCs. In contrast, transfer of a PPARγ-DN gene attenuated the effect of rosiglitazone. These effects are primarily, but not entirely, dependent on the PPARγ gene, which showed an ability to inhibit VSMC proliferation and to promote their sustained apoptosis. These results indicate that PPARγ overexpression is important in the protective effect of PPARγ ligand on the prevention of restenosis or atherosclerosis.

Acknowledgments

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