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Objective—Critical limb ischemia is a severe form of peripheral artery disease (PAD) for which neither surgical revascularization nor endovascular therapy nor current medicinal therapy has sufficient therapeutic effects. Peroxisome proliferator activated receptor-γ agonists present angiogenic activity in vitro; however, systemic administration of peroxisome proliferator–activated receptor-γ agonists is hampered by its side effects, including heart failure. Here, we demonstrate that the nanoparticle (NP)-mediated delivery of the peroxisome proliferator activated receptor-γ agonist pioglitazone enhances its therapeutic efficacy on ischemia-induced neovascularization in a murine model.

Methods and Results—In a nondiabetic murine model of hindlimb ischemia, a single intramuscular injection of pioglitazone-incorporated NP (1 μg/kg) into ischemic muscles significantly improved the blood flow recovery in the ischemic limbs, significantly increasing the number of CD31-positive capillaries and α-smooth muscle actin–positive arterioles. The therapeutic effects of pioglitazone-incorporated NP were diminished by the peroxisome proliferator activated receptor-γ antagonist GW9662 and were not observed in endothelial NO synthase–deficient mice. Pioglitazone-incorporated NP induced endothelial NO synthase phosphorylation, as demonstrated by Western blot analysis, as well as expression of multiple angiogenic growth factors in vivo, including vascular endothelial growth factor-A, vascular endothelial growth factor-B, and fibroblast growth factor-1, as demonstrated by real-time polymerase chain reaction. Intramuscular injection of pioglitazone (1 μg/kg) was ineffective, and oral administration necessitated a >500 μg/kg per day dose to produce therapeutic effects equivalent to those of pioglitazone-incorporated NP.

Conclusion—NP-mediated drug delivery is a novel modality that may enhance the effectiveness of therapeutic neovascularization, surpassing the effectiveness of current treatments for peripheral artery disease with critical limb ischemia. (*Arterioscler Thromb Vasc Biol*. 2012;32:2427-2434.)

Key Words: endothelium ■ nitric oxide synthase ■ peripheral arterial disease ■ nanoparticle ■ pioglitazone

Peripheral artery disease (PAD) is a common disorder that causes claudication, ischemic pain, and ulcers in the lower extremities and that often requires limb amputation when it develops into critical limb ischemia (CLI). Currently, there is no effective medicinal therapy for CLI. The standard therapy for CLI is lower-extremity revascularization, either by bypass surgery or by endovascular therapy, or lower limb amputation, which is associated with a poor prognosis when revascularization is not applicable. Surgical revascularization is associated with ≈5% perioperative mortality rate and a complication rate of 30% to 50%.[1,2] Endovascular therapy is an option for selected patients with CLI; however, this therapy is rarely an option and is associated with inferior patency. Hence, there is an unmet need for less invasive medical therapies to improve the quality of life and prognosis of patients with peripheral artery disease.

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The peroxisome proliferator activated receptor-γ (PPAR-γ) agonists thiazolidines (TZDs) are in clinical use for treating type 2 diabetes mellitus to improve insulin sensitivity in skeletal muscles. PPAR-γ is also expressed in vascular cells, and the TZDs troglitazone and pioglitazone stimulate the migration, proliferation, and survival of endothelial cells and endothelial progenitor cells via the expression of growth factors and cytokines in vitro.[3,4] This effect suggests that TZDs may enhance ischemic neovascularization. By contrast, the high-dose TZDs troglitazone and rosiglitazone suppress vascular endothelial growth factor (VEGF)–induced

endothelial cell proliferation via suppressing VEGF receptor 1 (Flt-1) and 2 (Flk/KDR) expression in vitro.[5] Likewise, there are controversial reports regarding in vivo neovascularization using different TZDs in different models.[6–8] Pioglitazone exerts therapeutic angiogenesis in vivo in hindlimb ischemia in a murine model;[9] however, it remains unclear whether pioglitazone-induced angiogenesis depends solely on PPAR-γ activation in endothelial cells or whether it depends on the improvement of hyperglycemia.

Despite its potential therapeutic effect on ischemic neovascularization, the systemic administration of pioglitazone is hampered by its undesirable side effects, including edema and heart failure, which may be optimized by a novel drug delivery system. Recently, we reported that polylactic–glycolic acid (PLGA) nanoparticles (NP) accumulate in the capillary and arteriolar endothelium after intramuscular injection in murine[10] and rabbit[11] models of hindlimb ischemia. The application of PLGA NP as a drug delivery system for pioglitazone may enhance its therapeutic efficacy and may reduce the possible side effects. In this study, we tested our hypothesis that pioglitazone induces therapeutic neovascularization in a nondiabetic murine model of hindlimb ischemia and that the NP-mediated delivery of pioglitazone enhances the therapeutic efficacy of pioglitazone.

Materials and Methods

Preparation of PLGA NP

We prepared PLGA NP incorporating pioglitazone (Pio-NP; Takeda Pharmaceutical Company Limited, Osaka, Japan) or fluorescein isothiocyanate (FITC) (FITC-NP) via the emulsion solvent diffusion method.[12] PLGA was quickly dissolved in a mixture of acetone and methanol, and pioglitazone or FITC was then added. The resultant polymer-FITC or polymer-pioglitazone solution was emulsified in polyvinyl alcohol solution under stirring at 400 rpm using a propeller-type agitator with 3 blades (Heidon 600G; Shinto Scientific, Japan). After agitating the system for 2 hours under reduced pressure at 40ºC, the entire suspension was centrifuged (20000g for 20 minutes at −20ºC). After removing the supernatant, purified water was mixed with the sediment. The wet mixture was then centrifuged again to remove the excess polyvinyl alcohol and the unencapsulated reagent that could not be adsorbed by the surfaces of NP. After repeating this process, the resultant dispersion was freeze-dried under the same conditions. The FITC-NP was 4.2% (wt/vol) FITC and Pio-NP was 3.7% (wt/vol) pioglitazone.

Animal Preparation and Experimental Protocol

The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines of the American Physiological Society. All mice were maintained in the Laboratory of Animal Experiments at Kyushu University. After anesthesia with an intraperitoneal injection of ketamine hydrochloride (70 mg/kg) and xyladine hydrochloride (3 mg/kg), we induced unilateral hindlimb ischemia in the mice by ligation and excision of the femoral arteries and veins, as previously described.[10] Immediately after inducing ischemia, the animals were divided into treatment groups and were euthanized on day 21. Hindlimb blood flow measurements were performed using a laser Doppler perfusion imaging (LDPI) analyzer (Moor Instruments, United Kingdom). The LDPI index was expressed as the ratio of the LDPI signal in the ischemic limb compared with that in the normal limb. In protocols 1, 2, and 3, the animals received an intramuscular injection of therapeutic agents into the left femoral and thigh muscles using a 27-gauge needle immediately after inducing ischemia, as indicated (Methods and Figure I in the online-only Data Supplement). In protocol 4, the animals received a daily oral treatment

of pioglitazone or vehicle (Methods and Figure I in the online-only Data Supplement).

Distribution of FITC-NP in Ischemic Hindlimb Tissues

Three, 7, 14, and 21 days after hindlimb ischemia and intramuscular injection of FITC-NP, the gastrocnemius muscle was isolated from ischemic and nonischemic limbs, and the FITC signals were examined under a fluorescent microscope. Frozen cross sections of the muscles 3 days after hindlimb ischemia and intramuscular injection of FITC or FITC-NP were then prepared and examined under a fluorescent microscope (Biozero; Keyence, Co, Osaka, Japan). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Shield, Vector Labs Ltd, United Kingdom) in some sections. Other sections were stained with anti-mouse platelet/endothelial cell adhesion molecule-1 antibody (CD31; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) as the primary antibody and anti-goat IgG (Alexa 555; Life Technologies, Grand Island, NY) as the secondary antibody.

Histological and Immunohistochemical Analyses

A histological and immunohistochemical evaluation was performed in 5-μm paraffin-embedded sections of the gastrocnemius muscle after hindlimb ischemia. To determine the capillary and arteriolar density, cross sections were stained with anti-mouse platelet endothelial cell adhesion molecule with anti–platelet/endothelial cell adhesion molecule-1 (CD31) antibody as a primary antibody (Santa Cruz Biotechnology, Inc) and anti-goat IgG antibody (Alexa 555; Life Technologies) as a secondary antibody. The nuclei were counterstained with DAPI (Vector Laboratories, Inc, Burlingame, CA) and α-smooth muscle actin antibody (DAKO, Glostrup, Denmark).

Measurements of Pioglitazone Concentration in the Serum and Muscle Tissue

The pioglitazone concentrations in the serum and the muscle were measured at predetermined time points using liquid chromatography coupled to tandem mass spectrometry. Additional details are provided in the Methods in the online-only Data Supplement.

PPAR-γ Activity Measurement in the Muscle Tissue

Nuclear extracts were prepared from the gastrocnemial muscle homogenates using a nuclear extract kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents; Thermo Fisher Scientific Inc, Rockford, IL) according to the manufacturer's instructions. The protein was measured using a BCA Protein Assay kit (Thermo Fisher Scientific Inc). PPAR-γ activation was assayed using ELISA-based PPAR-γ activation TransAM kit (Active Motif, Rixensart, Belgium), which was used according to the manufacturer's instructions. Ten micrograms of nuclear protein samples were incubated for 1 hour in a 96-well plate coated with an oligonucleotide that contains a PPAR response element domain (5′-AACTAGGTCAAAGGTCA-3′), to which the activated PPAR-γ contained in nuclear extracts specifically binds. After washing, PPAR-γ antibody (1:1000 dilutions) was added to these wells and incubated for 1 hour. After incubation for 1 hour with a secondary horseradish peroxidase–conjugated antibody (1:1000 dilution), specific binding was detected by colorimetric estimation at 450 nm with a reference wavelength of 655 nm.

Polymerase Chain Reaction Array

The total RNA was isolated from the gastrocnemial muscles using an RNeasy Fibrous Tissue Mini kit (QIAGEN Inc, Valencia, CA). cDNA synthesis was performed using 1 μg of the total RNA with a PrimeScript RT reagent kit (Takara-Bio, Inc, Shiga, Japan). For angiogenic factor expression analysis, quantitative real-time polymerase chain reaction analysis of 84 angiogenic factors was performed using mouse-specific angiogenic factors RT2 Profiler PCR Arrays (QIAGEN Inc),
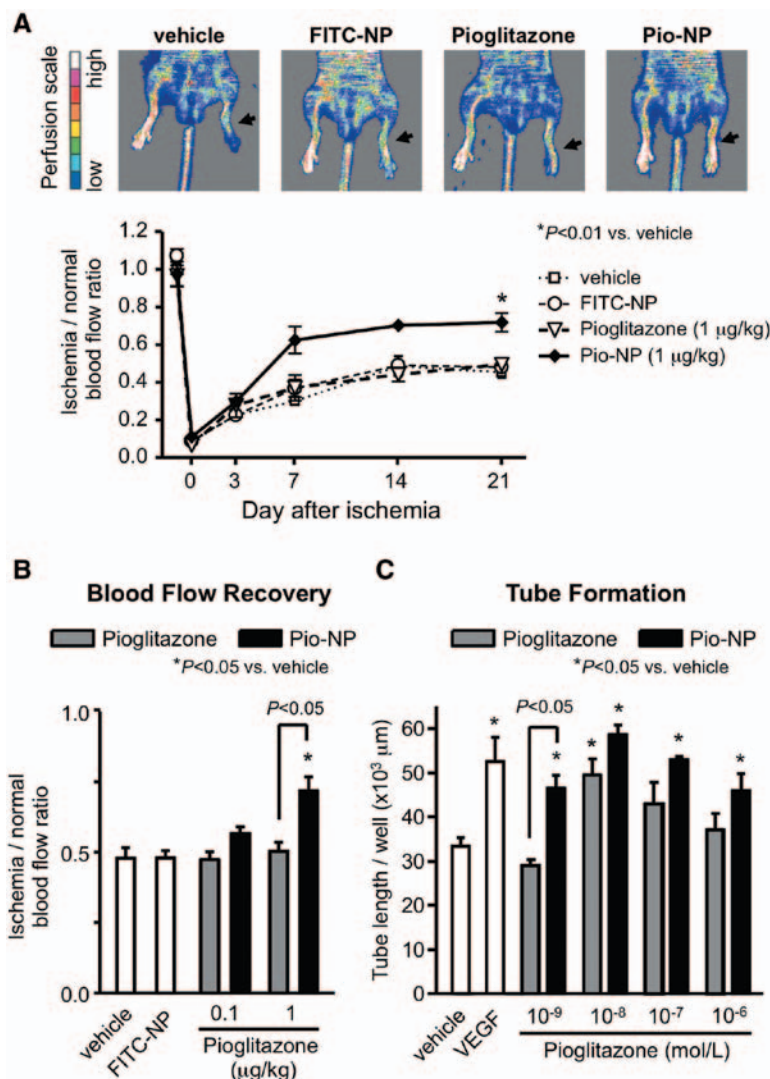
Figure 1. The effects of pioglitazone and pioglitazone-incorporated nanoparticle (Pio-NP) on ischemia-induced neovascularization. **A**, Representative laser Doppler perfusion imaging (LDPI) 21 days after inducing hindlimb ischemia (upper images). The arrow indicates ischemic limb. Quantification of LDPI-derived blood flow recovery. n=6 to 7. *$P<0.01$ vs no treatment group. The data were compared using 1-way ANOVA followed by Bonferroni multiple comparison tests. **B**, Quantification of LDPI-derived blood flow recovery 21 days after induction of hindlimb ischemia. n=6 to 7. The data were compared using 2-way ANOVA followed by Bonferroni multiple comparison tests. *$P<0.05$ vs control. **C**, The effects of Pio-NP or pioglitazone on the angiogenic capacity of human endothelial cells in vitro. Quantitative analysis of tube formation (tube length) of 4 independent experiments. The data were compared using 2-way ANOVA followed by Bonferroni multiple comparison tests. *$P<0.05$ vs control. VEGF indicates vascular endothelial growth factor; FITC, fluorescein isothiocyanate.

according to the manufacturer's protocol. The complete list of the genes analyzed is available online at http://www.sabiosciences.com/rt_pcr_product/HTML/PARN-030A.html. The relative gene expression levels were normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase-1. Data analysis was performed using the ΔΔCt-based fold-change method.

Angiogenesis Assay in Human Umbilical Vein Endothelial Cells

We performed a 2-dimensional Matrigel assay in human umbilical vein endothelial cells. Additional details are provided in the Methods in the online-only Data Supplement.

Western Blot Analysis

The homogenates of the muscle tissues were analyzed for immunoblotting 3 days after inducing hindlimb ischemia. The membrane was incubated with antibodies against phosphorylated Akt, phosphorylated endothelial NO synthase (eNOS), Akt (Cell Signaling, Danvers, MA), and eNOS (Thermo Fisher Scientific Inc).

Statistical Analysis

The data are expressed as the mean±SEM. The statistical analysis was assessed using analysis of variance and multiple comparison tests. P values <0.05 were considered to be statistically significant.

Results

Tissue Distribution of PLGA NP

We first examined the distribution of FITC after an injection of either FITC-NP or FITC into ischemic hindlimb muscles in a murine model. FITC fluorescence was detectable for 14 days in the FITC-NP–injected hindlimbs, whereas FITC fluorescence was undetectable at day 7 in FITC only–injected hindlimbs (Figure II in the online-only Data Supplement), suggesting NP-dependent modification of pharmacokinetics as shown in our previous study.[10]

Pio-NP Enhanced Ischemia-Induced Neovascularization

We examined the effect of Pio-NP on blood flow recovery after induction of acute hindlimb ischemia in a murine model using LDPI. Importantly, a single intramuscular injection of Pio-NP containing 1 μg/kg pioglitazone significantly enhanced blood flow recovery in the ischemic limbs, whereas an intramuscular injection of PBS (vehicle), FITC-NP, or 1 μg/kg pioglitazone had no therapeutic effects (Figure 1A and 1B). After injecting pioglitazone or Pio-NP into ischemic hindlimbs, we examined the tissue and serum concentration of pioglitazone at several

Table 1.   Tissue and Serum Pioglitazone Concentrations After Intramuscular Injection of Pioglitazone or Pioglitazone-NP

	Time After Injection			
	15 min	1 h	6 h	24 h
Pioglitazone only at 1 µg/kg				
Muscle, ng/g tissue	19±1	ND	ND	ND
Serum, ng/mL	1±1	ND	1±1	ND
Pioglitazone-NP containing 1 µg/kg pioglitazone				
Muscle, ng/g tissue	37±4*	5±2	2±1	ND
Serum, ng/mL	2±1	1±1	2±0	ND

ND indicates not detected (muscle <1 ng/g tissue; serum <1 ng/mL); NP, nanoparticle.

*$P<0.05$ vs pioglitazone.

time points. As shown in Table 1, Pio-NP remained detectable for 6 hours in the muscular tissue compared with pioglitazone alone. The concentration of pioglitazone in the muscular tissue was estimated to be 37±4 ng/g tissue 15 minutes after injecting Pio-NP containing 1 µg/kg pioglitazone and was below the detectable limit (≈1.0 ng/g tissue) after 24 hours. By contrast, pioglitazone was undetectable 1 hour after injecting 1 µg/kg pioglitazone alone (Table 1), suggesting that NP enhanced the accumulation of pioglitazone within the hindlimb tissue.

We then tested the angiogenic activity of pioglitazone in cultured endothelial cells in vitro. Pio-NP induced significant endothelial tube formation in human umbilical vein endothelial cells at 10^{-9} to 10^{-6} mol/L, which was equivalent to the effect induced by VEGF (10 ng/mL; Figure 1C). The induction of endothelial tube formation was significantly enhanced with Pio-NP compared with pioglitazone alone at the same doses, suggesting that NP modified the drug kinetics and efficacy. Pio-NP–induced blood flow recovery was accompanied by a significant increase in the number of CD31-positive endothelial cells surrounding the regenerating myocytes and an increase in number of capillary and arterioles accompanied by α-smooth muscle actin–positive smooth muscle cells 21 days after inducing ischemia (Figure 2).

Pio-NP Enhances Ischemic Neovascularization Through PPAR-γ Activation

We questioned whether Pio-NP–induced neovascularization is dependent solely on PPAR-γ activation. The blood flow recovery by Pio-NP was reversed in mice pretreated with the PPAR-γ antagonist GW9662, suggesting that PPAR-γ activation is critical for Pio-NP–induced blood flow recovery (Figure 3A). Indeed, Pio-NP significantly activated PPAR-γ DNA binding in the nuclear extract from the ischemic hindlimb tissues. Systemic pretreatment with GW9662 again reversed the Pio-NP–induced PPAR-γ activation in the hindlimb tissues (Figure 3B, left). By contrast, an intramuscular injection of Pio-NP in the ischemic limbs did not affect PPAR-γ transcriptional activity in the contralateral nonischemic limbs (Figure 3B, right). The serum levels of glucose, triglyceride, and insulin did not differ between the control group and the oral administration of pioglitazone groups. This result suggests that pioglitazone enhances

ischemic neovascularization independent of blood glucose or insulin, even when administered daily, in the nondiabetic murine model (Table I in the online-only Data Supplement).

Effect of Pio-NP on Endogenous Angiogenic Factor Expression

PPAR-γ may regulate several genes that contribute to ischemic neovascularization. Three days after inducing ischemia, we examined gene expression in the hindlimb tissues that were treated with Pio-NP. Real-time polymerase chain reaction analysis revealed a significant induction of VEGF-A, VEGF-B, VEGF receptor1/Flt-1, fibroblast growth factor-1 (FGF-1), FGF receptor 3, and platelet/endothelial cell adhesion molecule-1 in the ischemic muscle tissues treated with Pio-NP compared with vehicle-treated ischemic muscle tissues (Table 2).

Effects of Pio-NP on Neovascularization Depends on eNOS

The aforementioned angiogenic growth factors were demonstrated to induce the proliferation of endothelial cells, in
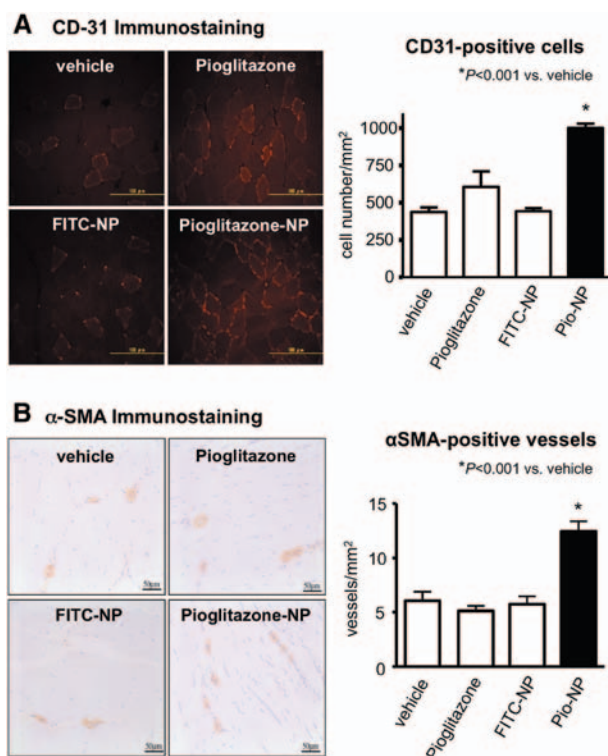
Figure 2. Quantitative analysis of angiogenesis and arteriogenesis. **A**, Immunofluorescent staining of cross sections from ischemic muscle at 21 days after inducing ischemia, stained for the endothelial marker CD31 (red; left). Scale bars, 100 µm. Quantitative analysis of angiogenesis (right; CD31-positive cells per mm²). **B**, Representative micrographs of ischemic muscle sections stained immunohistochemically with antibodies against α-smooth muscle actin (α-SMA) at 21 days after surgery (left). The nuclei were counterstained with hematoxylin. Scale bars, 100 µm. Quantitative analysis of arteriogenesis (right; α-SMA–positive vessels per mm²). The data were compared using 1-way ANOVA followed by Bonferroni multiple comparison tests. *$P<0.001$ vs untreated group. Pio-NP indicates pioglitazone-incorporated nanoparticle.

Figure 3. The essential role of peroxisome proliferator–activated receptor-γ (PPAR-γ) in the enhanced ischemia-induced neovascularization induced by pioglitazone-incorporate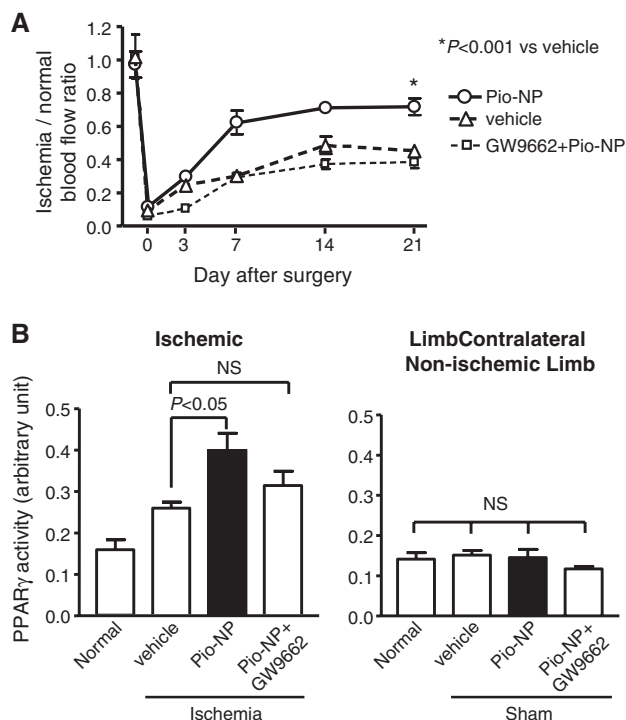d nanoparticle (Pio-NP). The increased neovascularization induced by Pio-NP was associated with PPAR-γ activity. **A**, Quantification of laser Doppler–derived blood flow recovery after surgery in wild-type mice with or without administration of GW9662, a PPAR-γ inhibitor. **B**, PPAR-γ activation in ischemic muscles 3 days after ischemia. The data were compared using 1-way ANOVA followed by Bonferroni multiple comparison tests. NS indicates not significant.

Table 2. Gene Expression Changes in Pioglitazone-Incorporated Nanoparticle–Treated Ischemic Muscle Tissue Compared With Vehicle-Treated Tissue (Real-Time Polymerase Chain Reaction Array)

Gene Name	Description	Epox/Control Normalized (Increase)	Reference Sequence
FGF-6	Fibroblast growth factor-6	6.04	NM_010204
VEGF-B	Vascular endothelial growth factor-B	3.39	NM_011697
FGF-1	Fibroblast growth factor-1	3.06	NM_010197
—	T-box–1	2.86	NM_011532
PECAM-1	Platelet/endothelial cell adhesion molecule-1	2.61	NM_008816
Agpt2	Angiopoietin-2	2.56	NM_007426
VEGF-A	Vascular endothelial growth factor-A	2.26	NM_009505
EGF	Epidermal growth factor	2.06	NM_010113
Galpha13/ AU024132	Guanine nucleotide binding protein, α13	1.96	NM_010303
VEGFR-1/Flt-1	Vascular endothelial growth factor receptor 1	1.90	NM_010228
FGFR-3	Fibroblast growth factor receptor-3	1.74	NM_008010
VEGF-D	Vascular endothelial growth factor-D	1.74	NM_010216

Gene Name	Description	Epox/Control Normalized (Decrease)	Reference Sequence
MK/Mek	Midkine	2.14	NM_010784
TGF-β1	Transforming growth factor-β1	1.78	NM_011577
CINC-2a/Gro2/ MIP-2//Mgsa-b/ Scyb2	Chemokine (C-X-C motif) ligand-2	1.62	NM_009140
ELF-2/Htk-L/ LERK-5	Ephrin-B2	1.60	NM_010111
TSP2/Thbs-2	Thrombospondin-2	1.52	NM_011581

which endothelial NO plays a critical role.[13] PPAR-γ activation induces NO release from endothelial cells, both dependently and independently of eNOS mRNA expression.[14,15] Hence, we examined the role of eNOS expression and its activation in Pio-NP–induced neovascularization in the ischemic hindlimb model. In eNOS$^{-/-}$ mice, femoral artery occlusion induced a relatively severe lesion compared with wild-type mice (data not shown), whereas blood flow recovery determined by LDPI was equivalent in untreated eNOS$^{-/-}$ mice compared with wild-type mice. Importantly, the Pio-NP–induced enhancement was not observed in eNOS$^{-/-}$ mice (Figure 4A), suggesting the critical role of eNOS in the therapeutic effects of Pio-NP. Pio-NP did not affect eNOS mRNA expression (data not shown) and the eNOS protein levels in the ischemic hindlimb tissues in wild-type mice (Figure 4B and 4C). The treatment with Pio-NP significantly increased the phosphorylation of eNOS in ischemic muscles compared with nonischemic control and nontreated ischemic muscles 3 days after treatment (Figure 4C). Pio-NP tended to increase Akt phosphorylation compared with no treatment, although the difference was not statistically significant.

Efficacy of the PLGA NP

Finally, we confirmed the therapeutic advantage of the NP-mediated intramuscular delivery of pioglitazone over pioglitazone alone or the oral administration of pioglitazone. The daily oral administration of pioglitazone (1 µg/kg per day) did not enhance blood flow recovery. Oral treatment with pioglitazone required as high as 1000 µg/kg per day to significantly enhance blood flow recovery equivalent to Pio-NP (Figure 5A). Finally, a single intramuscular injection of Pio-NP was significantly more effective compared with a single intramuscular injection of pioglitazone alone or daily oral administration of pioglitazone at the same single dose (1 µg/kg) (Figure 5B).

Discussion

There have been controversial results regarding the angiogenic effects of TZDs both in vitro and in vivo.[16] In the present study, we demonstrated that oral treatment with pioglitazone at clinically relevant dose (1000 µg/kg) enhanced blood flow recovery in a hindlimb ischemia model in nondiabetic mice independent of the changes in blood glucose
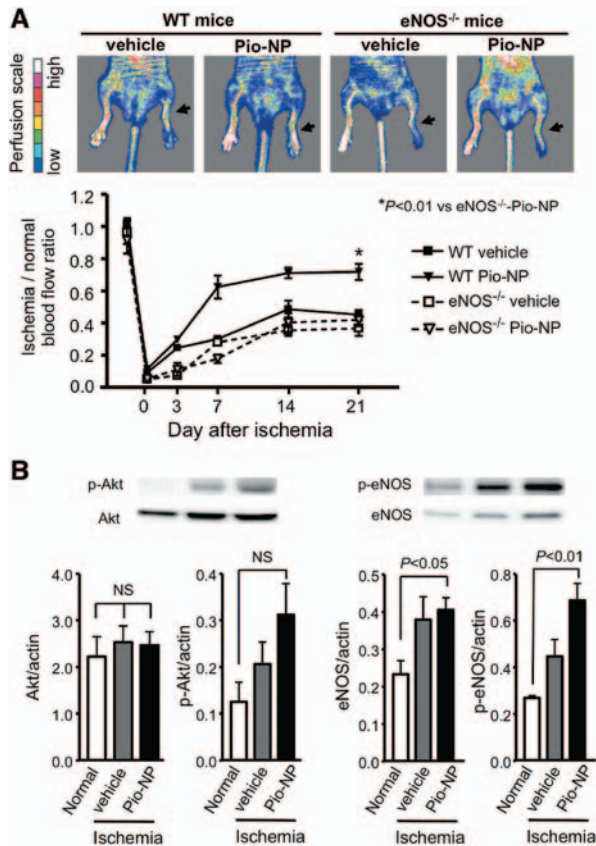
Figure 4. The essential role of endothelial nitric oxide synthase (eNOS) in the enhanced ischemia–induced neovascularization induced by pioglitazone-incorporated nanoparticle (Pio-NP). The increased neovascularization by the Pio-NP was associated with the increased expression of the active forms of eNOS and Akt. **A**, Quantification of laser Doppler–derived blood flow recovery after surgery in wild-type (WT) mice and in eNOS$^{-/-}$ mice. n=5 to 6. *P<0.01 vs no treatment group. **B**, Western blot analysis of phosphorylated Akt and eNOS in ischemic and nonischemic muscles 3 days after ischemia. The quantitative evaluation was expressed as a ratio of phosphorylated Akt, phosphorylated eNOS (p-eNOS), Akt, eNOS to actin. n=4 to 6. The data were compared using 1-way ANOVA followed by Bonferroni multiple comparison tests. NS indicates not significant.
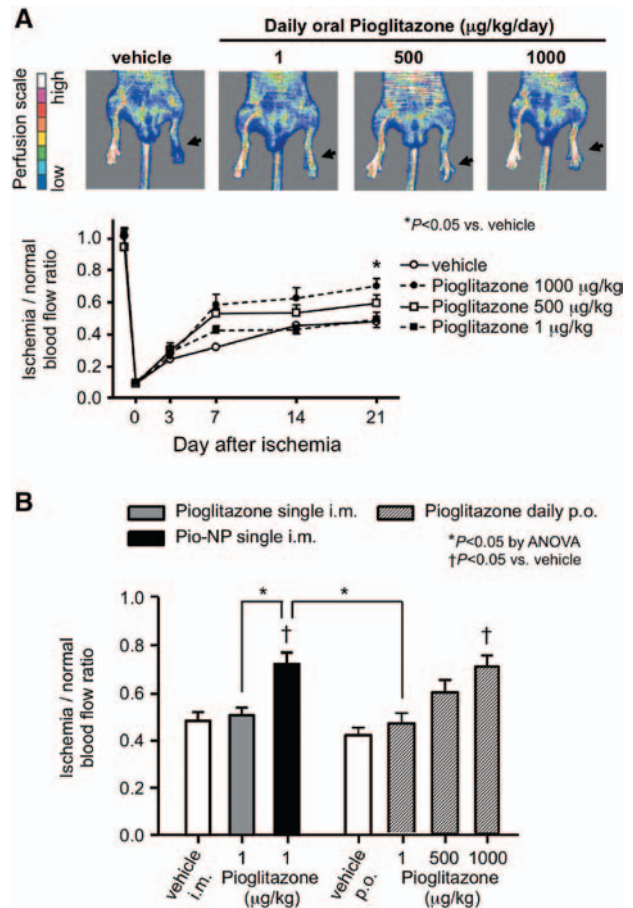

Figure 5. Effects of the daily oral administration of pioglitazone on ischemia-induced neovascularization. **A**, Quantification of laser Doppler perfusion imaging (LDPI)–derived blood flow recovery. n=7 to 8. *P<0.01 vs untreated group. **B**, The effects of single intramuscular injection of pioglitazone- incorporated nanoparticle (Pio-NP) compared with the daily oral administration of pioglitazone 21 days after ischemia. n=7 to 8.

or insulin levels, suggesting that pioglitazone directly acts on vascular cells to enhance ischemia-induced neovascularization. Despite its potential effectiveness, the oral administration of pioglitazone is hampered by its adverse effects, including edema and heart failure.[17] Recently, the potential link between pioglitazone and the risk of bladder cancer has been raised as a concern.[18]

Importantly, we demonstrated that a single intramuscular injection of Pio-NP at as low a concentration as 1 μg/kg had significant therapeutic effects on ischemia-induced neovascularization (Figure 1). The pioglitazone concentrations in the tissue and the serum suggest the local retention of Pio-NP in ischemic skeletal muscles in vivo. The distribution of FITC-NP (Figure II in the online-only Data Supplement) suggests that Pio-NP accumulates in the endothelial cells in the ischemic muscles. In our previous studies,[10,11] we have shown that PLGA NP accumulates preferentially in endothelial cells compared with skeletal muscle cells, relying on

clathrin-mediated endocytosis, which may explain the selective distribution in endothelial cells in the muscle tissues. A single intramuscular injection of PLGA NP may cover the therapeutic window for the blood flow recovery in the murine model (≈7 days; eg, Figure 1A), given that we have also demonstrated that PLGA NP (mean diameter =200 nm) gradually undergo hydrolysis over 21 days in a physiological fluid (pH 7.4; 32°C).[10] Importantly, Pio-NP exhibited a significant induction of endothelial tube formation at a wider range of doses (10^{-9} to 10^{-6} mol/L) compared with pioglitazone alone (Figure 1C), suggesting that PLGA NP–dependent changes in tissue distribution and release kinetics optimized the proangiogenic activity of pioglitazone on endothelial cells. Further studies are needed to clarify the effect of ischemia and the amount of PLGA NP on the mode of endosomal escape, intracellular localization, and drug release kinetics of PLGA NP used in the present study.[19]

PPAR-γ activation appears to be a critical step in therapeutic neovascularization by Pio-NP because we confirmed that Pio-NP induced PPAR-γ DNA binding to the PPAR response element (Figure 3B) and that the PPAR-γ antagonist GW9662 abolished the blood flow recovery by Pio-NP (Figure 3A).
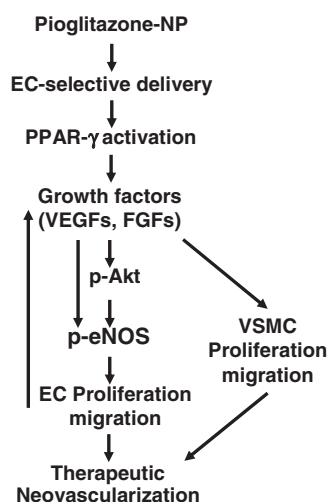
Pioglitazone-NP

↓

EC-selective delivery

↓

PPAR-γ activation

↓

Growth factors
(VEGFs, FGFs)

↓

p-Akt

↓

p-eNOS

VSMC
Proliferation
migration

↓

EC Proliferation
migration

↓

Therapeutic
Neovascularization

Figure 6. Schematic illustration of the effects of pioglitazone nanoparticles. The nanoparticle (NP)–mediated endothelial cell–selective delivery of pioglitazone induces peroxisome proliferator activated receptor-γ (PPAR-γ) activation. PPAR-γ activation with pioglitazone enhances the expression of multiple growth factors in endothelial cells and induced the phosphorylation of Akt and endothelial nitric oxide synthase (eNOS), resulting in enhanced endothelial cell proliferation and migration. Fibroblast growth factors (FGFs) stimulate smooth muscle cell proliferation and migration. The recruitment of endothelial cells and vascular smooth muscle cells (VSMCs) results in arteriogenesis as therapeutic neovascularization. VEGF indicates vascular endothelial growth factor; p-Akt, phosphorylated Akt; p-eNOS; phosphorylated eNOS; EC, endothelial cell.

Ligand-induced activation of PPAR-γ is known to modulate angiogenic factors including VEGF and FGFs via binding to the PPAR response element.[20] Blood flow recovery by Pio-NP was accompanied by arteriogenesis in ischemic hindlimb muscle, which is demonstrated as arterioles with α-smooth muscle actin–positive pericytes. Polymerase chain reaction array revealed that Pio-NP induced multiple genes that are involved in endothelial proliferation and migration (VEGF-A, VEGF-B, VEGF-D, and epidermal growth factor),[21–26] as well as vascular smooth muscle cell migration and proliferation (FGF and Gna13).[27–30] This coordinated induction of endothelial cells and VSMCs is critical for Pio-NP–induced mature arteriogenesis to form functional collaterals.

Recent phase II randomized clinical trials designed to prove the therapeutic neovascularization for ischemic vascular disease by administering exogenous angiogenic growth factors failed to demonstrate the clinical benefits, although each angiogenic growth factor induces angiogenesis (increased capillary density) in preclinical studies.[31,32] Arteriogenesis, defined as the enlargement of preexisting collateral arteries and their remodeling to conductance vessels, is considered to be more effective at inducing blood flow recovery.[33] In the present study, we performed a histological evaluation of the peripheral ischemic muscles and discovered an increased number of endothelial cells (capillaries), as well as smooth muscle cells forming arterioles (Figure 2) along with overall blood flow recovery. These data suggest that neovascularization in the peripheral muscles may induce arteriogenesis in the proximal regions, which remains to be clarified in larger animal models such as rabbits.[11]

eNOS activation appears to be essential for Pio-NP–induced neovascularization, as shown in the experiment using eNOS$^{-/-}$ mice (Figure 4). Several angiogenic factors including VEGF-A, VEGF-B, and FGF are known to activate eNOS phosphorylation in an Akt-dependent and Akt-independent fashion, and the increased endothelium-derived NO in turn mediates the proliferation of endothelial cells,[34–37] which may be a central mechanism of Pio-NP–induced neovascularization (Figure 6). The therapeutic effects afforded by Pio-NP were independent of the improvement in insulin resistance. We demonstrated that the intramuscular injection of Pio-NP did not affect glucose, insulin, or triglyceride levels in the serum. These findings suggest that Pio-NP acted locally on ischemic vascular endothelial cells to induce therapeutic neovascularization.

In conclusion, the NP-mediated endothelial cell–selective delivery of pioglitazone induces functionally mature collaterals through eNOS activation and the expression of multiple endogenous angiogenic growth factors. NP-mediated drug delivery is a novel modality that may advance therapeutic neovascularization over current medical treatment for severe peripheral artery disease, including CLI.

Disclosures

Dr Egashira holds a patent on the results reported in this study. The remaining authors report no conflict of interest.

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Supplementary Figure Legends

Supplementary Figure I. Animal experimental protocols.

In protocol 1, the treatment groups received intramuscular injections of FITC-NP (0.9 mg/100 μl PLGA; NP group), pioglitazone at 0.0025, 0.025 μg/100 μl (1 μg/kg; pioglitazone group), or pioglitazone-NP (0.067, 0.67 μg/100 μl PLGA containing 0.0025, 0.025 μg pioglitazone) into the left femoral and thigh muscles using a 27-gauge needle. In protocol 2, the effects of the intramuscular injections of pioglitazone-NP were examined in wild-type mice administered GW9662 (PPAR-γ antagonist) (Sigma). In protocol 3, the effects of the intramuscular injections of pioglitazone-NP were examined in endothelial nitric oxide synthase (eNOS) $^{-/-}$ mice. WT mice or eNOS$^{-/-}$ mice received single intramuscular injections of pioglitazone-NP at doses of 1 μg/kg immediately after inducing ischemia. In protocol 4, immediately after inducing ischemia, the animals were randomly divided into three groups; three other groups received a systemic daily oral administration of pioglitazone at doses of 1, 500 and 1000 μg/kg dissolved in 0.5% carboxymethyl cellulose by gavage from the day of surgery until the mice were euthanized on day 21.

Supplementary Figure II. Cellular distribution of NP in ischemic muscles.

A, Representative light (upper panels) and fluorescent (lower panels) stereomicrographs

of the gastrocnemius muscles isolated from a control, non-ischemic hindlimb and those

from an ischemic hindlimb injected with FITC-NP or FITC alone. B, Quantitative

analysis of the magnitude of intracellular FITC fluorescence signals in the

gastrocnemius muscles are shown. The data were compared using two-way ANOVA

followed by Bonferroni's multiple comparison tests. *$p<0.05$ versus control condition.

[†]$p<0.05$ versus FITC. C, Immunofluorescent staining of cross-sections from ischemic

muscle 3 days after a FITC-NP or FITC only injection stained with the endothelial

marker CD31 (red). Scale bars: 100 μm.

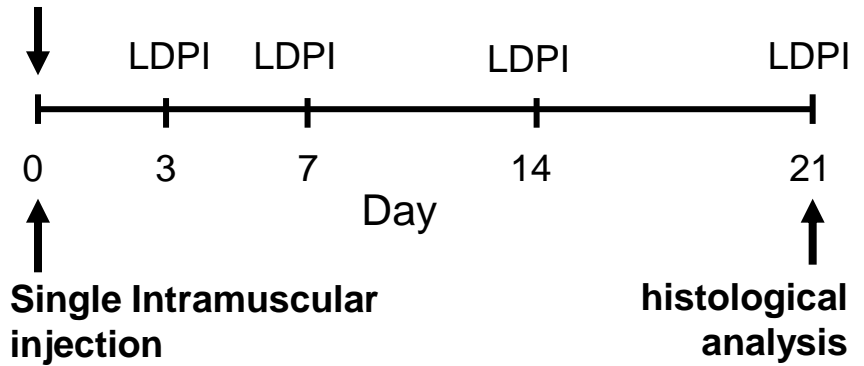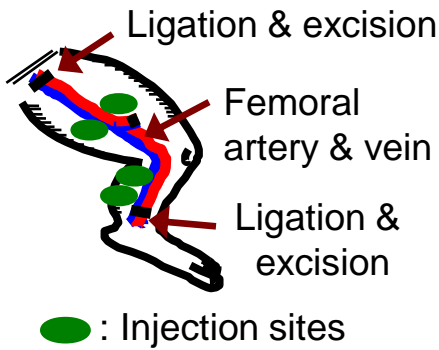**Supplemental Table I**. Serum concentration of glucose, triglyceride and insulin at 3 weeks after daily oral administration of pioglitazone

Oral Pioglitazone (mg/kg/day)	Body Weight (g)	Serum Glucose (mg/dL)	Serum Triglyceride (mg/dL)	Serum Insulin (µIU/mL)
0	24.4±0.6	303±33	39±2	ND
1	24.5±1.0	272±23	55±11	ND
500	24.4±1.2	309±114	60±12	ND
1000	23.6±0.9	280±48	63±10	ND

ND: not detected (<0.3 µIU/mL)

The data are shown as the mean±SEM (n=2-5 each).

**Surgical Induction of
Hindlimb ischemia**

Ligation & excision

Femoral
artery & vein

Ligation &
excision

● : Injection sites

LDPI   LDPI   LDPI   LDPI

0   3   7   14   21
Day

**Single Intramuscular
injection**

**histological
analysis**

Protocol 1.
•vehicle
•FITC-NP (control NP)
•Pioglitazone (0.1, 1 μg/kg)
•Pio-NP (0.1, 1 μg/kg as Pioglitazone)

Protocol 2.
•vehicle
•Pio-NP (1 μg/kg as Pioglitazone)
•GW9662 i.p. + Pio-NP (1 μg/kg as Pioglitazone)

Protocol 3.
•WT mice, vehicle
•WT mice, Pio-NP (1 μg/kg as Pioglitazone)
•eNOS-/- mice, vehicle
•eNOS-/- mice, Pio-NP (1 μg/kg as Pioglitazone)

**Surgical Induction of
Hindlimb ischemia**

Ligation & excision
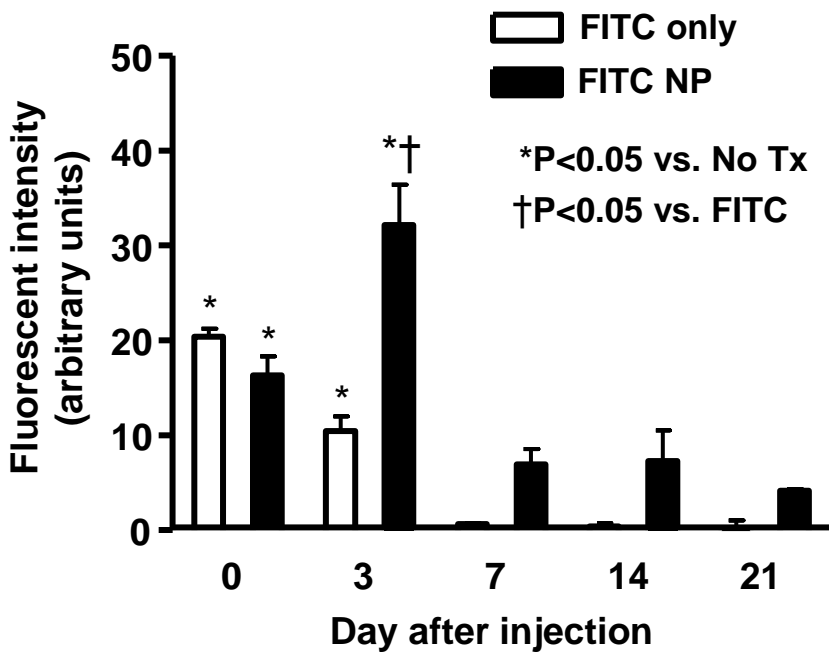
Femoral
artery & vein

Ligation &
excision

LDPI   LDPI   LDPI   LDPI

0   3   7   14   21
Day

Daily oral treatment of Pioglitazone
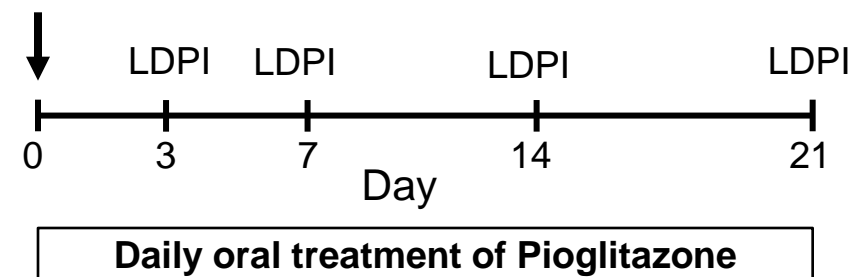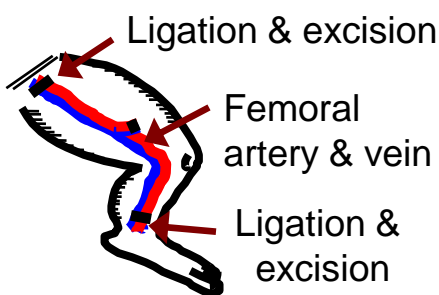
Protocol 4.
•Vehicle
•Pioglitazone (1, 500, 1000 μg/kg)

A

	No Tx	FITC only	FITC-NP			

Light

Fluorescence

3 **3** **7** **14** **21**

Day after injection

B

☐ FITC only
■ FITC NP

*P<0.05 vs. No Tx
†P<0.05 vs. FITC

Fluorescent intensity (arbitrary units)

50
40
30
20
10
0

0 3 7 14 21

Day after injection

C

FITC only

FITC-NP

Green: FITC
Red: CD31