Pioglitazone-Incorporated Nanoparticles Prevent Plaque Destabilization and Rupture by Regulating Monocyte/Macrophage Differentiation in ApoE−/− Mice

Soichi Nakashiro, Tetsuya Matoba, Ryuta Umezu, Jun-ichiro Koga, Masaki Tokutome, Shunsuke Katsuki, Kaku Nakano, Kenji Sunagawa, Kensuke Egashira

Objective—Inflammatory monocytes/macrophages produce various proteinases, including matrix metalloproteinases, and degradation of the extracellular matrix by these activated proteinases weakens the mechanical strength of atherosclerotic plaques, which results in a rupture of the plaque. Peroxisome proliferator–activated receptor-γ induces a polarity shift of monocytes/macrophages toward less inflammatory phenotypes and has the potential to prevent atherosclerotic plaque ruptures. Therefore, we hypothesized that nanoparticle-mediated targeted delivery of the peroxisome proliferator–activated receptor-γ agonist pioglitazone into circulating monocytes could effectively inhibit plaque ruptures in a mouse model.

Approach and Results—We prepared bioabsorbable poly(lactic-co-glycolic-acid) nanoparticles containing pioglitazone (pioglitazone-NPs). Intravenously administered poly(lactic-co-glycolic-acid) nanoparticles incorporated with fluorescein isothiocyanate were found in circulating monocytes and aortic macrophages by flow cytometric analysis. Weekly intravenous administration of pioglitazone-NPs (7 mg/kg per week) for 4 weeks decreased buried fibrous caps, a surrogate marker of plaque rupture, in the brachiocephalic arteries of ApoE−/− mice fed a high-fat diet and infused with angiotensin II. In contrast, administration of control-NPs or an equivalent dose of oral pioglitazone treatment produced no effects. Pioglitazone-NPs inhibited the activity of matrix metalloproteinases and cathepsins in the brachiocephalic arteries. Pioglitazone-NPs regulated inflammatory cytokine expression and also suppressed the expression of extracellular matrix metalloproteinase inducer in bone marrow–derived macrophages.

Conclusions—Nanoparticle-mediated delivery of pioglitazone inhibited macrophage activation and atherosclerotic plaque ruptures in hyperlipidemic ApoE−/− mice. These results demonstrate a promising strategy with a favorable safety profile to prevent atherosclerotic plaque ruptures. (Arterioscler Thromb Vasc Biol. 2016;36:491-500. DOI: 10.1161/ATVBAHA.115.307057.)

Key Words: macrophages ■ matrix metalloproteinases ■ nanoparticles ■ pioglitazone

Coronary artery disease (CAD) is the leading cause of death worldwide, and 375,295 people died of CAD in the United States in 2011.1 Myocardial infarction is the most severe form of CAD and has devastating effects on patient mortality and quality of life and medical economies. Recent advances in interventional devices and procedures have achieved low in-hospital mortality (<10%) after myocardial infarction,2,3 but more than half of the deaths caused by myocardial infarction occur before arrival at the hospital.4 Therefore, the prevention of acute myocardial infarction is the most feasible approach for improving the survival rate of patients.

According to several autopsy studies, ≈60% of acute myocardial infarction occurs as a result of rupture or fissuring of thin-capped fibroatheroma.5-6 Rupture-prone plaques are characterized by the abundant accumulation of innate immune cells (which mainly consist of monocytes/macrophages), lipid core formation, and induction of several proteinases that catalyze the extracellular matrix.6 Monocytes are functionally heterogeneous and are classified into at least 2 major subsets in mice: inflammatory monocytes (Ly-6ChighCCR2−CX3CR1low) and noninflammatory monocytes (Ly-6ChighCCR2 CX3CR1high).7 In humans, CD14+CD16− monocytes and CD14+CD16+ monocytes are described as their respective counterparts according to their chemokine receptor expression, although their functional similarities have not been fully determined. Monocyte-derived macrophages also consist of at least 2 subsets: classically activated macrophages (M1) and alternatively activated macrophages (M2). We have...
reported that adoptive transfer of Ly-6ChighCCR2+ inflammatory monocytes increases buried fibrous caps in the brachioccephalic arteries in a mouse model of plaque rupture. Therefore, manipulating these innate immune cells may be a promising therapeutic strategy to prevent plaque ruptures in patients with CAD.

We previously developed a nanoparticle-mediated drug delivery system (DDS) that consists of a bioabsorbable poly(lactic-co-glycolic acid) (PLGA) polymer. In our latest report, we used the nanoparticle-mediated DDS to deliver pitavastatin, a hydroxymethylglutaryl-CoA reductase inhibitor, in an atherosclerotic mouse model. Pitavastatin-incorporated nanoparticles were delivered to circulating monocytes and significantly decreased plaque rupture primarily by inhibiting the recruitment of Ly-6C high inflammatory monocytes to atherosclerotic lesions, which resulted in less macrophage content in the atherosclerotic lesions. Alternatively, modulating the polarity of monocytes and macrophages (ie, Ly-6C high/Ly-6C low monocytes and M1/M2 macrophages, respectively) toward a less inflammatory direction, rather than merely suppressing the recruitment of inflammatory monocytes, may be another potential strategy to prevent plaque destabilization and rupture.

Pioglitazone, one of the clinically approved thiazolidinediones, is a potent agonist of peroxisome proliferator–activated receptor-γ (PPARγ). PPARγ is a member of nuclear receptor family. Ligands, including pioglitazone, bind to PPARγ and form a heterodimer with another nuclear receptor, retinoid X receptor. This heterodimer binds to PPAR response element and regulates downstream gene expression. In addition to their antidiabetic effects, PPARγ agonists have been shown to affect macrophage polarity. More specifically, PPARγ activation enhances the alternative (M2) activation/differentiation of macrophages with expression of anti-inflammatory markers, such as CD206 (mannose receptor) and CD36 (a member of the class B scavenger receptor family). In an animal study, it was reported that oral administration of pioglitazone reduced macrophage content and matrix metalloproteinase (MMP) activity in murine carotid atherosclerosis. Several clinical studies have also suggested the atheroprotective effects of pioglitazone. In the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive trial), pioglitazone reduced recurrent myocardial infarction in type 2 diabetic patients. Because many blood glucose–lowering agents have failed to reduce macrovascular complications in diabetic patients, the atheroprotective effects of pioglitazone could be explained by its class-specific properties, namely PPARγ-dependent regulation of genes. However, it is unclear whether pioglitazone prevents plaque destabilization and rupture. Therefore, we tested the hypothesis that nanoparticle-mediated targeted delivery of pioglitazone into circulating monocytes/lesional macrophages regulates the polarity of those leukocytes and inhibits their activation, preventing plaque rupture in a mouse model.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Delivery of PLGA Nanoparticles to Peripheral Blood Monocytes and Aortic Macrophages

First, we analyzed the cellular distribution of PLGA nanoparticles using PLGA nanoparticles containing fluorescein isothiocyanate (FITC; FITC-NPs) and flow cytometry in the leucocytes of peripheral blood and the aorta. In ApoE−/− mice fed a high-fat diet and infused with angiotensin II, nanoparticles were taken up by monocytes, neutrophils, and lymphocytes in the peripheral blood. These hematopoietic lineages, the fluorescent signal was the highest in CD11b Lin monocytes as shown in Figure 1A. In the aorta, nanoparticles were predominantly delivered to monocytes/macrophages, and few particles were delivered to neutrophils and lymphocytes (Figure 1A). Immunohistochemical analysis showed that injected nanoparticles were delivered to plaque macrophages (Figure III in the online-only Data Supplement) 24 hours after injection. On the other hand, nanoparticles in the endothelial layer were much less when compared with macrophages and negligible in smooth muscle cells. As shown in Figure 1B, injected nanoparticles remained in monocytes/macrophages 7 days after injection in the aorta. The FITC fluorescence intensity was higher after treatment with FITC-NPs than with FITC alone (Figure 1A and 1B), showing the advantage of PLGA nanoparticles for drug delivery to circulating monocytes and tissue macrophages/macrophages. Next, we examined the pharmacokinetics of PLGA nanoparticles containing pioglitazone (pioglitazone-NPs). After intravenous administration of pioglitazone-NPs (containing 7 mg/kg pioglitazone), mice were euthanized at the indicated time points, and the pioglitazone concentrations in the plasma and each tissue were quantified. The pioglitazone concentration in the plasma peaked at 2 hours after injection and then declined in a time-dependent manner. The tissue concentration of pioglitazone was higher (not significant) in the aorta than in the spleen and kidneys at 48 hours after injection (Figure 1C).

#### Intravenous Administration of Pioglitazone-NPs Skews the Peripheral Monocyte Population Toward a Less Inflammatory State

We examined the effect of pioglitazone-NPs on the monocyte population in the peripheral blood. Flow cytometric analysis performed 2 days after a single intravenous administration of pioglitazone-NPs showed a significantly decreased number of Ly-6Chigh monocytes that represent the inflammatory monocyte subset, whereas Ly-6C low noninflammatory monocytes.
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showed a modest increased trend. The total monocyte count was not affected. As a result, the peripheral monocyte polarity defined as Ly-6Chigh monocytes/Ly-6Clow monocytes was significantly altered toward a less inflammatory state (Figure 2). Because Ly-6Chigh monocytes preferentially accumulate in atherosclerotic lesions and play causative roles in plaque destabilization, this result prompted us to test whether pioglitazone-NP treatment reduces atherosclerotic plaque ruptures.

On the other hand, intravenous administration of pioglitazone-NPs had no significant effects on the number and percentage of CD11b+Lin− premonocytes (Figure VI in the online-only Data Supplement). In addition, there was no significant difference in the percentage of Ly-6Chigh cells in these premonocytes.

Nanoparticle-Mediated Transfer of Pioglitazone Attenuates Features Associated With Plaque Ruptures in the Brachiocephalic Arteries of ApoE−/− Mice

To examine the effect of pioglitazone-NPs on atherosclerotic plaques, ApoE−/− mice were fed a high-fat diet and infused with angiotensin II. Angiotensin II accelerates monocyte/macrophage-mediated inflammation and shortens the period until the plaque rupture occurs when compared with high-fat diet alone. We treated these mice with weekly intravenous injections of pioglitazone-NPs or control-NPs for 4 weeks, and then the mice were euthanized for histological analyses (as shown in Figure I in the online-only Data Supplement). The body weight, lipid profile, serum glucose, and hemodynamic indices were similar among the groups (Table II in the online-only Data Supplement). Alternatively, another group of mice were treated with daily oral pioglitazone of an equivalent dose. The number of buried fibrous caps in the brachiocephalic arteries was determined as a surrogate marker of plaque rupture. Daily oral administration of 1 mg/kg pioglitazone did not significantly affect the number of buried fibrous caps, thickness of fibrous caps, or the plaque area (Figure 3A). Orally administered pioglitazone did not decrease macrophage accumulation as determined by Mac-3 staining. In contrast, nanoparticle-mediated delivery of an equivalent dose (7 mg/kg, weekly) of pioglitazone significantly decreased the number of buried fibrous caps and increased the fibrous cap thickness (Figure 3B). We also evaluated the plaque area at the aortic roots and whole aorta. There were no significant differences in the plaque area.

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<td>CD11b+Lin− premonocytes</td>
</tr>
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<td>Ly-6Chigh</td>
<td>Ly-6Chigh</td>
</tr>
<tr>
<td>Ly-6Clow</td>
<td>Ly-6Clow</td>
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</table>

Figure 1. Nanoparticle (NP)–mediated drug delivery to monocytes/macrophages. A, Distribution of NPs in peripheral blood leukocytes and the aorta. Peripheral blood and the aorta were analyzed by flow cytometry 2 days after injection of fluorescein isothiocyanate (FITC), FITC-NPs, or saline. Representative dot plots (left), histograms (middle), and quantitative data (right) are shown. B, Time course of the FITC signal £7 days after injection. **P<0.01 and ***P<0.001 vs the FITC group. C, Pioglitazone concentration in the plasma, spleen, kidney, and aorta (n=3 at each time point). Pioglitazone-NPs (7 mg/kg) were intravenously injected, and the mice were euthanized. 2, 12, and 48 hours later.
among each treatment group and the control group (Figures II and IVA in the online-only Data Supplement). Concomitant administrations of GW9662, a PPAR\(\gamma\) antagonist, abrogated the effect of pioglitazone-NPs, suggesting that the effect of pioglitazone-NPs was mediated via PPAR\(\gamma\) pathway (Figure V in the online-only Data Supplement).

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**Figure 2.** Anti-inflammatory effects of pioglitazone-nanoparticles (Pio-NPs) on peripheral blood monocytes. Flow cytometric analysis of peripheral blood 2 days after Pio-NP or control-NP injection. A, Representative dot plot and histogram. B, Cell counts of total monocytes, Ly-6C\(^{hi}\) monocytes, Ly-6C\(^{lo}\) monocytes, and peripheral monocyte polarity defined as Ly-6C\(^{hi}\) monocytes/Ly-6C\(^{lo}\) monocytes (n=5 per group). *\(P<0.05\). IV indicates intravenous.

**Figure 3.** Nanoparticle (NP)–mediated delivery of pioglitazone decreases buried fibrous caps in the brachiocephalic arteries. A and B, Photomicrographs of atherosclerotic plaques in the brachiocephalic arteries stained with Elastica van Gieson (EVG) and Mac-3 stained by immunohistochemistry (IHC) are shown (top) after 4 weeks of treatment. Scale bar, 200 \(\mu m\). Arrowheads indicate buried fibrous caps. The number of buried fibrous caps, fibrous cap thickness, plaque area, and Mac-3 positive area were compared (bottom) between oral pioglitazone (1 mg/kg, daily) and no treatment (A) and between pioglitazone-NPs (7 or 0.7 mg/kg, weekly) and control-NPs (B). Data are presented as the mean±SEM. Unpaired \(t\) tests (A) and 1-way ANOVA followed by Bonferroni multiple comparison test (B) were used for data analysis. *\(P<0.05\) and **\(P<0.01\) vs the control-NP group; n=10 per group. ns indicates not significant.
Collagen hue analysis indicated that pioglitazone-NPs had no significant effects on total collagen volume and the content of collagen (thin fiber or thick fiber; Figure VII in the online-only Data Supplement). Pioglitazone-NPs were also effective at a 0.1-fold dose (0.7 mg/kg, weekly). In the brachiocephalic arteries, pioglitazone-NPs did not decrease the plaque area or macrophage accumulation compared with control-NPs (Figure 3B). Macrophage accumulation, however, was inhibited by pioglitazone-NPs at the aortic root (Figure IVB in the online-only Data Supplement).

**Pioglitazone-NP Suppresses Proteinase Activity in the Brachiocephalic Artery**

The above results suggest that nanoparticle-mediated delivery of pioglitazone decreases buried fibrous caps by modulating macrophage functions rather than decreasing macrophage accumulation into the atherosclerotic plaques. Plaque macrophages produce several proteinases, including MMPs and cathepsins, which degrade the extracellular matrix and impair the mechanical strength of atherosclerotic plaques. To examine whether pioglitazone regulates these proteinases, we performed molecular imaging with near-infrared fluorescent probes and fluorescence reflectance imaging. As shown in Figure 4A, pioglitazone-NPs decreased MMP activity in brachiocephalic arteries. The activity of cathepsins, another relevant proteinase in atherosclerotic lesions, was also significantly decreased by pioglitazone-NPs (Figure 4B). We next analyzed the gene expression of aortic macrophages isolated from mice treated with pioglitazone-NPs to examine the effects of pioglitazone-NPs on macrophage functions in vivo. Pioglitazone-NPs tended to increase the expression of M2 markers, including arginase-1 and interleukin-10 (IL-10). In contrast, IL-6, MMP-9, and extracellular MMP inducer (EMMPRIN), an upstream protein that literally induces MMPs,24,25 tended to be suppressed by pioglitazone-NPs (Figure 4C). These data suggest that intravenously administered pioglitazone-NPs regulate macrophage polarity and proteinase activity in atherosclerotic lesions.

We examined whether intravenously administered pioglitazone-NPs regulate tissue macrophage polarity in another inflammatory model, thioglycollate-elicited peritonitis. Thioglycollate was injected 1 day before intravenous administration of pioglitazone-NPs. Three days later, peritoneal macrophages were collected, and a real-time polymerase chain reaction array (Mouse Atherosclerosis PCR Array; QIAGEN PAMM-038) was performed on RNA extracts. Real-time polymerase chain reaction revealed that intravenous pioglitazone-NPs induced a series of M2-related genes, such as endoglin, KLF2, ABCA1, and IL-4. Conversely, M1-related cytokines, including IL-1β, monocyte chemoattractant protein-1, and RANTES (regulated on activation, normal T cell expressed and secreted), as well as EMMPRIN (also known as basigin) and MMPs, were downregulated (Table). Flow cytometric analysis also demonstrated decreased EMMPRIN expression in the peritoneal macrophages from mice pretreated with pioglitazone-NPs (data not shown).

**Pioglitazone-NPs Regulate Macrophage Inflammatory Phenotypes and Functions**

Mouse bone marrow cells pretreated with pioglitazone-NPs were stimulated by interferon-γ and lipopolysaccharide to induce M1 polarization,26 and then the culture supernatants...
were analyzed using a cytokine array or quantitative ELISA. Interferon-γ and lipopolysaccharide indeed increased M1 macrophage markers, namely IL-1β, IL-6, and monocyte chemotactic protein-1, and decreased M2 macrophage markers, namely IL-4 and IL-10 (Figure 5A). Among these molecules, induction of IL-1β was significantly suppressed by pioglitazone-NP pretreatment, but the induction of IL-6 and monocyte chemoattractant protein-1 was not affected (Figure 5A). However, inhibition of an M2 cytokine, IL-4, was abrogated by pretreatment with pioglitazone-NPs (Figure 5A). Finally, we examined the expression of EMMPRIN in these bone marrow–derived macrophages by flow cytometry. Flow cytometric analysis revealed that bone marrow–derived macrophages expressed negligible levels of EMMPRIN in the resting state. M1 polarization by interferon-γ and lipopolysaccharide induced EMMPRIN expression, which was suppressed by pretreatment with pioglitazone-NPs (Figure 5B). M1 polarization significantly decreased CD206, a typical M2 macrophage marker, in macrophages, which were unaffected by pioglitazone-NPs (Figure 5C). Collectively, these data suggest that pretreatment of circulating monocytes with pioglitazone-NPs induces macrophage polarity toward a less inflammatory state and decreases EMMPRIN expression after differentiation into macrophages, which, in turn, leads to lower MMP activity in atherosclerotic lesions.

**No Significant Effects of Pioglitazone-NPs on the Expression of Epithelial Na⁺ Channel in the Kidneys**

In clinical practices, the use of pioglitazone is often hampered in patients with congestive heart failure because of the undesired side effect, that is, water retention. If nanoparticulation can avoid these undesired effects by reducing the dose of the

**Table. Gene Expression Change in Thioglycollate-Elicited Macrophages After Intravenous Administration of Pioglitazone-Nanoparticles**

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MMP indicates matrix metalloproteinase.

*P<0.05, **P<0.01.
incorporated drugs and changing in vivo distribution, clinical feasibility of this DDS increases. To address this point, we evaluated the expression of epithelial Na⁺ channel (ENaC) in the kidney. ENaC is one possible key molecule that is induced by PPARγ agonists and promotes reabsorption of urine sodium.27 Pioglitazone alone for 4 weeks increased the expressions of ENaCα and ENaCγ, whereas pioglitazone-NPs did not significantly increase the mRNA of these molecules (Figure 6).

**Discussion**

The novel findings of this study include the following: (1) PLGA nanoparticle–mediated intravenous administration of pioglitazone altered the inflammatory polarity of peripheral monocytes, (2) pioglitazone-NPs regulated tissue macrophage polarity toward less inflammatory (M2) phenotypes with suppression of the EMMPRIN/MMP pathway, and (3) pioglitazone-NPs inhibited atherosclerotic plaque destabilization and rupture more effectively than oral pioglitazone in a mouse model.

We have previously demonstrated that PLGA nanoparticles are taken up by various types of cells.6–10,23,28–32 For example, they were distributed in endothelial cells after injection into ischemic muscles, whereas intratracheal administration resulted in delivery to alveolar macrophages and small pulmonary arteries. When administered intravenously, nanoparticles were taken up by peripheral monocytes and neutrophils. In addition, a nanoparticle-mediated DDS showed an enhanced therapeutic efficacy in animals with chronic atherosclerosis, hindlimb ischemia, and pulmonary hypertension.8,28–32 The advantage of PLGA nanoparticle–mediated DDS in atherosclerotic animal models is an effective delivery of its contents to circulating monocytes that eventually migrate into aortic lesions and regulate inflammation. In this study, we demonstrated that intravenously administered PLGA nanoparticles were incorporated into circulating monocytes for 2 days and were observed in the tissue macrophages extracted from aortas at 2 and 7 days after administration. PLGA nanoparticles incorporated with pioglitazone skewed the Ly-6C<sup>high</sup>/Ly-6C<sup>low</sup> monocyte proportion. Flow cytometric analysis of bone marrow cells suggests that pioglitazone-NPs converted Ly-6C<sup>high</sup> monocytes into Ly-6C<sup>low</sup> not in peripheral blood.

Ly-6C<sup>high</sup> monocytes are characterized by high expression levels of CCR2 and rapidly enter inflammatory sites, including atherosclerotic plaques, where they give rise to macrophages. However, previous reports have suggested...
that Ly-6C^{low}CX3CR1^{high} monocytes also contribute to atherosclerotic plaque progression. Importantly, none of those studies focused on plaque stability or rupture. In this study, pioglitazone-NPs significantly altered the proportion of peripheral monocytes, whereas the total monocyte number did not decrease. Similarly, pioglitazone-NP–treated mice had significantly fewer buried fibrous caps, whereas plaque size and macrophage accumulation were unchanged. Our in vitro data show that the monocytes exposed to pioglitazone-NPs exerted lower proteolytic activity after subsequent differentiation into macrophages. Therefore, it is conceivable that the peripheral monocyte proportion skewed by pioglitazone-NPs is reflected in the less inflammatory and less proteolytic phenotypes of lesional macrophages. This concept is consistent with a previous report that found that PPARγ activation primes undifferentiated monocytes to become M2 macrophages, whereas the M1/M2 polarity of already differentiated macrophages in the plaque is not affected. Therefore, the beneficial effects of pioglitazone-NPs seem to be primarily because of their effects on circulating monocytes, which eventually differentiate into lesional macrophages. The observation that a negligible amount of nanoparticles was delivered to endothelial cells and smooth muscle cells supports our hypothesis.

Our in vivo data show apparent trends toward increased expression of several M2 markers, such as arginase-1 and IL-10 in aortic macrophages isolated from pioglitazone-NP–treated mice, whereas an M1 marker IL-6, MMP-9, and its inducer, EMMPRIN, tended to be suppressed. In accordance with these trends, local proteinase activities were significantly suppressed in the brachiocephalic arteries of pioglitazone-NP–treated mice. MMPs and cathepsins released by activated macrophages in plaques degrade the extracellular matrix, including collagens, and impair the mechanical strength of atherosclerotic lesions. In this study, total collagen and its content were not altered after the treatment with pioglitazone-NPs, suggesting that thinning of the superficial fibrous caps (Figure 3B) that comprise several fibers, including elastins and collagens, is critical for plaque ruptures rather than the total collagen amount. Indeed, clinical studies suggest that thin (<65 μm)–capped fibroatheroma is associated with cardiovascular events. Some reports show that PPARγ inhibits MMP activity in macrophages, but the detailed mechanism has not been clarified. Our results suggest a novel mechanism that PPAR activation inhibits MMP activity, at least in part, by suppressing its inducer, EMMPRIN, in inflammatory macrophages.

To the best of our knowledge, this is the first report to demonstrate that pioglitazone reduces features associated with atherosclerotic plaque ruptures. In this study, we used a nanoparticle-mediated DDS and found that clinically relevant low doses of pioglitazone (7 mg/kg and 0.7 mg/kg per week) could decrease the number of buried fibrous caps in the brachiocephalic arteries. By contrast, no significant therapeutic effect was observed in the oral treatment group (1 mg/kg per day), suggesting that nanoparticle-mediated DDS enhances the therapeutic effects by >10-fold because of optimized drug delivery, that is, effective delivery of pioglitazone into circulating monocytes.

The reason why the use of pioglitazone is hampered in the patients with cardiovascular diseases is that pioglitazone causes water retention and exacerbates heart failure. The detailed mechanisms of water retention are unclear, but ENaC-mediated sodium reabsorption in the collecting ducts is considered to be a possible mechanism. Our results showed that inductions of ENaCα and ENaCγ are abrogated by nanoparticulation of pioglitazone, suggesting that by using pioglitazone-NPs, undesired nonspecific systemic effects might be avoidable. In addition, we have quantified expressions of adiponectin and leptin, molecules associated with lipid metabolism/insulin sensitivity in the adipose tissue. At mRNA level, pioglitazone-NPs did not significantly change the expression of these molecules (data not shown), suggesting that pioglitazone-NPs decreased buried fibrous caps via monocyte/macrophage-mediated mechanisms. A possibility, however, cannot be excluded from the limited data that pioglitazone-NPs altered the expression of adiponectin or leptin in the adipose tissue and indirectly inhibited the progression of atherosclerosis.

As a limitation of this study, it is controversial whether buried fibrous caps really represent healed rupture. Some argue that such a layered structure can be explained by episodic plaque formation; therefore, this is not a well-established indicator of past plaque rupture. However, Rodgers et al adduced the following points supporting the notion that buried fibrous caps are healed ruptured plaques: (1) buried fibrous caps are often observed in the brachiocephalic arteries, where plaque rupture occurs frequently, but not in the aortic sinus where rupture is rare; (2) immunoreactive fibrin exists at the site of buried fibrous caps; and (3) pravastatin treatment or deletion of cathepsin S decreases the number of buried fibrous caps. These data suggest that buried fibrous caps are not merely the result of episodic plaque growth but evidence of healed ruptured plaques. To date, buried fibrous caps are the most convincing evidence of past plaque ruptures. Second, the bioavailability of oral pioglitazone is high but not 100% (81% in mice). Therefore, the dose of oral pioglitazone may not be completely comparable with dose of the intravenously administered pioglitazone. Further examinations, including dose optimization, are necessary in large animals to translate the mouse results into humans.

In conclusion, nanoparticle-mediated intracellular delivery of pioglitazone reduced plaque ruptures in mouse brachiocephalic arteries by decreasing circulating inflammatory monocytes and suppressing the proteinase activity of plaque macrophages. These results suggest that PPARγ-targeting therapy by PLGA nanoparticles is a clinically feasible and promising strategy to prevent acute thrombotic complications and improve the prognosis of patients with CAD.

**Acknowledgments**

We thank Eiko Iwata, Miho Miyagawa, and Satomi Abe for their excellent technical assistance.

**Sources of Funding**

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K. Egashira holds a patent on the results reported in this study. The other authors report no conflicts.

References


8. Nakashiro et al. Pioglitazone-NPs for Plaque Stabilization 499


One of the ultimate goals in the treatment of coronary artery disease is the prevention of acute myocardial infarction. Current medical therapies, including statins, are not sufficient, and substantial risks of major cardiovascular complications exist. Accumulating evidence suggests that macrophages play important roles in the pathobiology of plaque rupture by releasing proteinases that degrade the extracellular matrix. Here, we focused on peroxisome proliferator–activated receptor-γ, a nuclear receptor regulating cellular differentiation and metabolism. Peroxisome proliferator–activated receptor-γ induced polarity shifts of monocytes/macrophages toward less inflammatory phenotypes. Poly(lactic-co-glycolic-acid) nanoparticles, incorporating the peroxisome proliferator–activated receptor-γ agonist pioglitazone, were effectively delivered to circulating monocytes, as well as lesional macrophages, and decreased buried fibrous caps, a surrogate marker of plaque ruptures, in the brachiocephalic arteries of hyperlipidemic mice. Pioglitazone is currently used as an antidiabetic drug, whereas poly(lactic-co-glycolic-acid) is a clinically approved bioabsorbable polymer. Therefore, this combination could be a clinically feasible strategy to prevent acute thrombotic complications in patients with coronary artery disease.
Pioglitazone-Incorporated Nanoparticles Prevent Plaque Destabilization and Rupture by Regulating Monocyte/Macrophage Differentiation in ApoE−/− Mice
Soichi Nakashiro, Tetsuya Matoba, Ryuta Umezu, Jun-ichiro Koga, Masaki Tokutome, Shunsuke Katsuki, Kaku Nakano, Kenji Sunagawa and Kensuke Egashira

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Materials and Methods

Experimental animals
All study protocols were reviewed and approved by the Committee on the Ethics of Animal Experiments of Kyushu University Graduate School of Medical Sciences. ApoE<sup>-/-</sup> mice (B6.129P2-Apo<sup>em1Unc</sup>/J, stock No.002052, C57Bl/6J background) were purchased from Jackson Laboratory (Bar Harbor, ME. USA). All mice were maintained on a 12-hour light-dark cycle and fed either normal chow or a high-fat diet and water ad libitum.

Preparation of nanoparticles
PLGA-NPs containing indicated dose of pioglitazone with an average diameter of 247 nm were prepared by an emulsion solvent diffusion method as previously reported. The average molecular weight of PLGA was 20,000. PLGA-NPs containing fluorescein isothiocyanate (Dojindo laboratories, Kumamoto, Japan) (FITC-NPs) were prepared to examine the in vivo kinetics of nanoparticles.

Measurements of pioglitazone concentrations
For pharmacokinetic analyses, pioglitazone-NPs were intravenously injected. After euthanization at the indicated time points, tissue homogenates were prepared and diluted in methanol. Pioglitazone concentrations were determined by high-performance liquid chromatography-tandem mass spectrometry (LC/MS/MS) as follows: separation of the sample was performed on a 150 × 2 mm, 5 µm analytical column (Develosil C30-UG-5, Nomura Chemical) at 40 °C over 7 minutes with a flow rate of 0.2 mL/min. The high-performance liquid chromatography was next coupled to tandem mass spectrometry by positive turbo ion spray (5500 V).

Flow cytometric analysis
Flow cytometric data were acquired on a FACSCalibur (BD Biosciences, San Jose, CA. USA) or Gallios (Beckman Coulter, Fullerton, CA. USA) cytometer. For peripheral blood samples, red blood cells were removed by hemolysis (VersaLyse, Beckman Coulter) and centrifugation at 240×g for 5 minutes at 4 °C. Thereafter, the remaining leukocytes were washed once with Hanks’ balanced salt solution (HBSS) (Thermo Fisher Scientific, Waltham, MA). Bone marrow cells were isolated from the tibia as previously reported. After blocking the Fc receptor with anti-CD16/32 mAb (BD Pharmingen, San Diego, CA. USA) for 5 minutes at 4 °C, the cells were incubated with a mixture of fluorochrome-labeled monoclonal antibodies for 1 hour at 4 °C. Aorta samples were minced with fine scissors, placed into a cocktail of 450 U/ml collagenase type I, 125 U/ml collagenase type XI, 60 U/ml DNase I and 60 U/ml hyaluronidase (all enzymes were obtained from Sigma-Aldrich, St. Louis, MO. USA) in phosphate buffered saline (PBS) containing 20 mM Hepes and shaken at 37 °C for 1 hour. Then, the cells were washed once with HBSS and filtered through a 70 µm cell
strainer. The blocking and staining procedures were performed as described above. Data were analyzed with FlowJo or Kaluza software. Monocytes/macrophages were identified as CD11b\text{high} lineage markers (including Ly6G, NK1.1, CD90, CD49b, B220)\text{low} and Ly6C\text{high/low}, neutrophils were identified as CD11b\text{high} lineage markers\text{high}, and lymphocytes were defined as CD11b\text{low} lineage markers\text{high}. For in vitro experiments, the macrophage purity was confirmed by high expression of CD11b and F4/80 and low expression of lineage markers. The following antibodies were used: anti-CD90, 53-2.1 (BD Pharmingen), anti-B220, RA3-6B2 (BD Pharmingen), anti-CD49b, DX5 (BD Pharmingen), anti-NK1.1, PK136 (BD Pharmingen), anti-Ly6G, 1A8 (BD Pharmingen), anti-CD11b, M1/70 (BD Pharmingen), anti- Ly6C, AL-21 (BD Pharmingen), anti-mouse CD147, OX-114 (BioLegend, San Diego, CA. USA), anti-F4/80, BM8 (BioLegend) and anti-mouse CD206, C068C2 (BioLegend).

**Animal experiments**

Male ApoE\text{−/−} mice were fed a high-fat diet containing 21% fat from lard supplemented with 0.15% cholesterol beginning at 16 weeks of age. From 20 weeks of age, angiotensin II (Sigma Aldrich) was intraperitoneally administered at 1.9 mg/kg/day by an osmotic mini-pump (Alzet, Cupertino, CA. USA). Mice were euthanized at 24 weeks of age, and blood samples were collected from the apex of the right ventricle. After perfusion with saline with physiological pressure, the brachiocephalic arteries were dissected for histological analyses. In several experiments, aortas were excised for flow cytometric analysis or semi-quantitative real-time PCR. PPARγ antagonist, GW9662 (2.0 mg/kg) (Wako Pure Chemical Industries, Osaka, JAPAN) was intraperitoneally injected 3 times a week for 4 weeks to the animals indicated elsewhere.

**Histopathology and immunohistochemistry**

Serial paraffin sections were prepared for histopathological analyses. Briefly, the brachiocephalic artery was fixed overnight in 10% buffered formalin and then embedded in paraffin. Serial cross sections (3 µm thick) throughout the entire length of the artery were prepared, and lesion characteristics were analyzed every 36 µm along the vessel after Elastica van Gieson staining and hematoxylin-eosin staining. The sections were also subjected to immunostaining for macrophages using a rat anti-mouse Mac-3 (LAMP-2, M3/84) antibody (Santa Cruz Biotechnology, Dallas, TX. USA). Normal rat IgG (Santa Cruz Biotechnology) was used as a negative control. After overnight incubation with the primary antibody at 4 °C, the sections were washed 3 times with PBS and incubated with biotinylated goat anti-rat IgG (Santa Cruz Biotechnology). Then, the sections were incubated with streptavidin-peroxidase and diaminobenzidine (DAB) substrate for visualization and counterstained with hematoxylin. FITC was stained with anti-FITC antibody (American Research Products, Inc., Waltham, MA) and following secondary antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific).

In addition, aortic root sections (5 µm thick) were also prepared for histological analyses. Briefly, atherosclerotic plaques were evaluated at 3 sections, each separated by 100
μm, with the most proximal site starting from where at least two aortic valve leaflets first appeared. The area of each plaque was quantified, and the largest value was used to represent the atherosclerotic burden of the animal. Analysis was performed with a microscope with a computerized, digital image analysis system and ImageJ Software.

**Molecular imaging of proteinase activity**
Fluorescent reflectance imaging (FRI) was performed to evaluate macrophage accumulation and proteinase activity, including MMPs and cathepsins, in vivo as previously reported. We used the activatable fluorescent imaging agents ProSense 680 (Ex/Em=680/700 nm) and MMPSense 750 (Ex/Em = 749/775 nm) (PerkinElmer, Inc., Waltham, MA. USA). These agents were administered via the femoral vein 24 hours before imaging. After surgical exposure of the brachiocephalic arteries, fluorescent signals from the activated agents were quantified using an FRI system (FMT2000, PerkinElmer, Inc).

**Collagen hue analysis**
In sections stained with picrosirius red (Polyscience, Inc., Warrington, PA), fibrillar collagen was observed under a circularly polarized microscope with green and red optic filters (HQ535/50m, D605/55m), as previously described. As the thickness of collagen fiber increases, the color shifts from green to red.

**Preparation of primary macrophages**
Peritoneal macrophages were prepared for in vitro experiments as previously described. Brewer’s thioglycollate medium (BD Diagnostic Systems, Sparks, MD) was injected into the peritoneal cavity 3 to 4 days before macrophage collection. Ice-cold PBS was injected into the peritoneal cavity, and cells were harvested. Then, the cells were washed with PBS, resuspended in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and plated on culture dishes. Two hours later, nonadherent cells were removed, and the remaining adherent cells were cultured further in fresh medium. Bone marrow derived macrophages were prepared by stimulating bone marrow cells, which were harvested from the tibiae and femurs of donor mice with 20 ng/mL macrophage colony stimulating factor (M-CSF) in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Aortic macrophages were isolated as follows. The aorta was cut into small pieces and incubated with enzyme solution including 450 U/mL collagenase type I, 125 U/mL collagenase type XI, 60 U/mL DNase I and 60 U/mL hyaluronidase (all enzyme were obtained from Sigma-Aldrich) in PBS containing 20 mM Hepes at 37°C for 1 hr. After filtration through a 40-μm cell strainer (BD Pharmingen), cells were used for further experiments. In real-time PCR of isolated macrophages, F4/80 positive macrophages were isolated by magnetic sorting. PE-conjugated anti-F4/80 antibody (BioLegend, San Diego, CA) were used as the primary antibody to specifically select macrophages.
Semi-quantitative real-time PCR
Tissue macrophages were isolated from the digested aortas using PE-conjugated anti-mouse F4/80 antibody and EasySep mouse PE selection kit (STEMCELL Technologies, Vancouver, Canada). Kidney and visceral fat were homogenized by TissueLyserII (QIAGEN, Venlo, Netherlands). RNA was extracted from aortic macrophages, peritoneal macrophages or visceral fat with ISOGEN (Nippon Gene, Tokyo, Japan) or an illustra RNAspin Mini kit (GE Healthcare, PA, USA), and cDNA was synthesized with a PrimeScript RT reagent Kit (Perfect Real Time, TAKARA BIO Inc. Shiga, Japan). Semi-quantitative real-time PCR was performed with SYBR Premix DimerEraser (Perfect Real Time, TAKARA BIO Inc.) and a StepOnePlus real-time PCR system (Applied Biosystems, MA, USA). The primer sequences are listed in Supplemental Table 1. Data were calculated by the ΔΔCt method and expressed in arbitrary units that were normalized to the β-actin or GAPDH levels.

Statistics
All data are reported as the mean ± SEM. Statistical analysis of differences was performed using Student’s t-test for comparisons of two groups, and multiple groups were evaluated with one way ANOVA followed by Bonferroni’s or Dunnett’s multiple comparison tests unless otherwise stated. P values < 0.05 were considered statistically significant.
References


In Vivo Experimental Protocol

- Male apoE-/- mice
- High Fat Diet
- Angiotensin II

Treatment for 4 weeks:
- No treatment control
- Weekly IV of control NP
- Daily oral Pio (1mg/kg/day)
- Weekly IV of Pio-NP (7mg/kg/week)
- Weekly IV of Pio-NP (0.7mg/kg/week)

Supplemental figure I
Supplemental figure I. In vivo experimental protocol. At 16-18 weeks of age, male ApoE-/- mice began receiving high fat diet. Four weeks later, osmotic mini-pump (Alzet, Cupertino, CA, USA) was implanted and angiotensin II infusion at 1.9mg/kg per day was started. We treated them for 4 weeks with weekly injection of pioglitazone-NP, control NP, or daily oral pioglitazone. Systolic blood pressure and heart rate were measured by the tail-cuff method and body weight was measured. At the end of the treatment period (24 weeks of age), the mice were euthanized and the brachiocephalic arteries and aortic roots were excised for histological analysis.
Supplemental figure II

A

NT: No treatment
OP(1): Oral pio (1 mg/kg/day)

B

CN: Control NP
PN(0.7): Pio-NP (0.7 mg/kg/wk)
PN(7): Pio-NP (7 mg/kg/wk)
Supplemental figure II. Atherosclerotic plaque size at aortic root. Aortic root sections excised from mice treated with daily oral pioglitazone (A), and weekly intravenous pioglitazone-NP (B) were stained with EVG. Representative photomicrographs and quantitative analysis of plaque size are shown. Scale bar indicates 200nm.
Supplemental figure III. Distribution of PLGA nanoparticles after intravenous injection. PLGA incorporating fluorescent marker (FITC) was injected by tail vein and the brachiocephalic artery was observed 24 hours later. The left picture shows negative control without immunostaining of FITC. Right picture shows FITC stained by anti-FITC antibody. White arrow heads indicate macrophages incorporating FITC. Red arrow head indicates endothelial cells. Cont-NP; control nanoparticles, Pio-NP; pioglitazone nanoparticles. Scale bar 20 µm.
Supplemental figure IV

A. Plaque area (whole aorta, oil red O)

B. Mac-3 (aortic root)
Supplemental figure IV. Nanoparticle-mediated delivery of pioglitazone decreases macrophage accumulation in the aortic root. (A) En face oil red O staining of the aorta harvested from ApoE⁻/⁻ mice fed a HFD and infused angiotensin II (right). The left graph shows quantitative analysis of plaque area determined as the percentage of oil red O positive area per entire surface of the aorta. N=9 and 10. (B) Macrophage accumulation was determined by Mac-3 staining. N=6. Scale bar 100 μm.
Supplemental figure V

Number of buried fibrous caps

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of Caps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont-NP</td>
<td>3</td>
</tr>
<tr>
<td>Pio-NP</td>
<td>1</td>
</tr>
<tr>
<td>Pio-NP + GW9662</td>
<td>3</td>
</tr>
</tbody>
</table>

p < 0.01  p < 0.01
Supplemental figure V. The effect of pioglitazone nanoparticles was abrogated by GW9662. PPARγ antagonist, GW9662 (2 mg/kg) was intraperitoneally injected three times a week to ApoE−/− mice fed a HFD and infused angiotensin II. Upper panel shows pictures of the brachiocephalic arteries stained with elastica van Gieson staining. Lower panel shows quantitative data. N=9, 10 and 11. Scale bar indicates 200 µm.
Supplemental figure VI

A

Cont-NP

Ly-6C

CD11b

Lin

19.4±2.5%

91.7±3.4%

Pio-NP

Ly-6C

CD11b

Lin

19.7±3.8%

91.4±2.7%

B

CD11b^+^Lin^-^ cells

Ly-6C^{high} cells

NS

NS
Supplemental figure VI. Flow cytometry analysis of bone marrow premonocytes and Ly-6C expression. Bone marrow cells were isolated from ApoE£¬£¬ mice fed a HFD and infused angiotensin II. Weekly injection of Pioglitazone nanoparticles (Pio-NP) had no effect on the number of CD11b£¬Lin£¬ premonocytes and Ly-6C£¬ cells. NS; not significant, Cont-NP; control nanoparticles, Pio-NP; pioglitazone nanoparticles. N=6.
Supplemental figure VII

Cont-NP  

Pio-NP

Oral Pio

% Collagen

% Red

% Green

Cont-NP  Pioglitazone-NP  Oral pioglitazone
Supplemental figure VII. Collagen hue analysis. The brachiocephalic arteries were stained with picro-sirius red solution. Above pictures show the intima of brachiocephalic arteries observed under a circularity polarized microscopy (yellow). Collagen hue was analyzed with red (middle) and green (right) filters. Graphs show quantitative data indicating percentage of total collagen (left), thick collagen fibers (middle) and thin collagen fibers (right) in the intima of ApoE−/− mice treated with control nanoparticles (Cont-NP), pioglitazone nanoparticles (Pio-NP) or oral pioglitazone. N=8,14 and 6. Scale bar indicates 100 µm.
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<tr>
<th>Gene</th>
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<th>Reverse primer</th>
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<tr>
<td><strong>Arg</strong>1</td>
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<td><strong>Mmp9</strong></td>
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<td>5'-AGATAGCAAATTGAGCT-3'</td>
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<tr>
<td><strong>Bsg</strong></td>
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<td>5'-GCATCGGACTTGAGACCA-3'</td>
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<tr>
<td><strong>iNos</strong></td>
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<td><strong>Actb</strong></td>
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<td><strong>Gapdh</strong></td>
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**Supplemental Table II: Physical, metabolic and hemodynamic parameters**

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<th>No Treatment</th>
<th>Control NP</th>
<th>Pioglitazone-NP</th>
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<tr>
<td>Body weight (g)</td>
<td>29.8±1.2</td>
<td>28.2±0.7</td>
<td>28.0±1.0</td>
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<td>Glucose (mg/dl)</td>
<td>101.3±24.5</td>
<td>199.5±52.2</td>
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<td>Total cholesterol (mg/dl)</td>
<td>687.9±52.0</td>
<td>734.1±60.6</td>
<td>772.8±42.8</td>
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<tr>
<td>Triglyceride (mg/dl)</td>
<td>43.3±12.8</td>
<td>47.4±11.5</td>
<td>38.0±9.9</td>
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<tr>
<td>Heart rate (/min)</td>
<td>605.0±30.1</td>
<td>643.1±33.8</td>
<td>634.3±20.7</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>127.7±5.4</td>
<td>126.7±5.1</td>
<td>129.2±4.7</td>
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