Pioglitazone Attenuates Valvular Calcification Induced by Hypercholesterolemia

Yi Chu,* Donald D. Lund,* Robert M. Weiss, Robert M. Brooks, Hardik Doshi, Georges P. Hajj, Curt D. Sigmund, Donald D. Heistad

Objective—Development of calcific aortic valve stenosis involves multiple signaling pathways, which may be modulated by peroxisome proliferator-activated receptor-γ. This study tested the hypothesis that pioglitazone (Pio), a ligand for peroxisome proliferator-activated receptor-γ, inhibits calcification of the aortic valve in hypercholesterolemic mice.

Methods and Results—Low density lipoprotein receptor−/−apolipoprotein B100/100 mice were fed a Western-type diet with or without Pio (20 mg/kg per day) for 6 months. Pio attenuated lipid deposition and calcification in the aortic valve, but not aorta. In the aortic valve, Pio reduced levels of active caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labeling staining. Valve function (echocardiography) was significantly improved by Pio. To determine whether changes in gene expression are associated with differential effects of Pio on aortic valves versus aorta, Reversa mice were fed Western diet with or without Pio for 2 months. Several procollagen genes were increased by Western diet, and the increase was attenuated by Pio, in aortic valve, but not aorta.

Conclusion—Pio attenuates lipid deposition, calcification, and apoptosis in aortic valves of hypercholesterolemic mice, improves aortic valve function, and exhibits preferential effects on aortic valves versus aorta. We suggest that Pio protects against calcific aortic valve stenosis, and Pio or other peroxisome proliferator-activated receptor-γ ligands may be useful for early intervention to prevent or slow stenosis of aortic valves. (Arterioscler Thromb Vasc Biol. 2013;33:523-532.)

Key Words: calcific aortic valve stenosis ■ echocardiography ■ hypercholesterolemia ■ peroxisome proliferator-activated receptor-γ ■ valvular/vascular calcification

Calcification occurs in atherosclerotic lesions and in the aortic valve.1 The presence of osteoblasts in atherosclerotic lesions and in calcific aortic valve stenosis (CAVS) implies that calcification is an active, regulated process,2,3 as first proposed by Demer and colleagues.4 If calcification is active, from pro-osteogenic pathways, one might expect that development and progression of calcification could be inhibited. Several experimental findings suggest that peroxisome proliferator-activated receptor-γ (PPARγ) may protect against cardiovascular calcification. First, PPARγ in the vascular wall and several cell types protects against development of atherosclerosis.5-8 Second, PPARγ impairs differentiation of progenitor cells into osteoblasts,9 and inhibition of PPARγ increases differentiation of embryonic stem cells to osteoblasts.10 Third, oxidative stress and inflammation appear to play an important role in vascular calcification and CAVS,11-16 and PPARγ is antiinflammatory.17,18

Multiple signaling pathways appear to be important in the pathophysiology of vascular calcification and CAVS. PPARγ is an attractive intervention to inhibit cardiovascular calcification because, instead of targeting a single mechanism, PPARγ affects a cluster of genes,19,20 and thus may protect against calcification at multiple levels.

Activation of PPARγ by thiazolidinedione (TZD) ligands is used commonly for treatment of patients with impaired glucose tolerance and type II diabetes mellitus.21 To minimize the effect on metabolism, we used a relatively low dose of pioglitazone (Pio) at which no effect on plasma glucose or body weight was observed. The first goal of this study was to test the hypothesis that chronic administration of Pio, a TZD, inhibits calcification of the aortic valve in hypercholesterolemic mice. A unique aspect of this study was to examine calcification in both the aortic valve and aorta, in which mechanisms and functional consequences may differ. The second goal was to examine molecular mechanisms by which Pio may affect calcification in vivo. We examined mechanisms that mediate an osteogenic pathway,20,22 and measured levels of active caspase-3 (as a
reflection of a possible role of cell death in calcification). If Pio is effective in slowing CAVS, the findings would imply that a TZD could potentially be clinically useful in slowing the development of CAVS.

Materials and Methods

Animals

Female low density lipoprotein receptor–/–/apolipoprotein B100/100 (LA) mice were fed normal chow until 2 months of age, and then were fed normal chow, Western diet (WD; Teklad #TD88137), or WD+Pio (20 mg/kg per day). At 8 months of age, echocardiograms were performed, plasma was obtained, and aortic valves and ascending aorta were harvested for histological/immunohistological studies (Figure 1A).

Another study was performed to examine earlier effects of Pio on gene expression in aortic valves and aorta. We used Reversa mice, which are similar to LA, except with insertion oflox sites flanking the Mttp gene and the Cre gene under the control of the promoter of the interferon-inducible Mx-1 protein (Figure 1A).24

Evaluation of Aortic Valve Function

Aortic valve function was evaluated, as described previously. Briefly, mice were sedated with midazolam 0.15 mg subcutaneously and cradled in the left lateral recumbent position, while a 30-MHz linear-array probe was applied horizontally to the chest. The imaging probe was coupled to an imager (Vevo 2100, VisualSonics, Toronto) generating 200 frames (2-dimensional) per second in both short- and long-axis left ventricular planes. Images of the aortic valve were acquired in M-mode, at a nominal sampling rate of 1000 frames per second, with 2-dimensional images used for guidance.

Plasma Measurements

Total cholesterol was measured using the CHOD-DAOS method (Wako #439–17501). Plasma glucose was measured with AccuChek test strips (Roche). Plasma adiponectin was measured with ELISA (R&D). Serum amyloid A was measured in plasma using a mouse serum amyloid A ELISA kit (Invitrogen).

Histology, Immunofluorescence, and Immunohistochemistry

The aortic valve and aorta, embedded in optimal cutting temperature compound, were cut in 10-μm-thick sections, and stained with Alizarin Red (Sigma) for calcium, Oil Red O (Sigma) for lipid, and Masson trichrome or picrosirius red for collagen. The number of pixels expressing red (Alizarin Red, Oil Red O, or picrosirius red) or blue (Masson) staining was quantified, as described previously. Data are expressed as percentage of valve area that displays positive staining.

Immunofluorescence of osterix was performed, as described previously. Immunohistochemistry of osteocalcin (rabbit antibody purchased from ABBIOTECH) and active caspase-3 (rabbit antibody from Cell Signaling) were performed as given below. Sections were fixed with phosphate-buffered paraformaldehyde (4%), and treated with 3% hydrogen peroxide to inactivate endogenous peroxidase. After blockage with 10% BSA in TBS/0.3% Triton X-100, sections were incubated with a primary antibody, or normal rabbit IgG as negative control, at 4°C overnight. After rinses in PBS, sections were incubated with SignalStain Boost IHC Detection Reagent (horse radish peroxidase, Rabbit, Cell Signaling). After rinses in PBS, sections were incubated in 3,3-diaminobenzidine solution (Vector) for exactly 3 to 9 minutes. Slides were dehydrated, and mounted with coverslips. Images were digitally photographed, and brown staining was quantified, as described previously.

Figure 1. A, Summary of experimental protocol. B, Effects of pioglitazone on calcification of aortic valve and aorta of low density lipoprotein receptor–/–/apolipoprotein B100/100 (LA) mice. Calcification of the aortic valve and ascending aorta was measured as percentage of area stained with Alizarin Red in the base of aortic valves and the ascending aorta. Pioglitazone attenuated calcification of the aortic valve, but not ascending aorta, produced by Western diet (WD) in LA mice (n=9–13). C, Effects of pioglitazone on lipid deposition in aortic valve and ascending aorta of LA mice. Lipid deposition was measured as percentage of area stained with Oil Red O. Pioglitazone attenuated lipid deposition produced by WD in aortic valves, but not in the aorta (n=5–8). D, Effects of pioglitazone on collagen in aortic valve and ascending aorta of LA mice. Collagen was measured as percentage of area stained with Masson staining. Pioglitazone, not WD, had an effect on collagen (n=6–8). Values are mean±SE; *P<0.05 vs chow; and **P<0.05 vs WD.
Comparison of Gene Expression in Aortas Versus Aortic Valves

Total RNA of aorta and aortic valves was isolated using TriZol extraction followed by Qiagen miRNaseasy mini kit. Aortic valves were harvested from 80-µm-thick optimal cutting temperature compound-embedded sections under a stereo scope. Reverse transcription was performed, as described previously. Quantitative PCR for a gene of interest (6-carboxyfluorescein) with TaqMan primers ordered from Applied Biosystems (with a few ordered from integrated DNA technologies) was performed with the normalizer glyceraldehyde 3-phosphate dehydrogenase (VIC) in the same well.

Statistical Analyses

All data are reported as mean±SE. Significant differences (P<0.05) between groups were detected using 1-way ANOVA followed by Student-Newman-Keuls tests.

Results

Plasma Levels

Total plasma cholesterol and nonfasting plasma glucose were not altered by Pio (20 mg/kg per day; Table 1). Plasma adiponectin was increased by Pio, as expected. Plasma serum amyloid A, a systemic inflammation marker in mice, was increased in mice fed WD, and Pio attenuated the increase in serum amyloid A (Table 1).

Calcification, Lipid Deposition, and Fibrosis in Aortic Valves and Aorta

There were small amounts of calcium in the aortic valve of chow-fed LA mice. WD significantly increased calcification of the aortic valve (Figure 1B). Pio attenuated the increase in calcification of the valve (Figure 1B). In aorta, WD did not prevent the increase in calcium in LA mice fed WD (Figure 1B; see Figure I in the online-only Data Supplement for images of staining). Thus, Pio attenuates calcification in aortic valves, but not in aorta.

Lipid deposition in the aortic valve was greater when LA mice were fed WD than chow (Figure 1C). Pio attenuated the increase in lipid deposition produced by WD (Figure 1C; see Figure II in the online-only Data Supplement for images of staining). In the aorta, WD did not increase lipid deposition significantly, and Pio did not have an effect (Figure 1C).

Masson staining indicated no difference in collagen among the 3 groups in both aortic valves and aortas (Figure 1D), which suggests that fibrosis is not changed by Pio (see Figure III in the online-only Data Supplement for images of staining). As also studied with picrosirius red staining, fibrosis in aortic valves was not reduced by Pio (Figure IV in the online-only Data Supplement).

Protein Expression in Aortic Valve and Aorta

Expression in the aortic valve of osterix, a procalcific transcription factor, and osteocalcin, an osteoblast marker, was not increased by WD, and was not altered by Pio (Figure 2A). Active caspase-3, a marker for apoptosis, tended to increase in the aortic valve during WD, and Pio prevented the increase (Figure 2B; see Figure V in the online-only Data Supplement for images of staining). In contrast, there was no difference in active caspase-3 among the 3 groups in the aorta (Figure 2B). Double-staining confocal images suggest that active caspase-3–positive cells colocalized with several markers, and thus could be osteoblast-like cells (positive for Cbfa1), myofibroblasts (positive for α-smooth muscle actin), and macrophages (positive for F4/80; Figure 2C). Terminal deoxynucleotidyl transferase dUTP nick end labeling staining also indicated that Pio attenuated apoptosis in aortic valves (see Figure VI in the online-only Data Supplement).

Differences in Gene Expression in Aortic Valves Versus Aorta

Rbp7 responded similarly in aortic valves and aorta to WD and Pio, which suggests that Pio activated PPARγ in both tissues (Figure 3). Cbfa1 expression, reflecting osteoblastic transformation, increased markedly in response to WD in aortic valves, but not in aorta. Pio strongly attenuated the WD-induced increase in Cbfa1 expression in aortic valves (Figure 3). Expression of osteocalcin, an osteoblast-specific gene encoding a bone matrix protein, was significantly increased by the WD, both in aortic valves and aorta. Pio eliminated the increase in osteocalcin expression associated with the WD (Figure 3). Expression of the proinflammatory gene, interleukin (IL)-6, was significantly increased by the WD, and decreased by Pio, only in aortic valves (Figure 3). Among profibrotic genes, collagen 1a2 responded to WD and Pio only in aortic valves (Figure 3). Among the 36 genes examined, 18 genes demonstrated different expression patterns in aortic valves versus aorta (Table 2).

Table 1. Effects of Pioglitazone (20 mg/kg per day) on Plasma of LA Mice After 6 Months of Treatment

<table>
<thead>
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<th>Chow</th>
<th>Western Diet</th>
<th>Western Diet +Pio</th>
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<tr>
<td>Cholesterol, mg/dL</td>
<td>298±16</td>
<td>1233±67*</td>
<td>1167±37*</td>
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<td>Nonfasting glucose, mg/dL</td>
<td>247±7</td>
<td>254±11</td>
<td>264±10</td>
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<tr>
<td>Adiponectin, mg/dL</td>
<td>119±2.6</td>
<td>109±4.0</td>
<td>154±2.8**</td>
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<td>Serum amyloid A, µg/dL</td>
<td>8.0±0.03</td>
<td>13.2±0.9*</td>
<td>11.1±0.7**</td>
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</table>

LA indicates low density lipoprotein receptor–/-/apolipoprotein B100/100, and Pio, pioglitazone.

Values are mean±SE; n=9–13.

*P<0.05 vs chow.

**P<0.05 vs Western diet.
In LA mice fed a WD beginning at 2 months of age, treatment with Pio for 6 months resulted in ≈20% greater aortic cusp separation, a measure of aortic valve function that we have previously validated hemodynamically,14 compared with LA mice fed WD that did not receive Pio (Figure 4). The difference corresponds nominally to ≈44% greater systolic valve area in Pio-treated mice.

Pio did not affect heart rate, cardiac output, or left ventricular mass, but was associated with increased left ventricular end-systolic/diastolic volume and decreased left ventricular ejection fraction in LA mice that were fed a WD (Table II and Figure VII in the online-only Data Supplement).

Regarding left ventricular (LV) dilation and reduced systolic function, the midsystolic anteroposterior diameter of the aortic root at the level of the sinuses of Valsalva was 1.65±0.04 mm in LA mice receiving WD alone, and 1.70±0.06 mm in LA mice receiving WD+Pio (P=not significant). The diameters were measured using the same M-mode echo image sets, as were used to determine aortic valve cusp separation (Figure 4). Mid-systole and sinuses of Valsalva were chosen because they represent the time and anatomic location corresponding to maximal separation of aortic valve cusps. Thus, because there was no significant difference between Pio-treated and untreated LA mice, with respect to aortic root diameter, aortic root size does not explain protection of aortic valve function by Pio. In addition, diameter of sinus of Valsalva was greater than aortic cusp separation in both groups, which argues against restraint of cusp excursion by the aortic root, at the stage of disease reported here.

**Discussion**

The major findings in this study are that, in the aortic valve of hypercholesterolemic LA mice that are susceptible to CAVS, Pio (1) prevented deposition of lipids during WD, (2) attenuated apoptosis, and (3) attenuated calcification and impairment of cusp mobility in the aortic valve, but had no effect on fibrosis. The findings suggest that Pio protects against valvular calcification, in part, by inhibition of apoptosis. In the aorta, Pio failed to reduce lipid deposition or calcification, despite positive effects on gene expression of Rbp7 (a PPARγ target), proinflammatory cytokines tumor necrosis factor-α and IL-6, and bone morphogenetic protein 2 (a signaling molecule in a pro-osteogenic pathway; Table I and Figure VII in the online-only Data Supplement).

Comparison of gene expression in the aortic valve versus aorta indicates that many genes were expressed in a different pattern in the aortic valve versus aorta, which supports the observation of different responses to WD and Pio of the aortic valve and aorta.

**Pio and Lipid Deposition**

Early studies suggested that PPARγ might be proatherosclerotic, as oxidized linoleic acids in oxidized low density lipoprotein activate PPARγ, which upregulates CD36, which mediates uptake of oxidized low density lipoprotein.27,28 The
first in vivo study using rosiglitazone (a TZD) and GW7845 (a non-TZD activator of PPARγ) in WD-fed LDLr-deficient mice, however, indicated that activation of PPARγ is antiatherosclerotic in male, but not female, mice despite upregulation of CD36.29 Although the mechanisms for the sex difference are not known, PPARγ activation produced greater inhibition of TNFα and matrix metallopeptidase 9 in aortas of male than female mice, and PPARγ activation worsened the lipid profile in female mice.29 Subsequent studies in vivo, using other PPARγ agonists in WD-fed LDLr- or apoE-deficient mice, confirmed that PPARγ activation protects against development of atherosclerosis, but does not reverse advanced atherosclerosis.5,30

We studied female mice as a more stringent test for effects of PIO on the aortic valve. Our findings in the aortic valve that PIO attenuates calcification, lipid deposition, and apoptosis, and improves valve function, therefore, occurred even though one might expect smaller effects of TZDs on the valve, as well as in protection against atherosclerosis, in female mice.29 We have not performed systematic studies to determine whether severity of CAVS differs in male and female mice.

Cholesterol efflux from macrophages and possibly other cells may be a better marker than high-density lipoprotein levels in predicting severity of atherosclerosis.34 PIO attenuates lipid deposition by promoting cholesterol efflux, an effect that is not observed with a statin.31 Thus, we measured gene expression of molecules that mediate cholesterol efflux. In aorta of LA mice (Table I in the online-only Data Supplement), ATP binding cassette transporter A1, ATP binding cassette transporter G1, and apoE were upregulated by WD, regardless of treatment of PIO, whereas upregulation of apolipoprotein-AI by WD was inhibited by PIO. The effects were similar, with variations, in aortic valve and aorta of Reversa mice (Table 2). The most pertinent change is that CD36 was greatly upregulated by PIO in aortic valves of Reversa mice, and in aortas of LA and Reversa mice. In addition to mediating uptake of oxidized low density lipoprotein, CD36 may be involved in cholesterol efflux.32 Whether this great increase in CD36 expression by PIO contributes to decreased lipid deposition in the aortic valve warrants further investigation.

**Pio, Apoptosis, and Calcification**

Cell death may contribute to initiation of vascular and valvular calcification.33,34 Apoptosis is common in atherosclerotic arteries and stenotic aortic valves.35,36 In this study, active caspase-3, a marker for apoptosis, was increased in aortic valves of LA mice receiving WD, and was reduced by PIO to a level similar to that in chow-fed LA mice. Terminal deoxynucleotidyl transferase dUTP nick end labeling staining also was significantly decreased by PIO. The finding that PIO attenuated apoptosis is consistent with effects of PIO on lipid deposition and calcification, as colocalization studies indicate that cells that express active caspase-3 may be macrophage (positive for F4/80), myofibroblast (positive for α-smooth muscle actin), or osteoblast-like cells (positive for Cbfa1). This pattern of apoptotic cell types suggests that apoptosis may result from a general injury, perhaps by the atherosclerotic environment, including oxidized low density lipoprotein and inflammatory cytokines. PIO reduced apoptosis, perhaps through inhibition of inflammatory cytokines and reduction of intracellular lipid content. A recent study also suggests that activation of PPARγ inhibits secretion of telomeres, and thus inhibits apoptosis in endothelial cells in an injury model.37

The importance of cell death as a mechanism for calcification in CAVS and atherosclerosis is not known. In a study of the time course of calcification in a rabbit balloon injury model, calcium deposits occurred within 2 days after injury, and osteopontin and osteocalcin were detected only after 8 to 14 days, whereas osteonectin was undetectable at all time points.38 This finding suggests that apoptosis may be a primary cause of vascular calcification in that experimental model. Our finding that PIO inhibited both apoptosis and calcium deposition suggests that apoptosis may be important in valvular calcification induced by hypercholesterolemia. It is of interest that PIO increased macrophage apoptosis and plaque necrosis in advanced
### Table 2. Gene Expression in Aortic Valves and Aorta of Reversa Mice After 2 Months of WD±Pioglitazone

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<th>Chow</th>
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<td><strong>Aortic valves</strong></td>
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<tr>
<td>Plasma cholesterol, mg/dL</td>
<td>46±6</td>
<td>825±45*</td>
<td>701±45*,**</td>
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<tr>
<td><strong>PPARγ-related</strong></td>
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<td>PPARγ</td>
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<td>ND</td>
<td>ND</td>
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<td>Rbp7</td>
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<td>72±15*,**</td>
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<td>ND</td>
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<td>SOD3 (ECSOD)</td>
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<td><strong>Aorta</strong></td>
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<tr>
<td>PPARγ</td>
<td>1±0.26</td>
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(Continued)
The explanation for contrasting findings, that Pio is proapoptotic or antiapoptotic, is not clear. However, the dose of Pio differed (40 mg/kg per day in Reference 39 versus 20 mg/kg per day in our study), the age of mice differed (6 months of age in Reference 39 versus 8 months of age in our study), and the strain of mice differed.

Table 2. Continued

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<td>Rbp7</td>
<td>1±0.26</td>
<td>0.70±0.30</td>
<td>5.5±3.0**</td>
</tr>
<tr>
<td>ABCA1</td>
<td>1±0.10</td>
<td>4.0±1.1*</td>
<td>5.4±1.3*</td>
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<tr>
<td>ABCG1</td>
<td>1±0.24</td>
<td>9.3±3.4</td>
<td>26.3±6.9**</td>
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<tr>
<td>CD36</td>
<td>1±0.28</td>
<td>1.61±0.77</td>
<td>9.1±3.5**</td>
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<tr>
<td>Caveolin-1</td>
<td>1±0.16</td>
<td>0.70±0.16</td>
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<tr>
<td>ApoAI</td>
<td>1±0.33</td>
<td>1.73±0.51</td>
<td>1.33±0.51</td>
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<tr>
<td>ApoE</td>
<td>1±0.10</td>
<td>4.0±1.1</td>
<td>8.9±2.3**</td>
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**Inflammation**

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<td>TNFα</td>
<td>1±0.28</td>
<td>1.62±0.50</td>
<td>2.08±0.56</td>
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<td>IL-6</td>
<td>1±0.29</td>
<td>7.5±2.4*</td>
<td>6.1±1.5*</td>
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<td>IL-10</td>
<td>1±0.17</td>
<td>1.07±0.44</td>
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**Calcification-related**

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<tr>
<td>BMP2</td>
<td>1±0.08</td>
<td>0.85±0.14</td>
<td>0.84±0.09</td>
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<tr>
<td>Cbfa1</td>
<td>1±0.21</td>
<td>1.66±0.46</td>
<td>1.44±0.38</td>
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<td>Alkaline phosphatase</td>
<td>1±0.21</td>
<td>0.75±0.16</td>
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<td>Osteopontin</td>
<td>1±0.35</td>
<td>23.5±9.8*</td>
<td>30.5±8.5*</td>
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<td>Osteocalcin</td>
<td>1±0.17</td>
<td>1.79±0.44*</td>
<td>0.72±0.09</td>
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<td>Osteoprotegerin</td>
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<td>2.41±0.79</td>
<td>2.26±0.46</td>
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<tr>
<td>RANK</td>
<td>1±0.03</td>
<td>0.93±0.12</td>
<td>0.84±0.08</td>
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<td>RANK ligand</td>
<td>1±0.20</td>
<td>1.04±0.34</td>
<td>0.83±0.28</td>
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<tr>
<td>α-smooth muscle actin</td>
<td>1±0.12</td>
<td>0.69±0.14</td>
<td>0.53±0.07*</td>
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**Fibrosis-related**

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<td>Collagen3A1</td>
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**Macrophage**

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<td>Emr1 (F4/80)</td>
<td>1±0.19</td>
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**Oxidative stress**

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<td>1±0.06</td>
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<td>SOD2 (MnSOD)</td>
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<td>SOD3 (ECSOD)</td>
<td>1±0.12</td>
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<td>0.83±0.07</td>
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<tr>
<td>Catalase</td>
<td>1±0.07</td>
<td>0.94±0.12</td>
<td>1.19±0.12</td>
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<td>Nox2</td>
<td>1±0.19</td>
<td>3.3±1.0*</td>
<td>3.3±0.7*</td>
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<tr>
<td>Nox4</td>
<td>1±0.06</td>
<td>1.32±0.16*</td>
<td>0.85±0.08**</td>
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<td>p22phox</td>
<td>1±0.04</td>
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<td>NOS3 (eNOS)</td>
<td>1±0.16</td>
<td>0.93±0.11</td>
<td>0.85±0.05</td>
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ABCA1 indicates ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; apoAI, apolipoprotein AI; BMP2, bone morphogenetic protein 2; EMR, EGF-like module containing mucin-like hormone receptor-like 1; ND, not detectable; Nox2, NADPH oxidase 2; Pio, pioglitazone; PPARγ, peroxisome proliferator-activated receptor-γ; TNFα, tumor necrosis factor α; and WD, Western diet. Values are means±SE; n=11–12 (aortic valves); n=8–9 (aorta).

*P<0.05 vs Chow.

**P<0.05 vs Western diet.

P>0.05 by ANOVA in the groups without asterisk. Genes in bold face have different expression patterns in aorta vs aortic valve.
Pio and Osteogenic Calcification Pathways

Our previous studies demonstrated that protein expression of osteogenic calcification molecules (P-Smad1/5/8, Msx2, Osterix, and Cbfa1) increased in WD-fed Reversa mice, and was reduced after reversal to normocholesterolemia by a genetic switch. We have found that treatment with osteoprotegerin reduced osterix and osteocalcin, but not Cbfa1 (Runx2) in LA mice. The findings suggest that osteoprotegerin inhibits an osteogenic signaling step downstream to Cbfa1 and upstream to osterix.

In the present study, osterix, a critical transcription factor for differentiation to osteoblast, and osteocalcin, a marker protein produced by osteoblast-like cells, did not change with WD or Pio in aortic valves or aorta. Thus, the findings suggest that reduction of calcification in aortic valves by Pio in the present study may not be mainly through reduction of osteogenic signaling, as in our previous studies with reduction of cholesterol or administration of exogenous osteoprotegerin. We cannot conclude that Pio does not affect osteogenic signaling, however, because Pio reduced expression of an important osteogenic molecule, bone morphogenetic protein 2, in the aorta, even though reduction of calcification was not observed. Pio also may have indirect effects on osteogenic signaling, through reduction of inflammation and oxidative stress (TNFα, IL-6, serum amyloid A, adiponectin, EGF-like module containing mucin-like hormone receptor-like 1, NADPH oxidase 2), as demonstrated previously and as shown in Table 2.

One explanation for our finding that Pio did not alter expression of osterix and osteocalcin could be that osteogenic signaling molecules changed early in treatment with Pio, but not after 6 months. Thus, we measured gene expression in aortic valves and the aorta after only 2 months of treatment with Pio in Reversa mice. Indeed, expression in aortic valves of Cbfa1 (or Runx2), a transcription factor required for early differentiation to osteoblasts, was upregulated 9-fold by WD, and decreased significantly by Pio. Cbfa1 expression, however, did not change in aorta. In contrast, expression of osteocalcin was increased by WD and reduced by Pio in both aortic valves and the aorta. These findings clearly indicate that Pio inhibits osteogenic molecules. Thus, our finding of reduction of calcification in aortic valves after 6 months of treatment with Pio may have resulted, at least in part, from inhibition of osteogenic signaling by Pio at an earlier time.

Comparison of gene expression revealed that, among the genes examined, ≥50% have distinct expression patterns in aortic valves versus aorta in the 3 groups of mice (Table 2). In general, aortic valves are more sensitive than aorta in response to WD and Pio. The changes in gene expression are consistent with structural changes (lipid deposition and calcification) in the younger LA mice. From these experiments with LA and Reversa mice, we conclude that Pio attenuates calcification of aortic valves by reduction of both apoptosis and osteogenic signaling.

There are important limitations in our studies. Previous studies with porcine and human aortic valves demonstrated that gene expression patterns in endothelial cells are different on the aortic and ventricular sides. The mouse aortic valve is too small to allow isolation of cells on the 2 sides. Thus, our findings in both aortic valve and aorta represent an average of all cells. Although we found a detectable difference in many genes, it is likely that there are even greater differences on a particular side and in some regions.

PPARγ as a Therapeutic Approach to CAVS

Diabetes mellitus and metabolic syndrome are risk factors for CAVS. We chose a dose of Pio that does not affect plasma glucose levels, to focus on effects of PPARγ and to minimize effects of changes in plasma glucose. We cannot, however, exclude the possibility that the dose of Pio may have affected insulin resistance and plasma levels of insulin.

Studies in vitro and in vivo suggest that PPARγ modulates cardiovascular calcification by many mechanisms, including regulation of genes that modulate expression of osteoblasts, or are antiinflammatory. It is beyond the scope of this study to examine all of the mechanisms in depth, but we have examined several mechanisms by which Pio may modulate cardiovascular calcification.

First, Pio reduced expression of bone morphogenetic protein 2, and Cbfa1 and osteocalcin at an earlier time, which implies that Pio inhibits calcification, at least in part, by an effect on an osteogenic pathway, which is similar to pathways in bone. Second, Pio attenuated increases in serum amyloid A in plasma, and prevented increases in TNFα and IL-6 in...
aortic valves and the aorta. Thus, Pio attenuated inflammation, which may have contributed to inhibition of calcification.

One of the goals of this study was to test the hypothesis that a novel therapeutic approach might prevent, or slow the progression of, CAVS. As 3 clinical trials were published47–49 and failed to show that a statin slows the progression of CAVS, there is skepticism that any nonsurgical therapy will be beneficial in treatment of CAVS. We have shown that reduction of hypercholesterolemia in mice prevents the development of CAVS, but does not produce reversal of established CAVS.16

The finding that Pio prevents calcification of the aortic valve could lead to a novel approach to therapy. Administration of Pio to patients with impaired glucose tolerance delays the onset of diabetes mellitus. We speculate that a PPARγ ligand might also be useful in a high-risk population, such as patients with bicuspid aortic valve or impaired glucose tolerance, in prevention of CAVS.

One potential concern in consideration of using a TZD for prevention of CAVS is that TZDs in animals and postmenopausal women reduce bone density.51 It is possible that new PPARγ agonists, which do not have side effects on bone, may be developed. Another concern is that, although PPARγ ligands inhibit development of atherosclerosis, TZDs fail to slow progression of moderately severe atherosclerosis.30,39 Thus, it is possible that, although Pio may prevent development of CAVS, it may not slow progression after moderate CAVS has developed.

We found that LV ejection fraction was decreased in mice treated with Pio for 6 months, compared with mice that did not receive Pio. This decrease was associated with increased LV end-systolic and end-diastolic volumes, with preserved stroke volume. The findings suggest that Pio treatment is associated with development of dilated cardiomyopathy in mice. Studies of diabetic patients treated with TZDs (Pio or rosiglitazone) have demonstrated increased risk of heart failure, without concomitant increase in cardiovascular deaths.53 Our finding that Pio increased LV end-diastolic volume in mice is consistent with observations in patients treated with TZDs.54 In humans, however, TZDs have no effect on LV ejection fraction.53 Our finding that Pio reduced LV ejection fraction in mice thus may be an idiosyncratic effect in mice or because of nonequivalence of doses in humans and mice.

Another consideration, in potential use of a TZD to prevent development of CAVS, relates to concerns about safety of TZDs. Rosiglitazone, a TZD, has been reported to paradoxically increase the risk of cardiovascular diseases.56,57 PROActive studies,58,59 however, suggest that Pio does not increase the risk of myocardial infarction. The risk of cardiovascular events appears to be less with Pio than rosiglitazone.60 Thus, we speculate that Pio may be a better choice than rosiglitazone in testing the hypothesis that a PPARγ ligand protects against development of CAVS.

Acknowledgments

We thank Kathy Zimmerman and Melissa Davis for assistance in echocardiography; Arlinda LaRose, Teresa Ruggle, and Stephanie Brackey for assistance in preparation of the manuscript; the Central Microscopy Research Facility for use of equipment; and Katherine Walters for assistance.

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Disclosures

None.

References


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Yi Chu, Donald D. Lund, Robert M. Weiss, Robert M. Brooks, Hardik Doshi, Georges P. Hajj, Curt D. Sigmund and Donald D. Heistad

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Supplemental Material

**Dose studies.** In preliminary studies, we examined effects of two doses of pioglitazone, 20 and 60 mg/kg/day in LA mice fed a Western diet for 6 months. In contrast to the 20 mg dose of pioglitazone (See Table 1), 60 mg of pioglitazone produced a paradoxical increase in levels of serum amyloid A (a marker of inflammation) to 74±30 μg/ml, and failed to increase Rbp7 (a PPARγ responsive gene) mRNA in aorta (0.9 ±0.2 with 60 mg/kg vs 20±8 with 20 mg/kg pioglitazone [Figure 1]). Paradoxical effects of high vs low doses (with smaller effects with high doses) also have been observed with rosiglitazone, another TZD. These paradoxical effects of low and high doses contrast with effects of pioglitazone on non-fasting plasma glucose, which (as anticipated) was lower (p<0.05) with pioglitazone 60 mg/kg/day (226±10 mg/dl) than with 20 mg/kg/day (264±10).

Thus, we chose to examine effects of the lower dose of pioglitazone (20 mg/kg/day) because it appears to be more effective in increasing expression of Rbp7 and reducing serum amyloid A (SAA), and to avoid the hypoglycemic effects of the higher dose of pioglitazone (which would make interpretation of the findings more difficult).
Table I. Gene expression (mRNA levels) in the aorta of LA mice after 6 months of WD ± pioglitazone

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<td>1±0.11</td>
<td>1.36±0.27</td>
<td>1.00±0.13</td>
</tr>
<tr>
<td>Catalase</td>
<td>1±0.08</td>
<td>0.96±0.04</td>
<td>1.13±0.14</td>
</tr>
<tr>
<td>p22phox</td>
<td>1±0.10</td>
<td>1.20±0.07</td>
<td>1.04±0.05</td>
</tr>
<tr>
<td>Nox2</td>
<td>1±0.08</td>
<td>1.65±0.23*</td>
<td>1.28±0.07**</td>
</tr>
</tbody>
</table>

Values are mean±SE, n=9-13; * = p<0.05 vs chow; ** = p<0.05 vs. Western diet. p>0.05 by ANOVA in the groups without asterisk.
Table II. Echocardiographic assessment of left ventricular structure and function in LA mice after 6 months of WD±pioglitazone

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>Western Diet</th>
<th>Western Diet +Pioglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (per min.)</td>
<td>534±68</td>
<td>468±38</td>
<td>463±55</td>
</tr>
<tr>
<td>End-Diastolic Volume (μl)</td>
<td>45±6</td>
<td>48±6</td>
<td>81±8*</td>
</tr>
<tr>
<td>End-Systolic Volume (μl)</td>
<td>16±3</td>
<td>19±3</td>
<td>42±5*</td>
</tr>
<tr>
<td>Stroke Volume (μl)</td>
<td>29±3</td>
<td>29±4</td>
<td>39±4</td>
</tr>
<tr>
<td>Mass (mg)</td>
<td>102±3</td>
<td>116±11</td>
<td>111±7</td>
</tr>
<tr>
<td>Cardiac Output (ml/min)</td>
<td>15.4±2.0</td>
<td>13.3±1.3</td>
<td>16.7±2.6</td>
</tr>
<tr>
<td>Ejection Fraction</td>
<td>0.65±0.03</td>
<td>0.62±0.04</td>
<td>0.48±0.03*</td>
</tr>
</tbody>
</table>

Values are mean±SE, n= 5-6. * = p<0.05 vs Chow or WD. p>0.05 by ANOVA in the groups without asterisk.

Figure I. Alizarin red staining for calcium (red, arrow) of mouse aortic valves. Aortic valves are outlined with dashed lines.
Figure II. Oil red O staining for lipid deposition (red, arrow) of mouse aortic valves (within the dashed lines).

Figure III. Masson’s trichrome staining for fibrosis (blue) of mouse aortic valves (within the dashed lines).
Figure IV. Picrosirius red staining for collagen in aortic valves. There is a small decrease in picrosirius red staining during Western diet, and no effect of pioglitazone. n=7-11. * = p<0.05 vs. chow
Figure V. Active caspase-3 staining of mouse aortic valves (within the dashed line).

Immunofluorescence was performed with 1:3200 dilution of anti-active caspase-3 (Cell Signaling) and 1:250 dilution of Alexa Dye (568 nm)-conjugated anti-rabbit IgG. Punctate red signal indicates active caspase-3 (Red fibers resulted from autofluorescence of aortic walls).
Figure VI. TUNEL staining for apoptosis of mouse aortic valves. TUNEL staining was performed using an in situ cell death detection kit (Roche, Inc.) There was a significant decrease in TUNEL staining by pioglitazone. n=6-9. * = p<0.05 vs. chow; ** = p<0.05 vs. WD.
**Figure VII.** Effects of pioglitazone on mRNA expression in the aorta of LA mice at 8 months of age. Rbp7 and CD36, which are PPARγ responsive genes, were increased by pioglitazone. TNFα and IL-6, which are inflammatory cytokines, and Nox2, were increased by the Western diet (WD), and the increases were attenuated by pioglitazone. Pioglitazone decreased expression of BMP2, which promotes calcification. Values are mean±SE in 9-13 mice per group. * = p<0.05 vs. chow; ** = p<0.05 vs. WD.
피오글리타존(Pioglitazone)은 고지혈증에 의한 판막 석회화를 약화시킨다.

박철영 교수
성균관대학교 강북삼성병원 내분비내과

Summary

배경
대동맥 판막 협착증의 석회화 과정은 여러 신호전달경로를 통해 일어나며, peroxisome proliferator-activated receptor-γ (PPARγ)에 의해 조절될 수 있다. 이 연구는 PPARγ 리간드인 pioglitazone (Pio)이 고콜레스테롤 동물모델에서 대동맥 판막의 석회화 과정을 억제할 수 있다는 가설을 확인하고자 하였다.

방법 및 결과
Low density lipoprotein receptor<sup>−/−</sup>/apolipoprotein B<sup>100/100</sup> 쥐에게 서양식 먹이와 함께 Pio(20 mg/kg per day)를 같이 투여한 군과 그렇지 않은 군으로 나누어 6개월간 관찰하였다. Pio 투여군에서 대동맥 판막의 지질 침착과 석회화가 감소하는 것을 관찰하였지만 대동맥에서는 관찰되지 않았다. 대동맥 판막에서 Pio 투여군은 세포자멸사(apoptosis)의 표지자인 active caspase-3와 TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) 염색 정도를 감소시켰다. 심초음파로 관찰한 판막의 기능은 Pio 투여군에서 유의한 개선 효과를 보였다. Pio 투여에 따른 대동맥 판막과 대동맥의 다른 효과와 연관된 유전자 발현의 변화를 관찰하기 위해서 reversa mice를 이용하여 서양식 먹이를 2개월간 투여군과 비투여군으로 나누어 실험하였다. 대동맥 판막에서 석회화 과정의 유전자들이 서양식 먹이 투여군에서 증가하였고, Pio 투여군에서 는 악화되었지만, 대동맥에서는 그러한 변화를 관찰할 수 없었다.

결론
Pio 투여는 고지혈증 동물모델에서 대동맥 판막에서의 지질 침착, 석회화 및 세포자멸사를 약화시켰고 이는 대동맥 판막 기능을 개선시켰다. Pio는 대동맥 판막 협착증을 억제시킬 수 있으며, Pio 또는 다른 PPARγ 리간드도 대동맥 판막 협착을 예방하거나 경과를 억제하기 위한 초기 치료에 유용하게 사용될 수 있을 것으로 생각된다.
이 연구에서는 Pio를 20 mg/kg per day로 6개월 간 투여한 결과, 총콜레스테롤과 serum amyloid A의 유의한 감소효과를 보였으며 아디포넥틴의 유의한 상승을 관찰할 수 있었다. 이 연구에서 총콜레스테롤 외에 HDL-cholesterol, TG 등을 같이 측정할 수 있다면 좀 더 설득력 있는 결과가 되었을 것으로 생각되며, 아울러 이전 연구와의 연관성을 더 뒷받침할 수 있을 것이라 생각한다. Pio 투여군에서는 콜레스테롤의 개선 효과뿐 아니라 inflammation, macrophage, oxidative stress 관련 유전자의 개선 효과도 같이 관찰되었다(Table 1). Pio 치료에 따른 surrogate marker 연구나 outcome 연구들을 살펴보면 일관된 결과들을 관찰할 수 있다. Pio와 관련된 다른 연구들을 살펴보면 다음과 같다. CHICAGO (a study evaluating carotid intima-media thickness in atherosclerosis using pioglitazone) 연구에서는 제2형 당뇨병 환자 462명을 72주간 Pio 투여군과 glimepiride 투여군으로 나누어 CIMT (common carotid artery IMT)를 관찰하였다. Pio 투여군에서는 평균 0.026 mm, glimepiride 투여군에서는 0.002 mm 감소하여 두 군간에는 -0.024 mm (-0.042, -0.006)의 차이를 보였다. PERISCOPE (pioglitazone effect on regression of intravascular sonographic coronary obstruction prospective evaluation study) 연구

**Table 1. Gene Expression in Aortic Valves.**

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>Western Diet</th>
<th>Western Diet + Pio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma cholesterol, mg/dL</strong></td>
<td>46±6</td>
<td>825±45*</td>
<td>701±45**,</td>
</tr>
<tr>
<td><strong>PPARY-related</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rbp7</td>
<td>1±0.65</td>
<td>1.22±0.22</td>
<td>4.5±1.3**,</td>
</tr>
<tr>
<td>CD36</td>
<td>1±0.34</td>
<td>45±9*</td>
<td>72±15**,</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>1±0.26</td>
<td>3.25±0.41*</td>
<td>1.64±0.49**</td>
</tr>
<tr>
<td><strong>Calcification-related</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cbfa1</td>
<td>1±0.24</td>
<td>9.1±1.5*</td>
<td>4.5±1.2**</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>1±0.35</td>
<td>5.4±1.4*</td>
<td>0.66±0.27**</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>1±0.29</td>
<td>5.32±0.87*</td>
<td>1.43±0.56**</td>
</tr>
<tr>
<td><strong>Macrophage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginase 1</td>
<td>1±0.40</td>
<td>8.0±20*</td>
<td>3.1±1.2**</td>
</tr>
<tr>
<td><strong>Oxidative stress</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>1±0.16</td>
<td>2.11±0.18*</td>
<td>1.42±0.18**</td>
</tr>
<tr>
<td>p22phox</td>
<td>1±0.17</td>
<td>3.97±0.45*</td>
<td>3.04±0.29**,</td>
</tr>
</tbody>
</table>

*P<0.05 vs chow. **P<0.05 vs Western diet.
에서는 intravascular sonography를 반복하여 coronary artherosclerosis의 진행을 관찰하였다. 이 연구에서도 Pio 투여군에서 percent atheroma volume(%)의 유의한 개선효과를 보였다(0.73% vs -0.16%). 이 두 연구에서 모두 Pio 투여군은 glimepiride 투여군과 비교하여 HDL-C의 증가 및 TG 감소 소견을 보여 atherogenic dyslipidemia의 개선효과가 이런 차이에 주요한 원인으로 생각되었다. PROACTIVE (prospective pioglitazone clinical trial in macrovascular events) 연구에서는 5,238명의 대혈관질환을 동반한 제2형 당뇨병 환자를 대상으로 Pio 투여군과 비투여군에서의 심혈관질환 발생 위험도를 평가하였다. 제1차 복합 연구 목적[death from any cause, non-fatal myocardial infarction (including silent myocardial infarction), stroke, acute coronary syndrome, leg amputation, coronary revascularisation, or revascularisation of the leg]은 달성하지 못하였지만 사망, 심근경색증, 뇌졸중은 3년에 약 16%의 유의한 개선 효과를 보였다. 이 연구에서도 HDL-C의 유의한 증가, TG의 유의한 감소 소견을 보였다. 상기 임상연구 결과에서 공통적으로 low HDL-C, high TG의 atherogenic dyslipidemia의 질 패턴을 보이고 있어, 이 연구들의 연관성을 뒷받침 해주고 있다.

REFERENCES