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# Pioglitazone Increases Macrophage Apoptosis and Plaque Necrosis in Advanced Atherosclerotic Lesions of Nondiabetic Low-Density Lipoprotein Receptor–Null Mice

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**Background**—Thiazolidinediones (TZDs), which have actions that involve both peroxisome proliferator–activated receptor (PPAR)- $\gamma$ -dependent and –independent effects, improve insulin sensitivity in type II diabetes and inhibit early atherogenesis in mice. However, the effects of TZDs on advanced lesion progression are unknown.

**Methods and Results**—Pioglitazone and rosiglitazone enhanced macrophage apoptosis by a number of stimuli, including those thought to be important in advanced atherosclerosis. Macrophage death was not enhanced by non-TZD PPAR $\gamma$  activators, and TZD-induced apoptosis was still observed in PPAR $\gamma$ -deficient macrophages. In wild-type macrophages, death enhancement was associated with reduced activation of the cell-survival mediator nuclear factor- $\kappa$ B. TZDs also increased the ability of macrophages to phagocytically clear apoptotic cells, which is proposed to protect against plaque necrosis in advanced lesions. The mechanism of this effect was complex, involving both PPAR $\gamma$ -dependent and –independent mechanisms. To explore the net effect on advanced atherosclerosis in vivo, *Ldlr*<sup>–/–</sup> mice were fed a nondiabetogenic cholesterol-enriched diet to promote midstage lesions. Then, pioglitazone was administered with the diet for an additional 10 weeks. Aortic root lesions from the pioglitazone-treated mice showed a substantial increase in apoptotic cells and plaque necrosis compared with lesions from non–drug-treated mice.

**Conclusions**—The potential atheroprotective effects of TZDs conferred by insulin sensitization may be partially offset by adverse effects on advanced atherosclerosis. Because the mechanisms of the beneficial and proposed adverse effects may differ, these findings have potentially important implications for drug optimization. (*Circulation*. 2007;116:2182-2190.)

**Key Words:** apoptosis ■ atherosclerosis ■ macrophages ■ plaque ■ drugs

Thiazolidinediones (TZDs) are drugs that affect cells through activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)- $\gamma$  and through other “off-target” mechanisms.<sup>1</sup> In patients with type II diabetes mellitus, TZDs improve insulin sensitivity, an effect ascribed to PPAR $\gamma$  activation.<sup>2</sup> Because insulin resistance promotes atherogenesis,<sup>3</sup> it has been proposed that TZDs will be beneficial in decreasing the incidence of atherothrombotic vascular disease. In this context, a number of animal studies have shown antiatherogenic effects of TZDs, although the mechanisms are not always correlated with improved metabolic parameters.<sup>4–8</sup> Interestingly, other animal studies have failed to show an antiatherogenic effect of TZDs even in the setting of improved insulin sensitivity.<sup>9</sup> In humans, pioglitazone failed to show a statistically significant beneficial effect on a primary composite end point of all-cause mortality and cardiovascular disease in the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive).<sup>10</sup> Moreover, in

a recent meta-analysis of rosiglitazone trials in diabetic patients, the drug was associated with a 43% increase in myocardial infarction and a 64% increase in cardiovascular death.<sup>11</sup> On the other hand, pioglitazone was found to have an overall beneficial effect in diabetic patients on the individual endpoints of all-cause mortality, nonfatal myocardial infarction, and stroke in both the PROactive study and in a recent meta-analysis by Nissen and colleagues.<sup>10,12</sup>

## Clinical Perspective p 2190

The above findings highlight the uncertainty related to the overall mechanisms and consequences of TZDs on atherothrombotic disease. Although one might argue, on the basis of the aforementioned meta-analyses, that rosiglitazone has adverse effects on coronary disease that are unique to that 1 compound,<sup>11,12</sup> the effects of pioglitazone on advanced atherosclerosis are far from settled. Regarding potential antiatherogenic

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mechanisms, insulin sensitization involves PPAR $\gamma$  activation in the liver, adipose, and muscle.<sup>2,3</sup> On the other hand, PPAR $\gamma$  is expressed in atherosclerotic lesional cells, including macrophages.<sup>3</sup> Whereas TZDs were found to increase expression of the oxidized low-density lipoprotein (LDL) receptor CD36 in cultured macrophages,<sup>13</sup> TZDs reduced CD36 expression and uptake of oxidized LDL in a mouse model of insulin resistance, concomitant with improved insulin signaling in macrophages.<sup>14</sup> Furthermore, activation of PPAR $\gamma$  has been shown to enhance macrophage cholesterol efflux through transcriptional induction of LXR $\alpha$  and perhaps ABCA1.<sup>15</sup> and to suppress proinflammatory cytokine secretion from activated macrophages.<sup>16</sup>

To fully understand how TZDs might affect atherothrombotic disease, it is important to consider how these drugs might affect specific processes that promote advanced plaque progression. Two such processes are advanced lesional macrophage death and the phagocytic clearance of these apoptotic cells (efferocytosis). Macrophage apoptosis is increased in advanced lesions and can lead directly to plaque necrosis when these apoptotic cells are not efficiently cleared by neighboring macrophage phagocytes.<sup>17</sup> Plaque necrosis, in turn, promotes plaque disruption and subsequent acute thrombosis.<sup>18</sup> In vivo studies suggest that 1 mechanism of macrophage death unique to advanced lesions is that triggered by an excess of intracellular unesterified, or “free,” cholesterol (FC) delivered by atherogenic lipoproteins.<sup>19</sup> The lipoproteins and FC trigger a series of proapoptotic signal transduction pathways involving the type A scavenger receptor, toll-like receptor 4, the mitogen-activated protein kinase JNK, and the endoplasmic reticulum (ER) stress pathway known as the unfolded protein response (UPR).<sup>20,21</sup> Although the mechanisms of defective efferocytosis in advanced lesions are not known, possibilities include competitive inhibition of apoptotic cell-phagocyte interaction by oxidized lipoproteins and suppression of apoptotic cell engulfment by oxidative stress and hypoxia.<sup>17</sup>

In this context, we report here that TZDs enhance macrophage apoptosis induced by a number of stimuli and promote efferocytosis of apoptotic cells. Most important, when pioglitazone is administered to nondiabetic *Ldlr*<sup>-/-</sup> mice after midstage lesions have already been established, the net effect is increased advanced lesional macrophage apoptosis and plaque necrosis. Because the mechanisms of the beneficial and proposed adverse effects of TZDs may differ, these findings have potentially important implications for drug optimization.

## Methods

See the online-only Data Supplement for an expanded Methods section.

### Mice

Wild-type macrophages were obtained from 8- to 10-week-old female C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, Me). For the PPAR $\gamma$ -deficient studies, macrophages were from 8- to 10-week-old female PPAR $\gamma$ <sup>fl/fl</sup>×LysMCre mice (PPAR $\gamma$  <sup>$\Delta$ Mc</sup>), which have deficient PPAR $\gamma$  expression, or from control PPAR $\gamma$ <sup>fl/fl</sup> mice, which have normal PPAR $\gamma$  expression.<sup>22</sup> The PPAR $\gamma$  <sup>$\Delta$ Mc</sup> and PPAR $\gamma$ <sup>fl/fl</sup> mice are on the C57Bl/6N-FVB genetic background. *Ldlr*<sup>-/-</sup> mice on a C57Bl/6J background were purchased from Jackson Laboratories.

### Macrophage Incubations and Apoptosis Assays

Before FC loading, macrophages were preincubated with TZDs in dimethyl sulfoxide or dimethyl sulfoxide vehicle control for 18 to 24

hours as indicated. The macrophages were FC loaded by incubation with 100  $\mu$ g/mL acyl-LDL plus 10  $\mu$ g/mL 58035 (to inhibit acetyl-coenzyme A acetyltransferase-mediated cholesterol esterification). Externalization of phosphatidylserine, a sign of early to midstage apoptosis, was detected by quantitative microscopy and flow cytometry with Alexa-488-labeled annexin V (Molecular Probes, Carlsbad, Calif). Membrane leakiness, a sign of late-stage apoptosis, was detected by staining with propidium iodide. Micrographs were captured with an Olympus IX-70 inverted fluorescence microscope, and 5 representative fields ( $\approx$ 1000 cells total) per condition were used to quantify the number of annexin V-positive, propidium iodide-positive, and total cells. For flow cytometry, macrophages were rinsed in cold PBS, resuspended in annexin V-binding buffer (10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L CaCl<sub>2</sub>), and stained with Alexa-488-labeled annexin V for 15 minutes. Cells were then rinsed with binding buffer and subjected to flow cytometry as previously described.<sup>23</sup>

### Efferocytosis Assay

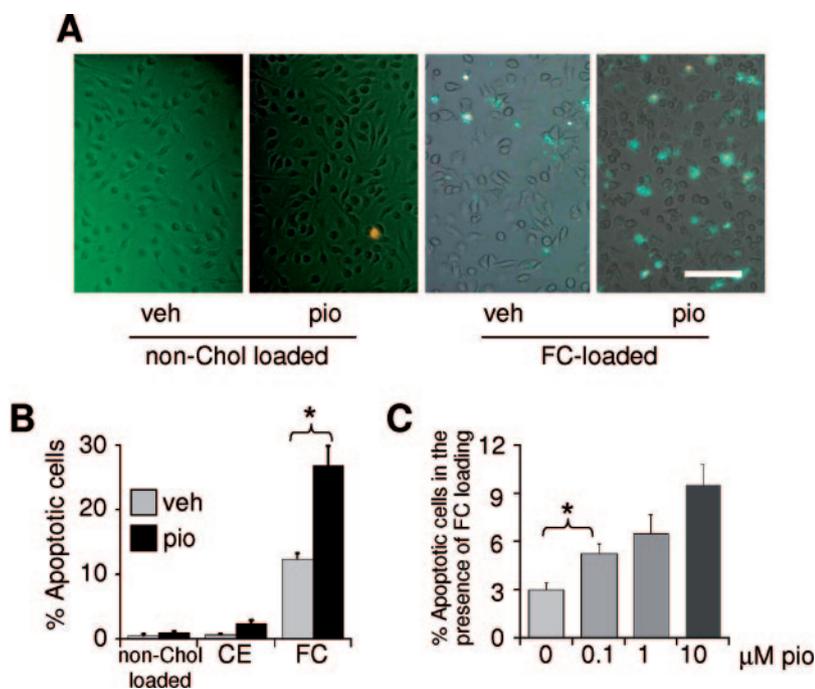
Efferocytosis was assessed as previously described<sup>24</sup> with minor modifications. The source of apoptotic cells, which were prelabeled with the green fluorescent dye calcein AM, was FC-loaded peritoneal macrophages or ultraviolet-treated J774 murine macrophages (15 minutes at 254 nm, 20 J/cm<sup>2</sup>). Before inducing apoptosis, the macrophages were fluorescently labeled with calcein AM (green) (Molecular Probes). The apoptotic cells were overlaid onto monolayers of octadecylrhodamine-labeled (red) macrophages (phagocytes) at a 1:1 ratio. After 30 to 45 minutes, noningested apoptotic macrophages were removed by vigorous agitation and rinsing. The adherent cells were then fixed in paraformaldehyde and viewed and imaged by fluorescence microscopy. These images were used to quantify phagocytic uptake, which was distinguished from external apoptotic cell-phagocyte binding by confocal microscopy.

### Pioglitazone-Atherosclerosis Study

At 6 weeks of age, *Ldlr*<sup>-/-</sup> mice were fed a gamma-irradiated, low-fat (10-kcal fat), high-cholesterol (0.5% or 5.3 g cholesterol/4057 kcal) semisynthetic (AIN76) Clinton/Cybulsky pellet diet (D00083101) from Research Diets (New Brunswick, NJ).<sup>25</sup> The mice were maintained on this diet for 8 weeks at  $\approx$ 3 g/d. The mice were then split into 2 groups; 1 group received pioglitazone in addition to the semisynthetic diet for an additional 10 weeks. According to food intake, the dose of pioglitazone was 40 mg/kg body weight per day. All animal protocols were approved by the Columbia University Institutional Animal Care and Use Committee.

### Lesion Analysis and Immunohistochemistry

For morphometric lesion analysis, sections were stained with Harris' hematoxylin and eosin. Total intimal lesion area (between internal elastic lamina to the lumen) and acellular/anuclear areas (negative for hematoxylin-positive nuclei) per cross section were quantified by taking the average of 6 sections spaced 30  $\mu$ m apart beginning at the base of the aortic root. Histomorphological analysis of collagen was performed with Masson's trichrome stain (Richard-Allan Scientific, Kalamazoo, Mich) and elastin stain (hematoxylin-iodine-ferric chloride, Sigma Chemical Co, St Louis, Mo). Images were viewed and captured with a Nikon Labophot 2 microscope equipped with a Sony CCD-Iris/RGB color video camera attached to a computerized imaging system with Image-Pro-Plus 3.0 software. For immunohistochemistry, antigens were retrieved via heating in an EDTA solution, followed by hydrogen peroxide/methanol blocking of endogenous peroxidase. Blocking was performed with immunoglobulin from the species of the secondary antibody. Macrophages were detected with a rabbit anti-macrophage antibody (AIA31240) from Accurate Chemical and Scientific Corporation (Westbury, NY). Smooth muscle cell actin was detected with Zymed's mouse anti-smooth muscle actin (1A4) following the protocol of Zymed's Histomouse-SP Kit (Invitrogen, Carlsbad, Calif). Secondary antibodies were biotinylated conjugates that were subsequently detected with streptavidin-horseradish peroxidase. The horseradish peroxidase substrate was diaminobenzidine. Images were viewed and captured as above. Apoptotic cells in atherosclerotic lesions were detected by



**Figure 1.** Pioglitazone enhances FC-induced macrophage apoptosis. A, Macrophages were pretreated for 18 hours with 10  $\mu\text{mol/L}$  pioglitazone (pio) or vehicle (veh) control and then incubated under nonloading or FC-loading conditions for an additional 12 hours with or without pioglitazone or vehicle control. The cells were then stained with annexin V (green) and propidium iodide (red) and viewed by fluorescence microscopy. Bar=100  $\mu\text{m}$ . B, Quantification of annexin V-positive, propidium iodide-negative (apoptotic) macrophages in nonloaded, cholesteryl ester (CE)-loaded, or FC-loaded macrophages with or without pioglitazone. C, Percent of apoptotic FC-loaded macrophages at the indicated doses of pioglitazone. Chol indicates cholesterol. \* $P<0.05$ .

Tdt-mediated dUTP nick-end labeling (TUNEL) after proteinase K treatment using the TMR-red kit from Roche (Nutley, NJ). The stringency methods of Kockx<sup>26</sup> were followed to avoid nonspecific staining. Nuclei were counterstained with Hoechst for 5 minutes. The slides were viewed and imaged by fluorescent microscopy. For quantitative data analysis, the stained areas in the images were obtained and quantified as described above.

### Statistical Analysis

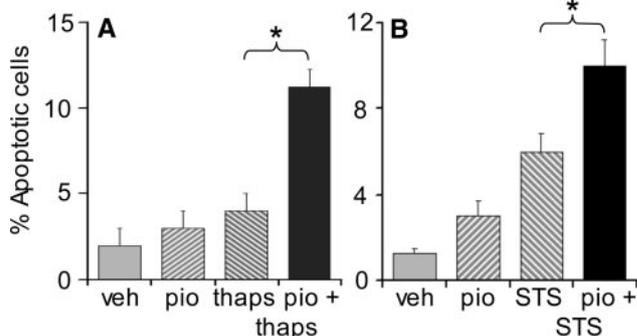
Data are presented as mean  $\pm$  SEM. The absence of error bars in the bar graphs signifies that SEM values were smaller than the graphic symbols. For paired groups, Student's *t* test was used. ANOVA was used for  $>2$  groups, and multifactor ANOVA was used under conditions of  $\geq 2$  independent variables. The post hoc analysis was the Tukey procedure.

## Results

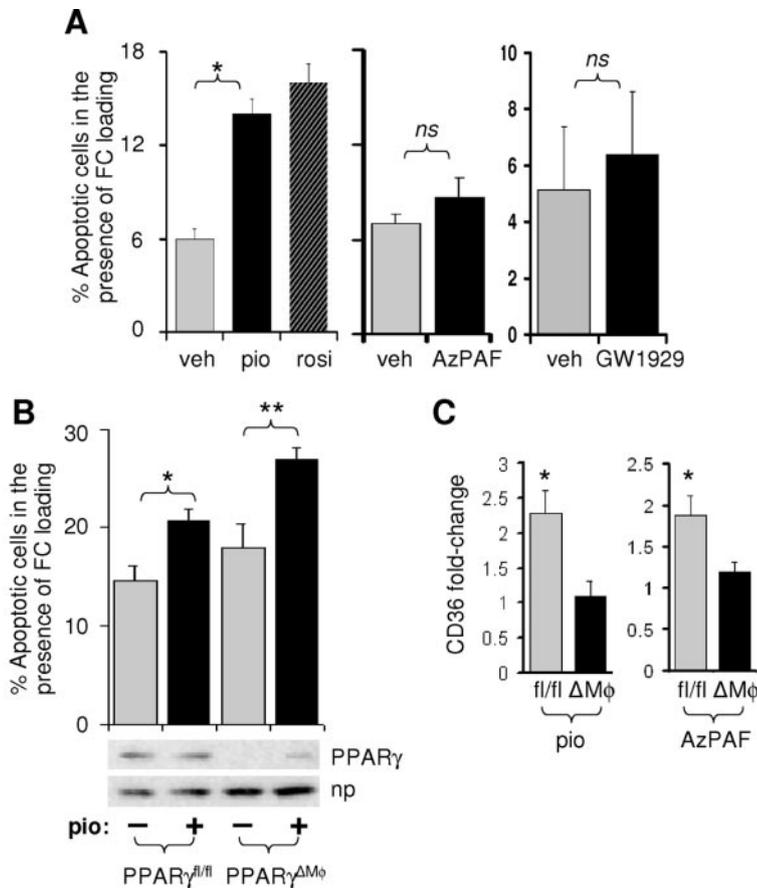
### TZDs Enhance Macrophage Apoptosis Induced by FC Loading and Other Inducers

To determine the effects of TZDs on macrophage apoptosis in a context relevant to advanced atherosclerosis, macrophages were preincubated for 18 hours in the absence or presence of 10  $\mu\text{mol/L}$  pioglitazone and then incubated for an additional 12 hours under control or FC-loading conditions with or without pioglitazone. The cells were then assayed for apoptosis with annexin V staining. As shown in Figure 1A and 1B, pioglitazone treatment led to a  $\approx 2$ -fold increase in FC-induced apoptosis. A similar increase in apoptosis was measured by annexin V flow cytometry (see Figure I of the online-only Data Supplement). Pioglitazone did not induce de novo apoptosis in macrophages that were not cholesterol loaded or in cholesteryl ester-loaded macrophages, which represent the state of most macrophages ("foam cells") in early atherosclerotic lesions (Figure 1B). Death enhancement by pioglitazone followed a direct dose-response relationship, with apoptosis enhancement observed even at the lowest dose of 100 nmol/L (Figure 1C). One possible mechanism for the enhancement of FC-induced apoptosis by TZDs could be

increased lipoprotein uptake, leading to increased delivery of lipoprotein-derived FC to the ER. The latter processes induced the UPR effector CCAAT/enhancer-binding protein-homologous protein (CHOP), which is required for apoptosis.<sup>27</sup> However, we found that pioglitazone pretreatment did not increase the uptake and processing of [<sup>125</sup>I]acetyl-LDL, the delivery of acetyl-LDL cholesterol to the ER, or the expression of CHOP (data not shown). Moreover, we found that pioglitazone was able to enhance macrophage apoptosis induced by 2 noncholesterol factors, the UPR activator thapsigargin and the protein phosphatase inhibitor staurosporine (Figure 2A and 2B). Thus, pioglitazone is a general enhancer of macrophage apoptosis and does not depend on FC loading per se.



**Figure 2.** Pioglitazone increases apoptosis in both ER and non-ER stressed macrophages. A, Macrophages were pretreated for 24 hours with 10  $\mu\text{mol/L}$  pioglitazone (pio) or vehicle (veh) control and then incubated for an additional 15 hours with 10  $\mu\text{mol/L}$  pioglitazone, 2.5 mmol/L thapsigargin (thaps), or both reagents. The cells were then stained with annexin V (green) and propidium iodide (red), viewed by fluorescence microscopy, and quantified for apoptosis. B, The same procedure was followed except that the macrophages were treated for 11 hours with 100 nmol/L staurosporine (STS) instead of thapsigargin where indicated. \* $P<0.05$ .



**Figure 3.** Enhancement of FC-induced macrophage apoptosis is not seen with a non-TZD PPAR $\gamma$  activator, and TZD-enhanced apoptosis occurs normally in PPAR $\gamma$ -depleted macrophages. A, Percent of apoptotic FC-loaded macrophages after treatment with 10  $\mu$ mol/L pioglitazone (pio), 5  $\mu$ mol/L rosiglitazone (rosi), 5  $\mu$ mol/L AzPAF, 1  $\mu$ mol/L GW1929, or vehicle control (veh). B, Peritoneal macrophages from PPAR $\gamma^{fl/fl}$  and PPAR $\gamma^{\Delta M\phi}$  mice were FC loaded after pretreatment with 10  $\mu$ mol/L pioglitazone or vehicle control and then assayed and quantified for apoptosis. The immunoblot below the graph shows nuclear PPAR $\gamma$  ( $\approx$ 57 kDa) and nucleophosmin (np), a nuclear protein that serves as a loading control. C, Fold increase in CD36 mRNA by pioglitazone and AzPAF versus vehicle control in macrophages from PPAR $\gamma^{fl/fl}$  and PPAR $\gamma^{\Delta M\phi}$  mice. Data are derived from quantitative polymerase chain reaction measurements of CD36 mRNA relative to 36B4 mRNA. \* $P < 0.05$  vs non-drug-treated control.

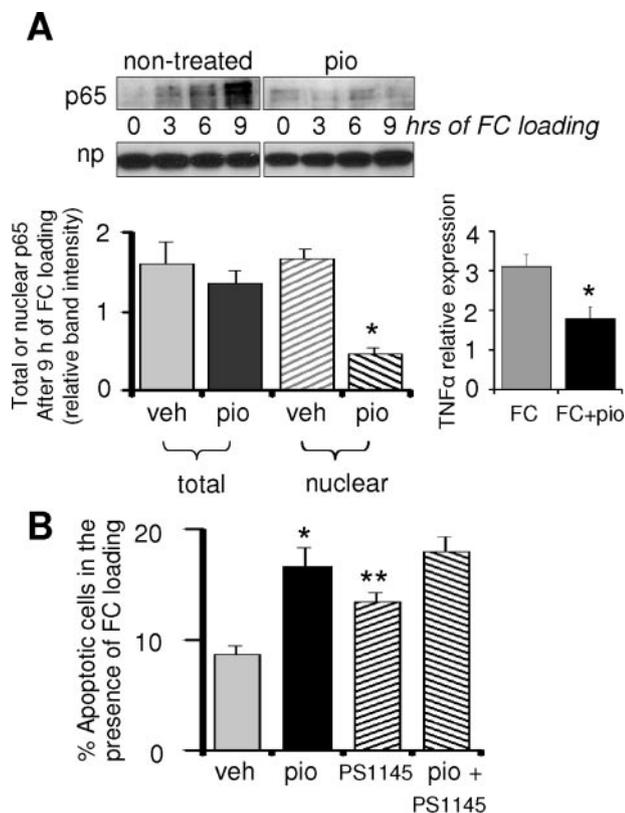
**Macrophage Death Is Not Enhanced by Non-TZD PPAR $\gamma$  Activators, and TZD-Enhanced Apoptosis Is Observed in PPAR $\gamma$ -Deficient Macrophages**

TZDs can affect cells through both PPAR $\gamma$ -dependent and -independent mechanisms.<sup>1</sup> To determine whether death enhancement was specific to pioglitazone or TZDs in general, we measured FC-induced apoptosis in the presence of the TZD rosiglitazone and the non-TZD PPAR $\gamma$  ligands and agonists azelaoyl PAF (AzPAF) and GW1929.<sup>28,29</sup> Enhancement of FC-induced apoptosis was observed with rosiglitazone but not with either AzPAF or GW1929 (Figure 3A). To verify that AzPAF in particular was acting as a PPAR $\gamma$  agonist in our system, we showed that mRNA for the PPAR $\gamma$ -dependent gene CD36 was induced 1.8-fold in the AzPAF-treated macrophages (Figure 3C). AzPAF also increased levels of adipose differentiation-related protein, an PPAR $\gamma$ -inducible protein (data not shown). These data raised the possibility that apoptosis enhancement was being mediated through a PPAR $\gamma$ -independent effect of TZDs. To further explore this possibility, we used peritoneal macrophages from mice carrying floxed alleles of PPAR $\gamma$  with (PPAR $\gamma^{\Delta M\phi}$ ) or without (PPAR $\gamma^{fl/fl}$ ) the macrophage-targeted LysMCre recombinase gene.<sup>22</sup> As expected, macrophages from PPAR $\gamma^{fl/fl}$  mice expressed PPAR $\gamma$ , whereas those from PPAR $\gamma^{\Delta M\phi}$  mice had undetectable or very low PPAR $\gamma$  expression (Figure 3B, immunoblot). Moreover, expression of CD36 mRNA was unchanged after pioglitazone or AzPAF treatment of PPAR $\gamma^{\Delta M\phi}$  macrophages compared with an  $\approx$ 2-fold induction in control PPAR $\gamma^{fl/fl}$  cells (Figure 3C). As

shown by the quantified apoptosis data in Figure 3B, pioglitazone enhanced FC-induced apoptosis in both PPAR $\gamma$ -expressing and PPAR $\gamma$ -deficient macrophages. These data, together with the AzPAF and GW1929 data above, suggest that enhancement of macrophage apoptosis by TZDs occurs via a PPAR $\gamma$ -independent mechanism.

**Pioglitazone Suppresses Nuclear Factor- $\kappa$ B-p65, a Cell-Survival Factor, in FC-Loaded Macrophages**

We next sought to probe the mechanism of pioglitazone-induced enhancement of apoptosis. We previously reported that FC loading activates the nuclear factor (NF)- $\kappa$ B pathway in macrophages through a mechanism that involves both ER stress and toll-like receptor 4.<sup>21,30</sup> NF- $\kappa$ B activation can drive prosurvival responses in many cell types,<sup>31</sup> and TZDs have been shown to suppress NF- $\kappa$ B activation in other scenarios.<sup>32</sup> We therefore hypothesized that pioglitazone enhances FC-induced apoptosis at least in part through inhibition of FC-induced NF- $\kappa$ B. In support of this hypothesis, pioglitazone markedly suppressed FC-induced nuclear translocation of NF- $\kappa$ B p65, a measure of NF- $\kappa$ B activation, but not total cellular p65 (Figure 4A). As predicted by the nuclear p65 data, pioglitazone treatment reduced mRNA of the NF- $\kappa$ B-dependent gene tumor necrosis factor- $\alpha$  (Figure 4A). Although the mechanism of suppressed nuclear p65 remains to be determined, we found that it was not associated with either reduced I $\kappa$  kinase activity or increased expression of the NF- $\kappa$ B inhibitor I $\kappa$  B $\alpha$  (data not shown). Importantly, when the cells were treated with the I $\kappa$  kinase  $\beta$  inhibitor PS-1145, which effectively suppresses NF- $\kappa$ B activa-

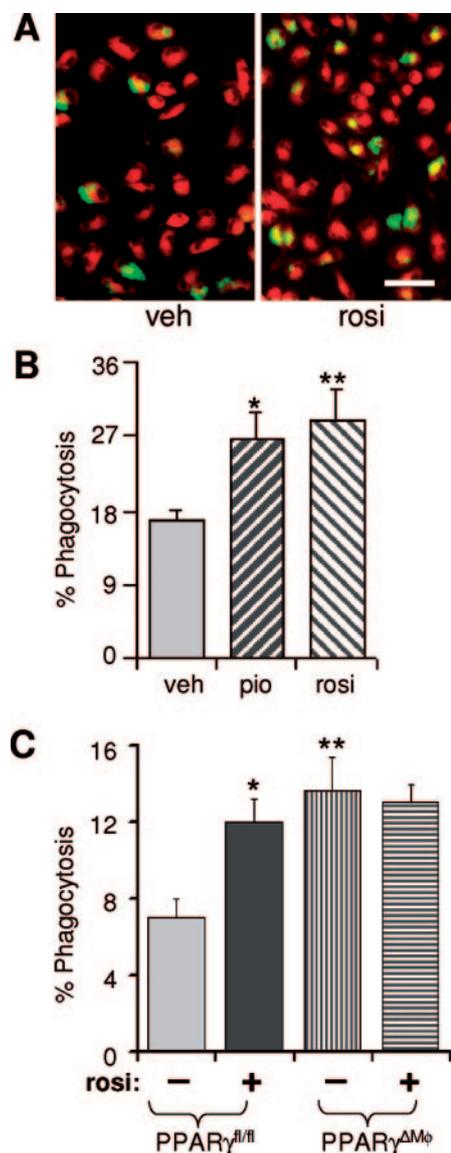


**Figure 4.** Pioglitazone suppresses nuclear NF- $\kappa$ B-p65, a cell-survival factor, in FC-loaded macrophages. **A**, Peritoneal macrophages were incubated with 10  $\mu$ M pioglitazone (pio) or vehicle control (veh); then, nuclear extracts from these cells were immunoblotted with anti-p65 at the indicated times after FC loading. Blots were stripped and reprobbed for nucleophosmin (np) as a nuclear extract protein loading control. The bar graph on the left shows quantification of total and nuclear p65 (normalized to tubulin and nucleophosmin, respectively) in macrophages treated with vehicle alone or pioglitazone and then FC loaded for 9 hours. The bar graph on the right shows the induction of tumor necrosis factor- $\alpha$  mRNA by FC loading with or without pioglitazone. The data are derived from quantitative polymerase chain reaction measurements of tumor necrosis factor- $\alpha$  mRNA relative to 36B4 mRNA. **B**, Macrophages were pretreated for 18 hours with vehicle control (veh), 10  $\mu$ M/L pioglitazone, 10  $\mu$ M/L PS-1145, or pioglitazone plus PS-1145. The cells were then loaded with FC (with or without the same reagents) for 11 hours and assayed for apoptosis. \* $P$ <0.05 vs vehicle control.

tion in FC-loaded macrophages,<sup>30,33</sup> enhancement of FC-induced apoptosis was similar to that seen with pioglitazone (Figure 4B). Coincubation of pioglitazone with PS-1145 did not cause an additive increase of apoptosis, consistent with a similar proapoptotic mechanism for NF- $\kappa$ B activation and pioglitazone treatment. Moreover, pioglitazone treatment did not inhibit the expression of a number of prosurvival molecules, including phospho-Akt, Bcl-2, and apoptosis inhibitor of macrophages in FC-loaded macrophages. These data suggest that the suppression of NF- $\kappa$ B contributes to the enhancement of apoptosis by TZDs in wild-type macrophages, but other mechanisms are likely involved (see Discussion section).

### TZDs Enhance Efferocytosis of Apoptotic Macrophages

Postapoptotic necrosis of apoptotic macrophages, resulting from inefficient efferocytosis of these cells by neighboring macro-



**Figure 5.** Efferocytosis of apoptotic macrophages is enhanced by TZDs and by PPAR $\gamma$  deficiency. **A**, Monolayers of red fluorescently labeled macrophages ("phagocytes") were treated for 18 hours with vehicle (veh) control or 1  $\mu$ M/L rosiglitazone. FC-AMs were then added to these phagocytes for 30 minutes. After vigorous rinsing to get rid of noninternalized FC-AMs,<sup>23</sup> the monolayers were viewed by fluorescence microscopy. **B**, Quantification of efferocytosis in phagocytes treated with vehicle control, 10  $\mu$ M/L pioglitazone, or 1  $\mu$ M/L rosiglitazone. \* $P$ <0.05 vs vehicle control. **C**, Peritoneal macrophages from PPAR $\gamma$ <sup>fl/fl</sup> or PPAR $\gamma$  <sup>$\Delta$ M $\phi$</sup>  mice were treated with vehicle control or 1  $\mu$ M/L rosiglitazone and then overlaid with FC-AMs and quantified for efferocytosis. \* $P$ <0.05 vs PPAR $\gamma$ <sup>fl/fl</sup> phagocytes.

phage phagocytes, is thought to be an important contributor to advanced lesional plaque necrosis.<sup>17</sup> To determine the effect of TZDs on efferocytosis, we first treated monolayers of red fluorescently labeled macrophages ("phagocytes") with or without pioglitazone or rosiglitazone. Green fluorescently labeled FC-induced apoptotic macrophages (FC-AMs) were then added to these phagocytes, and efferocytosis was assayed and quantified as described in Methods. As shown in Figure 5A and 5B, TZD-treated phagocytes ingested significantly more FC-AMs, as indicated by the number of red phagocytes stained with green

FC-AMs. TZDs also enhanced the efferocytosis of macrophages rendered apoptotic by ultraviolet treatment (data not shown). Enhancement of phagocytosis by TZDs was specific to apoptotic cells because there was no effect on phagocytosis of immunoglobulin-opsonized sheep erythrocytes (data not shown).

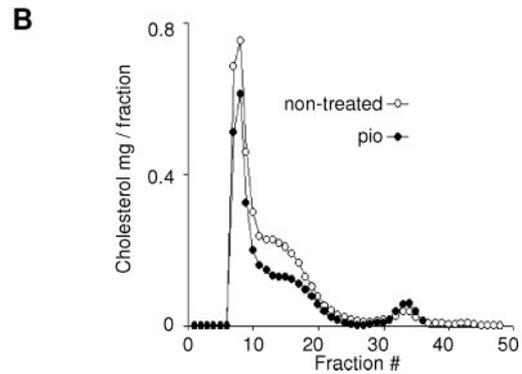
To determine the effect of phagocyte PPAR $\gamma$  depletion on efferocytosis, we compared efferocytosis by phagocytes from PPAR $\gamma^{fl/fl}$  and PPAR $\gamma^{\Delta M\phi}$  mice. A comparison of the first and third bars in Figure 5C revealed an unexpected result, namely that PPAR $\gamma$  depletion was associated with enhanced efferocytosis even in the absence of pioglitazone. This finding suggests that basal expression of PPAR $\gamma$  in macrophage phagocytes suppresses efferocytosis or that a secondary compensatory response to PPAR $\gamma$  depletion in macrophages triggers a pathway that enhances efferocytosis. On the other hand, TZD treatment of PPAR $\gamma$ -deficient phagocytes caused no further enhancement of efferocytosis (compare the third and fourth bars in Figure 5C). Although reconciling these data into a coherent mechanism requires further investigation, the data may suggest opposing effects of PPAR $\gamma$  expression per se versus TZD-mediated activation of PPAR $\gamma$  on efferocytosis.

**Pioglitazone Increases Plaque Necrosis in Advanced Atherosclerotic Lesions of LDL Receptor-Deficient Mice**

The enhancement of apoptosis by TZDs, in the setting of advanced lesions, would be predicted to promote plaque necrosis, whereas the enhancement of efferocytosis would be predicted to lessen plaque necrosis.<sup>17</sup> To determine the net effect in vivo in a setting in which the insulin-sensitizing effects of TZDs would be minimal, we chose to examine pioglitazone-treated *Ldlr*<sup>-/-</sup> mice fed a nondiabetogenic, semisynthetic, low-fat, high-cholesterol diet.<sup>25</sup> Importantly, the mice were administered pioglitazone only after midstage lesions had already developed so that the focus would be on the effect of the drug on advanced lesion progression. Specifically, 6-week-old *Ldlr*<sup>-/-</sup> mice were fed the cholesterol-rich diet for 8 weeks, and then the semisynthetic diet was continued for 10 additional weeks in the presence or absence of pioglitazone. We subsequently assessed plasma metabolic and lipid parameters and performed morphometric lesion analysis at the aortic root. The mice were moderately hyperinsulinemic, and although there was a trend toward lower insulin levels in the pioglitazone-treated group, the difference did not reach statistical significance (Figure 6A). There was no hyperglycemia in either group. The pioglitazone-treated mice had  $\approx 10\%$  decrease in plasma total cholesterol and  $\approx 25\%$  increase in high-density lipoprotein (HDL) cholesterol. Fast-performance liquid chromatography of plasma lipoproteins showed that the pioglitazone-treated mice had cholesterol reductions in very LDL and LDL fractions (Figure 6B).

Analysis of plaque morphology revealed substantial differences between the control and pioglitazone-treated groups. As illustrated by the trichrome- and hematoxylin and eosin-stained images in Figure 7A and the quantified data in Figure 7B, plaques from the pioglitazone-treated mice had substantially less collagen content and an increase in areas that were anuclear, afibrotic, and eosin negative. Immunohistochemis-

A	non-treated	pioglitazone	p
Body Weight (g)	32.7 $\pm$ 0.5	32.9 $\pm$ 0.6	0.78
Glucose (mg/dl)	76.1 $\pm$ 2.5	81.2 $\pm$ 2.0	0.12
Insulin (pg/ml)	1,513.6 $\pm$ 106.4	1,335 $\pm$ 131.5	0.09
Total Cholesterol (mg/dl)	1,138.2 $\pm$ 33.9	949.8 $\pm$ 39.3	<0.01
HDL-Cholesterol (mg/dl)	64.5 $\pm$ 3.9	90.7 $\pm$ 4.5	<0.01
Triglycerides (mg/dl)	130.4 $\pm$ 12.7	109.7 $\pm$ 15.9	0.32

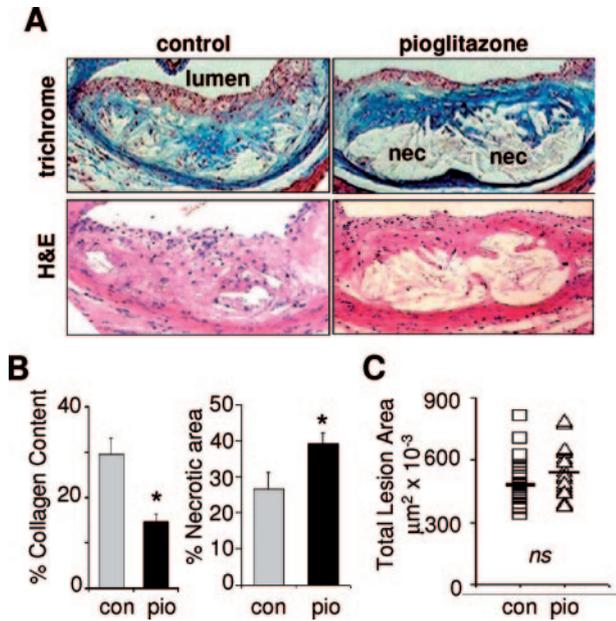


**Figure 6.** Metabolic parameters of control and pioglitazone (pio)-treated, cholesterol-fed *Ldlr*<sup>-/-</sup> mice. *Ldlr*<sup>-/-</sup> mice were placed on a semisynthetic, cholesterol-containing diet at 6 weeks of age. After 8 weeks on this diet, the mice were continued for 10 additional weeks on the same diet alone (control) or on a diet containing pioglitazone. A, Plasma from the mice was analyzed for the indicated metabolic parameters (n=25 for control, n=26 for pioglitazone). B, Pooled plasma samples from each group of mice were subjected to fast-performance liquid gel-filtration chromatography.

try showed the presence of macrophage debris in these regions (data not shown), consistent with “necrosis.” Elastin staining in plaques was similar between the 2 groups (data not shown). Importantly, total lesion area per se was not different between the 2 groups of mice (Figure 7C), so the differences in plaque morphology cannot be ascribed simply to differences in overall plaque size. Finally, nuclear-specific TUNEL staining revealed an increase in macrophage apoptosis in the lesions of pioglitazone-treated mice, which occurred in the macrophage-rich areas of these lesions (Figure 8). Pioglitazone treatment did not increase the number of TUNEL-positive macrophages in nonatherosclerotic tissues, eg, in lung and spleen (data not shown). In summary, nondiabetic *Ldlr*<sup>-/-</sup> mice treated with pioglitazone after prior development of midstage lesions showed a marked decrease in plaque collagen and a substantial increase in plaque necrosis and macrophage apoptosis. These morphometric parameters, which indicate increased plaque progression in the pioglitazone-treated group, occurred despite a slight improvement in metabolic parameters by the drug and no difference in overall lesion area per se.

**Discussion**

The insulin-sensitizing effects of TZDs would be expected to lessen the incidence of atherothrombotic macrovascular dis-

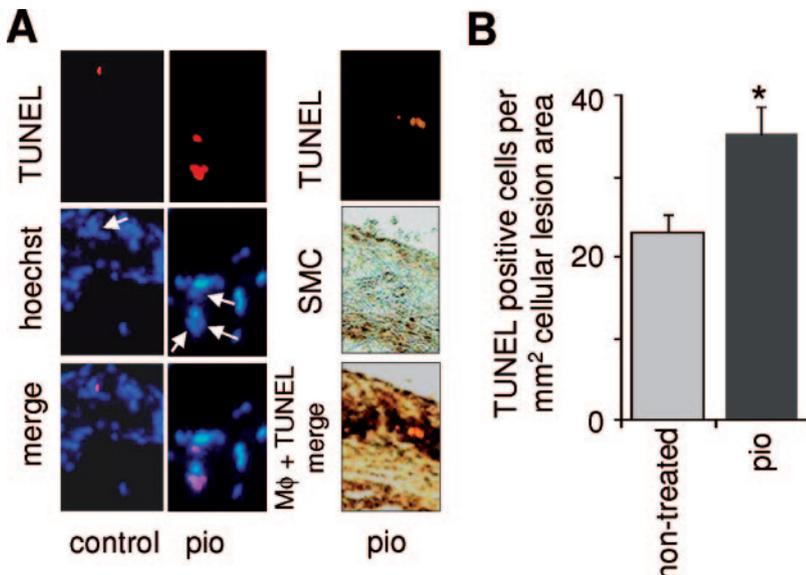


**Figure 7.** Total lesion area, necrosis, and collagen content in aortic roots from pioglitazone-treated, cholesterol-fed *Ldlr*<sup>-/-</sup> mice. **A**, Representative sections of aortic roots from control (con) and pioglitazone-treated (pio) mice were stained with Masson's trichrome stain and with hematoxylin and eosin. Collagen stains blue and cytoplasm stains red in the trichrome method. **B**, Quantification of percent collagen content and percent anuclear, afibrotic, and eosin-negative (necrotic) area per total plaque area (n=20 plaques for control, n=20 plaques for pioglitazone). **C**, Quantitative analysis of atherosclerotic lesion area (n=25 for control, n=26 for pioglitazone). Nec indicates plaque necrosis; ns, not significant. \**P*<0.05.

ease in subjects with type II diabetes. Indeed, although the benefit of pioglitazone in macrovascular disease in diabetics was not statistically significant in the PROactive trial using a composite primary end point, analysis of important individual end points suggested a beneficial effect both in this study and in a recently published meta-analysis.<sup>10,12</sup> However, as with any drug, there are likely to be multiple effects. Some of the adverse effects of rosiglitazone on coronary artery disease

probably reflect specific effects of this 1 compound.<sup>11,12</sup> In that sense, it was extremely fortunate that the present plaque necrosis study used pioglitazone and not rosiglitazone, because a study showing that rosiglitazone increased plaque necrosis could have simply reflected the specific adverse effect of that 1 compound, with no relevance to TZDs that are likely to be used in the future. However, even in the case of pioglitazone, the overall beneficial effect on acute coronary syndromes in diabetics may reflect a balance between protective mechanisms (eg, insulin sensitization and antiinflammatory processes) and adverse processes that promote plaque necrosis. It was in this context that we sought to explore the effects of TZDs in the following 2 settings: specific cellular events thought to be associated with plaque progression and an in vivo model that emphasizes effects on advanced plaque progression while de-emphasizing effects on either early atherogenesis or the insulin-sensitizing effects of TZDs.

The key finding was the plaque morphology data in the *Ldlr*<sup>-/-</sup> mouse study. Pioglitazone, when administered to mice with pre-established lesions, resulted in plaques that had signs of increased necrosis, decreased collagen content, and increased macrophage apoptosis despite lower plasma total cholesterol, increased HDL cholesterol, and unaltered overall lesion area. The number of apoptotic macrophages in the lesions of pioglitazone-treated mice was a relatively small percentage of total lesional macrophages, but these levels are consistent with previous studies in which increased apoptosis was associated with increased plaque necrosis.<sup>34</sup> Moreover, the apoptotic cells were found mostly near the edges of expanding necrotic cores. Note that TUNEL staining reflects the number of apoptotic cells at 1 point in time, whereas plaque necrosis likely results from the gradual accumulation over a much longer period of time of apoptotic macrophages that become secondarily necrotic as a result of failure of phagocytic clearance.<sup>17</sup> Overall, the in vivo data in this report are consistent with the conclusion that pioglitazone can promote advanced plaque progression in a model in which the beneficial insulin-sensitizing effects do not come into play.



**Figure 8.** Macrophage apoptosis is increased in advanced aortic root lesions of pioglitazone-treated, cholesterol-fed *Ldlr*<sup>-/-</sup> mice. **A**, Representative images show TUNEL-positive cells (red) in sections of aortic root lesions from control and pioglitazone-treated, cholesterol-fed *Ldlr*<sup>-/-</sup> mice; the sections also were stained with Hoechst nuclear dye (blue). Also shown are sections of an aortic root lesion from pioglitazone-treated mice that were stained for TUNEL (top), smooth muscle cells (middle), and macrophages (Mφs)+TUNEL (bottom). **B**, Quantification of nuclear-specific TUNEL data (n=25 for control, n=26 for pioglitazone). \**P*<0.05.

Two points relative to our cellular mechanistic studies deserve comment. First, Chinetti et al<sup>35</sup> reported that TZDs induce apoptosis of nonactivated differentiated macrophages in vitro. In our hands, pioglitazone did not induce apoptosis de novo but rather enhanced cell death in response to apoptosis inducers such as FC enrichment of macrophages. This is an important distinction because cholesteryl ester-rich foam cells predominate in early lesions, whereas FC-loaded macrophages are a feature of advanced lesions.<sup>19</sup> The lack of de novo apoptosis induction by TZDs in cholesteryl ester-loaded macrophages is consistent with our finding of no increase in macrophage apoptosis in aortic root lesions from a small group (n=6) of mice treated with pioglitazone during early lesion development (data not shown). Second, our data suggest that suppression of NF- $\kappa$ B participates in the enhancement of apoptosis by TZDs in wild-type macrophages, but the mechanism is undoubtedly more complex. For example, the fact that non-TZD PPAR $\gamma$  agonists do not enhance apoptosis (Figure 3A) but probably still suppress NF- $\kappa$ B<sup>32</sup> raises the possibility that TZDs have additional proapoptotic mechanisms not shared with non-TZD PPAR $\gamma$  agonists or that non-TZDs actively promote cell-survival signaling in a manner that counteracts the suppression of NF- $\kappa$ B. Moreover, we found that pioglitazone did not suppress NF- $\kappa$ B in PPAR $\gamma$ -deficient macrophages (data not shown), despite being able to enhance apoptosis in these cells (Figure 3B). These data suggest that in the special case of PPAR $\gamma$ -deficient macrophages, a mechanism other than suppression of NF- $\kappa$ B is involved in the enhancement of apoptosis by TZDs. This alternative proapoptotic mechanism may represent some sort of “compensatory” response to the chronic absence of PPAR $\gamma$  in these cells. Future mechanistic studies are required to sort out these additional complexities.

Our cell culture studies also showed an enhancing effect of TZDs on efferocytosis of apoptotic macrophages. From a number of studies, this effect, if translated in vivo, might be expected to lessen plaque necrosis.<sup>17</sup> The fact that the overall in vivo effect of pioglitazone was increased, not decreased, plaque necrosis may indicate that this action of pioglitazone does not occur in the setting of advanced atherosclerosis or that other plaque-promoting effects of pioglitazone such as enhancement of macrophage death play a dominant role. Nonetheless, pending further mechanistic studies, future drug refinement may be able to take advantage of this potentially beneficial effect of TZDs.

In summary, the data in the present report reveal an action of TZDs that promotes advanced plaque progression in *Ldlr*<sup>-/-</sup> mice through a mechanism that may involve enhancement of advanced lesional macrophage apoptosis. Key future goals are to determine whether TZDs promote advanced lesional macrophage apoptosis and plaque necrosis in PPAR $\gamma$ -deficient *Ldlr*<sup>-/-</sup> mice, as predicted, and to further probe cellular and molecular mechanisms of TZD and PPAR $\gamma$  effects on apoptosis and efferocytosis. Our overall contention is that PPAR $\gamma$ -dependent improvement in insulin resistance has the potential to decrease cardiovascular disease in diabetic patients but that this benefit will be optimally realized only if the potential detrimental effects of even

“good” TZDs such as pioglitazone be eliminated through drug optimization.

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

Thiazolidinediones (TZDs) are widely prescribed drugs that improve insulin sensitivity in patients with type II diabetes mellitus. Because insulin resistance is linked to accelerated atherogenesis, TZDs have the potential to decrease the incidence of atherothrombotic vascular disease. Despite evidence that TZDs reduce early atherosclerotic lesions in mice, their effects on advanced atherosclerosis, which is more relevant to acute coronary syndromes in humans, are not known. On the one hand, the Prospective Pioglitazone Clinical Trial in Macrovascular Events failed to show a beneficial effect of pioglitazone in diabetic patients on the primary composite end point of all-cause mortality, nonfatal myocardial infarction, stroke, acute coronary syndrome, and peripheral vascular disease. Moreover, a recent meta-analysis by Nissen's group has linked the TZD rosiglitazone with a greater risk of heart attack. On the other hand, pioglitazone was found to have an overall beneficial effect in diabetic patients on the individual end points of all-cause mortality, nonfatal myocardial infarction, and stroke in both the Prospective Pioglitazone Clinical Trial in Macrovascular Events and in a recent second meta-analysis by Nissen and colleagues. How can one reconcile these findings? The current study in nondiabetic mice showing that pioglitazone accelerates advanced plaque development and that the mechanism appears to be independent of peroxisome proliferator-activated receptor- $\gamma$  suggests a possible explanation. First, Nissen's 2 recent meta-analyses suggest that rosiglitazone has adverse cardiovascular effects that are probably specific to this 1 compound. In that sense, it was extremely fortunate that the current plaque necrosis study used pioglitazone and not rosiglitazone, because a study showing that rosiglitazone increased plaque necrosis could have simply reflected the specific adverse effect of that 1 compound, with no relevance to TZDs that are likely to be used in the future. In terms of pioglitazone, the current plaque necrosis study raises the possibility that the overall beneficial effect of pioglitazone reflects a balance between protective mechanisms (eg, insulin sensitization and antiinflammatory processes) and adverse processes that promote advanced plaque necrosis. Moreover, the adverse effects may be due to non-peroxisome proliferator-activated receptor- $\gamma$  off-target effects of the drug. Therefore, the 'good' TZDs like pioglitazone may be made even better if their potential detrimental effects are eliminated through drug optimization. Whether such optimization could also eliminate the more widely observed adverse effects of TZDs on heart failure remains to be seen.