Pioglitazone Suppresses Inflammation In Vivo in Murine Carotid Atherosclerosis

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Objective—To investigate the effects of pioglitazone (PIO), a peroxisome proliferator–activated receptor γ agonist, on plaque matrix metalloproteinase (MMP) and macrophage (Mac) responses in vivo in a molecular imaging study.

Methods and Results—In vitro, PIO suppressed MMP-9 protein expression in murine peritoneal Macs (P<0.05). To assess PIO’s effects on plaque inflammation, nondiabetic apolipoprotein E−/− mice receiving a high-cholesterol diet (HCD) were administered an MMP-activatable fluorescence imaging agent and a spectrally distinct Mac-avid fluorescent nanoparticle. After 24 hours, mice underwent survival dual-target intravital fluorescence microscopy of carotid arterial plaques. These mice were then randomized to HCD or HCD plus 0.012% PIO for 8 weeks, followed by a second intravital fluorescence microscopy study of the same carotid plaque. In the HCD group, in vivo MMP and Mac target-to-background ratios increased similarly (P<0.01 versus baseline). In contrast, PIO reduced MMP and Mac target-to-background ratios (P<0.01) versus HCD. Changes in MMP and Mac signals correlated strongly (r ≥0.75). Microscopy demonstrated MMP and Mac reductions in PIO-treated mice and a PIO-modulated increase in plaque collagen.

Conclusion—Serial optical molecular imaging demonstrates that plaque MMP and Mac activity in vivo intensify with hypercholesterolemia and are reduced by PIO therapy. (Arterioscler Thromb Vasc Biol, 2010;30:1933-1939.)

Key Words: atherosclerosis ■ pioglitazone ■ inflammation ■ molecular imaging ■ fluorescence

Inflammation is involved in all stages of atherosclerosis, including foam cell formation, plaque progression, and ultimately plaque disruption and thrombus formation. Systemic inflammation correlates with an increased rate of fatal cardiovascular disease events, even in statin-treated patients. Despite ex vivo evidence linking PIO to reduced atheroma inflammation, the anti-inflammatory effects of PIO on plaque MMP activity and Macs have not been spatially mapped, tracked, and quantified in vivo. In addition, questions exist regarding potential clinical differences among the TZDs in terms of cardiovascular effects, suggesting that in vivo approaches may be needed to resolve such issues. Furthermore, although PPAR-γ agonists, such as PIO, decrease destabilizing MMP expression in the atheromata, it is unknown whether this reflects primarily (1) reduced MMP expression from a stable number of plaque Macs or (2) reduced numbers of Macs, the cells responsible for most of the MMP expression in the atheromata.

To address these open questions, we harnessed serial dual-target fluorescence molecular imaging to (1) perform longitudinal spatial assessment of PIO- and hypercholesterolemic-modulated alterations of plaque inflammation and (2)
investigate whether changes in plaque MMP activity track with changes in plaque Mac activity, as assessed by in vivo imaging and ex vivo molecular analyses. By using this novel strategy, our findings provide unequivocal in vivo evidence that PIO reduces plaque inflammation and that decreases in plaque MMP activity correlate strongly with reductions in plaque Mac phagocytic activity.

Methods
In vitro studies (Western blotting), fluorescence microscopy, image analysis, histopathological features, plasma measurements, and MMP and Mac molecular imaging agents are detailed in the supplemental data (available online at http://atvb.ahajournals.org).

Serial Dual-Target Fluorescence Molecular Imaging of Plaque Inflammation
PIO’s effects on carotid plaques were investigated in apolipoprotein E (apoE)/−/− mice (n=16 female mice; Jackson Laboratories, Bar Harbor, Me). Mice consumed a high-cholesterol diet (HCD; TD88137; 42% milk fat and 0.2% cholesterol; Harlan Teklad, Madison, Wis) from the age of 10 to 26 weeks. Twenty-four hours before imaging, MMP and Mac agents were coinjected (tail vein). Next, apoE/−/− mice underwent intravital fluorescence microscopy (IVFM 1) after surgical exposure of the right carotid artery. Briefly, the distal right common carotid artery was carefully separated from the periadventitial tissues by blunt dissection. For coregistration, a phantom was placed underneath the carotid artery bifurcation. After the initial IVFM study, the incisions of the 16 apoE/−/− mice were surgically closed. Mice recovered from surgery without incident and were allowed water and their specified diet ad libitum. An additional group of wild-type C57BL/6J mice fed a normal chow diet (n=6) were injected with MMP and Mac imaging agents; these mice served as controls.

ApoE/−/− mice with carotid atheroma detected on the first IVFM study (IVFM 1) were then randomized to either continued HCD or HCD supplemented with PIO, 0.012% wt/wt, ad libitum, for 8 weeks. After 8 weeks (when the mice were aged 34 weeks), apoE/−/− mice were reimaged with the same dosages of MMP and Mac imaging agents. Twenty-four hours later, the same carotid atheroma was visualized in IVFM 1 and the mice underwent a second IVFM (IVFM 2) before euthanasia and histopathological analyses. The Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, approved all procedures.

Intravital Fluorescence Microscopy
IVFM studies used a multichannel laser scanning fluorescence microscope (supplemental data). The ×4 objective (numerical aperture, 0.15) used provided an in-plane resolution of 13×13 μm. A plastic tube phantom (PE-10 tubing; Becton Dickinson, Franklin Lakes, NJ) was placed underneath the carotid artery bifurcation and served to coregister the imaging fields of the 2 IVFM data sets. The plaque target-to-background ratio (TBR) was calculated as the ratio of plaque signal intensity to the adjacent vessel background signal intensity (supplemental data).

Results
PIO Represses Cytokine-Induced MMP-9 Expression In Vitro
To determine whether PPAR-γ activation by PIO represses MMP-9 protein expression in vitro, isolated mouse peritoneal Macs were plated and cultured in the presence of lipopolysaccharide and/or PIO, 10 μmol/L. PIO pretreatment attenuated the lipopolysaccharide-augmented protein expression of MMP-9, as seen on immunoblotting: (data expressed as mean±SEM) control, 34.1±2.0 arbitrary units; lipopolysaccharide, 46.8±2.2 arbitrary units; and lipopolysaccharide plus PIO, 19.1±2.9 arbitrary units (P<0.05) (supplemental Figure I). Ponceau S staining confirmed equal supernatant protein gel loading (data not shown).

Effect of PIO Therapy on Body Weight and Metabolic Parameters
PIO-treated apoE/−/− mice, 13 mg/kg per day, showed reduced plasma cholesterol levels and a similar body weight and plasma glucose, insulin, and triglyceride levels as HCD-treated mice (supplemental Table), consistent with a prior study.13

Dual-Target Fluorescence Molecular Imaging of Carotid Atherosclerosis Reveals Abundant In Vivo MMP Activity and Mac Signals Compared With Healthy Vessels
Survival IVFM in cholesterol-fed 26-week-old apoE/−/− mice (n=16) was performed to assess in vivo carotid plaque inflammation at baseline. Twenty-four hours before imaging, dual-targeted and spectrally resolved MMP activity (MMPSense680) and Mac phagocytic activity (cross-linked iron oxide [CLIO]-cyanine 7 [Cy7]) near infrared fluorescence (NIRF) imaging agents were coinjected. At the beginning of the imaging session, a third spectrally distinct intravascular agent (fluorescein isothiocyanate–dextran) was administered and multichannel high-resolution IVFM was performed.

Plaques were located in the distal common carotid artery and its bifurcation. Discrete MMP (green) and Mac (red) plaque NIRF signals were confirmed to be intravascular by fluorescein isothiocyanate–dextran (blue)–based carotid arterial angiograms. The angiogram provided precise identification of the plaque boundaries to enable accurate region of interest analyses of the molecular imaging targets of MMP and Mac activity (Figure 1). The carotid arteries of control C57BL/6J mice injected with the same agents showed mild near infrared (NIR) fluorescence diffusely throughout the vessel wall; no plaques were evident, as expected. Z stacks demonstrated significantly more MMP and Mac signals in apoE/−/− mice compared with C57BL/6J mice: MMP TBR, 1.9±0.1 versus 1.2±0.1 (P<0.001); and Mac TBR, 2.4±0.1 versus 1.1±0.1 (P<0.001).

PIO Suppresses Plaque MMP Activity and Macs In Vivo
To assess the in vivo inflammation-modulating effects of hypercholesterolemia and PIO therapy, a second follow-up IVFM study was performed to measure changes in carotid plaque MMP and Mac activity over time. At the age of 26 weeks, 13 (81%) of the 16 apoE/−/− mice had carotid arterial plaques on baseline IVFM imaging, and were randomized to either continued HCD (group 1, HCD; n=6) or HCD admixed with PIO (group 2, HCD plus PIO; n=7). The baseline MMP and Mac plaque activities were similar between these 2 groups (baseline MMP TBR of group 1 versus group 2, 1.8±0.1 versus 2.0±0.1 [P=0.35]; and baseline Mac TBR of group 1 versus group 2, 2.1±0.2 versus 2.5±0.2 [P=0.19]).

After 8 additional weeks, the same plaque that was imaged in IVFM 1 underwent repeat multichannel IVFM 2. The plaques of mice with continued HCD (group 1) demonstrated
significantly increased MMP and Mac signals over baseline (MMP TBR at 26 weeks versus 34 weeks, 1.8±0.1 versus 2.5±0.1 [P=0.002]; and Mac TBR at 26 weeks versus 34 weeks, 2.1±0.2 versus 3.0±0.2 [P=0.002]) (Figure 2B and Figures 3A and D). In contrast, plaques in the PIO group (group 2, HCD plus PIO, Figure 2C) showed trends of reduction for both plaque MMP and Mac activity (MMP TBR at 26 weeks versus 34 weeks, 2.0±0.1 versus 1.7±0.1 [P=0.26], Figure 3B; and Mac TBR at 26 weeks versus 34 weeks, 2.5±0.2 versus 2.0±0.1 [P=0.16], Figure 3E).

The natural history of in vivo plaque MMP and Mac activity during the 8-week study period differed significantly between

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**Figure 1.** High-resolution in vivo fluorescence molecular imaging of multiple inflammatory targets in the murine carotid atheromata. A and B, IVFM of carotid arteries revealed discrete plaque-specific MMP and Mac activity in apoE<sup>−/−</sup> mice (A) in contrast to healthy carotid vessels of age- and sex-matched C57BL/6J mice (B). C, The in vivo plaque MMP TBR in apoE<sup>−/−</sup> mice was 55% higher than in C57BL/6J mice (1.9±0.1 vs 1.2±0.1; P<0.001). D, Similarly, the in vivo plaque Mac TBR was 110% greater in apoE<sup>−/−</sup> mice (2.4±0.1 vs 1.1±0.1; P<0.001). Images within each channel were processed identically. FITC indicates fluorescein isothiocyanate.

**Figure 2.** Longitudinal in vivo tracking and quantification of multicomponent plaque inflammation in apoE<sup>−/−</sup> mice. The same right carotid plaque from each animal underwent serial IVFM. A, Study schema. After 16 weeks of an HCD, IVFM was performed. Next, mice with carotid plaques (n=13) were randomized to continued HCD, with or without 0.012% PIO, corresponding to a PIO dose of 13 mg/kg per day. After 8 weeks, a second IVFM study of the same plaque was performed. B, In HCD-treated mice, plaque MMP and Mac phagocytic activities (green and red, respectively) increased significantly over 8 weeks. C, In contrast, PIO treatment reduced plaque MMP and Mac activities. Representative images processed identically.
Plaque MMP Activity Exhibits a Linear Relationship With Plaque Macs

To better understand the relationship between plaque MMP activity and plaque Mac activity in vivo, Pearson correlation coefficients between the ΔMMP TBR and ΔMac TBR were derived (Figure 4). In the entire cohort, a strong relationship existed between the ΔMMP TBR and ΔMac TBR (r = 0.96, P < 0.001). Subgroup analyses revealed a stronger correlation in the PIO-modulated group (r = 0.92, P = 0.003) compared with the HCD group (r = 0.75, P = 0.08).

PIO Reduces Corresponding Histological, Microscopic, and Molecular Measures of Plaque Inflammation

Compared with carotid atherosclerotic plaques of HCD-fed apoE<sup>−/−</sup> mice, plaques of PIO-treated HCD-fed apoE<sup>−/−</sup> mice contained reduced MMP-9 and Mac presence: percentage MMP-9 staining for HCD versus HCD plus PIO, 42.6 ± 4.0% versus 14.3 ± 2.8% (66% reduction) (P < 0.001); and percentage Mac staining for HCD versus HCD plus PIO, 38.3 ± 4.2% versus 14.1 ± 2.4% (63% reduction) (P < 0.001) (Figure 5). On multichannel fluorescence microscopy, carotid plaque sections revealed colocalization of MMP activity and Mac molecular imaging signals. In contrast to animals fed an HCD, PIO-treated animals had substantially reduced plaque MMP activity and Mac signals. Similar levels of NIR autofluorescence emanated from the medial fibers of plaques from both groups.

Next, immunoblots of MMP-9 and Mac expression were examined to evaluate whether PIO reduced aortic vessel wall MMP-9 and Mac-specific protein levels. Consistent with the IVFM, histological, and fluorescence microscopy studies, PIO reduced aortic MMP-9 and Mac-3 protein levels in apoE<sup>−/−</sup> mice (63% and 67% reductions: P = 0.01 and P = 0.005, respectively) (Figure 6).

PIO Increases Plaque Collagen Content Without Altering Lesion Size

Given the reduced plaque inflammatory composition after PIO therapy, we investigated whether PIO might induce a compensatory increase in plaque collagen content. In fact, the Masson trichrome stain for collagen demonstrated increased carotid and aortic plaque collagen in the PIO group, with a 72% increase in the aortic intimal lesion collagen content (percentage collagen area for HCD versus HCD plus PIO, 17.3 ± 2.6% versus 29.7 ± 3.2%; P = 0.01) (Figure 5E and supplemental Figure II). Similar levels of collagen staining in the media were noted. No reduction in aortic plaque area was found (HCD versus HCD plus PIO, 0.42 ± 0.03 mm² versus 0.45 ± 0.02 mm²; P = 0.45).

Discussion

By using serial dual-targeted fluorescence molecular imaging coupled to ex vivo molecular methods, this study provides novel in vivo evidence that PIO reduces inflammation in atherosclerosis. Plaque MMP and Mac activity progressively increased in vivo in hypercholesterolemic apoE<sup>−/−</sup> mice.
and was reduced by the TZD PPAR-γ agonist PIO. Furthermore, this study provides additional insight into the temporal relationship of MMP and Mac activities in atherosclerosis. Specifically, we found that alterations in plaque MMPs were strongly linked to alterations in plaque Macs, as assessed by in vivo activity imaging, fluorescence microscopy, immunohistochemistry, and immunoblotting.

MMPs, zinc-dependent endopeptidases that digest collagen, elastin, and extracellular proteins, are implicated in plaque destabilization by promoting both expansive remodeling and fibrous cap rupture. In this study, in vivo functional MMP activity, rather than the sole presence of MMPs, was imaged using an MMP-activatable NIRF substrate, in concert with optimized IVFM. Serial IVFM demonstrated that carotid plaque MMP activity increased by 42% in the HCD group (Figures 2 and 3). In contrast, PIO treatment reduced in vivo plaque MMP activity by 13% (P < 0.05 versus the HCD control group), extending a prior ex vivo PIO study into the in vivo realm. MMP-9 was directly investigated for 3 reasons: (1) MMP-9 is a well-established destabilizing factor in atherogenesis, promoting both expansive remodeling and fibrous cap disruption; (2) prior stud-
ies suggest that TZDs may downregulate MMP-9 expression in cellular models, including Macs; and (3) MMP-9 substantially activates the MMP imaging agent (MMPSENSE) in atherosclerosis. Recent MMP9 gene deletion studies further support MMP-9 as a dominant activator of the NIRF imaging agent. Corroborating the in vivo molecular imaging findings, we found that PIO reduced MMP-9 expression by 66% and 63% on respective immunohistochemical and immunoblotting studies of atherosclerotic tissues (Figures 5 and 6).

The observed reductions in plaque MMP activity in vivo could be because of the combination of the following factors: (1) suppression of MMP expression from resident Macs, as demonstrated in vitro for various PPAR-γ agonists and herein specifically for the TZD PIO (supplemental Figure 1); (2) reductions in present or recruited plaque Macs; (3) changes in MMP inhibitor levels independent of any regulation of MMP mRNA expression or protein levels; and/or (4) a change in other non-Mac cells exerting a paracrine effect. To elucidate the relative contributions of these possibilities, we simultaneously imaged Mac phagocytic activity using spectrally distinct dextran-coated fluorescent nanoparticles validated for detecting plaque Macs. These unmodified nanoparticles are similarly absorbed by resting and activated Macs in vitro and do not target apoptotic cells. In the HCD group, we observed a 43% TBR increase in plaque Mac activity (Figures 2 and 3), similar to the 42% TBR increase in plaque MMP activity. In the PIO group, PIO reduced Mac activity by 19%, similar to the observed 13% reduction in MMP activity. Concurrent fluorescence microscopy, immunohistochemical, and immunoblotting analyses confirmed that PIO reduced plaque Mac content, as previously noted ex vivo. Notably, PIO-mediated reductions in plaque MMP activity were highly similar to reductions in plaque Macs by all in vivo and ex vivo measures.

To further assess the relationship between in vivo plaque MMP and Mac activity, we derived Pearson correlation coefficients (Figure 4). A significant relationship existed between the ∆MMP TBR and the ∆Mac TBR (r=0.96), with a stronger correlation evident in the PIO subgroup (r=0.92) compared with the HCD subgroup (r=0.75). The ability to assess and correlate these concomitant changes in plaque inflammation stemmed from the serial 2-point imaging method of the same carotid plaque for a given subject.

The integrated in vivo molecular imaging, microscopic, histological, and protein immunoblotting results suggest that PIO-mediated reductions in plaque MMP activity and presence are predominantly because of the reduced number of Macs that furnish MMPs, rather than reduced MMP expression from a numerically static Mac population. TZD-relevant mechanisms that may underlie reductions in plaque Macs, while beyond the scope of this investigation, include the following: (1) reduced monocyte recruitment via decreased expression of leukocyte adhesion molecules and/or decreased monocyte chemotaxis; and (2) increased TZD-mediated apoptosis of Macs.

From a translational imaging agent perspective, both Macs and MMP activity are viable clinical atherosclerosis molecular imaging targets. Iron oxide magnetic nanoparticles are already clinically used in noninvasive pharmacological MRI studies of Mac responses in the atherosclerotic plaques of carotid arteries. In addition, the backbone of the MMP-activatable agent has been safely tested in clinical trials, and a related cysteine protease-activatable NIRF agent is planned for clinical trials.

From a clinical technology perspective, NIR fluorescence molecular imaging is positioned well to interrogate the human coronary arteries via clinically translatable intravascular catheters that detect NIRF plaque inflammation through blood in coronary-sized arteries. From a noninvasive perspective, fluorescence molecular tomographic–based systems (3D noninvasive imaging systems that reconstruct fluorescence quantitatively deep in tissue) can be scaled up for interrogating the human carotid arteries, with and without ultrasonographic integration. Advances in either of these 2 arenas, coupled with Food and Drug Administration approval of appropriate NIRF imaging agents, may enable a clinical investigation of the anti-inflammatory effects of PIO in atherosclerosis.

Additional study findings merit further discussion regarding PIO’s effects on atherosclerosis. In contrast to a prior key atherosclerosis investigation of PPAR-γ agonists by Li et al., showing nonsignificant reductions in inflammatory plaque markers in female mice, this investigation found significant reductions in plaque MMP-9 and Mac levels. Potential explanations for this difference might include the use of rosiglitazone in the prior study as opposed to PIO and the prior use of low-density lipoprotein receptor–deficient mice rather than apoE–/– mice. PIO also reduced low-density lipoprotein levels, as noted by other researchers, which may also have contributed to the changes seen. Further mechanistic studies are needed to distinguish the relative contributions of PPAR activation and cholesterol alterations in the reductions of plaque inflammation observed herein. In addition, although not the focus of this in vivo inflammation investigation, PIO administered at a 3 times higher dose (ie, 40 mg/kg per day) has been reported to promote necrotic core formation but was not found at the lower dose used herein. The histological analysis of the carotid artery, a vessel much less investigated in murine atherosclerosis studies, as opposed to the aortic root or the inominate artery, was also important. Immunohistochemical analyses of carotid plaque MMP-9 and Macs were supplemented with more established plaque measurements, including whole aorta immunoblots, which also demonstrated reduced MMP-9 and Mac content and plaque area. The collagen content also increased, as seen by others. Last, experimental murine PIO dosages are higher than human dosages (<1 mg/kg per day); therefore dedicated clinical studies will be necessary to assess whether PIO can reduce human plaque inflammation.

Ultimately, additional clinical outcome trials in both non-diabetic and diabetic human subjects are needed to determine the net clinical and dose-dependent effect of PIO and distinctions between different PPAR-γ agonists. More specific in vivo assessments are required given the complexity of both the atherosclerotic disease process and transcriptional modifiers, such as PPAR agonists, which have complex effects and both stabilizing and destabilizing actions. Integrated biological and imaging studies in vivo in both mice and humans hold the potential to provide more specific and detailed information regarding antiatherosclerotic strategies.
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Disclosures

Drs Weissleder and Jaffer have equity interest and are consultants for VisEn Medical; and Dr Plutzky is a consultant for Roche, Novo Nordisc, Takeda, and Amylin Pharmaceuticals.

References

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SUPPLEMENTAL MATERIALS

Supplemental Methods

In vitro assessment of pioglitazone on MMP-9 protein expression in murine macrophages

Mouse peritoneal macrophages (MPMs) were isolated from C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). MPMs were isolated 4 days after intraperitoneal injection of 4% aged thioglycollate broth (Sigma, St. Louis, MO). MPMs were pelleted and resuspended in RPMI 1640 medium (Sigma). Cells of equal number (a total of $5 \times 10^6$ cells in each well) were seeded in 6-well plates and incubated at 37°C for 2 hours before gentle rinsing to remove nonadherent cells. Adherent MPMs were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum.

To examine the effect of pioglitazone on MMP-9 protein secretion from murine macrophages, MPMs were pretreated with with pioglitazone hydrochloride (10 µM, Takeda Pharmaceuticals North America, Lincolnshire, IL) for 18 hours, and then stimulated with lipopolysaccharide (LPS, 100 ng/mL, Sigma) for a period of 24 hours (PIO+,LPS+). Separate control groups using equal numbers of cells included 1) MPMs without any manipulation (PIO-, LPS-) and 2) MPMs stimulated with LPS for 24 hours (PIO-, LPS+). Cell culture supernatants from each of the three groups were collected and concentrated to the same volume using Amicon ultra-15 filters (Millipore, Billerica, MA). A total of 20 µL of concentrated culture supernatants was loaded into each lane for
immunoblotting. Experiments were performed in triplicate. Band intensity was quantified by densitometry of immunoblots using ImageJ (version 1.36b, NIH Bethesda, MD). Equal supernatant protein loading from each group of was verified by Ponceau S staining of membranes.

Spectrally distinct MMP and macrophage imaging agents for in vivo fluorescence molecular imaging.

Carotid plaque MMP activity was imaged using a near-infrared fluorescence (NIRF) MMP-activatable probe (MMPSense680, 200 nmol/kg, ex/em 680nm/700nm, Visen Medical, Bedford, MA) with avidity for plaque gelatinases.¹ Macrophage phagocytic activity was detected utilizing a spectrally distinct macrophage-avid dextranated nanoparticle (CLIO-Cy7, 10 mg Fe/kg, ex/em 750 nm/800 nm, MGH-CMIR, Boston, MA).² At the time of imaging, a third spectrally resolved fluorescence agent, FITC-dextran (MW 2,000,000, 10 mg/kg, ex/em 494 nm/521 nm, Sigma), was injected to provide a vascular angiogram outlining carotid arterial plaques.

Intravital fluorescence microscopy

IVFM studies employed a multichannel laser scanning fluorescence microscope (IV 330, Olympus Corp, Tokyo, Japan) equipped with 3 laser lines (488-, 633-, and 748-nm excitation) and optimized for intravital imaging. The utilized 4x objective (NA 0.15) provided an in-plane resolution of 13×13 µm. Z-stacks (30-50 slices) were obtained at 10 µm steps through the vessel. A plastic tube phantom (PE-10 tubing, Becton Dickinson, Franklin Lakes, NJ) was placed underneath carotid artery bifurcation and
served to co-register the imaging fields of the 2 IVFM datasets. Excitation with three laser lines and image collection of the different channels were interleaved to minimize crosstalk between channels. All image settings were kept constant for all time points and samples.

**Image analysis**

Image analysis of IVFM data sets was performed by compiling z-stacks into a 2-dimensional summation image (ImageJ, version 1.36b, Bethesda, MD). Plaques were further colocalized as filling defects on FITC-dextran generated angiograms. Regions-of-interest (ROIs) were manually traced within the plaque and the adjacent normal vessel as previously.\(^{3-5}\)

The total NIRF signal in the ROI was calculated as the summation of the signal intensity (SI) of all pixels in the ROI. The plaque target-to-background ratio (TBR) was calculated as the ratio of plaque SI to the adjacent vessel background SI. The plaque MMP and macrophage TBRs were measured in each respective channel. If a carotid plaque was not visualized on baseline IVFM, the animal was excluded from the study. The change in TBR (\(\Delta\)TBR) for each respective NIRF channel was defined as the difference in TBR between 2\(^{nd}\) IVFM and 1\(^{st}\) IVFM.

**Histopathology**

After sacrifice, mice were perfused with 0.9% saline (20 mL) via the left ventricle. For histopathological analysis, excised right carotid arteries and aortic roots were
embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), and the remainder of the aorta was snap-frozen for Western blot analysis. Serial 6-μm-thick cryostat sections were obtained from embedded aortic roots and carotid arteries for fluorescence microscopy. Adjacent sections were stained with hematoxylin and eosin (H&E) for general morphology and Masson’s trichrome for collagen.

Immunohistochemistry on adjacent sections was performed for macrophages (anti-mouse Mac3, BD Biosciences, San Jose, CA) and MMP-9 (anti-mouse MMP-9, Abcam Inc., Cambridge, MA), using avidin-biotin peroxidase method. Briefly, sections treated with 0.3% hydrogen peroxide were incubated for 60 minutes with a primary antibody, followed by respective biotinylated secondary antibody. The reaction was visualized with a 3-amino-9-ethyl-carbazol substrate (AEC, Sigma), and counterstained with Harris hematoxylin solution. Adjacent sections treated with nonimmune IgG provided controls for antibody specificity.

**Quantitative histological measurements of plaque sections**

Stained tissue sections were viewed with a microscope (Nikon Eclipse 50i, Tokyo, Japan), and images were digitally captured with a CCD-SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI). Measurements were performed on IPLab imaging software (version 3.9.9; Scanalytics Inc., Rockville, MD). The %MMP-9 and %macrophage areas were defined as the respective positive immunostained areas divided by the total carotid plaque area (2 sections from each of the 13 animals, 26 total sections). Quantification of aortic lesion areas (mm²) was performed using the software
program ImageJ (NIH). Calibration of digital aortic plaque images was performed using an image of a hemacytometer slide, where 1 mm was determined to equal to 1600 pixels. The aortic plaque area was calculated as the area between the internal elastic lamina and the lumen on H&E sections (3 sections from each of the 13 animals, 39 total sections). The percent collagen area on aortic plaque sections was defined as the percentage of positive staining area per total plaque area in Masson’s trichrome stained sections.

**Fluorescence microscopy**

Fluorescence microscopy was performed on fresh-frozen carotid atheromata sections using an upright epifluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan) interfaced with a cooled CCD camera (Cascade, Photometrics, AZ). Fluorescence images were obtained at a wavelength of far red (filter, 650±22.5 nm excitation; 710±25 nm emission, Q680LP bandpass, exposure time 1.0 second), or near-infrared (filter, 775±25 nm excitation; 845±27.5 nm emission, Q810LP bandpass, exposure time 30.0 seconds), to visualize the distribution of MMPsense680 (MMP activity) or CLIO-Cy7 (macrophage activity) fluorescence in plaque sections, respectively.

**Immunoblot analysis**

For mouse peritoneal macrophages, MMP-9 immunoblotting was performed using a goat monoclonal antibody (1:1000, R&D Systems, Minneapolis, MN) applied to concentrated supernatants.
For immunoblotting of aortic vessel lysates, frozen aortas were first pulverized, added to RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with freshly added protease inhibitors, and centrifuged (10 minutes, 13,000 rpm, 4°C). Next the protein extract was boiled in electrophoresis buffer for 10 minutes, followed by the separation on 10% polyacrylamide gels under reducing conditions (β-mercaptoethanol) and transfer onto Immobilon-P membranes (Millipore) using semi-dry transfer (2 hr, 18V). After nonspecific binding sites were blocked for 1 hour with 5% delipidated milk in TBST (20 mM Tris, 55 mM NaCl, and 1% Tween 20), immunoblotting for MMP-9 (1:1000, R&D Systems) and macrophages (anti-mouse LAMP-2, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) was performed using chemiluminescence (PerkinElmer Life Sciences, Boston, MA).

Measurement of plasma metabolic and lipid parameters

At the time of sacrifice, blood was obtained for the measurement of plasma glucose, cholesterol and triglyceride levels using commercial colorimetric assay kits (Wako chemicals, Richmond, VA). Plasma insulin was measured with a murine ELISA kit (Crystal Chem, Downers Grove, IL) according to the manufacturer’s protocol.

Statistical analyses

Statistical analyses were performed with GraphPad Prism (v5.0; GraphPad Software, San Diego, CA). Data are expressed as mean±SEM. Densitometry measurements of MMP-9 signal on MPM immunoblots were assessed by ANOVA testing and followed by a post-hoc Tukey’s test for multiple comparisons. The unpaired Student’s’ t-test was
used to test differences in the $\Delta$TBR between the two groups, and was used for histological and immunoblotting parameters. The paired Student’s’ t-test was used to assess differences in the plaque TBRs between IVFM#1 and IVFM#2 within each group (HCD and HCD+PIO). Pearson correlation coefficients (r-values) were calculated for correlations between the $\Delta$MMP TBR and $\Delta$Mac TBR for the entire group, the HCD+PIO group, and the HCD group. A p-value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL REFERENCES


**SUPPLEMENTAL TABLE**

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<th>HCD</th>
<th>HCD+PIO</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>25.4±0.3</td>
<td>25.5±0.5</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Glucose (mg/dL)</strong></td>
<td>98.7±7.9</td>
<td>93.9±9.4</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Insulin (pg/mL)</strong></td>
<td>335.9±41.9</td>
<td>226.5±31.3</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Total Cholesterol</strong></td>
<td>747.7±32.3</td>
<td>519.6±39.7</td>
<td>0.0009</td>
</tr>
<tr>
<td><strong>Triglyceride (mg/dL)</strong></td>
<td>86.3±6.0</td>
<td>92.8±8.8</td>
<td>0.56</td>
</tr>
</tbody>
</table>

**TABLE.** Metabolic parameters of control (HCD) and pioglitazone-treated (HCD+PIO) apoE⁻/⁻ mice. Values are expressed as mean±SEM. HCD= high cholesterol diet, PIO=pioglitazone.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. In vitro assessment of pioglitazone on matrix metalloproteinase (MMP)-9 protein expression in murine peritoneal macrophages (MPMs). (A) Immunoblot analysis of MMP-9 protein expression in supernatants of MPMs pre-treated with or without pioglitazone (10 µM, 18hr) before lipopolysaccharide (LPS; 100 ng/mL, 24hr) stimulation. The immunoblot is representative of three independent experiments. (B) Densitomtery analyses revealed that LPS-mediated MMP-9 expression was attenuated by pioglitazone pretreatment (P<0.05).

Supplemental Figure S2. Pioglitazone reduced plaque collagen content (as assessed by Masson’s trichrome stain) in both (A) carotid and (B) aortic atheromata. HE,H&E-hematocytin and eosin stain; MT=Masson’s trichrome stain for collagen.