Enzyme-Sensitive Magnetic Resonance Imaging Targeting Myeloperoxidase Identifies Active Inflammation in Experimental Rabbit Atherosclerotic Plaques

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Background—Inflammation undermines the stability of atherosclerotic plaques, rendering them susceptible to acute rupture, the cataclysmic event that underlies clinical expression of this disease. Myeloperoxidase is a central inflammatory enzyme secreted by activated macrophages and is involved in multiple stages of plaque destabilization and patient outcome. We report here that a unique functional in vivo magnetic resonance agent can visualize myeloperoxidase activity in atherosclerotic plaques in a rabbit model.

Methods and Results—We performed magnetic resonance imaging of the thoracic aorta of New Zealand White rabbits fed a cholesterol (n=14) or normal (n=4) diet up to 2 hours after injection of the myeloperoxidase sensor bis-SHT-DTPA(Gd) [MPO(Gd)], the conventional agent DTPA(Gd), or an MPO(Gd) analog, bis-tyr-DTPA(Gd), as controls. Delayed MPO(Gd) images (2 hours after injection) showed focal areas of increased contrast (>2-fold) in diseased wall but not in normal wall (P<0.84) compared with both DTPA(Gd) (n=11; P<0.001) and bis-tyr-DTPA(Gd) (n=3; P<0.05). Biochemical assays confirmed that diseased wall possessed 3-fold elevated myeloperoxidase activity compared with normal wall (P<0.01). Areas detected by MPO(Gd) imaging colocalized and correlated with myeloperoxidase-rich areas infiltrated by macrophages on histopathological evaluations (r=0.91, P<0.0001). Although macrophages were the main source of myeloperoxidase, not all macrophages secreted myeloperoxidase, which suggests that distinct subpopulations contribute differently to atherogenesis and supports our functional approach.

Conclusions—The present study represents a unique approach in the detection of inflammation in atherosclerotic plaques by examining macrophage function and the activity of an effector enzyme to noninvasively provide both anatomic and functional information in vivo. (Circulation. 2009;120:592-599.)

Key Words: magnetic resonance imaging ■ atherosclerosis ■ inflammation ■ myeloperoxidase ■ contrast media

Atherogenesis is a complex, multistage process that culminates in rupture of a vulnerable plaque, leading to acute arterial occlusion and life-threatening clinical end points.1,2 Diagnosis of atherosclerosis can occur relatively late in the disease process, just preceding or even after the occurrence of an acute event. Delayed diagnosis limits the range and application of effective evidence-based intervention strategies to reduce morbidity and mortality of atherosclerosis.3 Therefore, there exists a pressing need to develop new biomarkers and imaging techniques to detect and evaluate vulnerable plaques before their rupture and to better risk stratify these patients.

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Imaging of inflammation has been proposed as a way to improve patient stratification and therapy monitoring, because this would allow localization of inflamed plaques, and the degree of inflammation in individual plaques could be tracked over time after treatment. Ultrasmall superparamagnetic iron oxide nanoparticles and other nanoparticles have...
been used to detect accumulation of phagocytic cells in atherosclerotic plaques.\(^5\) However, nanoparticles may be taken up by both active and resting macrophages,\(^6\) as well as other cell types such as neutrophils, endothelial cells, lymphocytes, and smooth muscle cells.\(^7\) In addition, different phagocytic cells play diverse roles in inflammation, with some cell types having more attenuated inflammatory or even antiinflammatory properties.\(^8\) Thus, imaging only phagocytes could overestimate the severity of plaque inflammation.

An emerging biomarker of plaque instability and future acute events is the enzyme myeloperoxidase. Elevated plasma myeloperoxidase concentrations are found in stroke patients\(^12\) presenting with acute chest pain\(^13\) and in apparently healthy individuals.\(^14\) Within advanced human atherosclerotic vulnerable plaques, myeloperoxidase is expressed predominantly by activated macrophages and macrophage-derived foam cells\(^15\) and consumes hydrogen peroxide to generate hypochlorite and other reactive oxygen species that contribute to plaque progression and rupture (online-only Data Supplement for diet details and plaque characterization; Figure IA).\(^18\) In the present study, we examined the utility of a unique functional imaging sensor of myeloperoxidase activity\(^19\) called MPO(Gd), which was previously shown to be highly sensitive and specific to myeloperoxidase activity in mouse models of multiple sclerosis\(^22\) and myocardial infarction,\(^23\) to identify inflamed plaques in a rabbit model of atherosclerosis on a clinical magnetic resonance imaging (MRI) scanner. Our hypothesis was that once inside the diseased wall, myeloperoxidase-mediated activation would cause the agent to oligomerize and bind to resident proteins, which would result in higher magnetic resonance (MR) signal and prolonged retention of the activated agent within myeloperoxidase-rich plaque (online-only Data Supplement Figure IB and IC).

### Methods

#### Animal Protocol

A total of 18 male New Zealand White (NZW) rabbits were used for the present study. Eleven rabbits were fed 100 g of cholesterol-supplemented rabbit chow per day for 28 to 29 months to promote the formation of aortic lesions, as described previously.\(^24\) The cholesterol level in the diet was titrated between 0.125% and 0.25% (wt/wt) for the length of the experiment. (Please see the online-only Data Supplement for diet details and plaque characterization; Figure IIA). Four age-matched male NZW rabbits were used as controls and were fed normal chow. Three additional cholesterol-fed NZW rabbits (17 months on diet) were used to establish the vessel-wall signal kinetics of the imaging agents. Animals were cared for in accordance with guidelines of the Canadian Council on Animal Care.

#### Imaging Agents

The myeloperoxidase sensor bis-5-hydroxytryptamide-diethylentriamine-pentaacetate gadolinium [bis-SHT-DTPA(Gd)] and the analog agent bis-try-DTPA(Gd) were synthesized according to a modified protocol based on the work of Querol et al.\(^19\) Chemicals were purchased from Sigma-Aldrich (St. Louis, Mo). DTPA-bisanyhydrine was reacted with serotonin (5HT) or tyramide (tyr) in dimethylformamide in the presence of an excess of triethylamine. The product bis-SHT-DTPA was isolated by recrystallization from methanol and acetone. Complexation with gadolinium was performed in the presence of 5% citric acid (wt/wt) and purified by high-performance liquid chromatography. Purity of the agents was confirmed by mass spectroscopy (matrix-assisted laser desorption/ionization time-of-flight spectroscopy). DTPA(Gd) was purchased from Berlex Laboratories (Wayne, NJ).

#### Magnetic Resonance Imaging

Animals were sedated via an intramuscular injection of stock anesthetic (ketamine 23.4 mg/kg, xylazine 1.3 mg/kg, and glycopyrrolate 0.0075 mg/kg) followed by intravenous administration of a 10-fold dilution (in saline) of this stock at a rate of \(\sim 3\) to 12 mL/h. Anesthetized rabbits were imaged in the supine position with a clinical 3T MRI scanner (GE Sigma HD 12x, GE Healthcare, Waukesha, Wis) interfaced with a custom-made, 2-channel, phased-array surface radiofrequency coil.\(^25\) Axial images of the thoracic aortas of 11 cholesterol-fed rabbits and 4 control rabbits were collected before and up to 2 hours after intravenous injection of either DTPA(Gd) 0.2 mmol/kg or MPO(Gd) 0.2 mmol/kg with a T1-weighted quadruple-inversion-recovery fast-spin-echo sequence developed previously for quantitative contrast-enhanced imaging of atherosclerotic vessels.\(^26\) Three cholesterol-fed rabbits were scanned up to 4 hours after and 24 hours after MPO(Gd) administration. In 3 additional cholesterol-fed rabbits, either MPO(Gd), DTPA(Gd), or bis-try-DTPA(Gd) (all at 0.1 mmol/kg) was administered, and imaging was performed on a 1.5T MRI scanner (GE Sigma HD 12x). A minimum of 3 days was allowed to pass between each imaging agent administration. Finally, ex vivo T1-weighted imaging was performed on perfusion-fixed (10% formalin) excised aortic specimens from a cholesterol-fed animal (17 months of feeding) euthanized 2 hours after injection of MPO(Gd). (See online-only Data Supplement for technical specifications of MRI.)

#### MR Image and Data Analysis

MR images were analyzed with an OsiriX DICOM reader (version 2.7.5, Geneva, Switzerland) by 2 independent readers (JAR and JWC). In each image, the inner and outer vessel-wall boundaries were traced to determine average wall signal intensity (SI\(_{\text{wall}}\)). Regions of interest were placed in both the paraspinal muscle adjacent to the aorta and in a motion-free region outside the animal (air) to determine average muscle signal intensity (SI\(_{\text{muscle}}\)) and the standard deviation of the noise signal (\(\sigma_{\text{noise}}\)), respectively. Contrast-to-noise ratios (CNR) between the wall and adjacent muscle were calculated (CNR\(_{\text{baseline}}\)=\((\text{SI}_{\text{wall}}-\text{SI}_{\text{muscle}})/\sigma_{\text{noise}}\)) and differences between baseline (CNR\(_{\text{baseline}}\)) and imaging after injection of contrast agent (CNR\(_{\text{enh}}\)−CNR\(_{\text{baseline}}\)). For signal kinetics experiments, a region outside the animal was not within the field of view; therefore, contrast (SI\(_{\text{wall}}\)-SI\(_{\text{muscle}}\)) at each time point was calculated and normalized to baseline values. Enhancement ratios (ERs) were determined (ER\(_{\text{enh}}\)=\((\text{SI}_{\text{wall}}-\text{SI}_{\text{muscle}})/\text{SI}_{\text{wall,baseline}}\)) as described previously.\(^27\) \(\Delta\text{CNR}\) was also calculated after tracing of select regions of interest within the wall that appeared noticeably bright on images collected 2 hours after MPO(Gd) administration compared with images collected before contrast and 2 hours after DTPA(Gd) administration. For correlational analyses, regions of interest were placed to measure plaque size and the areas positive for myeloperoxidase, macrophages, lipid, and collagen in histopathological sections (see below), with the reader blinded to the other imaging results.

#### Histology

After imaging, animals were euthanized with an intravenous injection of ketamine (200 mg) and perfused transcardially under pressure with \(\sim 1.5\) L of heparinized (1 IU/mL) Hanks’ balanced salt solution. The imaged aortic segments were isolated carefully, marked on the ventral surface with Evan’s blue dye for matching to MRI, and dissected. Fresh-frozen sections were collected from a portion (~1 mm of the 3-mm total thickness) of each block. Sections were then immunostained for plaque constituents, including myeloperoxidase and macrophages (RAM-11). Negative control staining was also performed. Please see the online-only Data Supplement for tissue block preparation, additional plaque characterization (Figure IIA), and peroxidase activity (Figure IIB) staining, as well as full staining descriptions. Images of stained sections were taken with a...
Figure 1. A, RAM-11 and myeloperoxidase immunostaining on adjacent sections revealed that macrophages and macrophage-derived foam cells at the surface of plaques expressed significant amounts of myeloperoxidase (MPO). In contrast, macrophages found in the deeper parts of the plaques often weakly expressed myeloperoxidase (arrows; scale bar represents 100 μm). B, On average, myeloperoxidase activity of diseased wall was 3-fold higher than in normal wall (n=8, 23 sections analyzed for diseased wall; n=4, 12 sections analyzed for normal wall).

Myeloperoxidase Activity Assay
To quantify myeloperoxidase activity in detergent extracts, we performed the guaiacol myeloperoxidase activity assay on a Beckman Coulter DU 530 UV/vis spectrophotometer (Fullerton, Calif). Briefly, the portion of each aortic block that remained after sectioning (~2 mm) was homogenized in 1% cetyltrimethylammonium bromide extraction buffer, followed by centrifugation for 10 minutes at 10 000 rpm. The supernatant (detergent extract) was collected, and protein concentration was determined with a standardized biuret uric acid assay (Pierce, Rockford, Ill). The myeloperoxidase activity assay solution consisted of 3 mL of 0.1 mol/L NaPO₄ buffer, supplemented with 48 mL of guaiacol and 100 μL of 0.1 mol/L H₂O₂. To this, 25 μg of protein was added to a final volume of 0.6 mL and assayed at 25°C. The units of activity were computed according to the following formula: Activity = (ΔOD × V × T × Δ)/E = sample volume, E (extinction coefficient) = 26.6 mmol/L-1, and Δt = change in time.

Statistical Analysis
A 2-tailed t test was performed for comparison between animal groups for the peroxidase activity assay (Figure 1B). For comparisons of MR data between animal groups, either a 2-way repeated-measures ANOVA (if normal and diseased animals were tested; Figure 2D) or a 1-way repeated-measures ANOVA (Figure 3A and 3B) was performed. For the above tests in which multiple measurements were collected per animal (eg, MR slices), the data were averaged to generate a single measurement per animal before the test was performed. Error bars in figures represent SEM. Pearson correlational analysis was performed between both histological measures and rabbit identity (multiple plaques imaged per rabbit) on MPO(Gd) MRI values (Table). The nominal level of significance for all tests was P<0.05. Both GraphPad Prism 4.0a (GraphPad Software Inc, San Diego, Calif) and SAS (SAS Institute Inc, Cary, NC) software were used for statistical analysis.

Results
Hypercholesterolemic Rabbits Develop Atherosclerotic Aortic Plaques That Contain Significant Numbers of Myeloperoxidase-Expressing Macrophages
We performed myeloperoxidase immunostaining on aortic sections from cholesterol-fed rabbits and confirmed that the diseased wall expressed myeloperoxidase (Figure 1A). As in humans, we found that the major source of myeloperoxidase in these plaques was from macrophages and macrophage-derived foam cells (Figure 1A). Most plaques contained more macrophage-positive areas than myeloperoxidase-positive areas on histopathological analysis (Figure IIIA in the online-only Data Supplement), which matched our observation that macrophages in deeper portions of plaques expressed less myeloperoxidase (Figure 1A). Myeloperoxidase activity in diseased aortic sections from cholesterol-fed rabbits was more than 3-fold higher than in corresponding normal aortic sections from control rabbits (Figure 1B; P<0.01). Furthermore, we found moderate positive correlations between the size of individual plaques and both the area of myeloperoxidase (r=0.78; P<0.001) and macrophage (r=0.76; P<0.001) immunostaining and myeloperoxidase activity (r=0.58; P<0.05; Figure IIIB, IIIC, and IIID in the online-only Data Supplement, respectively). Therefore, similar to previous findings, these results suggest that as myeloperoxidase expression and activity increase, so does plaque size.

MPO(Gd) Imaging Demonstrates Increased and Prolonged Contrast of Atherosclerotic Aortic Wall
To understand the behavior of MPO(Gd) in diseased walls, we performed dynamic imaging after MPO(Gd) injection in 3 rabbits fed a cholesterol diet for 17 months (Figure 2). We then compared the kinetics of the MR signal intensity change within individual plaques (compared with muscle) using MPO(Gd) with those obtained using DTPA(Gd), which has no molecular specificity (Figure 2B). We found that both DTPA(Gd) and MPO(Gd) had similar contrast enhancement over the first 20 minutes of imaging (Figure 2A and 2C), but in certain areas of the plaques, MPO(Gd) started to demonstrate higher contrast by 30 minutes of imaging (Figure 2C). By 2 hours, an unequivocal, large difference in contrast existed between DTPA(Gd) and MPO(Gd) (Figure 2B and 2C; online-only Data Supplement Figure IVA and IVB), and this difference persisted at nearly the same level over at least 4 hours of imaging (Figure 2C; online-only Data Supplement Figure IV A). This is consistent with the activated agent being retained in myeloperoxidase-rich regions. Furthermore, we noted that intraplaque areas that did not enhance strongly on early- or late-phase DTPA(Gd) or early-phase MPO(Gd) images were clearly enhanced on delayed MPO(Gd) images.
By 24 hours, the signal in the plaques had returned to baseline levels (online-only Data Supplement Figure IVB). Note that the same dose (0.2 mmol/kg) was administered for both agents and that in the absence of myeloperoxidase, MPO(Gd) had similar $r_1$ relaxivity as DTPA(Gd) (both approximately $4 \text{s}^{-1}\text{mmol/L}^{-1}$ at 1.5 T) and blood half-life (in mice, ≈6 minutes). Furthermore, it was noted that the increased enhancement was often not homogeneous within the vessel wall but was selective, showing focal areas of enhancement that persisted in the late MPO(Gd)-enhanced images but not in the late DTPA(Gd)-enhanced images (online-only Data Supplement Figure IVA).

MPO(Gd) Imaging Results in More Than a 2-Fold Increase in Contrast in Areas With Increased Myeloperoxidase Activity

On the basis of our dynamic imaging results, precontrast and 2-hour postcontrast imaging with MPO(Gd) and DTPA(Gd) were performed in 8 rabbits fed a cholesterol diet for 28 to 29 months and 4 age-matched controls. Analysis of the imaging data (Figure 2D) revealed that in focal areas within the diseased wall that displayed increased signal, there was more than a 2-fold increase in the difference in the contrast-to-noise ratio at the 2-hour time point ($\Delta\text{CNR}$) compared with the same regions imaged with DTPA(Gd) ($P<0.001$). For normal wall, no significant difference in $\Delta\text{CNR}$ was noted between the 2 agents ($P=0.84$), and we obtained similar $\Delta\text{CNR}$ values of the normal and diseased wall imaged with DTPA(Gd) ($P=0.92$). Representative images of normal wall after administration of either MPO(Gd) or DTPA(Gd) over the entire 2-hour period are available in the online-only Data Supplement (Figure IVC). To avoid potential bias, we also performed the same analysis over the entire wall (Figure 2D). Despite averaging in areas without increased myeloperoxidase activity, similar results were obtained, with a 58% increase in $\Delta\text{CNR}$ between diseased and normal walls imaged with MPO(Gd) (Figure 2D; $P<0.01$). Finally, enhancement

Figure 2. A, As-acquired (5-cm field-of-view; top) and higher-magnification (bottom; centered around aorta) early-phase (~10 minutes) MR images of diseased wall in rabbit fed a cholesterol diet for 17 months, taken after administration of DTPA(Gd) (left) and MPO(Gd) (right). Similar levels of enhancement were seen at this time point with both agents. B, Delayed MPO(Gd) images (~2 hours; right) showed substantially increased enhancement compared with both late-phase (~2 hours; left) and early-phase (seen in A) DTPA(Gd) images. C, Kinetics study. Increased and prolonged contrast was found with MPO(Gd) compared with DTPA(Gd) (n=3 animals). D, Analysis of precontrast and 2-hour postcontrast [MPO(Gd) and DTPA(Gd)] MR images of rabbits fed a cholesterol diet for 28 to 29 months (n=8) and age-matched controls (n=4). $\Delta\text{CNR}$ in both focal wall areas (left) and the entire wall (right) revealed no difference from MPO(Gd) and DTPA(Gd) in normal wall (n=4, 11 sections analyzed). Diseased wall imaged with DTPA(Gd) (n=8, 24 sections analyzed) also showed no difference compared with normal wall imaged with either agent. In distinction, diseased wall imaged with MPO(Gd) (n=8, 24 sections analyzed) showed significantly higher $\Delta\text{CNR}$ (~2×) than all other values.
ratio analysis over the entire vessel wall was performed, which revealed no significant difference between MPO(Gd) and DTPA(Gd) imaging for normal wall (P=0.56), whereas MPO(Gd) administration significantly and consistently enhanced diseased wall to a greater degree than DTPA(Gd) administration (P<0.0001; online-only Data Supplement Figure IVD).

To further verify that the focal enhancement observed with MPO(Gd) imaging was not from nonspecific accumulation of MPO(Gd) but from activation by myeloperoxidase, we synthesized an analog of MPO(Gd), bis-tyr-DTPA(Gd), which is a substrate for peroxidases such as horseradish peroxidase but is not activatable by myeloperoxidase. We performed comparative imaging in 3 rabbits with MPO(Gd), bis-tyr-DTPA(Gd), and DTPA(Gd) (Figure 3). This experiment showed that only MPO(Gd) imaging demonstrated significantly increased ΔCNR and ER was found with MPO(Gd) compared with both control agents, which supports the concept that the increased enhancement observed is the result of myeloperoxidase activation of MPO(Gd). No significant (NS) differences were observed between the analog and DTPA(Gd). C, Representative 2-hour delayed images demonstrated that only the activatable MPO(Gd) was able to show focal increased enhancement to confirm myeloperoxidase activity. The window/level settings were determined on the precontrast images to ensure that the paraspinal muscles appeared similar between images for each agent, and then these settings were applied to the postcontrast images. D, Myeloperoxidase immunohistochemistry (IHC) confirmed MPO(Gd) imaging findings.

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### Table. Multivariable Regression Parameters for Positive Areas of MPO(Gd) Imaging Based on Model That Included Rabbit Identity and Various Histological Measures of Plaque Composition

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Regression Parameters</th>
<th>r² (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO IHC</td>
<td>P=0.0001</td>
<td>0.82</td>
</tr>
<tr>
<td>Mac IHC</td>
<td>NS</td>
<td>...</td>
</tr>
<tr>
<td>Lipid</td>
<td>NS</td>
<td>...</td>
</tr>
<tr>
<td>Collagen</td>
<td>NS</td>
<td>...</td>
</tr>
<tr>
<td>Rabbit</td>
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MPO IHC indicates myeloperoxidase immunohistochemistry; Mac IHC, macrophage immunohistochemistry; and NS, not significant (P>0.15).

*Pearson correlation coefficient in analysis of histological measures and positive areas of MPO(Gd) imaging.

**Figure 3. Imaging with a nonactivatable analog of MPO(Gd).** ΔCNR (A) and enhancement ratio (ER) analysis (B) of diseased walls in cholesterol-fed rabbits (n=3 rabbits, 11 sections analyzed) imaged after administration of DTPA(Gd), MPO(Gd), and a nonactivatable analog of MPO(Gd) (bis-tyr-DTPA(Gd)), with each agent administered at least 3 days apart. Significantly increased ΔCNR and ER was found with MPO(Gd) compared with both control agents, which supports the concept that the increased enhancement observed is the result of myeloperoxidase activation of MPO(Gd). No significant (NS) differences were observed between the analog and DTPA(Gd). C, Representative 2-hour delayed images demonstrated that only the activatable MPO(Gd) was able to show focal increased enhancement to confirm myeloperoxidase activity. The window/level settings were determined on the precontrast images to ensure that the paraspinal muscles appeared similar between images for each agent, and then these settings were applied to the postcontrast images. D, Myeloperoxidase immunohistochemistry (IHC) confirmed MPO(Gd) imaging findings.

**MPO(Gd) Imaging Reveals Areas of Increased Myeloperoxidase Activity in Atherosclerotic Plaques**

To identify areas of increased myeloperoxidase activity, and thus “active” inflammation, we compared MR images taken 2 hours after injection of MPO(Gd) with corresponding sections immunostained for myeloperoxidase. Two representative sections (Figure 4A) showed that the areas highlighted by MPO(Gd) imaging matched extremely well to myeloperoxidase-positive staining. A scatterplot (Figure 4B) that compared the positive areas identified on MPO(Gd) imaging with areas of positive myeloperoxidase immunostaining further validated the imaging findings across all the animals studied. Multivariable regression analysis was performed to investigate any effects of rabbit identity (multiple plaques were analyzed per rabbit) and the various histological measures of plaque composition on the variability in the MR data (Table). This analysis revealed a significant relationship between the area of myeloperoxidase immunostaining and positive MPO(Gd) (P<0.0001). No other measures achieved significance. To further validate the imaging findings, we performed correlation analysis and found that the areas highlighted during MPO(Gd) imaging at 2 hours correlated well to areas that were stained positive on myeloperoxidase histopathology (Table; r=0.91, P<0.0001). We further compared MPO(Gd) imaging results with macrophage, lipid, and collagen histopathology (Table). We found a mild positive
correlation of MPO(Gd) imaging with lipid histopathology ($r=0.66$, $P<0.05$) but no significant correlation with macrophage or collagen histopathology.

To learn about the regions of the plaque where MPO(Gd) accumulated, we also performed ex vivo imaging of fixed aortic segments from a rabbit euthanized at 2 hours after MPO(Gd) administration (Figure 5). We obtained nearly exact matches between the brightest areas on in vivo and ex vivo images. Interestingly, MPO(Gd) accumulated most often in the shoulder regions of the plaque, an area of high mechanical stress. In addition, MPO(Gd) appeared not only to be retained in the intima and fibrous cap but also to have penetrated into the plaque core.

**Discussion**

In the present study, we showed that myeloperoxidase activity in rabbit atherosclerotic plaques, and thus biologically relevant active inflammation, can be detected noninvasively with a clinical MR scanner by use of the myeloperoxidase-activatable agent MPO(Gd). Myeloperoxidase-rich areas were selectively enhanced by MPO(Gd) and easily identified 120 minutes after administration. We verified that the foci of increased intensity on MPO(Gd) imaging colocalized and correlated with myeloperoxidase-rich areas infiltrated by macrophages on histopathological evaluations. Biochemical assays showed that atherosclerotic plaques possessed elevated myeloperoxidase activity compared with normal arterial walls and that myeloperoxidase activity correlated positively with plaque size. The results demonstrate that in vivo myeloperoxidase activity is well associated with atherosclerotic plaque development and progression.

The animal model in the present study develops arterial plaques that exhibit several plaque features that have been described as markers of plaque vulnerability in human plaques, including neovascularization and extensive macrophage infiltration. Interestingly, some areas within the plaques were rich in macrophages but not in myeloperoxidase (Figure 1; Figure III in the online-only Data Supplement), which agrees with the concept of distinct macrophage phenotypes within plaques. This was supported by the strong correlation of MPO(Gd) imaging to myeloperoxidase immunostaining but not macrophage staining, because areas that contain myeloperoxidase-poor macrophages should not be highlighted by the agent we used (Table). There was also a mild positive correlation of MPO(Gd) imaging with lipid histopathology but not with collagen histopathology (Table),
which is consistent with the concept that lipid cores are associated with more advanced unstable plaques and that many of the macrophages that produced myeloperoxidase were rich in lipid (foam cells). These findings underscore the importance of our functional approach. Furthermore, the plaques in the present rabbit model expressed substantially lower amounts of myeloperoxidase (mean 7.7 U/mg protein) than did human atherosclerotic tissue (mean 251 U/mg protein). Although this underscores some differences between rabbit and human plaques, it also has important implications for the translation of this agent to the imaging of human plaques, because the increased myeloperoxidase content of human plaques should result in substantially greater signal intensity at sites of MPO(Gd) activation, which could allow lower doses of the agent to be used, as well as increasing the sensitivity for detecting plaque vulnerability at the earliest stages of development.

Previous enzyme-sensitive MRI agents were based on a cleavage mechanism to remove masking groups that limited water access, albumin-binding groups, or solubility. These studies represented significant advances in imaging agent design. However, to date, these prototypical agents have not been shown to be effective in vivo without significant manipulations of the test animals (which had been limited to invertebrates and small mammals), and unlike MPO(Gd), they cannot be administered intravenously, which is important for clinical translation. MPO(Gd) achieves signal amplification by enzymatic addition instead of cleavage. In a mouse model of myocardial infarction treated with atorvastatin, we found that MPO(Gd) was sufficiently sensitive to detect a decrease in myeloperoxidase activity and inflammation. Furthermore, MPO(Gd) discriminated between wild-type mice with full myeloperoxidase expression, myeloperoxidase-heterozygous mice with intermediate myeloperoxidase expression, and myeloperoxidase-knockout mice with no myeloperoxidase expression. Because myeloperoxidase is highly conserved across mammalian species, these results and the results of the present study confirm the high sensitivity and specificity of the agent for myeloperoxidase activity.

In summary, key advantages of this molecular technology lie in its ability to enable clinical MRI scanning and T1-weighted sequences to identify pathology noninvasively and to localize and track harmful oxidative reactions in atherosclerotic lesions. An additional advantage is the short readout delay between injection and imaging (90 to 120 minutes). The present study provides initial proof-of-principle for a new, more specific approach to the imaging of inflammation in atherosclerotic plaques. An additional advantage is the short readout delay between injection and imaging (90 to 120 minutes). This technology thus can localize plaques with significant active inflammation before devastating thromboembolic events occur. Consequently, it could change clinical standards by enabling earlier diagnosis and improved risk stratification, as well as allowing the ability to track the effects of timely, patient-specific interventions.

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Disclosures

None.

References

Inflammatory cells, particularly macrophages, are believed to undermine the stability of atherosclerotic plaques, promoting plaque rupture and subsequent life-threatening thrombosis. The present study represents a novel approach in imaging inflammation in atherosclerosis by imaging macrophage function and the activity of a key effector enzyme, myeloperoxidase, which is known to trigger oxidative reactions. Unlike measurement of the presence of phagocytes, measurement of myeloperoxidase activity in plaques is likely to have greater specificity to identify vulnerable plaques. This molecular imaging technology can potentially localize plaques with active inflammation before devastating thromboembolic events occur.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animal Protocol

To promote the formation of aortic atherosclerotic plaques New Zealand white (NZW) rabbits were fed a cholesterol (CH)-supplemented diet in which the amount of CH varied between 0.125 and 0.25% (w/w). At first, rabbits were fed the 0.25% CH-diet resulted in an average serum CH level of ~1200 mg/dl at 7 months. Thereafter, diets were varied between 0.125% and 0.25% (w/w) CH to maintain the animal’s eating habits and health. Average serum CH levels maintained between ~400 and ~700 mg/dl from 13 months to the end of the experiment. This diet regimen was originally developed to produce aortic lesions that resemble late-stage human atheroma (American Heart Association (AHA) type IV) after 6 months of feeding \(^1, 2\) and we have recently shown that MR detectable lesions are visible at 8 months of feeding, with increased lesion complexity detectable beyond 16 months \(^3\).

Magnetic Resonance Imaging

A standardized protocol was used for imaging anesthetized rabbits in the supine position. This consisted first of a 2D time-of-flight (TOF) MR angiogram (repetition time (TR)/echo time (TE) = 25/5.1 ms; flip angle (FA)= 45º; bandwidth (BW) = ±15.63 kHz; field of view (FOV) = 8 cm; matrix = 256 x 256; thickness = 1.5 mm; excitations = 1; 50 slices; scan time = 5:28). Next, 3 axial images were prescribed using the angiogram by placing the 1\(^{st}\) slice 2 cm above the celiac bifurcation and thereafter using a constant gap of 7 mm between each successive image, allowing images at 2, 3 and 4 cm above the
celiac bifurcation to be collected. Care was taken to ensure that the RF coil was positioned in the same portion of the animal during each imaging session. Images were collected pre- and up to 2 hours post-injection of either DTPA(Gd) (0.2 mmol/kg) or MPO(Gd) (0.2 mmol/kg) using a T1-weighted (T1w) quadruple inversion recovery fast-spin-echo (QIR-FSE) sequence (TR/TE = 800/21.77 ms; inversion time (TI) = 520 ms; echo train length (ETL) = 6; BW = ±11.9 kHz; FOV = 5 cm; matrix = 320 x 320; resolution = 0.156 x 0.156 x 3 mm$^3$; excitations = 14; scan time = 30:20, ~10 min per slice acquired serially). For signal kinetic experiments, to maintain good visualization of the vessel wall for quantification of enhancement, while increasing the temporal resolution (10 slices in ~9 minutes), we sacrificed some spatial resolution (0.195 x 0.260 x 5 mm$^3$). Here 10 axial QIR-FSE images were acquired with the following parameters (TR/TE = 800/16.8 ms; TI = 520 ms; ETL = 6; BW = ±11.9 kHz; FOV = 5 cm; matrix = 256 x 192; excitations = 2; scan time = 8:50, 53 seconds per slice acquired serially). A chemically-selective fat-suppression pulse was used to null the signal from periadventitial fat$^4$ in all QIR-FSE images. QIR-FSE was previously developed specifically to allow quantification of vessel wall enhancement following the intravenous injection of Gd-based (T1 shortening) contrast agents$^5,6$. Finally, high-resolution (0.1 x 0.1 x 0.3 mm$^3$) \textit{ex vivo} imaging was performed on aortic specimens from a rabbit sacrificed 2 hours after MPO(Gd) administration. This was performed at 3T using a combination of both customized insertable gradient (peak strength: 500 mT/m, peak slew rate: 3200 T/m/s) and solenoidal RF coils, and a 3D fast spoiled gradient echo (fSPGR) pulse sequence (TR/TE = 5.8/2.6 ms; FA = 30$^\circ$; BW = ±31.25 kHz; FOV = 2 cm; matrix = 200 x 200; excitations = 9; scan time = 28:29).
Histology

After animal sacrifice and perfusion, animals were dissected in the supine position (similar to the position during MR imaging). The aorta was exposed and the celiac bifurcation was identified. The celiac was used as our primary anatomical marker since this was easily identifiable during the MR angiogram performed and was used to prescribe the axial T1w MR images. Next, the ventral surface over the entire length of the aorta was marked with Evan’s blue dye to facilitate future rotation of microscope images of aortic sections allowing correct matching of the orientation between histological sections and MR slices. Finally, the 3 mm aortic tissue blocks at 2, 3 and 4 cm above the celiac bifurcation (corresponding to the MR imaged segments) were carefully dissected and snap frozen in OCT using liquid nitrogen and isopentane.

Perfusion fixation was avoided to allow both MPO immunostaining of plaque cryosections and assessment of the level of MPO activity of the imaged plaques, which requires fresh tissue. Axial cryostat sections (10 µm) of a portion of each tissue block (~1 mm) were collected, and both the left over block (~2 mm) and tissue sections were stored at -80°C until detergent extraction and staining, respectively. Adjacent sections of each plaque (10 µm) were immunohistochemically stained for both macrophages and MPO. Sections were fixed in 10% formalin (5 minutes), pre-incubated with 10% horse sera (30 minutes), and incubated with either mouse anti-human MPO IgG2a (0.04 mg/ml; Cedarlane Laboratories Limited, Hornby, ON) or mouse anti-rabbit macrophage IgG1 (RAM-11; 0.0164 mg/ml; DakoCytomation, Mississauga, ON) primary antibodies in 10% horse sera (24 hours). Negative control sections were incubated with 10% horse sera only. Sections were then incubated with biotinylated horse anti-mouse IgG (0.015
mg/ml; Vector Laboratories, Burlington, ON) secondary antibodies in 10% horse sera (30 minutes), followed by an ABC-alkaline phosphatase complex solution (30 minutes; Vector Laboratories, Burlington, ON). Finally, the stain was developed by addition of a Vector black substrate solution to each section (30 minutes; Vector Laboratories, Burlington, ON), and each slide was rinsed, dehydrated, and mounted. Similarly, macrophage and neutrophil immunostaining was also performed on adjacent sections using RAM-11 and a mouse anti-rabbit neutrophil defensin-5 antibody (1:20 dilution; clone R3; Abcam Inc.; Cambridge, MA). Sections were then incubated with a biotinylated horse anti-mouse IgG secondary antibody (0.006 mg/ml; Vector Laboratories, Burlington, ON), processed using an ABC-HRP complex solution for 30 minutes (Vector Laboratories, Burlington, ON), and visualized with 3'-diaminobenzidine (50 mg/100 ml; Sigma-Aldrich Canada, Oakville, ON; countered with hematoxylin for 1 minute).

AHA classification of aortic lesions was performed using several staining techniques. Nearly adjacent sections were stained for various components found in human plaques using either: 1) a combination of Oil red O (lipid), Von Kossa silver stain (calcium) and countered with hematoxylin (1 minute); 2) picrosirius red (collagen) staining imaged with either transmitted (Zeiss Axioplan 2ie; Carl Zeiss Canada, Toronto, ON) or circularly polarized microscopy (Nikon Canada Inc.; Mississauga, ON); or 3) CD-31 (endothelial cells) immunohistochemistry using a mouse anti-human CD-31 monoclonal IgG1 primary antibody (clone JC/70A; 0.003 mg/ml; Abcam Inc., Cambridge, MA), a biotinylated horse anti-mouse IgG secondary antibody (0.006 mg/ml; Vector Laboratories, Burlington, ON), processed using an ABC-HRP complex solution.
for 30 minutes (Vector Laboratories, Burlington, ON), and visualized with 3’-diaminobenzidine (50 mg/100 ml; Sigma-Aldrich Canada, Oakville, ON; countered with hematoxylin for 1 minute).

Finally, peroxidase activity staining was performed on sections as previously described. Briefly, sections were incubated in a diaminobenzidine tetrahydrochloride (DAB, D5905; Sigma-Aldrich Canada, Oakville, ON) solution containing hydrogen peroxide for 20 min (10 ml TRIS buffer, pH 8.2; 6 mg DAB; and 20 ml of 6% hydrogen peroxide). The slide was then covered using an aqueous mounting medium (Aquatex, VWR International, Mississauga, ON).

**Supplemental Results**

**Plaque Characterization**

Our model consisted of feeding NZW rabbits a low-level (0.125% to 0.25% w/w) cholesterol (CH) diet over 2 years without mechanical injury. These animals develop atherosclerotic plaques throughout the aorta \(^1,2\) and at 28 to 29 months of feeding the plaques we imaged were composed largely of fibrosis with smooth muscle cells overlying lipid cores interspersed with macrophage foam cells (Fig. S2A). At the subendothelial surface of many plaques a significant number of macrophages and macrophage-derived foam cells were found (Fig. 1A). Regions of calcification, vasa vasorum expansion, and intraplaque neovascularization were also apparent (Fig. S2A). Hence, the majority of plaques found at this time point in this rabbit model resemble AHA class type Va or Vb fibroatheroma found in humans \(^8\).
Peroxidase Activity Staining

Staining for endogenous peroxidase (ie. MPO) activity also revealed that areas positive for MPO immunostaining matched well to areas positive for peroxidase activity, suggesting the majority of peroxidase in the plaque was MPO and supporting the use of MPO immunostaining as a surrogate marker of MPO activity (Fig. S2B). Again, macrophages found deeper in plaques were often negative for both MPO immunostaining and peroxidase activity staining.
**Supplemental Figures and Figure Legends**

**Fig. S1:** Biological role of myeloperoxidase (MPO) in atherogenesis, MPO sensing mechanism. (A) Granulocyte macrophage colony-stimulating factor (GM-CSF) present in plaques is a regulator of the expression of MPO by macrophages. Secreted MPO consumes hydrogen peroxide (H$_2$O$_2$) to catalyze the formation of a variety of oxidative species, resulting in a cascade of anti-atherogenic events linked to the progression and destabilization of plaques. (B) MPO(Gd) is an MPO-responsive MR agent. Without MPO present, MPO(Gd) exists as monomers with an $r_1$ relaxivity similar to its parent compound DTPA(Gd). In the presence of MPO activity, MPO(Gd) is activated and forms oligomers with increased $r_1$ (increased MR signal). Activated agent can also bind to proteins, further increasing its $r_1$ and promoting retention of the agent. (C) Within MPO-rich vulnerable plaques (top), MPO secreted by active macrophages can activate MPO(Gd), causing oligomerization and protein binding. In contrast, in more stable plaques with minimal or no MPO present (bottom) MPO(Gd) is not activated and will more rapidly wash-out of the plaque.

**Fig. S2:** (A) Prolonged CH-feeding promotes the formation of complex plaques containing MPO-expressing macrophages. Plaques in this rabbit model have structural similarities to AHA type Va and Vb human plaques (top left – lipid, calcium and nuclei; top right – collagen; bottom – endothelial cells (CD-31), and nuclei; scale bars: low mag – 500 µm; high mag - 100 µm). (B) Differential MPO expression and activity within distinct macrophage populations within plaques. Low magnification images (top row) reveal that areas rich in macrophages (left column) in general corresponded well with
both areas positive for MPO immunohistochemistry (MPO IHC-middle column), and areas positive for peroxidase (i.e. MPO) activity (brown stain – right column; scale bar represents 500 μm). At higher magnification (bottom row) positive macrophage staining at the surface often corresponded well to both MPO protein and activity staining (asterisks). Importantly, deeper regions within plaques (dashed circles) that were positive for macrophages were often found to be weakly staining or negative for both MPO protein and activity (scale bar represents 200 μm).

**Fig. S3:** (A) Analysis of the area immunostained for MPO and macrophages in adjacent sections (n=8 animals, 17 sections analyzed). In general, plaques contained more macrophage positive areas than MPO positive areas suggesting some macrophages did not express MPO, supporting our qualitative observations. The area of (B) MPO (n=8, 16 sections) and (C) macrophage (n=8 rabbits, 17 sections analyzed) positive immunostaining both moderately correlated with the size of individual plaques (Pearson correlational analysis). (D) MPO activity of diseased vessel wall (neointima, media and minimal adventitia) was determined by the Klebanoff method (n=8 animals, 16 sections analyzed). Pearson correlational analysis of these two variables revealed that the level of MPO activity within individual plaques positively correlated with plaque size.

**Fig. S4:** (A) Dynamic MRI of diseased wall in a rabbit fed CH-diet for 17 months using MPO(Gd) and DTPA(Gd). Delayed images revealed distinct foci within diseased wall that appeared brighter after MPO(Gd) versus DTPA(Gd) injection (white arrows). Note that baseline images were similar in appearance and MPO(Gd) is cleared from the plaque
by 24 hours after injection. (B) Higher magnification of both early-phase (10 min.) and late-phase (2 hrs.) images seen in A shows intraplaque areas that are clearly enhanced in late-phase MPO(Gd) images but not early-phase and late-phase DTPA(Gd) images or early-phase MPO(Gd) images. (C) Dynamic MRI of normal wall in rabbit fed normal chow diet for 28 months using both agents. Similar signal kinetics and enhancement patterns were apparent throughout the 2 hour post-contrast imaging using both agents. (D) Vessel wall enhancement ratio (ER) analysis for both normal wall (left) and diseased wall (right) 2 hours after DTPA(Gd) and MPO(Gd) administration. Normal wall ER values were not significantly different between the two agents. In contrast, diseased wall ER values were significantly (paired t-test; p<0.001) and consistently higher after administration of MPO(Gd) compared to DTPA(Gd) values.
Figure S1

Macrophages & Foam Cells
GM-CSF → MPO

Catalytic Products
- Hypochlorite
- Chlorine
- Tyrosyl radicals
- Aldhydes
- Nitrogen Dioxide

Proatherogenic Events
- LDL oxidation
- Protein nitration
- HDL modification
- MMP activation
- Lipoprotein carbamylation

Adverse Outcomes
- Leukocyte chemotaxis
- Foam cell formation
- Endothelium dysfunction
- Endothelial cell apoptosis
- Fibrous cap breakdown
- Dysfunctional HDL

MPO Vulnerable Plaque
- Plaque
- Lumen

No MPO Stable Plaque

MPO(Gd)

Oligomerization

Protein Immobilization

RI ~ 4.5 (mMsec)^{-1}
RI ~ 9 (mMsec)^{-1}
RI ~ 25 (mMsec)^{-1}

Figure S2

Lipid (Red)
Calcium (Black)

Collagen

Mac IHC

MPO IHC

MPO Activity

Endothelial Cells
Figure S3

A

Area (pix)

Plaque #

B

Plaque size (pix)

MPO area (pix)

R²=0.61, p<0.001

C

Plaque size (pix)

Macrophage area (pix)

R²=0.58, p<0.001

D

Plaque size (pix)

MPO activity (U/mg protein)

R²=0.33, p<0.05
Figure S4

A) Diseased Wall
- DTPA(Gd) baseline, 10 min, 120 min
- MPO(Gd) baseline, 10 min, 120 min, 240 min, 24 hrs

B) DTPA(Gd) - 10 min, 2 hrs
- MPO(Gd) - 10 min, 2 hrs

C) Normal Wall
- DTPA(Gd) baseline, 10 min, 40 min, 70 min, 100 min
- MPO(Gd) baseline, 10 min, 40 min, 70 min, 100 min

D) Normal Wall, Diseased Wall
- Graph showing different parameters with p<0.001
Supplemental References


