p38 MAPK Inhibition Reduces Aortic Ultrasmall Superparamagnetic Iron Oxide Uptake in a Mouse Model of Atherosclerosis

MRI Assessment

Joanne B. Morris, Alan R. Olzinski, Roberta E. Bernard, Karpagam Aravindhan, Rosanna C. Mirabile, Rogely Boyce, Robert N. Willette, Beat M. Jucker

Objective—Ultrasmall superparamagnetic iron oxide (USPIO) contrast agents have been used for noninvasive MRI assessment of atherosclerotic plaque inflammation. The purpose of this study was to noninvasively evaluate USPIO uptake in aorta of apoE−/− mice and to determine the effects of Angiotensin II (Ang II) infusion and chronic antiinflammatory treatment with a p38 MAPK inhibitor on this uptake.

Methods and Results—ApoE−/− mice were administered saline or Ang II (1.44 mg/kg/d) for 21 days. In vivo MRI assessment of USPIO uptake in the aortic arch was observed in all animals. However, although the Ang II group had significantly higher absolute iron content (103%, P<0.001) in the aortic arch compared with the saline group, the p38 MAPK inhibitor (SB-239063, 150 mg/kg/d) treatment group did not (6%, NS). The in vivo MRI signal intensity was significantly correlated to the absolute iron content in the aortic arch. Histological evaluation of the aortic root lesion area showed colocalization of USPIO with macrophages and a reduction in USPIO but not macrophage content with SB-239063 treatment.

Conclusion—The present study demonstrates that noninvasive assessment of USPIO uptake, as a marker for inflammation in murine atherosclerotic plaque, is feasible and that p38 MAPK inhibition attenuates the uptake of USPIO in aorta of Ang II–infused apoE−/− mice. (Arterioscler Thromb Vasc Biol 2008;28:000-000.)

Key Words: atherosclerosis • inflammation • magnetic resonance imaging • USPIO • apoE−/− • p38 MAPK

Although experimental evidence linking inflammatory processes to the fate of the atherosclerotic plaque development exists, it is difficult to examine these processes noninvasively and to provide convincing links between preclinical and clinical studies. In this regard, ultrasmall superparamagnetic iron oxide (USPIO) contrast agents, optimized for uptake by the mononuclear phagocytic system, are of particular interest for translational cardiovascular research. These agents have been used for noninvasive MRI assessment of atherosclerotic plaque inflammation in humans as well as hypercholesterolemic balloon-injured NZW rabbits. However, descriptions of USPIO use in extensively-studied murine genetic models of atherosclerosis have been limited. Investigators have used other targeted and nontargeted approaches to noninvasively assess plaque burden or inflammation in atherosclerotic mouse models. Additionally, superparamagnetic iron oxide (SPIO) has been used to quantify monocyte recruitment histologically in atherosclerotic lesions of the apolipoprotein E–deficient (apoE−/−) mouse. In that study, acute administration of tumor necrosis factor (TNF), interleukin (IL)-1β, and interferon (INF)-γ was shown to stimulate SPIO uptake by 4-fold in plaque. Although this study supports the hypothesis that plaque targeting of the USPIO contrast agent in mouse is facilitated by monocyte recruitment or activation of macrophage phagocytosis, noninvasive assessment of USPIO uptake in mouse atherosclerotic lesions has not been reported.

Recent evidence suggests that a particularly aggressive model of atherosclerotic plaque development can be created by elevating circulating Angiotensin II (Ang II) levels in apoE−/− mice. The addition of Ang II to apoE−/− mice promotes a marked increase in the number of macrophages present in the adventitia associated with vascular lesions. In the vessel wall, Ang II–induced vascular inflammation is mediated by AT type-1 receptors which in turn activate inflammatory signaling pathways, generate reactive oxygen species, and decrease NO bioavailability. Evidence suggests that reciprocal activation of p38 MAPK and NADPH oxidase represents an important amplification loop in vascular inflammation induced by Ang II. Therefore, the purpose of this
study was to noninvasively evaluate USPIO uptake in aorta of apoE−/− mice and to determine the effects of Ang II infusion and chronic antiinflammatory treatment with a p38 MAPK inhibitor.

Materials and Methods

Animals

All procedures were approved by the Animal Care and Use Committee of GlaxoSmithKline and were specifically designed to minimize animal discomfort. Twenty-eight–week-old male apolipoprotein E–deficient (apoE−/−) background strain C57BL/6 mice (Taconic Farms, NY) were used in the study. ApoE−/− mice (n=28) were subjected to a subcutaneous infusion of either Angiotensin II (Ang II) or saline for a period of 21 days (Figure 1) via Alzet osmotic minipumps (model 2004; DURECT Corp). Minipumps were implanted subcutaneously in the mid scapular region of mice anesthetized with 2% isoflurane (Abbott) delivered in 100% oxygen. All animals also received a single preoperative analgesic dose of banamine of 1.1 mg/kg, s.c.

Study Groups and Experimental Protocol

Animals were assigned to 1 of 3 groups. Group 1 received a saline infusion (Saline, n=9), group 2 received an Ang II infusion delivered at 1.44 mg/kg/d (Ang II, n=10), and group 3 received Ang II delivered at 1.44 mg/kg/d + p38 MAPK inhibitor, SB-239063 (150 mg/kg/d, dietary dosing, n=10). Dosing of the p38 MAPK inhibitor was initiated 1 week before Ang II administration (Figure 1). ApoE−/− mice were housed individually and were allowed free access to chow. Osmotic minipumps were implanted on Day 0, and body weights and food consumption were recorded throughout the study. Indirect blood pressure was collected on Day 18 before USPIO administration. On Days 19 and 20 all animals received an i.v. administration of USPIO. The following day (Day 21), in vivo MRI was performed. After in vivo imaging, whole blood was collected (EDTA) for determination of plasma oxygen and isoflurane (1% to 2%) anesthesia. Imaging was performed using a 9.4 Tesla imaging system (vertical, 89 mm bore diameter, Bruker Medical). Both respiratory and ECG signal were monitored, and the ECG signal was used to trigger the MRI pulse sequence. The animal’s core body temperature was maintained by adjusting the water cooled gradient set to 37°C. During each imaging session a series of transverse, sagittal, and coronal scout images through the heart (FLASH sequence, TR/TE=502.7 ms, FOV=3×3 cm, matrix=128×128, slice thickness=0.5 mm, number of averages=4) were acquired. An oblique 2D Time Of Flight (TOF) image slice was positioned such that the image slice would capture the entire aortic arch from the root to the left subclavian arterial branch. A final high-resolution image was acquired in CINE (FLASH sequence, TR/TE=12/2.8 ms, FOV=2.5×2.5 cm, matrix=256×256, slice thickness=1.0 mm, number of averages=8).

Ex Vivo MRI

To provide optimal spatial resolution, ex vivo imaging of the plaque associated with the aortic root was performed on the same 9.4 Tesla system used for the in vivo studies. The perfused fixed heart was flushed with 0.2% Gd-DTPA (Magnevist, Berlex Labs) and high-resolution images were acquired through the entire root using a transverse multi-slice spin echo (TR/TE=2000/10 ms, FOV=10×10 mm, matrix=256×256, slice thickness=0.3 mm, number of averages=2) sequence.

Image Analysis

All images were analyzed using Analyze AVW software (AnalyzeDirect). Images were reconstructed to 512 pixels×512 pixels before semiautomated trace analysis. The in vivo image analysis was performed on the single oblique slice containing the aortic arch. The data are presented as the signal loss area (%) with respect to the entire aortic arch area defined from the root to the left subclavian artery. The in vivo MRI signal loss thresholding criteria was determined for each aorta by selecting a ROI in a region of aorta devoid of signal loss. From the measured signal intensity in this ROI, a 95% confidence interval was determined (i.e., ±2×SD). This confidence level was now used as the thresholding level by which signal loss area (>2×SD signal loss) was determined. The ROI tracing of the aorta remained similar for each aorta. All ex vivo aortic root plaque volume measurements were performed by analyzing the region of plaque deposition at the level of the aortic valve and the slice immediately cranial and caudal to it. Manually segmented tracing of the aortic root plaque in all 3 slices was performed.

Blood Pressure, Histology, and Analytical Procedures

Please find these additional methods and materials in the online data supplement section (http://atvb.ahajournals.org).

Statistical Analysis

All data are expressed as mean±SEM. Survival curves were analyzed using the Kaplan–Meier method. All other multiple comparisons were made by ANOVA followed by a Tukey post hoc test. All statistical tests were performed using Prism software (Graphpad Software) and a value of P<0.05 was considered to be significant.
Table. Plasma Lipid and Cytokine Profile at End of Study

<table>
<thead>
<tr>
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<th>Saline</th>
<th>Ang II</th>
<th>Ang II + SB-239063</th>
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<tbody>
<tr>
<td>Cholesterol, mg/dL</td>
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<td>619±42</td>
<td>607±21</td>
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<td>HDL, mg/dL</td>
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<tr>
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<td>MCP-1, pg/ml</td>
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<td>34.5±6.0</td>
</tr>
<tr>
<td>MIP-1β, pg/ml</td>
<td>27.4±2.7</td>
<td>62.1±15.1†</td>
<td>20.3±2.7</td>
</tr>
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</table>

Plasma lipids and cytokines were measured in apoE−/− mice after 21 days of Saline or Ang II administration. Data are presented as mean±SEM. *P<0.05 vs Saline, †P<0.01 vs Saline and Ang II + SB-239063 groups.

Plasma Cytokines

A variety of inflammatory cytokines (IL-1α, IL-1β, TNF-α, IL-6, G colony stimulating factor [CSF], MCP-1, MIP-1β, etc) were examined in plasma samples obtained from all groups at the end of study. Only MCP-1 and MIP-1β were increased by Ang II infusion (Table). The significant elevation of MCP-1 in the Ang II group (43.2±8.6 pg/mL) versus the Saline group (24.7±1.7 pg/mL, P<0.05) was blunted in the Ang II + SB-239063 treatment group (34.5±6.0 pg/mL). Additionally, SB-239063 treatment prevented the increase in MIP-1β observed in the Ang II group (20.3±2.7, 62.1±15.1, and 27.4±2.7 pg/mL in Ang II + SB-239063, Ang II, and Saline groups, respectively; P<0.01).

In Vivo MRI and Fe Analysis

2D white blood, TOF, images that captured the aortic arch including the aortic root, ascending, and the proximal descending aorta are shown in Figure 2A and 2B. In an apoE−/− mouse that had not received any USPIO, the signal intensity of the arch is homogeneous and bright (Figure 2A). Figure 2B illustrates the nature of the focal signal loss regions associated with USPIO deposition in atherosclerotic plaque regions of the aorta in an apoE−/− mouse that had received USPIO. Figure 2C and 2D illustrates a transverse slice orientation through the aortic root at the level of the valves in representative mice that did not receive or did receive USPIO, respectively. The signal loss region in the root was homogeneous and represented a significant area of the root that was associated with atherosclerotic plaque burden. Because of the promiscuous nature of macrophage deposition into regions outside of the vasculature including in the mediastinal lymph node and attributable to the “blooming” nature of signal loss.

**Figure 2.** In vivo imaging of the aorta in an Ang II-infused apoE−/− mouse. Bright blood TOF imaging was performed such that a 2D oblique slice was obtained through the aortic arch (A, B) and aortic root (C, D). Representative images from both control (−USPIO) (A, C) as well as (+USPIO) (B, D) administered mice are shown. In some in vivo images of the aorta, signal loss was detected in the pulmonary artery (B) which was likely attributable to the susceptibility artifact because of the significant iron uptake in the plaque from the lesser curvature of the aorta.
arising from USPIO particles, a group of C57BL/6 mice were administered USPIO and imaged to determine the degree of signal loss obtained in nonatherosclerotic aortas. Image analysis was performed by tracing the aortic arch from the level of the aortic root to the left subclavian artery. The percent signal loss area, using a signal loss threshold of $>2\times$SD of blood pool signal intensity, in this region is shown in Figure 3A. Using this thresholding criteria, 2 groups of control mice (apoE$^{-/-}$—USPIO and C57BL/6+USPIO) showed minimal signal loss (3±1 and 5±3%, respectively). Although Ang II administration resulted in a 36±7% signal loss compared with saline administration (19±4% signal loss), Ang II+SB-239063 administration resulted in a normalization of the in vivo MRI signal loss (21±3% signal loss, P<0.05). The absolute iron in the aortic arch was measured as a surrogate measure of USPIO deposition (Figure 3B). The absolute iron content in the aortas from the control mice (apoE$^{-/-}$—USPIO and C57BL/6+USPIO) was relatively low (178±25 and 165±65 ppm, respectively). The Ang II group (729±107 ppm) had a significantly higher iron level in the aortic arch compared with the Saline group (358±33 ppm, P<0.001). However, the Ang II+SB-239063 group had a significantly lower Fe content (380±74 ppm, P<0.001) which was comparable to the Saline group. Additionally, there was a significant correlation between the absolute iron content of the aortic arch and the proportion of in vivo MRI signal intensity loss present in the aortic arch (r=0.80, P<0.001; Figure 3C) indicating that the in vivo image analysis was sensitive to differences in aortic uptake.

**Ex Vivo MRI**

Ten 0.3-mm-thick transverse MRI slices were obtained through the aortic root at the level of the valve in the ex vivo formalin fixed hearts. The predominantly macrophage laden atherosclerotic plaque lesion was clearly visible and discernable from surrounding tissue in the MRI image (Figure 4A and 4B). The atherosclerotic plaque signal contrast with surrounding tissue was visible in hearts of apoE$^{-/-}$ mice that did not receive USPIO (Figure 4A), but this contrast was significantly increased in the hearts of those mice that did receive USPIO (Figure 4B). There were no significant differences in aortic root plaque volume between the Saline (0.23±0.01 mm$^3$), Ang II (0.24±0.02 mm$^3$), and Ang II+SB-239063 (0.21±0.01 mm$^3$) groups.
Histological Evaluation

Histological assessment of USPIO deposition by Perl staining and immunohistochemical assessment of macrophages by Cat-S staining was performed within the atherosclerotic lesions in the aortic root. Figure 5A illustrates, via Perl staining, the association of the USPIO with the plaque in the aortic root. This pronounced staining was consistent with the signal loss observed in this region by both in vivo (Figure 2D) and ex vivo (Figure 4B) MRI. The positive staining for Cat-S shown in Figure 5B illustrates that the lesion observed in the aortic root predominantly consisted of macrophage. The regions staining the contiguous 5-μm sections presented in Figure 5A and 5B are consistent with the notion that the USPIO deposition was associated with macrophage cells. On semiquantitative histological evaluation in a subgroup of study animals, there were no differences in lesion area between groups (Figure 5C). It was observed that there was a trend for an increase in macrophage content on administration of Ang II (Figure 5D), although there was no difference between Ang II and Ang II+SB-239063 groups. Further-

Figure 5. Immunohistochemical analysis of atherosclerotic plaque associated with the aortic root. 5-μm contiguous histological sections were taken in a similar orientation to the ex vivo MRI of the aortic root. Perl staining was used to demonstrate colocalization of iron positive area (A) and Cat-S (B) positive cells. The regions of interest in the 10× magnification images are shown below at 40× magnification. Lesion cross-sectional area (C), lesion area staining positive for Cat-S (D), and lesion area staining positive for Perl (E) is presented. Subgroup sizes were as follows: Saline, n=9; Ang II, n=6; and Ang II+SB-239063, n=4). Data are presented as mean±SEM. *P<0.05 vs Saline group.
more, whereas the area staining positive for iron increased in the Ang II group (P<0.05), this area was attenuated with SB-239063 treatment (Figure 5E).

Discussion

The present study demonstrates that noninvasive assessment of USPIO contrast agent uptake, used as an imaging marker of atherosclerotic plaque inflammation, is feasible in apoE−/− mice administered Ang II. Additionally, although numerous investigators have evaluated USPIO uptake in both animal7–10 and human11–14 atherosclerotic plaque noninvasively using MRI, the present study is the first to demonstrate a reduction of USPIO uptake in aorta after chronic treatment with a novel antiinflammatory therapy.

In the present study chronic Ang II administration was used to accelerate vascular inflammation in apoE−/− mice. As has previously been shown,21 Ang II had neither an effect on blood pressure nor on the plasma lipid profile in the present study. However, there were profound effects of Ang II administration on the morbidity and mortality observed in the study. Additionally, all of the animals that died or were moribund had evidence of aortic rupture or aneurysm formation, respectively. These findings are not surprising as Daugherty et al21 have shown that a relatively short Ang II (1000 ng/kg/min) exposure duration (28 days) promoted rapid atherosclerotic lesion development in the aorta, with a discernable morphology in the abdominal aorta consistent with characteristics of an aneurysm (ie, medial rupture, gross macrophage, and collagen deposition). Although we observed a 30% to 44% incidence of morbidity/mortality in the present study, there was no benefit of the p38 MAPK inhibitor on this parameter. In rodent models of end organ dysfunction, p38 MAPK inhibitors have been shown to be extremely effective in eliciting a survival benefit in part via promoting endothelial protection.20,22,23 However, in the present study, the deaths in the Ang II infused apoE−/− mice were most likely attributable to aneurysm rupture initiated by matrix metalloproteinase (MMP)chymase activation.21,24,25

Ang II has been shown to activate the p38 MAPK signaling pathway in a number of vascular cell types,18,19,26 including macrophages,27 and is an important signaling pathway for the recruitment of leukocytes.28 Additionally, the p38 MAPK system plays an important role in vascular inflammation and remodeling particularly in the medial and adventitial lamina of blood vessels.27,29 The mechanism by which Ang II regulates p38 MAPK signaling and promotes vascular inflammation is, in part, related to activation of vascular NADPH oxidase (NOX). In a variety of cellular systems, Ang II–mediated AT-type 1 receptor modulation activates NOX and generates superoxide anion required for redox-sensitive activation of p38 MAPK.19,26 Recent in vivo evidence suggests that activation of vascular NOX and p38 MAPK is likely to be a reciprocal event, ie, the generation of reactive oxygen species (ROS) activates p38 MAPK which in turn leads to further ROS production.30 In that study, the increased vascular production of ROS and enhanced expression of NOX induced by hypertensive doses of Ang II were abolished after chronic treatment with a p38 MAPK inhibitor. Once activated, proinflammatory p38 MAPK–dependent processes, ie, production of adhesion molecules (ie, vascular cell adhesion molecule [VCAM]-1, intercellular adhesion molecule [ICAM]-1), chemokines (ie, MCP-1, MIP-1β) and cytokines (ie, IL-1, TNF-α), can contribute to monocyte recruitment, macrophage activation, and vascular remodeling.17,27,28 In addition, elevated ROS generation may also contribute to further oxidation of lipoproteins and reduced bioavailability of nitric oxide, both of which contribute to the development of atherosclerosis.31 Taken together, these observations suggest that p38 MAPK inhibitors reduce USPIO uptake in atherosclerotic plaque by reducing vascular inflammation.

Although the percent MRI signal loss area detected in the aortic arch of the Ang II+SB-239063 group was reduced versus the Ang II group and normalized to the Saline group consistent with the notion that there was decreased aortic lesion associated with the p38 MAPK inhibitor treatment, one cannot rule out the distinct possibility that a difference in macrophage phagocytic activity rather than a difference in the number of macrophage present was responsible for this observation. There was no reduction in the Cat-S staining for macrophage while the Perl iron staining for USPIO was reduced on p38 MAPK inhibition in the aortic root lesion in the present study. Therefore the results suggest that the p38 MAPK inhibitor treatment reduced phagocytic activity of the resident macrophages. USPIO phagocytosis by macrophage has been shown in part to be facilitated via scavenger receptor A (SR-A),3 and p38 MAPK inhibition reduces SR-A expression on toll-like receptor stimulation in macrophages.32,33 However, it has been shown that Ang II administration to apoE−/− mice results in preferential promotion of lesion development in the aorta rather than in the aortic root.34 Indeed, there was no promotion of aortic root lesion development in the presence of Ang II assessed in the present study. Although we did not have a quantitative readout of atherosclerotic lesion development in the aorta because of the logistics of the study (ie, ICPMS analysis of aorta), on gross inspection the wet weight of the aorta was significantly elevated after Ang II administration and normalized with p38 MAPK inhibitor treatment. However, it is not clear whether the increased adventitial hypertrophy or increased atheroma development in the aorta was responsible for these dynamic changes after Ang II administration. Additionally, Ang II infusion in the absence of USPIO administration did not increase iron deposition in the aorta (data not shown). Therefore medial rupture of the vessel and thrombus development in the area of interest as a consequence of Ang II administration21 was not likely to have occurred as this would have increased the absolute iron content measured.

In summary, the present study illustrates for the first time that noninvasive MR imaging using USPIO as a contrast agent to target macrophage cells can be applied to a mouse atherosclerosis model. In addition, an antiinflammatory, experimental therapeutic agent was beneficial in reducing the vascular uptake of the USPIO agent. These findings are unique and may reflect an exciting new opportunity for noninvasive evaluation of therapeutic agents targeting macrophage presence or activity in the clinic.
Acknowledgments

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Disclosures

None.

References

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**Materials and Methods Supplement**

**Blood Pressure Measurements**

Indirect blood pressure was determined in isoflurane anesthetized mice via tail cuff measurement using a non-invasive blood pressure system (Harvard Apparatus, Holliston, MA) after 18 days of Ang II or saline infusion (Figure 1). Animals were acclimated to the device for two days prior to collection and measurements were recorded on the third day (Day 18). A total of five readings per animal were recorded and the mean value for each individual was analyzed.

**Histological Procedures**

Histology was performed on 5 µm sections cut through the aortic root in the heart. After sections were deparaffinized, rehydrated and placed in 0.05M Tris buffer (pH=7.6), they were stained on a DAKO Autostainer (DAKO Corporation, Carpenteria, CA) using reagents from the Catalyzed Signal Amplification system provided by DAKO. Sections were incubated with a cathepsin-S (Cat-S) goat polyclonal antibody, a marker of macrophage cells (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Additional 5 µm sections were placed in hydrochloric acid-potassium ferrocyanide working solution for 30 minutes and counterstained with nuclear fast red for the Perl’s iron staining. Quantification of lesion size and staining was performed using the Image-Pro Plus version 4.1 software (Media Cybernetics, Inc., MD). Data are reported as a mean analysis of three sections per heart including the aortic valve. Co-registration of Cat-S and Perl’s positive staining areas was assessed in serial sections of atherosclerotic lesion.
Analytical Procedures

Absolute iron content was measured in the same section of aortic arch that was assessed by in vivo MRI via inductively coupled plasma-mass spectrometry (ICP-MS) analysis (Elemental Research Inc., North Vancouver, Canada) on nitric acid digested tissue extracts. The absolute iron content was normalized to the dry tissue weight and reported in parts per million (ppm). Plasma lipids were measured using an Olympus AU640 chemical analyzer (Olympus Optical Company, Melville, NY). Pharmacokinetic analysis of SB-239063 was performed on whole blood and a 50:50 mixture of blood: H₂O was used for LC/MS/MS analysis. Plasma cytokine concentrations were measured using a Bio-Plex Mouse 23-Plex Panel Cytokine Assay (Bio-Rad Laboratories, Inc., Hercules, CA).