Molecular Imaging of Inflammation and Platelet Adhesion in Advanced Atherosclerosis

Effects of Antioxidant Therapy With NADPH Oxidase Inhibition

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Background—In atherosclerosis, local generation of reactive oxygen species amplifies the inflammatory response and contributes to plaque vulnerability. We used molecular imaging to test whether inhibition of NADPH oxidase with apocynin would reduce endothelial inflammatory activation and endothelial–platelet interactions, thereby interrupting progression to high-risk plaque phenotype.

Methods and Results—Mice deficient for both the low-density lipoprotein receptor and Apobec-1 were studied at 30 weeks of age and again after 10 weeks with or without apocynin treatment (10 or 50 mg/kg per day orally). In vivo molecular imaging of vascular cell adhesion molecule-1 (VCAM 1) P-selectin, and platelet glycoprotein-Ibα (GPIbα) in the thoracic aorta was performed with targeted contrast-enhanced ultrasound molecular imaging. Arterial elastic modulus and pulse wave transit time were assessed using ultrahigh frequency ultrasound and invasive hemodynamic measurements. Plaque size and composition were assessed by histology. Molecular imaging in nontreated mice detected a 2-fold increase in P-selectin expression, VCAM-1 expression, and platelet adhesion between 30 and 40 weeks of age. Apocynin reduced all of these endothelial events in a dose-dependent fashion (25% and 50% reduction in signal at 40 weeks for low- and high-dose apocynin). Apocynin also decreased aortic elastic modulus and increased the pulse transit time. On histology, apocynin reduced total monocyte accumulation in a dose-dependent manner as well as platelet adhesion, although total plaque area was reduced in only the high-dose apocynin treatment group.

Conclusions—Inhibition of NADPH oxidase in advanced atherosclerosis reduces endothelial activation and platelet adhesion, which are likely responsible for the arrest of plaque growth and improvement of vascular mechanical properties.

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Key Words: contrast ultrasound ◼ microbubbles ◼ oxidative stress ◼ platelets

Oxidative stress plays a key role in both the progression of atherosclerotic disease and susceptibility to acute atherothrombotic complications.1,2 Local production of reactive oxygen species (ROS) is thought to contribute to pathophysiology, in part, through upregulation of endothelial adhesion molecules that mediate the inflammatory response and the promotion of platelet adhesion,2–5 although methods for quantifying these important responses in vivo have not been readily available. Membrane-bound NADPH oxidase is a major source for superoxide production not only by leukocytes, but also by vascular cells, such as endothelial and smooth muscle cells.6,7 Its causal role in atherosclerosis is supported by data showing that gene-targeted deletion of cytosolic components of NADPH oxidase reduces plaque size in atherosclerotic mouse models.8 Although statins and angiotensin-converting enzyme inhibitors may provide protection partly through their inhibition of NADPH oxidase activity, there are no clinically approved therapies that are specifically targeted against this enzyme complex.

Clinical Perspective on p 82

Apocynin is a plant-derived inhibitor of NADPH oxidase that has been used to treat inflammatory diseases, such as asthma and arthritis.7 It is thought to reduce oxidative stress by preventing the translocation of cytosolic oxidase subunits of NADPH oxidase, such as p47phox.7,9 Apocynin has been

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shown to reduce atherosclerosis, when given early in disease in animal models. As the production of ROS is a mechanism by which the inflammatory response self-amplifies, a key question is whether apocynin has a beneficial effect in late-stage disease to reduce plaque vulnerability by reducing endothelial cell adhesion molecules and subsequent recruitment of monocytes, and platelets or platelet microparticles, which are recognized to have proinflammatory properties.

In this study, we used advanced in vivo imaging methods to investigate whether long-term administration of apocynin, when administered at a stage of moderate-to-advanced disease, would reduce vascular inflammation and arrest the progression of disease. Contrast-enhanced ultrasound (CEU) molecular imaging of endothelial adhesion molecule expression and platelet adhesion were performed together with ultra-high frequency ultrasound assessment of vascular mechanical properties in a murine model of advanced atherosclerosis.

Methods

Study Design

The study was approved by the Animal Care and Use Committee of the Oregon Health & Science University. Wild-type C57Bl/6 and double knockout (DKO) mice with age-dependent atherosclerosis produced by gene-targeted deletion of the low-density lipoprotein receptor and ApoB1-1 on a C57Bl/6 background were studied. Wild-type mice and DKO mice were also studied at 40 weeks of age with a preceding 10 weeks of either no therapy (n=11) or apocynin (acetovanillone, Sigma) given in the drinking water at low dose (10 mg/kg day, n=8) or high dose (50 mg/kg day, n=8). Drinking water concentrations were calibrated according to intake and changed daily to minimize light degradation. Measurements included transarterial echocardiography; CEU molecular imaging for P-selectin, VCAM-1, and platelet GPIbα; ultrasound assessment of plaque size and vascular mechanical properties; and invasive arterial hemodynamics. Histology of the aorta was performed for each of the treatment groups at 40 weeks.

Microbubble Preparation

Biotinylated, lipid-shelled decafluorobutane microbubbles were prepared by sonication of a gas-saturated aqueous suspension of distearoylphosphatidylcholine, polyoxyethylene-40-stearate, and distearoylphosphatidylethanolamine-PEG (2000) biotin. Microbubbles targeted to platelet GPIbα (MBGPIb), P-selectin (MBp), or VCAM-1 (MB) were prepared by conjugating the following ligands to the shell surface, as previously described: dimeric recombinant A1 domain (amino acids 445–709) of mouse von Willebrand factor (VWF); rat antimouse monoclonal IgG1 against P-selectin (RB4.34), or VCAM-1 (MK 2.7). Control microbubbles (MB) were prepared using rat isotype control antibody (R3-34, Pharmingen Inc). Microbubble concentration was measured by electrono sensing (Multisizer III, Beckman-Coulter).

Flow Chamber Assessment of Microbubble–Platelet Interactions

To validate targeted attachment of MBGPIb, in vitro flow chamber studies were performed to assess microbubble–platelet interactions. Capillary tubes were incubated with fibrillar type-I collagen (100 μg/mL) for 1 hour, then blocked with denatured BSA (5 mg/mL). Human whole-blood anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (40 μmol/L) was drawn through capillary tubes for 3 minutes at a wall shear rate of 500 s⁻¹ for 3 minutes. Tubes were washed with PBS and the number of microbubbles attached was averaged from 10 to 15 randomly selected nonoverlapping optical fields (0.09 mm²) on fluorescent microscopy. Experiments were performed in triplicate for each condition.

Molecular Imaging

Mice were anesthetized with inhaled isoflurane (1.0–1.5%). CEU of the ascending aorta and proximal aortic arch was performed with a linear-array probe (Sequioa, Siemens Medical Systems Mountain View, CA) using a right parasternal window. Multipulse phase-inversion and amplitude-modulation imaging was performed at 7 MHz and a mechanical index of 0.97. Images were acquired 8 minutes after intravenous injection of targeted or MB (1x10⁶ per injection) performed in random order. Signal from retained microbubbles was determined from the first frame obtained. As previously described, signal from the few freely circulating microbubbles was eliminated by digital subtraction of several averaged frames obtained after complete destruction of microbubbles in the imaging field (Figure 1 in the online-only Data Supplement). Intensity was measured from a region-of-interest placed on the ascending aorta and proximal arch, and extending into the origin of the brachiocephalic artery. Region selection was guided by fundamental 2-dimensional imaging at 14 MHz, acquired after each CEU imaging sequence.

Ultrasound Assessment of Plaque Size and Aortic Mechanical Properties

Plaque size was assessed by high-frequency (30 MHz) ultrasound imaging (Vevo 770, VisualSonics Inc, Toronto, Canada) performed from a right parasternal window. Measurements of vessel wall thickness at lesion-prone sites of the lesser curvature of the aortic arch and the origin of the brachiocephalic artery (near wall) were made at end-diastole. M-mode imaging perpendicular to the middescending aorta was performed, and aortic strain was calculated as the change in systolic and diastolic diameter divided by diastolic diameter (ΔD/Dₙ₀). A micrometer (SPR-1000, Millar Instruments, Inc, Houston, TX) was inserted into the aorta via the carotid artery for arterial pressure measurement and aortic elastic modulus (e₀) was calculated by:

\[ \Delta P \times D_{n0} / \Delta D \]

where \( \Delta P \) is pulse pressure expressed in 10⁵ N m⁻². Pulse wave transit time (PTT) assessment of arterial stiffness was assessed by the time delay of the onset of systolic pressure rise measured by pulse-wave Doppler in the proximal ascending aorta and the femoral artery approximately 0.5 mm from the pudendoeipagastic trunk, using the ECG as a time reference. Measurements were averaged for 3 cardiac cycles.

Echocardiography

Echocardiography (30 MHz) was performed using parasternal views. Left ventricular fractional shortening and percentage area change were assessed in the midventricular short-axis plane. Stroke volume was calculated by the product of left ventricle outflow tract area and left ventricle outflow tract time-velocity integral on pulsed-wave Doppler. Aortic centerline velocity was measured by pulsed-wave Doppler in the distal aortic arch.

Histology

Perfusion-fixed short-axis sections from 2 sites, the middescending aorta and arch just proximal to the brachiocephalic artery, were evaluated at 40 weeks of age (n=5 animals for each treatment group). Masson trichrome stain was performed to assess the plaque area calculated by the vessel tissue area within the internal elastic lamina. Immunohistochemistry was performed using goat polyclonal primary antibodies against mouse Mac-2 (M3/38, eBioscience San Diego, CA) for identifying monocytes and macrophages, or against GPIbα integrin (sc20234, Santa Cruz Biotechnology, Santa Cruz CA) for the fluorophores DiO, mixed with either PBS or anticoagulated whole blood, and drawn through the flow chamber at shear rates of 300, 500, or 1000 s⁻¹ for 3 minutes. Tubes were washed with PBS and the number of microbubbles attached was averaged from 10 to 15 randomly selected nonoverlapping optical fields (0.09 mm²) on fluorescent microscopy. Experiments were performed in triplicate for each condition.
### Table. Hemodynamic and Echocardiography Data (mean±SD)

<table>
<thead>
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<th>Systolic BP, mm Hg</th>
<th>Diastolic BP, mm Hg</th>
<th>Pulse pressure, mm Hg</th>
<th>Fractional shortening, %</th>
<th>LVEF, %</th>
<th>Stroke volume, μL</th>
<th>Aortic internal diameter, mm</th>
<th>Aortic peak systolic velocity, m/s</th>
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<td>39±2</td>
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<tr>
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Wild-type 30 wk (n=5) DKO 30 wk (n=5) Untreated Control (n=10) Low-Dose Apocynin (n=8) High-Dose Apocynin (n=6) DKO 40 wk

BP indicates blood pressure; DKO, double knockout mice; and LVEF, left ventricular ejection fraction.

*P<0.05 versus wild–type.
†P<0.05 versus DKO 30 wk.
‡P<0.05 versus untreated DKO group at 40 wk.

### Results

#### Blood Pressure and Left Ventricular Function

Arterial systolic and diastolic pressures in DKO mice at 30 weeks of age were higher than in wild-type mice, whereas pulse pressure was not significantly different (Table). In untreated DKO mice, arterial systolic pressure and diastolic pressure both increased between 30 and 40 weeks of age. This increase was prevented by apocynin in treatment as a dose-dependent fashion. Moreover, for the high-dose apocynin treatment group systolic arterial pressure and pulse pressure were lower at 40 weeks than 30 weeks of age.

On echocardiography, left ventricular fractional shortening, left ventricular ejection fraction, and stroke volume were not significantly different for wild-type and DKO mice at 30 weeks of age (Table). For DKO mice, there was a non-significant trend for an increase in these parameters between 30 weeks and 40 weeks of age, but all were similar between treatment groups at 40 weeks. Aortic internal diameter at 30 weeks of age was greater for DKO compared with wild-type mice and progressively increased by 40 weeks of age in all treatment groups. Aortic peak systolic flow velocities were not different between treatment groups at 40 weeks suggesting similar hemodynamic conditions and shear rates, which can potentially influence targeted microbubble attachment.

#### Aortic Mechanical Properties

Elastic modulus of the aorta (E_{Ao}) was significantly higher for DKO than wild-type mice at 30 weeks, and significantly increased between 30 and 40 weeks of age in untreated DKO mice (Figure 1A and 1B), indicating an age-dependent worsening of aortic elastic properties. The age-dependent increase in E_{Ao} was attenuated by apocynin. In the high-dose treatment cohort, E_{Ao} in DKO mice at 40 weeks was similar to that at 30 weeks of age. Although PTT was not different between DKO and wild-type mice at 30 weeks of age, there was an age-dependent decrease in PTT in untreated DKO mice, indicating a reduction in vascular compliance (Figure 1C and 1D). The age-dependent decrease in PTT was attenuated by apocynin. Again PTT in the high-dose treatment cohort was similar to that in mice at 30 weeks of age.

#### In Vitro Assessment of Targeted Microbubble Attachment

Binding properties have previously been characterized for microbubble molecular imaging agents targeted to P-selectin (MB_{P}) and VCAM-1 (MB_{V})

but not for MB_{GPIb}. Accordingly, flow chamber studies were performed to assess in vitro microbubble–platelet interactions. Across a range of shear rates, attachment of MB_{GPIb} in PBS to platelet aggregates was significantly greater than for MB_{GPIb}, lacking a targeting ligand (Figure 2A). Attachment of MB_{GPIb} was shear-dependent. When suspended in whole blood, attachment of MB_{GPIb} was also significantly greater than for microbubbles bearing a mutated nonbinding form of VWF A1-domain (MB_{GPIb-mut}) (Figure 2B). Attachment for MB_{GPIb-mut} was almost exclusively to platelet aggregates (Figure 2C and 2D). Platelet attachment for MB_{GPIb}, when suspended in whole blood, was similar to that when suspended in PBS, indicating minimal competitive inhibition from plasma VWF or from other endogenous GPIbα ligands.
Molecular Imaging of Inflammation and Platelet Adhesion

Molecular imaging signal for P-selectin, VCAM-1, and platelet GPIbα were all much greater (>5-fold higher) in DKO than wild-type mice at 30 weeks of age (Figure 3). In untreated DKO mice, signal for all 3 targets had increased by more than 2-fold between 30 and 40 weeks of age. At 40 weeks of age, P-selectin, VCAM-1, and platelet GPIbα molecular imaging signal were...
all reduced in a dose-dependent fashion by apocynin treatment. Signal in high-dose apocynin-treated DKO mice at 40 weeks was not significantly different to that measured in 30 weeks DKO mice. Signal for all targeted agents in the untreated DKO mice at 30 and 40 weeks was significantly greater ($P<0.01$) than signal from control nontargeted microbubbles.

### Plaque Size and Content

On high-frequency ultrasound, vessel thickness at the lesion-prone sites of the lesser curvature of the aorta and the proximal brachiocephalic artery was greater for DKO than wild-type mice at 30 weeks of age (Figure 4). In DKO mice, vessel wall thickness increased between 30 and 40 weeks of age, consistent with the described age-dependent worsening of atherosclerosis in the DKO model.12,14 Discrete raised plaques could be identified in the brachiocephalic artery in all untreated animals at 40 weeks of age. In DKO mice at 40 weeks, apocynin prevented the increase in vessel thickness only when given at the high dose, which resulted in vessel thickness similar to that measured at 30 weeks.

Histology in untreated DKO mice at 40 weeks of age demonstrated atherosclerotic lesions with abundant Mac-2–positive cells (monocytes and macrophages) encroaching well into the aortic lumen in all mice (Figure 5). Consistent with ultrasound imaging results, plaque size by histology in 40 weeks DKO mice was significantly reduced only in the high-dose apocynin treatment group (Figure 5A and 5B). In this group, however, discrete raised plaques were still observed (Figure 5G). Although low-dose apocynin did not reduce plaque area by ultrasound or histology, it did reduce monocyte and macrophage infiltration (Figure 5C). The degree to which macrophage infiltration was reduced was even greater for the high-dose group. Because plaque size was substantially reduced only in the high-dose group, monocyte and macrophage infiltration as a percentage of total plaque area was not different for the low-dose and high-dose apocynin groups (Figure 5D). Immunohistochemistry for GPIIb revealed the presence of platelets on the endothelial cell surface (Figure 6). Regions where site-density of platelets on the endothelial surface was high were seen only in the untreated mice. Interestingly, GPIIb staining was occasionally detected deep within large complex plaques in untreated mice. Staining for VCAM-1 confirmed a reduction in the spatial extent of VCAM-1 staining on both the endothelial surface and also within the plaque.

### Discussion

In this study, state-of-the-art noninvasive imaging methods for characterizing vascular, molecular, ultrastructural, and elastic phenotype in vivo were used to evaluate the effect of

![Figure 3. A to D, Molecular imaging signal (mean±SEM) for P-selectin, VCAM-1, or GPIbα, and for control nontargeted microbubbles. E, Example from an untreated 40 week old or wk old DKO mouse of a 2-dimensional ultrasound image of the aortic arch used to define regions-of-interest; and contrast-enhanced ultrasound (CEU) molecular imaging from the same mouse after injection of either (F) GPbα-targeted or (G) control microbubbles (color scale at bottom). As previously described,17 an ultrasound beam elevation (out-of-plane) dimension larger than the aortic diameter produced volume averaging and extension of molecular imaging signal enhancement into the apparent lumen. Illustration of method for background-subtraction are provided in the online-only Data Supplemental Figure I. *$P<0.05$ versus 30 weeks wild-type; †$P<0.05$ versus 30 weeks DKO; ‡$P<0.05$ versus untreated (control) 40 week old or wk old double knockout (DKO).](http://circimaging.ahajournals.org/content/78/1/78)
prolonged therapy with the NADPH oxidase inhibitor apocynin. Our results indicate that prolonged treatment with apocynin, when initiated at a mid- to late-stage of atherosclerotic disease: (a) prevented the progressive worsening of mechanical elastic properties of large arteries, (b) arrested any further increases in endothelial activation, (c) reduced platelet adhesion and monocyte infiltration, and (d) prevented plaque growth, when given at high dose.

Oxidative stress is a key factor involved in the pathogenesis of atherosclerosis and, in many respects, is a self-amplifying process. The local vascular production of ROS, such as superoxide, hydroxyl radicals, and hydrogen peroxide, is thought to be important for promoting the advancement of disease from fatty streak to unstable plaque.\(^1\)\(^,\)\(^2\) Oxidative stress is an important regulator of proinflammatory gene expression mediated largely through the transcription factor nuclear factor-κB.\(^4\) There is strong evidence that through these mechanisms oxidative stress will increase expression of endothelial cell adhesion molecules, such as P-selectin and VCAM-1; and production of proinflammatory chemokines.\(^5\)\(^,\)^\(^9\)\(^,\)\(^18\)\(^,\)\(^19\) It is also well established that inactivation of nitric oxide by ROS can lead to acceleration of atherosclerosis and susceptibility to atherothrombotic events by interfering with anti-inflammatory, antiplatelet, and antiproliferative effects of nitric oxide.\(^2\)

Self-amplification of oxidative stress in atherosclerosis has been attributed to the stimulation of the inflammatory cascade, which itself is a source of ROS. However, it is increasingly evident that ROS generation is not solely from monocytes and foam cells. Many of the resident vascular cells, such as endothelial cells, smooth muscle cells, and fibroblasts, possess membrane-bound NADPH oxidase, which serves as a major source for superoxide production in atherosclerosis.\(^6\)\(^,\)\(^7\) Again, self-amplifying mechanisms can be identified, whereby many proinflammatory cytokines, thrombin, and angiotensin II further activate NADPH oxidase in vascular cells.\(^1\)\(^2\)\(^0\)\(^-\)\(^2\)\(^2\) The role of NADPH oxidase specifically in the development of atherosclerosis is strongly supported by the observation that gene deletion of the p47\(^\text{phox}\) subunit of the NADPH oxidase complex in apolipoprotein-E–deficient mice reduces atherosclerotic lesion size in the descending aorta.\(^8\)

In this study, we studied whether oral treatment with apocynin, an inhibitor of NADPH oxidase, could arrest the progression of atherosclerosis, when initiated at mid- to late-stage. Apocynin is an \(\alpha\)-methoxyphenol extract from several plant sources that has been shown to have several biological effects, which include the prevention of NADPH oxidase assembly by inhibiting membrane translocation of the p47\(^\text{phox}\) and gp91\(^\text{phox}\) subunits.\(^7\) In vitro studies have demonstrated that reduction of ROS production from NADPH oxidase reduces endothelial VCAM-1, monocyte–endothelial interactions, platelet adhesion, and smooth muscle cell proliferation, in response to various stimuli.\(^5\)\(^,\)\(^9\)\(^,\)\(^18\) Apocynin specifically has been shown to reduce selectin and VCAM-1 expression, and platelet adhesion in vivo studies of acute inflammatory lung injury.\(^2\)\(^5\) However, there are few studies that have examined the effect of apocynin in atherosclerosis. Unpublished data has reported that apocynin, when given at the time of initiation of a hypercholesterolemic diet in New Zealand white rabbits, can attenuate atherosclerotic plaque development independent of any effects on lipid-lowering.\(^2\)\(^6\)

In order to mimic a clinically relevant situation, we chose to introduce apocynin after allowing sufficient time for the development of advanced lesions in a gene-targeted model of age-dependent atherosclerosis in mice. Our data indicate that apocynin can arrest plaque progression. These data complement prior studies showing a reduction in plaque size with prolonged inhibition of NADPH oxidase in apolipoprotein-E–deficient mice.\(^9\) We found that apocynin also reduced total plaque macrophage content. Although both plaque size and macrophage content were lower in the high-dose apocynin group, we cannot say for sure that this represents conversion...
to a more stable phenotype, as the ratio of monocytes to plaque area was significantly lower statistically from controls only in the low-dose group.

Molecular imaging data were useful for defining at least 1 potential mechanism by which inflammatory cell accumulation was reduced. Apocynin seemed to prevent the progressive and marked increase in the expression of 2 key endothelial adhesion molecules involved in monocyte recruitment, P-selectin, and VCAM-1. The use of CEU and targeted microbubbles with a mean diameter of approximately 2 μm is an advantage, in that allowed us to examine adhesion molecule expression only on the luminal surface. These results indicate that apocynin had a beneficial effect, in part, through interrupting one of the main mechanisms by which ROS is thought to contribute in a self-amplifying manner to the pathogenesis of unstable atherosclerotic disease.

To assess platelet adhesion in this study, a new probe for molecular imaging of platelets with CEU was developed. A recombinant protein with the amino acid sequence corresponding to the A-1 domain of mouse VWF was used as a targeting ligand, as its interaction with the platelet GPIbα receptor can occur in high-shear stresses. Our flow chamber studies indicated that this targeting ligand, when displayed on microbubbles, is not substantially affected by potential competitive inhibitors in plasma.

There were several reasons for examining platelet–endothelial interactions in this study, besides simply evaluating apocynin’s effect on thrombotic susceptibility. Platelet or platelet microparticle interactions with the vessel wall play a role in promoting inflammation in atherosclerosis through several mechanisms. Platelets are a source for local release of many proinflammatory factors, including C–C motif chemokines (RANTES, macrophage inflammatory protein-1β) and platelet factor-4, and can potentiate the adhesive interactions between monocyte and the activated endothelium. Platelet adhesion to the intact endothelium in DKO mice was demonstrated both by GPIb immunohistochemistry in this study and by electron microscopy in previous studies. These interactions were...
As reviewed elsewhere, oxidative stress in animal models prevented in a dose-dependent fashion by apocynin we observed is likely multifactorial. The improvement in the arterial elastic properties and blood pressure with apocynin therapy did not simply reflect the beneficial effects of apocynin and were several orders of magnitude lower than lower than doses where toxicity occurs. Although we have reported P-selectin signal as a marker for endothelial activation, a portion of this signal may have been attributable to adherent platelets. This possibility does not necessarily detract from the overall message that apocynin decreases endothelial inflammatory phenotype. P-selectin immunohistochemistry was not performed, as this molecule is stored preformed within endothelial Weibel–Palade bodies and, accordingly, its presence on histology does not necessarily reflect luminal expression. We also did not specifically assess the effects of apocynin on monocyte function largely because previous studies have suggested that macrophage adhesive function and migration are not affected in p47phox−/− mice. On molecular imaging, although MB signal was very low, it followed a similar pattern as targeted agents with an increasing with age in the DKO mice and a reduction with high-dose apocynin. This likely represents low-level interaction of lipid microbubbles with leukocytes on the vascular surface or direct complement-mediated interactions with the vascular endothelium. As we have previously described, although there are regions where atherosclerosis becomes particularly severe in this mouse model, beam volume-averaging precludes us from making any conclusions on regional heterogeneity of molecular imaging signal. Finally, these studies do not necessarily confirm that the beneficial effects of apocynin are all attributable to its effect on NADPH oxidase.

In summary, our results have demonstrated several beneficial vascular effects of apocynin, when administrated relatively late in the development of atherosclerotic disease. Apocynin arrests progressive worsening of atherosclerotic disease, in terms of plaque severity, degree of endothelial activation, and number of monocytes in the vessel wall, and may be a potential therapeutic approach to reducing risk for clinical events related to unstable atherosclerotic disease.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Oxidative stress plays a critical role in the development of atherosclerosis and complications, such as acute coronary syndrome and stroke. Results from clinical trials using antioxidants that are designed to quench reactive oxygen species have been disappointing. In this study, we examined the effects of inhibiting one of the main enzymatic sources for oxidative stress in atherosclerosis, NADPH oxidase. Ultrasound molecular imaging of endothelial inflammatory activation (VCAM-1 and P-selectin expression) and platelet adhesion (GPIb signal) were used together with advanced ultrasound imaging of vascular stiffness in a mouse with atherosclerosis treated with apocynin, an inhibitor of NADPH oxidase. When given at an intermediate stage of atherosclerosis for 10 weeks, apocynin in a dose-dependent fashion reduced endothelial activation and platelet adhesion. We think these effects were, in part, responsible for the reduction in plaque area over time, reduction in macrophage number, and improve- ment in vascular mechanical properties observed with apocynin treatment. These results suggest that potent antioxidant thera- pies aimed at preventing production of reactive oxygen species may be effective at arresting atherosclerosis. They also highlight that molecular imaging may be a valuable asset for the preclinical assessment of new therapies for atherosclerosis, and may possibly be used in the future for identifying patients who will benefit from these new therapies, according to disease phenotype.
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Supplemental Material

Supplemental Figures

Supplemental Figure A. Example showing image processing algorithm. A 2-D B-mode image (non-contrast setting) is shown for anatomic reference at the left. The first frame obtained 8 min after injection of P-selectin-targeted microbubbles (FCE) was used to represent signal from adhered microbubbles as well as any freely circulating microbubbles. Frames obtained >3 seconds after destroying all microbubbles with high power ultrasound (FBG) were used to detect signal from any freely circulating microbubbles alone and were used as background. Several background frames were averaged and digitally subtracted from the first frame to obtain signal from retained microbubbles alone as well as the background-subtracted color-coded images (BS=CC, color scale at right).
Supplemental Figure B. Control immunohistochemistry conditions. The top row illustrates Mac-2 staining with either Alexa Fluor-488 or Alexa Fluor-594 secondary, or isotype control primary IgG1 with the Alex Fluor-488 secondary antibody. These images show lack of staining with isotype control antibody. They also illustrate a similar pattern of staining in near-adjacent sections when using higher wavelength secondary antibody (Alex Fluor-594) which is less prone to autofluorescent background. The bottom panels show low-level autofluorescence in the plaque as well as autofluorescence of the elastic laminae when using only secondary antibody, similar to the appearance of isotype control antibody conditions in the upper left panel. The area defined as positive Mac-2 staining was performed by identifying the high-intensity staining region within the plaque identified by non-lumen tissue within the internal elastic lamina.
Supplemental Figure C. Examples on Masson’s trichrome of the aortic arch in DKO mice in a 30 wk DKO mouse in comparison to the 40 wk DKO mice with and without high-dose apocynin shown in Figure 5.